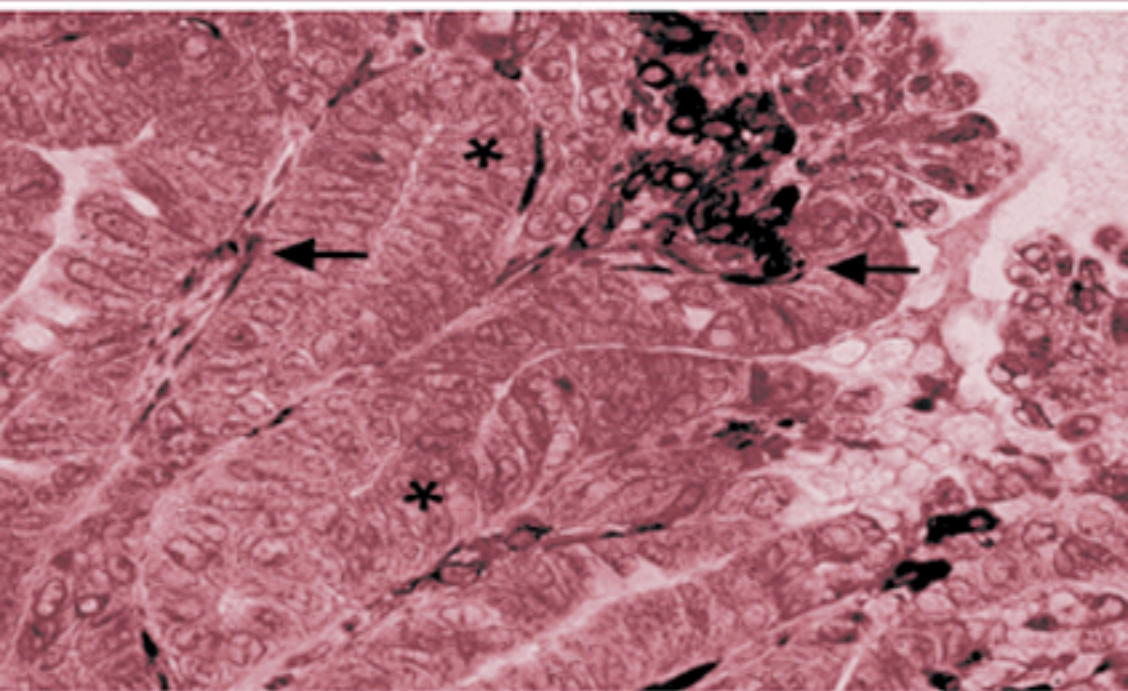


CRC Series in MODERN NUTRITION

Nutrient–Gene Interactions *in* Health *and* Disease



Edited by Naïma Moustaid-Moussa
Carolyn D. Berdanier

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Series Preface for Modern Nutrition

The CRC Series in Modern Nutrition is dedicated to providing the widest possible coverage of topics in nutrition. Nutrition is an interdisciplinary, interprofessional field par excellence. It is noted by its broad range and diversity. We trust the titles and authorship in this series will reflect that range and diversity.

Published for a scholarly audience, the volumes in the CRC Series in Modern Nutrition are designed to explain, review, and explore present knowledge and recent trends, developments, and advances in nutrition. As such, they will also appeal to the educated general reader. The format for the series will vary with the needs of the author and the topic, including, but not limited to, edited volumes, monographs, handbooks, and texts.

Contributors from any bona fide area of nutrition, including the controversial, are welcome.

We welcome the contribution of the volume Nutrient–Gene Interactions in Health and Disease edited by Naïma Moustaid-Moussa and my long time, and much respected, colleague Carolyn D. Berdanier. This book is a worthy companion to two other CRC Press books on related subjects: *Nutrition and Gene Expression* edited by C.D. Berdanier and L.L. Hargrove and *Nutrients and Gene Expression, Clinical Aspects* edited by C.D. Berdanier. Taken together they make a splendid resource on the cutting edge topic of nutrient–gene interactions. This book will be useful to a broad spectrum of nutritionists and life scientists of all walks.

Ira Wolinsky, Ph.D.
University of Houston
Series Editor

Preface

A century ago biochemistry was in its infancy. Early biochemists were interested in the vital amines, later called vitamins, and how living cells work. In the 1930s biochemists and physiologists formed a scientific group devoted to identifying and understanding nutrient needs. Over the subsequent 60 years the essential nutrients were identified one by one and feeding studies were conducted to determine how much of each nutrient was required by a wide variety of species. Of interest was the fact that there was a high degree of inter-animal variability. In some instances the need for a given nutrient by one animal (including humans) could be twice that needed by another animal of the same age, sex, and breed. Researchers began to realize that this diversity in nutrient need was likely due to variations in the genetic backgrounds of the individual animals. Genetic diversity was utilized by animal and plant breeders to produce (through selective breeding) animals that efficiently use nutrients for specific purposes. Hence, rapidly growing, energy- and protein-efficient meat animals were produced that allowed farmers to bring their produce to market at a reduced feed cost. Chickens, for example, can be brought to market weight in half the time and feed expense by capitalizing on this selective breeding. These are but a few examples of nutrient–gene interactions using traditional methods of species improvement through selective breeding.

Today this method of species improvement has entered a new era. Techniques are now available to probe the genome to tease out those genetic messages that dictate nutrient need and tolerance. We have come to realize that optimal nutrient intake is determined by very specific genetic messages. This realization has meant that an entirely new approach to understanding nutrition is needed. With the completion of the whole genome sequence for several organisms including a draft of the human genome project, new genes with novel functions in nutritional diseases will be uncovered. With the advent of new technology, exploration of nutrient effects on gene expression will expand the identification of these new genes. The use of microarrays allows researchers to examine thousands of genetic messages simultaneously. Functional genomics and proteomics have given nutritionists a new way to examine nutrient response, especially with respect to nutrition-related diseases. Obesity, diabetes, heart disease, alcoholism, and anemia are but a few of the diseases that have both a nutrition component and a genetic one.

The present book and its two predecessors were compiled to update the reader on specific nutrient–gene interactions. The first book, edited by James Hargrove and Carolyn Berdanier, and entitled *Nutrition and Gene Expression*, was published in 1993. The second, *Nutrients and Gene Expression: Clinical Aspects*, edited by Berdanier, addressed some of the clinical conditions that

arise as a result of nutrient–gene interactions. The present volume revisits the current basic science concerning nutrient-gene interactions. Reviews of examples of macronutrients and micronutrients as they affect gene expression are presented. The next volume will address nutrition from the functional genomics and proteomics point of view. We hope that you, the reader, enjoy reading about these important areas of research.

Näima Moustäïd-Moussa
Carolyn D. Berdanier

Acknowledgments

We would like to dedicate this book to the American Society for Nutritional Sciences, especially to the Nutrient–Gene Interaction Research Interest Section and to our respective departments for promoting the field of molecular nutrition.

We would like to thank Dr. Ira Wolinsky, the series editor, who played a key role in getting the publisher's approval for this edition.

We express appreciation to our respective families, Hanna, Sami, and Zaina Moussa and Reese Berdanier for their love and patience during the preparation of this book, with special appreciation to Lynn Berdanier for her efforts in creating some of the figures.

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Her research has been supported by NIH, USDA, and various commodity groups. She is a member of the American Society for Nutritional Sciences, the Society for Experimental Biology and Medicine, the American Diabetes Association, and several honorary societies. She has served on the editorial boards of the *Journal of Nutrition*, *FASEB Journal*, *Nutrition Research*, and *Biochemical Archives*. Current research interests include studies on the role of nutrients in the control of mitochondrial gene expression.

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1

Dietary and Hormonal Regulation of the Mammalian Fatty Acid Synthase Gene

Kristin Morris, Yanxin Wang, Suyeon Kim
and Naïma Moustaid-Moussa

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1.1 Introduction

Fatty acids in adipocytes can be derived from circulating lipoproteins via lipoprotein lipase, or they can be synthesized via *de novo* lipogenesis from carbohydrate precursors such as glucose. Excess glucose promotes lipogenesis by increasing the glycolytic flux and generating acetyl CoA. Acetyl CoA is the primary substrate for the synthesis of long-chain saturated fatty acids. These fatty acids are then used for esterification of α -glycerol phosphate-generating triacylglycerols, the major form of energy storage in adipose tissue.

Several enzymes contribute to lipogenesis. These enzymes include ATP-citrate lyase, which produces acetyl CoA from citrate; acetyl CoA carboxylase (ACC), which carboxylates acetyl CoA to generate malonyl CoA; fatty acid synthase (FAS), which catalyzes the synthesis of long-chain saturated fatty acids from acetyl CoA and malonyl CoA using NADPH generated by malic enzyme, glucose 6-phosphate dehydrogenase, 6-phosphogluconate, and citrate dehydrogenase (Fig. 1.1).

Fatty acid biosynthesis *per se* involves two of these enzymes, ACC and FAS. ACC catalyzes the first and rate-limiting step in *de novo* lipogenesis. The multifunctional FAS enzyme complex then catalyzes the following reaction:

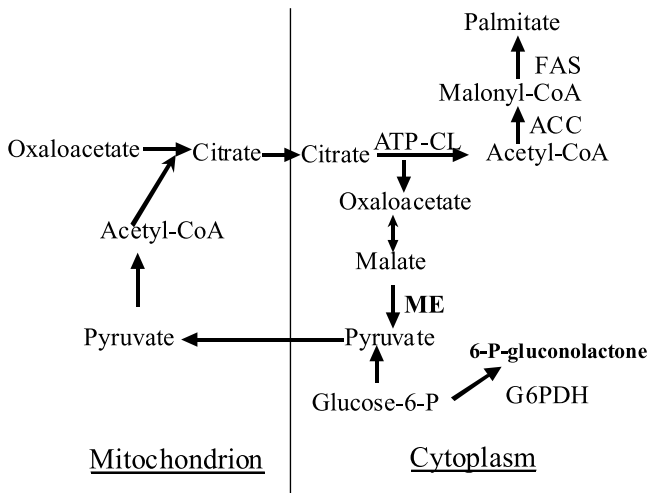
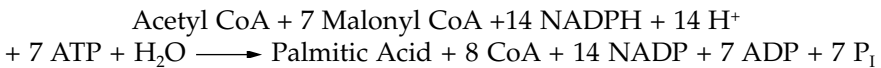


FIGURE 1.1

Key enzymes and intermediates of lipogenesis. ATP-CL, ATP-citrate lyase; FAS, fatty acid synthase; ACC, acetyl CoA carboxylase; ME, malic enzyme; G6PDH, glucose 6-phosphate dehydrogenase.

While ACC is subject to short-term regulation via allosteric modification of the enzyme, FAS is primarily regulated by the amount of mature protein.¹ FAS enzyme content generally correlates with the amount of FAS mRNA that is primarily controlled at the transcriptional level.^{1,2} This review will focus on the mammalian FAS gene and its dietary and hormonal regulation.

1.2 Organization and Function of the Multifunctional Fatty Acid Synthase

The functional fatty acid synthase complex is a homodimer of two identical subunits with an apparent molecular mass of approximately 250 kD.³ The multiple catalytic properties of FAS are responsible for the *de novo* synthesis of the long-chain fatty acid, palmitate. The structure of the rat and human FAS complexes is shown in Fig. 1.2. FAS involves seven different catalytic enzymes plus the acyl carrier protein, all of which are found in the FAS complex. These activities are arranged head-to-tail within discrete boundaries.³ Such an arrangement facilitates the product of one reaction binding to the catalytic site and stimulating the activity of the subsequent enzyme activity. Free palmitate is the major fatty acid resulting from the catalytic activities of FAS *de novo*, accounting for 70% of all long-chain fatty acids synthesized by the fatty acid synthase complex.

Proceeding from the N-terminus of FAS, three domains containing the catalytic activities have been identified.³ Domain I consists of the acetyl/malonyl transferase and β -ketoacyl synthase activities and is responsible for entry of the substrates, acetyl CoA and malonyl CoA, into the FAS enzyme complex by channeling them to the proper thiols. Domain II encodes the acyl carrier protein, which constitutes the eighth, albeit nonenzymatic, activity of the

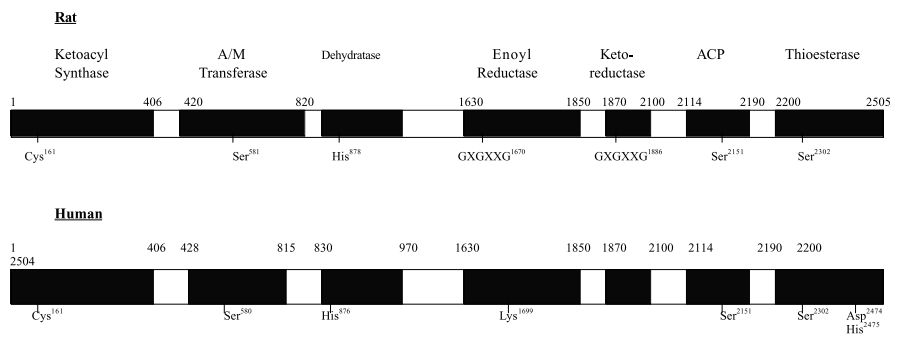


FIGURE 1.2 Structure of the rat and human fatty acid synthase multienzyme complex. Shown are the putative active sites of condensing enzyme (Cys¹⁶¹), acetyl/malonyl transacylases (Ser⁵⁸⁰), dehydratase (His⁸⁷⁶), and thioesterase (Ser²³⁰², Asp²⁴⁷⁴, and His²⁴⁷⁵). Also shown: Point of attachment of 4'phosphopantetheine (Ser²¹⁵¹).

fatty acid synthase complex. β -ketoacyl reductase, enoyl reductase, and dehydratase activities are also found in the second domain. The third domain is situated at the carboxyl end of the protein and contains a single enzymatic activity, thioesterase. Dissociation of the native enzyme to individual monomers results in loss of palmitate synthesis even though six of the seven catalytic activities remain in the monomer.³

1.3 Structure of the Fatty Acid Synthase Gene

Fatty acid synthase is a multifunctional enzyme complex encoded by a single copy gene that produces a single mRNA species in mice, humans, and pigs and two mRNA species in rats, chickens, and geese.⁴ This is consistent with the presence of two polyadenylation sites in the rat and avian sequences. The mammalian fatty acid synthase gene exhibits several characteristics consistent with mammalian genes: intron/exon junctions that follow GT/AG rules for splice sites, a 5' flanking non-coding exon separated by a large intron from the second exon which contains the transcription initiation site.⁴

The murine fatty acid synthase was identified by differential screening of cDNA libraries derived from liver mRNA of rats that were fasted, or fasted rats then refed a high carbohydrate diet. Northern blot analysis identified a single 8.2-Kb mRNA species encoding the murine FAS.⁵ This was consistent with the single mRNA species coding for FAS previously identified from the murine 3T3-L1 cell line.⁵ One polyadenylation site was identified, in contrast to rat, goose, and duck sequences, which contains two polyadenylation signals, both of which are derived from a single gene.⁶ Sequence comparison of the mouse cDNA found it to be 97% homologous to rat sequence.

FAS exhibits widespread tissue distribution in the mouse.^{2,3,5} The highest concentration of mRNA encoding for FAS is found in the liver, adipose tissue, and mammary glands.⁵ However, FAS is also expressed at lower levels in other tissues.

Sequence analysis of goose FAS cDNA has found that it contains an additional 74 amino acids at its amino terminus that are not present in the chicken cDNA. This has been determined to be the result of a single nucleotide deletion in the 5' end of goose DNA that alters the reading frame without affecting the functional activity of the enzyme.⁶

The partial sequence of the human fatty acid synthase gene has also been reported,⁷ and fundamental differences exist between the human and rodent or avian FAS gene. Three transcription initiation sites (Ti1, Ti2, and Ti3) were described in the human FAS gene, but only one of them (Ti1) was preceded by a classical TATA and CAAT boxes. Promoter II, situated upstream of Ti2, was shown to inhibit transcription from promoter I (upstream of Ti1).⁷ Promoter 1 was shown to increase reporter gene activity in transfection assays, while promoter II represses this activity.⁷

1.4 Fatty Acid Biosynthesis in Humans

There are species differences in where lipogenesis occurs.⁸ In rodents and humans, lipogenesis occurs in both adipose tissue and liver, while in birds, lipogenesis occurs primarily in the liver. In humans, the lipogenic capacity of the liver is significantly lower compared to other species. This is most likely attributable to the high fat content of the human diet. Rodents as well as other laboratory species are routinely fed low (~5 en %) fat diets. The major energy source in these diets is carbohydrate which, in turn, serves as the substrate for lipogenesis. Consistent with this, humans sustained parentally by high glucose solutions have much higher rates of lipogenesis than humans eating the typical Western diet.⁹

Despite its apparent low level, human lipogenesis has been shown to be regulated by several nutritional and hormonal factors including fasting, carbohydrates, and insulin.^{10,11} Fasting inhibited the lipogenic effect of insulin by mechanisms involving both transport and metabolism of glucose.¹⁰ In addition, when isolated human adipocytes were incubated with labeled glucose, 80% of intracellular radioactivity was incorporated into lipids.¹¹ *De novo* lipogenesis, measured by indirect calorimetry, was not detectable on low-carbohydrate diets but is increased on high-carbohydrate diets.¹² Results from these studies suggested that in depleted patients given hypercaloric high-carbohydrate diets, adipose tissue may account for up to 40% of whole body lipogenesis. In these subjects, total fat synthesis in adipose tissue was equal to or greater than in the liver on a per weight basis. Several studies have suggested that hepatic lipogenesis is a quantitatively minor pathway; however, these studies did not investigate adipose tissue lipogenesis.¹³ A recent study confirmed that the liver plays a minor role in human lipogenesis and that adipose tissue is more likely the main site for *de novo* fatty acid synthesis in humans.¹⁴ Using cultured human adipocytes, we demonstrated that insulin increases the expression of several lipogenic genes.¹⁵ In addition, we found that human expression of FAS varies among adipose tissue depots, ranging from very low levels to very high levels that equal or surpass FAS expression in rodents (unpublished data) and that the combination of insulin and glucocorticoids leads to as much as 40% of glucose being converted to fatty acids (unpublished data).

1.5 Regulation of the Fatty Acid Synthase Gene

The rate of fatty acid biosynthesis is primarily determined by the availability of glucose.¹⁶ Consuming a diet rich in carbohydrate elevates circulating glucose, which in turn signals the secretion of hormones which affect *de novo* synthesis of fatty acids.¹⁶ Among these hormones, insulin and thyroid

hormone (T3) are increased, and glucagon is decreased. Conversely, glucagon is decreased during feeding and elevated during starvation. Glucose, insulin, T3, agouti, and angiotensin II stimulate fatty acid synthesis and the expression of the fatty acid synthase gene while glucagon and polyunsaturated fatty acids (PUFA) downregulate FAS gene expression. We will review below the data available on the mechanisms of regulation of fatty acid synthase by these nutritional and hormonal factors.

The 5'-flanking region is critical for the regulation of FAS gene expression in cultured cells transfected with FAS-reporter fusion genes and in transgenic mice expressing this transgene. Several transcription factors, which bind specific cis-acting response elements in the FAS gene, mediate its dietary and hormonal regulation. Major regulatory sequences in the rat, human, and chicken genes are shown in Fig. 1.3 and are discussed in more detail in the following sections.

The cis-acting elements necessary for tissue-specific, nutritional, and hormonal regulation of FAS expression were identified using transgenic mice expressing FAS promoter linked to the chloramphenical acetyltransferase (CAT) reporter gene. The necessary sequences are contained mostly in the 2.1 kb of 5'-flanking DNA.¹⁷ There is strong positive correlation between mRNA levels and the tissue-specific gene expression patterns of the reporter and the endogenous FAS in transgenic mice.¹⁷ Fasting and refeeding, insulin, dibutyryl cAMP, and glucocorticoids regulated expression of the reporter gene and

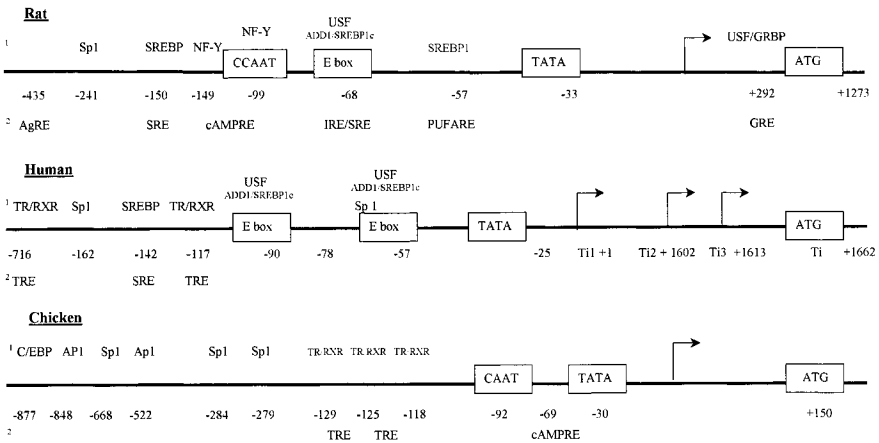


FIGURE 1.3

Major regulatory sequences in the rat, human, and chicken FAS gene. ADD1, adipocyte determination and differentiation factor; AgRE, agouti response element; cAMPRE, cAMP response element; CCAAT, inverted CCAAT box; C/EBP, CCAAT element binding protein; GRE, glucose response element; GRBP, glucose response element binding protein; NF, nuclear factor; PUFARE, polyunsaturated fatty acid response element; RXR, retinoid X receptor; SRE, sterol response element; SREBP, sterol response element-binding protein; TATA, TATA box; Ti: transcription initiation site; TRE: thyroid hormone response element, TR: thyroid hormone receptor, USF: upstream stimulatory factors.

¹ Transcription factors, ² Response elements.

the endogenous FAS gene in a similar manner. In contrast, PUFAs such as menhaden oil, which is rich in long-chain n-3 fatty acids, dramatically suppressed endogenous FAS mRNA in both the liver and adipose tissue of transgenic mice compared to those fed oleic acid. However, CAT activities in the tissues of PUFA-treated mice were only 50% lower than in transgenic mice fed the oleic acid diet.¹⁷ Thus, the stabilization of FAS mRNA that is observed during 3T3-L1 adipocyte differentiation and high-glucose or insulin treatment in HepG2 cells may also contribute to regulation of FAS mRNA levels by PUFA.^{15,18,19} Alternatively, elements not contained in the first 2.1kb of the FAS 5'-flanking DNA may be necessary for full suppression of FAS transcription by PUFA. Identification of the cis-acting nucleotide sequences and trans-acting factors involved in the nutritional and hormonal regulation of the FAS gene will greatly facilitate the understanding of the underlying molecular mechanisms regulating expression of lipogenic genes.

1.6 *In Vivo* Effects of Fasting, Refeeding, and Aging

Fatty acid synthase is regulated in animals in response to nutrient and hormonal signals. Fasting in rodents results in decreased glucose conversion into fatty acids; when these animals are subsequently refed a low-fat, high-carbohydrate diet there is a rapid and efficient increase in production of fatty acids and triacylglycerols.² Such changes are mediated by hormonal changes in response to diet.² Changes in nutrient intake that increase circulating glucose levels elevate circulating insulin and thus promote lipogenesis. Conversely, dietary changes that lower circulating blood glucose levels invoke glucagon secretion and suppression of *de novo* lipogenesis. Suppression of FAS activity by glucagon is mediated by elevations in intracellular cAMP levels. The liver and adipose tissue undergo the most dramatic metabolic changes in response to fasting and refeeding.^{2,5,17,20,21}

Activation of whole body lipogenesis and induction of the fatty acid synthase activity can be observed when animals are transitioned from a high-fat, low-carbohydrate diet to a low-fat, high-carbohydrate diet.²² This can be mimicked by refeeding previously fasted animals.²² Feeding a high-carbohydrate diet is accompanied by an increase in circulating insulin levels, both of which increase the transcriptional rate of fatty acid synthase, as well as other lipogenic genes.^{22,23} Interestingly, this effect is attenuated by aging. While FAS mRNA was dramatically and rapidly induced by fat-free diets in young rats,²⁴ this response was much slower in aged rats, which did not exhibit significant elevations in FAS mRNA above fasting levels for 6 h. In addition to delaying transcriptional activation, maximum rates of gene transcription were not achieved until animals had been maintained on a fat-free diet for 24 h.²⁴ This implies that aging is a factor in the regulation of lipogenic gene transcription.²⁵

1.7 Effects of Insulin

Feeding a low-fat, high-carbohydrate diet results in elevations in circulating insulin, which along with glucose, induce the enzymes of fatty acid synthesis including FAS, concomitant with suppression of the enzymes of fatty acid oxidation. FAS mRNA is low in animals made insulinopenic by administration of streptozotocin (STZ), a pancreatic β -cell toxin, compared to untreated animals. This effect is countered by insulin administration. Insulin treatment results in the rapid induction of FAS mRNA and gene transcription. Within 6 h of insulin administration to STZ-treated animals, FAS mRNA levels were induced to a level comparable to that observed when refeeding a low-fat, high-carbohydrate diet to previously fasted normal mice. Cyclohexamide, an inhibitor of protein synthesis, completely abolished the effect of insulin, demonstrating that transcriptional regulation of FAS by insulin requires ongoing protein synthesis.²⁶

The murine 3T3-L1 adipocyte cell line is widely used to study regulation of lipogenic gene expression.² Insulin treatment of fully differentiated 3T3-L1 adipocytes upregulates FAS protein content and mRNA level. In contrast, treatment of 3T3-L1 adipocytes with dibutyryl cAMP dramatically reduced both FAS mRNA and the rate of enzyme synthesis.²⁰ Transcriptional regulation of FAS by insulin was observed in both rodent and human cultured adipocytes.^{15,27} However, in human hepatoma HepG2 cells, glucose or insulin appear to increase FAS transcription by stabilizing FAS mRNA.^{15,18}

Since FAS is not regulated allosterically but primarily at the transcriptional level, efforts have focused on identifying and characterizing regulatory sequences in the FAS gene, primarily in the 5'-flanking region, which may mediate hormonal and nutritional regulation of this gene. Insulin is the most extensively studied and best understood regulator of FAS gene expression.

1.7.1 Insulin Responsive Sequences in the FAS Gene

We generated constructs containing progressive deletions in the 5'-flanking region of the FAS gene linked to either the CAT or luciferase reporter genes. The constructs were then transfected into 3T3-L1 adipocytes.²⁸ In the presence of glucose and physiological concentrations of insulin, transfections of plasmids containing 5' deletions within -2100/-67 to +67, relative to the transcription start-site induced insulin responsiveness of the reporter gene.²⁸ However, deletions of the region between -67 and -25 abolished insulin responsiveness of the luciferase gene.²⁸ Additional observations led to the localization of the insulin responsive sequences (IRS) in the FAS gene in the -68/-52 region.²⁸ DNase I footprinting analysis revealed a protected region from -68/-52. In addition, gel shift mobility assays demonstrate specific interactions between the protected region and nuclear factors isolated from mouse liver and 3T3-L1 nuclear extracts.²⁸ Finally, linking the heterologous

SV40 promoter to three tandem repeats corresponding to the -68/-52 region was sufficient to confer insulin responsiveness on the luciferase reporter gene (Fig. 1.3).²⁸ This mechanism may account for the stimulatory effects of carbohydrate feeding on the FAS gene. However, this does not preclude the existence of additional insulin responsive elements within the 5'-flanking region, which were indeed identified in upstream regions of the FAS promoter.²⁹

1.7.2 Transcription Factors

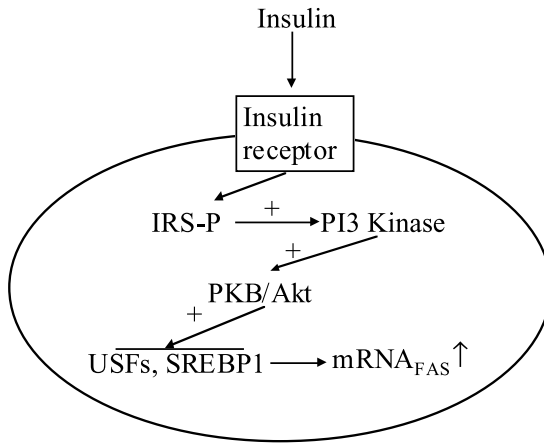
1.7.2.1 Upstream Stimulatory Factors

An E-box DNA-binding motif (5'-CATGTG-3') is located at -65/-60 bp within the IRS of the FAS gene.²⁹ This particular DNA sequence is recognized by upstream stimulatory factors 1 and 2 (USF1 and USF2), members of the basic helix-loop-helix family of transcription factors. This E-box sequence is important for basal- and insulin-stimulated activity of the FAS promoter. When deletions are introduced in the -73/-43 region in the first 150 bp of the 5'-flanking region, as much as 75% of basal promoter activity is lost. In addition to its role in basal transcription, interactions between USF transcription factors and the insulin responsive E-box of the FAS-IRS are functionally required for insulin regulation. Transfection assays demonstrated that mutations in the E-box sequence which abolish interactions with the USFs result in loss of insulin responsiveness. Furthermore, cotransfection of 3T3-L1 adipocytes with dominant negative USFs blunted the response of the FAS gene to insulin stimulation.³⁰

Insulin regulates a wide variety of biological responses through a cascade of signaling events.³¹ Insulin stimulation of the adipocyte FAS promoter is mediated by the phosphatidylinositol 3-kinase (PI3-K) pathway and protein kinase B (PKB)/Akt as the downstream effector in this cascade (Fig. 1.4).³² In agreement with these studies, IRS-1/PI3-K/Akt activation has also been demonstrated to be essential for insulin stimulation of lipid synthesis in brown adipocytes.³³

1.7.2.2 SREBP-1/ADD-1

Sterol regulatory element-binding proteins (SREBPs) are members of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors and regulate enzymes responsible for the synthesis of cholesterol, fatty acids, and triglycerides.³⁴ To date, three SREBP isoforms, SREBP-1a, -1c, and -2, have been identified and characterized. In addition to the USFs, SREBPs reportedly bind the FAS 5'-flanking DNA.³⁵ *In vitro*, SREBP binds to two regions of the FAS 5'-flanking DNA. The first region resides at -150/-140 bp and matches the SRE-1 sequence found in the LDL receptor 5'-flanking DNA. However, this region has proven dispensable for sterol regulation of FAS mRNA. The second SRE is within the FAS IRS and includes two tandem copies of SREBP binding sites that split the -65/-60 E-box.³⁵ Binding of a USF to

**FIGURE 1.4**

Proposed mechanism of insulin regulation of the fatty acid synthase gene. IRS-P: phosphorylated insulin receptor substrate, PI3 Kinase: phosphatidylinositol 3 kinase, PKB: protein kinase B, USFs: upstream stimulatory factors, SREBP1: sterol response element binding protein 1, FAS: fatty acid synthase.

the –65/–60 E-box and SREBP to the flanking sites appears to be distinct processes.³⁰ Because the USF- and SREBP-binding sites overlap, distinct transcription factors may be utilized to regulate FAS gene transcription under different regulatory conditions. Overexpression of SREBP-1a in mice results in markedly elevated levels of FAS mRNA in hepatocytes and the concomitant accumulation of cholesterol and triglycerides in the liver.³⁶ However, feeding mice sterol-depleted or -enriched diets does not cause a consistent change in FAS mRNA level.³⁷ In HepG2 cells, FAS mRNA levels were decreased by sterols while overexpression of active SREBP stimulates FAS promoter activity.³⁸

SREBP-2 is a relatively selective activator of cholesterol synthesis (as opposed to fatty acid synthesis) in liver and adipose tissue of mice and in cultured human cells, while SREBP-1c is a major mediator of insulin action on hepatic expression of glucokinase and lipogenic genes, including FAS.^{38–40} Changes in the expression of lipogenic genes parallel changes in SREBP-1 expression.³⁶ The demonstration that hepatic expression of lipogenic genes is non-responsive to carbohydrate feeding in SREBP-1 knockout mice suggests a critical role for SREBP-1 in induction of hepatic lipogenesis.

SREBP-1c is also called adipocyte determination and differentiation factor (ADD1).⁴¹ Insulin stimulates SREBP-1c expression at the level of transcription in cultured rat hepatocytes, while glucagon exerts an inhibitory effect on this gene.^{42,43} The effect of insulin on stimulation of nuclear SREBP-1c has also been observed in adipocytes, both *in vivo* and *in vitro*.^{42,43} Molecular dissection of the FAS promoter has shown that ADD1/SREBP-1c acts through an E-box motif at nucleotides –64 to 59, which is identical to the region previously recognized as the major IRE.^{28,42} In normal rats, the activation of this E-box is countered by the presence of a transcriptionally inactive sterol response

element (SRE) binding site at 150, which limits the availability of nuclear ADD1/SREBPs. In obese Zucker *fa/fa* rats, overproduction of SREBP-1 can compensate for ADD1/SREBP-1 and bind to the inactive SRE, thus overcoming the negative effects of the inactive SRE.⁴⁴ Furthermore, ADD1/SREBP-1 regulation of FAS promoter activity in adipocytes was antagonized by dominant negative members of the HLH family in rat adipocytes and mature 3T3-L1 adipocytes.⁴⁵ In summary, SREBP-1c/ADD1 is a key transcription factor not only in fatty acid synthesis but in other aspects of lipid metabolism in both the liver and adipose tissue, acting in part via the insulin responsive E-box to regulate lipogenic gene transcription.

1.8 Glucose Regulation of FAS Expression and Activity

Since glucose increases circulating insulin levels, it is often difficult to differentiate between the effects of glucose metabolism and those of insulin in mediating the regulation of gene expression. Both glucose-dependent and glucose-independent effects of insulin have been reported.¹⁶ A high level of glucose is not only required for insulin stimulation of FAS transcription but is also involved in stabilizing FAS mRNA as shown in studies using HepG2 cells and adipocyte differentiation studies.^{18,19,46} In USF1 or USF2 knockout mice, induction of hepatic FAS gene expression by refeeding a carbohydrate-rich diet was severely delayed, whereas expression of SREBP-1 expression was almost normal. The insulin response was unchanged, suggesting USF transactivators, especially USF1/USF2 heterodimers, are essential to sustain dietary induction of the FAS gene in liver (Fig. 1.5).⁴⁷ ADD1/SREBP-1 has

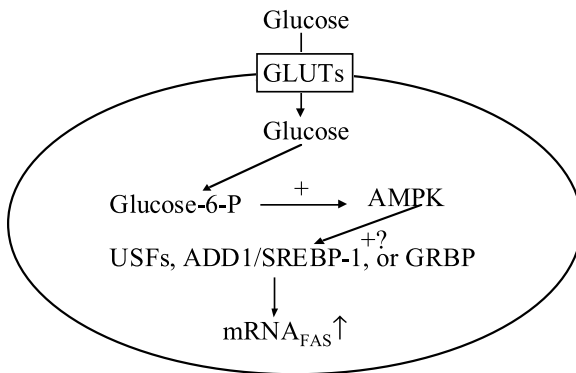


FIGURE 1.5

Proposed mechanism of glucose regulation of the fatty acid synthase gene. GLUTs: glucose transporter, AMPK: AMP-activated protein kinase, USFs: upstream stimulatory factors, ADD1/SREBP1: adipocyte determination and differentiation factor1/sterol response element binding protein 1, GRBP: glucose response binding protein, FAS: fatty acid synthase.

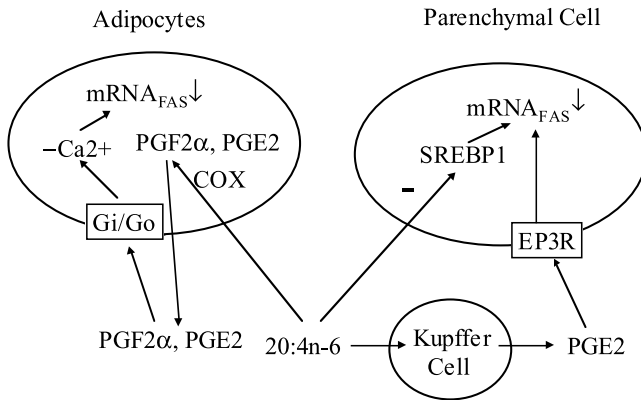
also been suggested to mediate glucose-induced expression of FAS as well as other hepatic genes (Fig. 1.5).^{41,48} An upstream stimulatory factor (USF)-binding site at +292/+297 bp in the first intron was reported to be a positive regulatory element involved in glucose responsiveness of the FAS gene.⁴⁹

Glucose-6-phosphate has been postulated to be the signaling molecule mediating glucose/insulin stimulation of several lipogenic genes including pyruvate kinase, spot 14, and FAS.^{50,51} However, xyulose 5-phosphate, an intermediate in the pentose phosphate pathway, is also suggested to play this role.⁵⁰ Additionally, stimulation of FAS and S14 mRNA levels by glucose and xylitol in rat hepatocyte cultures is more closely correlated with changes in intracellular glucose 6-phosphate levels than xyulose 5-phosphate levels, supporting the hypothesis that glucose 6-phosphate is likely the critical metabolite for regulation of lipogenic gene expression by glucose.^{51,52} However, the signaling pathway subsequent to glucose/glucose metabolites which mediate the transcriptional effects on gene expression, is still poorly understood. Glucose response-binding protein (GRBP) binds to the CACGTG motif of the glucose response element and has been identified as a transcription factor mediating the effects of glucose (Fig. 1.5). This protein is activated by high glucose concentrations *in vivo*.⁵⁰ Furthermore, the DNA-binding activity of GRBP was found to be inhibited by low glucose concentrations *in vivo* and by cAMP in cultured hepatocytes.⁵⁰

1.9 Regulatory Effects of Polyunsaturated Fatty Acids

Polyunsaturated fatty acids of the n-3 and n-6 families are known to suppress hepatic mRNA levels of several lipogenic genes, including FAS.⁵³ With the exception of glucose-6-phosphate dehydrogenase, PUFAs exert their regulatory effects at the level of transcription.⁵³ PUFAs exert dominant negative effects on many of the genes of lipogenesis and are known to override the stimulatory effects of insulin, carbohydrates, and thyroid hormones. Furthermore, the direct effects of PUFAs on the liver do not require extrahepatic factors.

PUFAs are known to suppress hepatic gene expression through three distinct pathways: (1) a peroxisome proliferator activated receptor (PPAR)-dependent pathway, (2) a prostanoid pathway, and (3) a PPAR and prostanoid-independent pathway. Although fibrates and prostanoids have modest inhibitory effects on FAS gene expression, the PUFA-mediated suppression of this gene in the liver does not require either PPAR α activation or cyclooxygenase conversion of PUFA to eicosanoids (Fig. 1.6).^{54–56} However, the n-6 PUFA arachidonic acid inhibits several lipogenic genes, including FAS in 3T3-L1 adipocytes, through a prostanoid pathway.⁵⁷ Furthermore, cyclooxygenase products from non-parenchymal cells can act on parenchymal cells through a paracrine process and mimic the effect of n-6 PUFAs on lipogenic gene expression.⁵⁷

**FIGURE 1.6**

Proposed mechanism of polyunsaturated fatty acid regulation of the hepatic fatty acid synthase gene. FAS: fatty acid synthase, PGF2 α : prostaglandin F2- α , PGE2: prostaglandin E2, COX: cyclooxygenase, 20:4n-6: arachidonic acid, SREBP1: sterol response element binding protein.

Several labs have investigated the mechanisms of PUFA regulation of FAS gene expression. Feeding PUFAs resulted in a decrease in the mature form of SREBP-1 in liver nuclei, which paralleled changes in FAS and ACC mRNA in wild-type mice. Studies using transgenic mice confirmed that the suppressive effect of PUFAs on hepatic FAS expression is due to a decrease in the mature form of SREBP-1 protein.⁵⁸ Other labs have also demonstrated the suppressive effects of dietary PUFAs on SREBP-1 expression mediate the subsequent regulation of FAS expression.^{59,60} This has been confirmed by feeding fish oils, which downregulate the mature form of SREBP by decreasing SREBP-1c mRNA expression and leads to the concomitant decrease in hepatic FAS mRNA.^{61,62} Studies using cultured hepatocytes linked fatty acid peroxidation to the effects of PUFAs on gene expression.⁶³ These findings suggest that the *in vivo* inhibitory effects of PUFAs on lipogenic genes could be mediated indirectly by a peroxidative mechanism. Further studies will be necessary to explore this signaling pathway in the liver.

1.10 Effects of Glucagon and cAMP

Fasting increases glucagon levels, which may contribute to downregulation of lipogenesis. Insulin presumably mediates the marked increase in the rate of FAS transcription that occurs subsequent to refeeding fasted animals. This elevation can be completely blocked by administration of glucagon or dibutyryl cAMP during the refeeding period. This implies that glucagon, via its second messenger cAMP, antagonizes the stimulatory effects of insulin on

FAS expression.²¹ In support of this theory, *in vitro* studies using H4IIE hepatoma cells demonstrate that glucagon antagonizes the effect of insulin on FAS transcription by increasing intracellular levels of cAMP. Studies using the H4IIE hepatoma cell line with a CAT-linked FAS promoter, demonstrate that cAMP is an effective inhibitor of insulin-stimulated FAS transcription.²⁰ Progressive deletions of sequences from the FAS 5'-flanking region led to identification of a cAMP response element between -149 and +68. The FAS IRS was required for cAMP antagonism of insulin action.¹⁶ An inverted CCAAT box was subsequently identified in the -99/-92 region of the FAS gene. Mutations in this region abolished cAMP responsiveness, suggesting this region is also responsible for mediating the effects of cAMP. Consistent with the localization of the FAS IRS to the -68/-52 bp region, insulin responsiveness was not affected by mutations in the -99/-92 region.²⁰ The identification of the cAMP response sequence of FAS as an inverted CCAAT box puts the FAS gene in a small group of cAMP-regulated genes that do not use the more common CREB- or ATF-1-binding sites for transcriptional regulation. In fact, the basal transcription factor NF-Y and related proteins bind to the inverted CCAAT box of the FAS promoter *in vitro*.⁶⁴

1.11 Regulatory Effects of Thyroid Hormones

In addition to changes in the levels of circulating insulin and glucagon, thyroid hormone (T3) is also elevated during refeeding of fasted animals. Thyroid hormone stimulates FAS expression through a mechanism that is independent of insulin. Administration of thyroid hormone to rats for 7 days doubled FAS activity in liver.⁶⁵ Furthermore, hypothyroidism reduced hepatic FAS activity.⁶⁶ These effects can be demonstrated *in vitro* as well. FAS activity can be stimulated 2- to 3-fold in primary cultures of rat and chick embryo hepatocytes.^{67–69} This is due to an increase in gene transcription and is accompanied by a 5-fold increase in FAS mRNA.

Adipocytes have also been demonstrated to be responsive to T3.⁶⁹ When mature 3T3-L1 adipocytes were treated with 10 nM T3, the relative rate of FAS synthesis, the steady-state mRNA level, and the transcriptional rate all increased within hours and could be sustained at this level for 24 h.⁶⁹ Thyroid hormone exerts its effects on FAS transcription by heterodimerization of ligand-bound thyroid hormone receptor (TR) with the retinoid receptor, RXR. The TR/RXR heterodimer binds to the consensus thyroid hormone response element (TRE) and promotes gene transcription.^{70–72} Transfections of fusion constructs of the human FAS promoter linked to the luciferase reporter gene into cultured human cells have identified two TREs, TRE1 (GGGTTAcgtcCGGTCA at -716 to -731) and TRE2 (sequence GGGTCC, at -117 to -112).⁷³

1.12 Insulin-Like Effects of Angiotensin II

In addition to its synthesis in the classical renin angiotensin system, the hypertensive hormone angiotensin II (AII) is secreted from adipose tissue.^{74,75} In adipocytes, AII has an insulin-like effect and acts as a lipogenic hormone to increase fatty acid and triglyceride synthesis.⁷⁶ We have demonstrated that AII increases FAS enzyme activity and mRNA content by upregulating FAS gene transcription.⁷⁶ Recently, in an attempt to identify AII regulatory sequences in the FAS gene, we found that AII targets insulin regulatory sequences that were previously identified²⁸ as E-box motifs.³⁰ Furthermore, we found that ADD1 is a potential transcription factor mediating transcriptional regulation of the FAS gene by AII.⁷⁷

1.13 Regulatory Effects of the Obesity Genes

1.13.1 Leptin

Leptin, the *ob* gene product, is a protein specifically secreted from adipose tissue, and is transported to the hypothalamus where it binds specific receptors resulting in a decrease in appetite.^{78,79} Since its identification, leptin has been recognized as a hormone which induces satiety and increases basal energy expenditure. Food intake, specifically dietary carbohydrate, results in the rapid and specific induction of *ob* mRNA levels in rat adipose tissue.^{80,81} The effects of leptin on FAS may be mediated by glucose, polyunsaturated fatty acids, or both. Administration of leptin to rats consuming a high-carbohydrate, fat-free diet suppressed the mRNA expression for several lipogenic enzymes compared to rats consuming a diet rich in corn oil. Corn oil, which is rich in polyunsaturated fatty acids, suppressed lipogenic enzyme expression while concomitantly increasing leptin expression.⁷⁹

Using hepatocytes and adipocytes from Wistar fatty rats, Fukuda et al.⁸² investigated the transcriptional regulation of the FAS gene by insulin/glucose, PUFAs, and leptin, and compared them to lean controls. The region of -57/-35 of the FAS gene, which has previously been identified as insulin responsive, was linked to the CAT-reporter gene containing a heterologous promoter and transfected into these cells.^{28,82} In the presence of insulin, there was marked stimulation of reporter gene activity in hepatocytes from lean rats, but there was not a significant increase in hepatocytes from obese rats. Stimulation of the reporter gene by insulin was reduced in leptin-treated cells and in cells from lean rats containing an expression vector encoding leptin. However, leptin-treated cells from obese rats did not respond to insulin.

These effects were mediated by leptin-dependent reductions in the insulin-binding capacities of the hepatic and adipose tissue receptors.⁸²

1.13.2 Agouti

Agouti is a paracrine factor normally secreted within hair follicles during the neonatal hair growth period. However, this hormone is also expressed in human adipose tissue,⁸³ suggesting its possible involvement in lipid and energy metabolism. The mechanism of the action of this hormone in both coat color and weight regulation is discussed in detail by Zemel et al. in Chapter 10 of this book. We will discuss only the transcriptional effects of agouti on the FAS gene.

We have demonstrated that agouti acts on adipocytes to increase lipogenesis via a calcium-dependent mechanism.⁸⁴ Obese viable yellow mice which over-express the agouti protein have significantly elevated levels of intracellular calcium and FAS gene expression compared to normal mice.⁸⁵ Furthermore, results from these studies determined that treatment of these animals with the calcium channel blocker, nifedipine, resulted in a dramatic increase in adipose FAS activity. This effect may be due, in part, to reduced plasma insulin levels in the nifedipine-treated viable yellow mice.⁸⁵ To test the possibility that increased FAS activity in adipose tissue of obese viable yellow mice was due to the direct effects of agouti, Jones et al. treated 3T3-L1 adipocytes *in vitro* with recombinant agouti protein.⁸⁶ Agouti treatment resulted in a 1.5-fold increase in FAS mRNA levels. In addition, FAS activity and triglyceride content were 3-fold higher in agouti-treated 3T3-L1 cells relative to controls. These effects were attenuated by simultaneous treatment with nifedipine. Together these data demonstrate that the agouti protein can directly increase lipogenesis in adipocytes via a calcium dependent mechanism.⁸⁶ Furthermore, we have identified an agouti-responsive region at the –435/–415 in the FAS promoter which is upstream of the previously identified insulin-responsive E-box.⁸⁷

1.14 Regulation of the FAS Gene by Other Factors

Like glucagons and cAMP, growth hormone (GH) decreases FAS mRNA abundance by decreasing both gene transcription and mRNA stability.^{88,89} GH-mediated inhibition of lipogenesis in adipocytes of growing pigs was due to decreased insulin sensitivity.⁹⁰ However, GH did not alter early events in the insulin signaling cascade, such as receptor binding and receptor kinase activation, suggesting that GH alters insulin signaling downstream of receptor activation.⁹⁰

Other nutrients, including dietary protein and minerals, regulate FAS gene expression. Hepatic FAS mRNA abundance in Wistar fatty rats was

significantly lower after feeding soybean protein compared to feeding casein.⁹¹ The regulation of FAS by essential amino acids was also reported in HepG2 cells.⁹² A deficiency of dietary copper is accompanied by a 2-fold increase in hepatic fatty-acid biosynthesis.⁹³ Dietary copper deficiency was demonstrated to increase hepatic FAS activity associated with a reduction in gene transcription. FAS gene transcription was suggested to be dependent on the hepatic thiol redox state.⁹³ This mechanism may mediate the effects of dietary copper on FAS activity and gene expression.

1.15 Conclusions and Implications

Despite the reduction in dietary fat consumption through massive promotion of low-fat foods, obesity has not ceased to increase.⁹⁴ This suggests that carbohydrates may be contributing to fat accumulation in humans. This is, in part, addressed by increased interest in the role of glycemic index in energy metabolism, obesity, and diabetes.

In a typical American diet (relatively high in fat), fatty acids would be derived primarily from lipoprotein triglycerides via the action of the lipoprotein lipase (LPL). As discussed in this chapter, although lipogenesis in humans has been considered as very limited for a long time, available studies clearly demonstrate that fatty acid biosynthesis is important enough in humans to be studied for its relevance to disorders of lipid metabolism. Limited data are available concerning regulation of genes of fatty acid biosynthesis in humans. Studies of the highly regulated fatty acid synthase gene as a marker in this pathway provide useful information on how lipogenesis may be regulated in humans. Inhibition of this enzyme has been shown to be beneficial for reductions of body weight⁹⁵ as well as tumorigenesis⁹⁶ (an aspect that we did not discuss in this chapter).

Thus, studies of this gene and other genes in fatty acid synthesis may bring additional insights into human diseases linked to abnormal fatty acid synthesis (such as cirrhosis, LPL deficiency).

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2

Nutrition and Adipocyte Gene Expression

Ron F. Morrison and Stephen R. Farmer

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2.1 Introduction

Excess adipose tissue, a condition commonly referred to as obesity, is associated with an increased risk of developing diabetes, hypertension, hyperin-

sulinemia, and cardiovascular disease.¹ Until recently, the adipocyte was viewed as a passive player in the development of obesity in much the way an oil can stores its contents. New discoveries implicating a more active role for the adipocyte in the regulation of energy homeostasis and body composition as well as other non-metabolic processes have given new incentives toward understanding the complexities of adipocyte differentiation. Current knowledge of this process includes a cascade of transcriptional events involving diverse families of transcription factors that cooperate to regulate, directly or indirectly, the gene expression necessary for the development and function of the mature adipocyte. Hormonal and nutritional signaling that impinges on these *trans*-acting factors provides a molecular link between extracellular mediators and adipocyte gene expression important for glucose and lipid homeostasis.

The contents of this chapter present three important aspects concerning nutrition and adipocyte gene expression. First, functions of the adipocyte are briefly discussed with the emphasis on defining this cell as a central mediator of lipid and glucose homeostasis as well as endocrine functions that are conveyed by its secretory products. Second, major transcription factor families considered to mediate adipocyte gene expression are presented in the context of a cascade of transcriptional events regulating the process of adipogenesis. Third, functional characteristics of these *trans*-acting factors are discussed in detail regarding mechanisms through which their transcriptional activities are modulated, as well as specific modes through which they function, singularly or cooperatively, to regulate adipocyte gene expression. Furthermore, current knowledge of the mechanisms through which dietary constituents and hormones mediate transcriptional activity and adipocyte gene expression will be discussed.

2.2 Pleiotropic Functions of the Adipocyte

As illustrated in Fig. 2.1, adipocyte functions can be generally grouped into three categories with potentially overlapping modalities. The classic role of this cell in lipid metabolism involves storage of energy in the form of triglycerides during times of plenty and its release as free fatty acids for vitally important processes such as myocardial contractions during times of need. The adipocyte is also central to glucose metabolism through the secretion of glycerol and fatty acids that play an important role in hepatic and peripheral glucose homeostasis. Furthermore, adipose tissue along with heart and skeletal muscle are the only tissues known to express and regulate the insulin-dependent glucose transporter, Glut4, that facilitates the entry of glucose into these cells and out of circulation postprandially. Emerging data suggest that the adipocyte also plays an important role in numerous processes through its secretory products and endocrine functions. In this regard, leptin has a wide

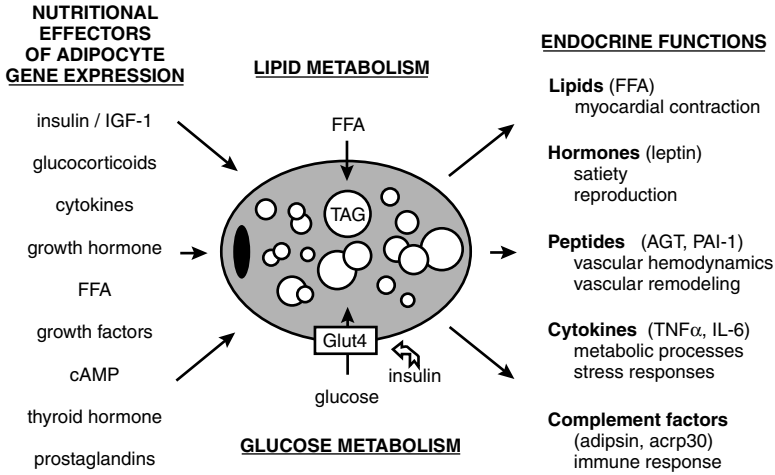


FIGURE 2.1

Pleiotropic functions of the adipocyte. Adipocytes play major roles in lipid metabolism in the storage of free fatty acid (FFA) as triacylglycerol (TAG) and in glucose metabolism through expression of the insulin-dependent glucose transporter, Glut4. Endocrine functions include the secretion of angiotensinogen (AGT), plasminogen activator inhibitor type 1 (PAI-1), tumor necrosis factor α (TNF α), interleukin-6 (IL-6), adipsin and adipocyte complement related protein (acrp30). Various nutritional effectors of adipocyte gene expression and potential functions associated with secretory products from the adipocyte are illustrated.

spectrum of biological activities including a hormonal role in mediating satiety and possibly other effects on fertility, reproduction, and hematopoiesis. In addition to this hormone, adipose tissue secretes a variety of peptides, cytokines, and complement factors in which their various functions are linked inseparably to the adipocyte as a source for their production.²

While the adipocyte is vitally important to energy homeostasis, adipose tissue may also play a central role in many of the pathologies associated with obesity and its related disorders. Genetic mutations that alter the release of leptin from the adipocyte or suppress its interaction with receptors in the hypothalamus are well-known causes of obesity in mice. Cytokines and lipids released from adipose tissue may lead to a decrease in glucose utilization in skeletal muscle and enhance glucose production by the liver, both of which contribute to high levels of glucose in the peripheral circulation, a hallmark of non-insulin-dependent diabetes mellitus. Furthermore, cytokines from the adipocyte may play a role in activating various inflammatory responses that are considered important mediators of cardiovascular disease. In addition, the development of atherosclerotic lesions is likely to be compounded by hyperlipidemia contributed to by the release of fatty acids from fat-laden adipocytes.

Numerous hormones, cytokines, growth factors, and synthetic compounds have been investigated for their potential to modulate adipogenesis.² Regulation of adipocyte differentiation or adipocyte gene expression by any extracellular effector is likely to play a role in regulating any of the diverse

adipocyte functions discussed above. A greater appreciation for the complexities of adipogenesis as well as newly ascribed adipocyte functions will lead to a greater understanding of the molecular mechanisms whereby nutrition may play an active role in lipid and glucose homeostasis through the regulation of adipocyte gene expression.

2.3 Cascade of Transcriptional Events Mediating Adipogenesis

Acquisition of function that develops during adipogenesis is estimated to involve both positive and negative changes in the expression of a great number of functional proteins.³ While post-transcriptional regulation has been demonstrated, many of these changes occur at the gene expression level through a series of molecular events involving several transcription factor families that exhibit diverse modes of activation and function. The more prominent *trans*-acting factors currently considered to play a regulatory role in the process of adipogenesis^{2–7} include peroxisome proliferator-activated receptor gamma (PPAR γ) and members of the CCAAT/enhancer-binding proteins (C/EBPs), particularly, C/EBP α . Also included are members of the signal transducers and activators of transcription (STATs) and adipocyte determination and differentiation factor-1 (ADD-1), also known as sterol regulatory element binding protein-1c (SREBP-1c). Lesser understood roles for other transcription factors in mediating adipocyte gene expression have recently been reported⁸ or reviewed elsewhere.²

Much of our knowledge concerning the sequence of transcriptional events mediating adipogenesis has evolved from cultured cell lines (e.g., 3T3-L1, 3T3-F442A) that differentiate from determined, fibroblastic-like cells into functionally mature adipocytes resembling those found in white adipose tissue, *in vivo*. A schematic of transcriptional events known to occur during differentiation of 3T3-L1 preadipocytes is illustrated in Fig. 2.2. Differentiation is initiated following exposure of post-confluent preadipocytes to a cocktail of mitogen and hormonal agents including fetal bovine serum, insulin (at concentrations known to activate the IGF-1 receptor), dexamethasone (a synthetic glucocorticoid), and methylisobutylxanthine (an agent that increases cAMP levels). Among the earliest transcriptional events following exposure to these inducers is the dramatic, but transient elevation in C/EBP β and C/EBP δ gene expression.⁹ While ectopic expression of these C/EBPs in non-progenitor fibroblasts results in significant adipocyte conversion,^{10–12} their early and transient expression in lieu of the delayed and sustained expression of most adipocyte genes led to the hypothesis that these C/EBPs play a role in regulating the expression of other transcription factors that initiate and/or maintain the gene expression constituting the adipocyte phenotype. Considerable evidence now

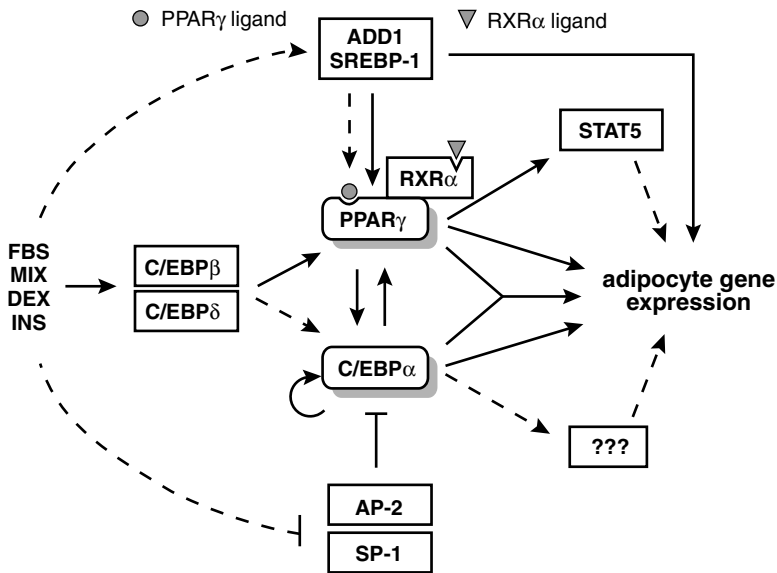


FIGURE 2.2

Cascade of transcriptional events mediating adipocyte differentiation. Activation of adipocyte differentiation culminates in the expression of PPAR γ and C/EBP α that regulate, directly or indirectly, much of the gene expression necessary for the functionally mature adipocyte. Broken lines represent transcription factor interactions or pathways that are poorly understood.

suggests that these trans-acting factors contribute to the process of adipogenesis, at least in part, by transactivating the expression of PPAR γ ^{10,13} and possibly C/EBP α .¹⁴ The kinetics of expression, gain- and loss-of-function studies, and identification of functional consensus sequences in the promoters of many adipocyte genes position PPAR γ and C/EBP α as central regulators of adipogenesis (discussed below). Evidence suggests that the expression of PPAR γ and C/EBP α is continuously maintained despite the ensuing decay of C/EBP β and C/EBP δ , primarily through cross-regulation.¹⁵ While data suggest that C/EBP α has the capacity to autoregulate its own expression,^{14,16,17} a recent study indicates that this is probably not the case for PPAR γ , as ectopic PPAR γ expression was not capable of activating endogenous gene expression in C/EBP α deficient fibroblasts.¹⁸ Once expressed, PPAR γ and C/EBP α transactivate much of the adipocyte gene program as a function of either *trans*-acting factor alone or through the cooperative efforts of both (discussed below).

Additional early events are known to be important for the activation of PPAR γ and C/EBP α gene expression. For example, exposure of preadipocytes to mitogens and hormonal agents that induce differentiation also leads to an early upregulation of ADD-1/SREBP-1c gene expression.¹⁹ It has been postulated that this SREBP family member plays a role in upregulating PPAR γ gene expression.²⁰ Moreover, evidence suggests that

ADD-1/SREBP-1c may be involved in gene expression that leads to the production of endogenous PPAR γ ligands needed for transcriptional activity.²¹ Other studies have demonstrated that undifferentiated preadipocytes express a number of inhibitory proteins that must be repressed or functionally inactivated for the process of differentiation to proceed. For example, exposure of preadipocytes to the differentiation agents leads to repression of AP-2 α and Sp1 transcriptional activity, events that are necessary for C/EBP α gene expression.^{22–24} Inhibitory molecules may function to maintain the preadipocyte phenotype until hormonal and nutritional conditions are supportive for adipocyte differentiation. Similar paradigms are now considered dogma in cell proliferation pathways where numerous proteins serve checkpoint functions regulating commitment to cell cycle progression.

Although PPAR γ and C/EBP α play a regulatory role in the development of the mature adipocyte, only a limited number of the exhaustive list of genes encoding for proteins mediating adipocyte function are known to contain active consensus sequences for either PPAR γ or C/EBP α . It is conceivable that these potent adipogenic transcription factors modulate the expression of other genes, indirectly, through the activation of intermediary *trans*-acting factors. In this regard, recent evidence indicates that the differentiation-dependent induction of STAT1, STAT5A, and STAT5B, are regulated downstream of PPAR γ in the differentiation paradigm.²⁵ Although upregulated in a differentiation-dependent fashion, the precise function of STATs in modulating adipogenesis has yet to be determined. While future studies will undoubtedly identify other unknown transcription factors downstream of PPAR γ and C/EBP α , activation of STAT expression represents the only known regulation of *trans*-acting factors by either of these adipogenic mediators.

2.4 Nutritional Regulation of Adipocyte Gene Expression

Nutrition has long been considered to play an active role in signaling metabolic tissues about the need for energy storage vs. energy expenditure. Until recently, the molecular mechanisms whereby these signals may regulate adipocyte gene expression to ensure energy homeostasis have been poorly understood. Even less has been known concerning the role of nutrition in regulating gene expression which leads to alterations in energy balance that culminate in pathological states such as diabetes, hyperlipidemia, and obesity. The following discussion will address specific characteristics regarding transcription factor families considered important mediators of adipocyte gene expression. Our understanding of the mechanisms whereby lipid-related compounds along with the hormone insulin may mediate the activity of these *trans*-acting factors *in vitro* and *in vivo* will be included.

2.4.1 Peroxisome Proliferator-Activated Receptors

2.4.1.1 General Characteristics

The peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear hormone receptors whose transcriptional activities depend on binding of specific ligands to the receptors and heterodimerization with other members of this superfamily prior to DNA binding and transactivation. The three known PPAR family members, PPAR α , PPAR γ , and PPAR δ , bind similar peroxisome proliferator response elements (PPREs), but exhibit different transactivating functions that are mediated, in part, by tissue distribution, ligand specificity, and coactivator recruitment.⁷ PPAR γ presents a more restricted pattern of expression than PPAR α and PPAR δ .²⁶ This receptor has two isoforms, $\gamma 1$ and $\gamma 2$,^{27,28} which are derived from the same gene by alternative promoter usage and splicing.^{29,30} Both isoforms are expressed predominantly in adipocytes of brown and white adipose tissue. In mice and humans, PPAR $\gamma 1$ and PPAR $\gamma 2$ are also expressed at lower levels in skeletal muscle to an extent that is not likely due to adipose contamination.^{31,32} The PPAR $\gamma 1$ isoform is also expressed in a variety of other cell types including macrophages³³ and epithelial cells of the breast, bladder, and colon.^{34,35} Binding of either PPAR γ isoform to the promoter of a target gene is dependent upon association with its obligate heterodimeric partner, retinoic X receptor alpha (RXR α).³⁶

Numerous studies have demonstrated an upstream, regulatory role for PPAR γ in mediating the process of adipogenesis. The expression of PPAR γ kinetically precedes the onset of most adipocyte gene expression and characteristic lipid accumulation.¹² Functional PPREs have been identified in the promoters of several adipocyte genes including the fatty acid binding protein, aP2, lipoprotein lipase (LPL), and phosphoenolpyruvate carboxykinase (PEPCK).⁷ Ectopic expression of PPAR γ in non-progenitor fibroblasts²⁹ and myoblasts³⁷ in combination with adipogenic inducers and synthetic ligands or activators for PPAR γ has been shown to result in nearly complete morphological and biochemical adipocyte differentiation. Synthetic ligands specific for PPAR γ also have been shown to enhance the hormonally induced differentiation of 3T3-L1³⁸ and 3T3-F442A³⁹ preadipocytes.

Several loss-of-function studies also support a regulatory role for PPAR γ during adipogenesis. For example, expression of a dominant negative mutant of PPAR γ in primary human preadipocytes has been shown to block differentiation mediated by a standard induction cocktail that included a synthetic ligand for PPAR γ .⁴⁰ Synthetic compounds that bind to the receptor and exhibit little⁴¹ or no⁴² PPAR γ agonist activity *in vitro* are known to block the ability of adipogenic cell lines to undergo hormone-mediated cell differentiation in the absence of exogenous ligands. Gene ablation studies have been complicated by early embryonic lethality due to placental defects. Supplementing PPAR γ null embryos with wild-type placentas via aggregation with tetraploid embryos produced one mouse that survived to term before dying of other complications. This study reported that brown adipose tissue

was absent in the neonate, but the contribution of PPAR γ to white adipose tissue could not be determined because death occurred before the normal development of this tissue.⁴³ Heterozygous PPAR γ -deficient mice, generated by the same technique, survived birth and were described as having smaller adipocytes and a decreased fat mass.⁴⁴ Another means of circumventing the placental defect was approached by generating chimeric mice from wild-type and PPAR γ null cells. In this study, little or no contribution of null cells to adipose tissue was observed.⁴⁵ The requirement of PPAR γ for adipose tissue development is further supported by the reduced capacity of embryonic stem cells⁴⁵ or mouse embryo fibroblasts⁴⁴ to differentiate *in vitro* under strong adipogenic conditions. Collectively, the results from gain- and loss-of-function studies provided strong evidence supporting an obligatory role for PPAR γ in adipocyte differentiation, *in vitro* and *in vivo*.

2.4.1.2 Natural and Synthetic Ligands

PPARs, originally classified as orphan receptors without identified ligands, are now considered to be receptors activated and bound by a broad range of both natural and synthetic molecules (Fig. 2.3). Compounds that are able to

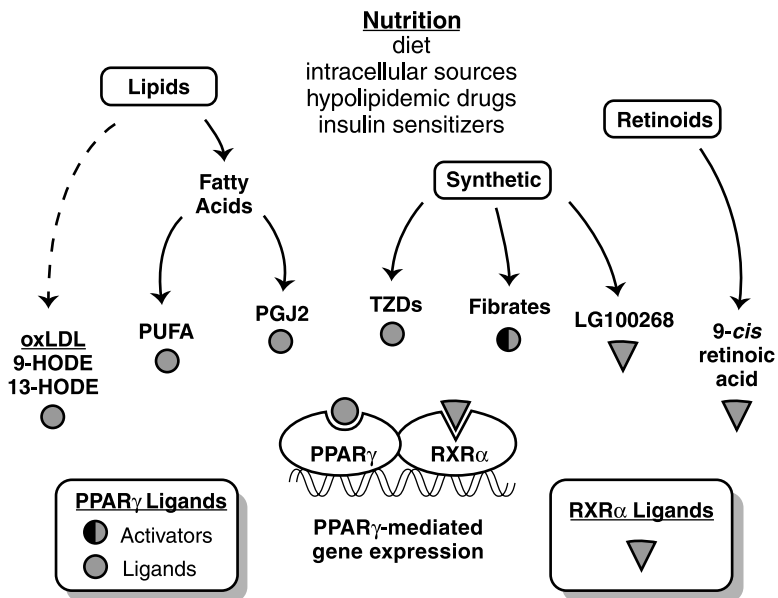


FIGURE 2.3

Natural and synthetic ligands modulating PPAR γ -RXR α transcriptional activity. Lipid-related molecules, such as metabolites of oxidized low-density lipoproteins (oxLDL), polyunsaturated fatty acids (PUFA), and prostaglandin J2 (PGJ2) are natural ligands for PPAR γ . Synthetic ligands or activators of PPAR γ include various insulin-sensitizing and hypolipidemic drugs such as thiazolidinediones (TZDs) and fibrates. The natural ligand for RXR α , 9-*cis*-retinoic acid, is derived from retinoids and vitamin A. Unknown involvement of specific ligands in adipocyte gene expression are represented by broken lines.

induce PPAR-mediated transactivation, but not yet proven to directly bind to the receptor are called activators. Ligands, on the other hand, are molecules that interact with the ligand-binding domain of the receptor and modulate transcriptional activity. Prior to the discovery of these receptors, a variety of diverse agents such as fibrate hypolipidemic drugs, phthalate ester plasticizers, and herbicides were observed to cause a massive proliferation in peroxisomes in rodent hepatocytes.⁴⁶ The observation that nutrients, such as high-fat diets, could also induce peroxisome proliferation, coupled with the knowledge that most of the major pathways of lipid metabolism are mediated by PPAR-regulated genes led to the discovery that various fatty acids and their metabolites function as natural ligands for PPARs. Several long-chain, polyunsaturated fatty acids (PUFA) bind to PPAR γ *in vitro* and can stimulate lipid lowering and enhanced insulin sensitivity effects similar to those reported for synthetic PPAR ligands.⁴⁷ Fibrates,^{48,49} fatty acids,^{29,49,50} and eicosanoids (i.e., arachidonic acid metabolites)^{51,52} can induce differentiation of cultured preadipocytes presumably through enhancing PPAR γ activity, suggesting that PUFAs and various prostaglandins, especially 15-deoxy prostaglandin J2 (PGJ2),^{53–55} function as natural PPAR γ ligands in the adipocyte. The uptake of oxidized low-density lipoproteins (oxLDL) through scavenger receptors has been shown to enhance the expression of PPAR γ in macrophages known as foam cells residing within atherosclerotic lesions of the arterial wall. In these cells, lipid components of oxLDL, 9-hydroxyoctadecadienoic acid (9-HODE), and 13-HODE also function as endogenous ligands of PPAR γ .^{56,57} While it is unknown if these molecules serve any role in the adipocyte, these data support the notion that lipid-related compounds are most likely the natural ligands for PPAR γ . The fatty acids and lipid metabolites that have thus far been characterized as natural ligands have weaker binding kinetics than ligands for other nuclear hormone receptors with Kd values in the micromolar vs. nanomolar range. While these kinetics have generated some concern as to the physiological role of these ligands, it should be noted that the level of plasma fatty acids is sufficiently high for PPAR γ activation. With this notion in mind, it has been postulated that it may not be a requirement for lipids, whose concentrations are naturally high and higher still in lipid overload, to bind PPAR γ with high affinity.⁵⁸

A synthetic class of compounds known as thiazolidinediones (TZDs) selectively bind PPAR γ with high affinity (nanomolar range) and potentially enhance transcriptional activity.⁵⁹ Interestingly, these compounds were developed and used as lipid-lowering and insulin-sensitizing agents before the discovery that they are also potent ligands for PPAR γ . As antidiabetic agents, TZDs appear to work by either mimicking or enhancing insulin action without stimulating β -cell insulin secretion⁶⁰ and have been demonstrated as very effective in improving insulin sensitivity and glucose tolerance in diabetic patients.^{61–63} Studies using various animal models of obesity and diabetes have also reported that these synthetic compounds are effective in lowering hyperglycemia and hyperinsulinemia and improving insulin sensitivity.^{64–66} The notion that TZDs produce these effects through

modulating PPAR γ activity is strongly supported by the observed close correlation between the relative binding kinetics of TZDs to PPAR γ and their antidiabetic action *in vivo*.^{54,59} It is interesting to note that synthetic ligands (e.g., LG100268) for RXR- α , obligatory heterodimeric partner PPAR γ , enhance the insulin-sensitizing effects of TZDs when administered to obese, insulin-resistant mice.^{67,68} In fact, the combination of PPAR γ and RXR α ligands appears to induce a greater transcriptional activation than either ligand alone,⁶⁹ suggesting that a combination of synthetic ligands may be more efficacious in the treatment of hyperlipidemia and insulin resistance. The physiological role of the natural ligand for RXR α , 9-*cis*-retinoic acid, derived from retinoids and vitamin A, in mediating adipocyte gene expression has not been investigated extensively.

2.4.1.3 Other Nutritional Considerations

It is not surprising that PPAR γ , which plays a significant role in mediating adipogenesis, is regulated during altered states of nutrition and obesity. Elevated levels of PPAR γ 2 mRNA in adipose tissue of human obese patients have been reported where there was a strong positive correlation between the ratio of PPAR γ 2/ γ 1 and body mass index.³² Restricting caloric intake for 10% weight loss in these patients resulted in a 25% decrease in PPAR γ 2 mRNA that reverted to pretreatment levels after 4 weeks of weight maintenance.³² Similar nutritional effects were observed in mice where high-fat diets or fasting were shown to produce a 50% increase or 80% decrease in adipose PPAR γ 2 mRNA levels, respectively.³¹ These nutritionally linked changes in mRNA levels suggest the possibility that PPAR γ gene expression may be regulated, directly or indirectly, by hormones such as insulin. This notion is supported by the observation that PPAR γ mRNA levels are suppressed in insulin-dependent diabetic mice and can be partially restored with insulin treatment.³¹ While cause and effect have not been established, it could be hypothesized that hormonally-mediated changes in gene expression in conjunction with nutrient-mediated changes in transcriptional activity position PPAR γ as an important molecular link between changes in diet and the effect on gene expression modulating energy homeostasis.

2.4.2 CCAAT/Enhancer-Binding Proteins

2.4.2.1 General Characteristics

The CCAAT/enhancer-binding proteins (C/EBPs) belong to a large family of transcription factors containing a leucine zipper motif that functions in forming homo- or hetero-dimeric complexes with other C/EBP family members. Three of these family members, C/EBP α , C/EBP β , and C/EBP δ , are expressed in both brown and white adipocytes as well as a variety of other cell types and have been implicated as major role players in regulating the gene expression necessary for adipocyte differentiation.^{4,70–72}

C/EBP α is expressed immediately prior to the transcription of a number of adipocyte-specific genes. Analyses of the upstream regulatory regions of aP2, stearoyl CoA desaturase 1 (SCD1), Glut4, PEPCK, leptin, and the insulin receptor have revealed functional C/EBP consensus sequences in the proximal promoters.² Ectopic expression of C/EBP α in 3T3-L1 preadipocytes has been shown to be sufficient for inducing adipogenesis without the use of external hormonal inducers.⁷³ Furthermore, ectopic expression of C/EBP α or C/EBP β has resulted in potent induction of adipogenesis in non-progenitor fibroblasts under adipogenic conditions.^{10,11,74} The observation that antisense expression of C/EBP α inhibits differentiation of cultured preadipocytes provides further evidence supporting a regulatory role for C/EBPs during adipogenesis.¹⁶ Mice targeted for C/EBP α gene ablation die within 8 h postpartum due, in part, to hypoglycemia since administration of glucose can rescue these animals for up to 40 h.⁷⁵ Within this time frame, lipid droplets appeared in white and brown adipose tissue of control animals. In contrast, C/EBP α deficient mice are suppressed in their ability to develop brown adipocytes and completely devoid of characteristic white adipose tissue. Gene ablation studies that target both C/EBP β and C/EBP δ also demonstrate a reduced propensity for adipogenesis, with deficient animals developing markedly less adipose tissue compared to wild-type littermates.⁷⁶ Collectively, these data demonstrate a prominent role for C/EBP family members during the development of adipocyte differentiation, *in vitro* and *in vivo*.

2.4.2.2 Translational and Post-Translational Modifications

Translational and post-translational modifications are thought to play a regulatory role in C/EBP transcriptional activity. Both C/EBP α and C/EBP β genes give rise to multiple protein isoforms through the regulated use of alternate translational start sites.¹⁷ The first and third in-frame AUG of C/EBP α yields two proteins of 42 kDa and 30 kDa, where the former is more efficient at inducing adipogenesis and the post-mitotic growth arrest associated with terminal differentiation.^{17,77} Changes in the ratio of C/EBP α isoforms during the course of differentiation has led to the notion that regulated alternate translational products may have a role in regulating C/EBP function.¹⁷ Similar changes have been observed for alternate translational products of C/EBP β proteins, 32 kDa and 20 kDa, referred to as liver activator protein (LAP) and liver inhibitor protein (LIP), respectively. In this case, the truncated 20-kDa isoform lacks a transactivation domain and functions as a dominant negative when heterodimerized with other C/EBPs.⁷⁸ Post-translational modification may also be an important mode of regulating C/EBP functional activity where C/EBP α protein is phosphorylated on as many as six amino acid residues in fully differentiated adipocytes.⁷⁹ Signaling pathways that lead to selective dephosphorylation of specific sites may serve an important function in regulating adipocyte gene expression (discussed below).

2.4.2.3 Cooperative functions of C/EBP α

Although PPAR γ and C/EBP α are considered the most prominent adipogenic transcription factors, much evidence now suggests that complete adipocyte differentiation requires cooperation of their transcriptional activities. While many adipocyte genes may be regulated by one or the other of these transcription factors, the promoters of aP2 and PEPCK contain functional binding sites for both PPAR γ and C/EBP α . One approach to understanding the unique contribution between these *trans*-acting factors is to ectopically express one under conditions not permissive for the other and examine the effect on adipogenesis or adipocyte function. For example, differentiation of 3T3-L1 preadipocytes by the standard induction cocktail does not require supplement of exogenous PPAR γ ligands for complete differentiation. In contrast, differentiation of C/EBP α -defective NIH-3T3 fibroblasts ectopically expressing PPAR γ is dependent upon exogenous ligands unless C/EBP α and PPAR γ are co-expressed.⁸⁰ These data indicate that the state of PPAR γ ligand independence is mediated, directly or indirectly, by C/EBP α . Cooperation is also noted in studies where PPAR γ was ectopically expressed in C/EBP α knockout mouse embryo fibroblasts¹⁸ or in NIH-3T3 fibroblasts that are defective for C/EBP α expression.⁸¹ Under potent adipogenic conditions including PPAR γ ligand supplement, fibroblasts in either case formed characteristic lipid droplets and expressed many genes associated with adipocyte differentiation. However, these fibroblasts were insulin resistant regarding glucose uptake. Rescue of this defect with co-expression of C/EBP α clearly demonstrates synergy among these adipogenic transcription factors within a program of events involving many proteins necessary for the complex process of insulin sensitivity. Similar studies in PPAR γ -deficient fibroblasts ectopically expressing C/EBP α will provide additional information regarding the cooperation and unique functions of these adipogenic transcription factors once cells become available.

2.4.2.4 Nutritional Considerations

Numerous studies have demonstrated a positive correlation between Glut4 and C/EBP α gene expression, suggesting that acquisition of insulin sensitivity in the adipocyte is tightly coupled to the function of this transcription factor. Adipose tissue Glut4 gene expression increases and decreases during states of high-fat feeding and fasting, respectively,⁸² suggesting that both gene expression and transporter localization to the membrane may be under hormonal control. While the effects of fasting and refeeding on C/EBP α gene expression *in vivo* have not been reported, it could be hypothesized that a coordinate change in C/EBP α would occur under similar nutritional conditions. It has been shown that C/EBP α mRNA is overexpressed in adipose tissue of obese Zucker rats, suggesting a possible role for this *trans*-acting factor in regulating lipid-related gene expression associated with obesity.⁸³ Paradoxically, insulin has been shown to cause a coordinate decrease in both Glut4 gene expression and C/EBP α activity when exposed to fully differen-

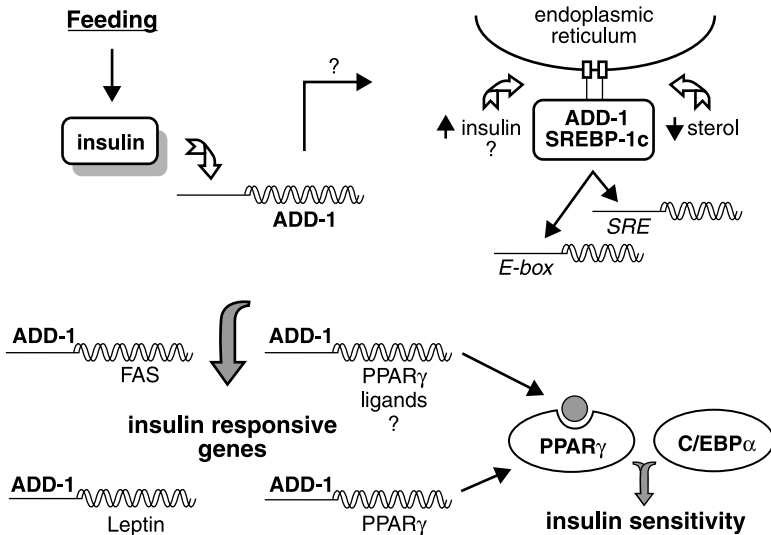
tiated 3T3-L1 adipocytes in culture. The kinetics of repression and the functional C/EBP-binding site in the Glut4 promoter lead to the possibility that the effects of insulin on Glut4 gene expression in culture are mediated by coordinate changes in C/EBP α activity. It has been reported that the mechanism by which insulin induces this repression on C/EBP α transcriptional activity may involve at least three mechanisms: suppression of C/EBP α gene expression, rapid dephosphorylation of C/EBP α protein, and inactivation of C/EBP α through induction of the dominant negative form of C/EBP β (i.e., LIP).^{79,84,85} Additional evidence suggests that insulin stimulates the dephosphorylation of C/EBP α through inactivation of glycogen synthase kinase 3.⁸⁶ While these results are unlikely to account for the positive effects of insulin on transporting glucose into the adipocyte postprandially, they may provide a transcriptional mechanism coupling the disease states of hyperinsulinemia and insulin resistance. Further studies *in vitro* and *in vivo* are required to ascertain the physiological effects of nutrition on C/EBP α -mediated gene expression.

2.4.3 Adipocyte Determination and Differentiation Dependent Factor-1

2.4.3.1 General Characteristics

Sterol regulatory element binding proteins (SREBPs) are known to modulate transcription of numerous genes encoding proteins that function in both cholesterol and fatty acid metabolism.⁸⁷ The SREBP family consists of three proteins, designated SREBP-1a, -1c, and -2, that are encoded by two independent genes. In humans and mice, SREBP-1a and SREBP-1c are produced from a single gene using alternate transcription start sites. Adipocyte determination and differentiation dependent factor-1 (ADD-1), cloned independently from a rat adipocyte cDNA library,⁸⁸ is homologous to human SREBP-1c. SREBPs contain two transmembrane domains that anchor the protein to the endoplasmic reticulum in an inactive state (Fig. 2.4). When sterol levels are low, two proteolytic events result in the cleavage and release of the cytoplasmic N-terminal fragment that translocates to the nucleus, binds to the promoters of target genes, and regulates transcription.^{89,90} The functional fragment of SREBPs (i.e., amino terminus) contains a basic-helix-loop-helix leucine zipper domain that exhibits dual specificity for classic E-box motifs as well as non-E-box sterol regulatory elements (SREs). While all three SREBPs are capable of activating similar gene expression, evidence suggests that regulation of gene expression important in fat metabolism is primarily mediated by SREBP-1a and ADD-1/SREBP-1c. SREBP-2 is more associated with cholesterol metabolism.⁹¹ Adipose tissue, *in vivo*, predominantly expresses ADD-1/SREBP-1c over other forms of SREBPs.

A role for ADD-1 in adipogenesis was indicated when ectopic expression of a constitutively active form was shown to enhance adipocyte gene expression in non-progenitor NIH-3T3 fibroblasts under differentiation conditions. In addition, expression of a dominant negative form of this *trans*-acting factor

**FIGURE 2.4**

Potential role of ADD-1 in mediating insulin action in the adipocyte. Insulin activation of cell surface receptors leads to an increase in ADD-1 gene expression through an unidentified mechanism. This protein is initially processed as an inactive molecule that is tethered to the endoplasmic reticulum. Following proteolytic cleavage, the amino terminus of ADD-1 translocates to the nucleus and activates insulin-responsive gene expression. Gene targets for ADD-1 also include PPAR γ and potentially genes mediating the production of endogenous PPAR γ ligand. Gene programs leading to insulin sensitivity with regard to glucose uptake are mediated by the cooperative efforts of both PPAR γ and C/EBP α .

suppressed 3T3-L1 preadipocyte differentiation.⁹² As discussed above, it appears that ADD-1 functions, in part, through cooperation with PPAR γ by increasing its expression²⁰ and by regulating the production of a lipid ligand²¹ that modulates its transcriptional activity (Fig. 2.4). In a parallel fashion to PPAR γ , ADD-1/SREBP-1c also functions directly in the transcription of several genes important for lipid homeostasis, such as fatty acid synthase (FAS) and leptin.⁹³ While ablation of the SREBP-1 gene (mice lacking both SREBP-1a and SREBP-1c) has been reported to have little effect on white adipose tissue mass, redundancy of function (i.e., SREBP-2 expression) has not been ruled out.⁹⁴

2.4.3.2 Nutritional Considerations

New insights into the role of ADD-1 in mediating the hormonal effects of insulin in both adipocytes and hepatocytes have recently been reported.⁹⁵ This anabolic hormone is generally considered the major regulator of energy homeostasis during the feed state. Insulin action at the level of the adipocyte includes regulation of insulin-sensitive processes such as uptake of glucose from the circulation as well as activation of numerous lipogenic genes important for energy storage. Although insulin-responsive elements have been

identified in the promoters of many anabolic genes, the identification of adipogenic transcription factors through which insulin may mediate gene expression has been elusive. Recent reports indicate that the mRNA levels of ADD-1 and two of its transcriptional target genes, FAS and leptin, decrease in adipose tissue when animals are fasted and increase upon refeeding (Fig. 2.4). These nutritional effects can be duplicated in culture through exposure to insulin.⁹³ Interestingly, the insulin-responsive element in the FAS promoter contains an E-box motif that binds ADD-1.^{93,96}

Analogous studies have been reported for cultured hepatocytes where insulin directly stimulates ADD-1 mRNA accumulation.⁹⁷ Furthermore, the expression of a dominant negative form of ADD-1 in hepatocytes blocks the ability of insulin to induce the expression of insulin-responsive genes. In addition, the expression of a dominant positive mutant of ADD-1 stimulates the expression of these insulin-responsive genes in hepatocytes even in the absence of insulin.⁹⁸ As discussed above, hepatic ADD-1 is initially tethered to the endoplasmic reticulum in an inactive form until proteolytic cleavage is induced by a decrease in cellular sterol levels. If ADD-1 is initially synthesized in an inactive form in adipocytes as well as hepatocytes, it will be interesting to determine if elevated insulin levels also mediate the release of ADD-1 in a fashion similar to that induced by low sterol levels (Fig. 2.4). It appears that ADD-1/SREBP-1c is positioned to mediate insulin action in the mature adipocyte directly through regulating insulin-responsive gene expression important for carbohydrate and lipid homeostasis as well as indirectly through the transcriptional activities of PPAR γ and C/EBP α that are essential for the establishment of insulin sensitivity during adipogenesis (Fig. 2.4).

2.4.4 Signal Transducers and Activators of Transcription

2.4.4.1 General Characteristics

Signal transducers and activators of transcription (STATs) comprise a family of cytoplasmic proteins that are activated and mediate gene expression in response to extracellular effectors that target receptors with intrinsic kinase activity or receptors to which Janus kinases (JAKs) are bound.⁹⁹ Ligand-mediated dimerization of the receptor results in phosphorylation of the associated kinase, which subsequently phosphorylates the cytoplasmic tail of the receptor, which then serves as a docking site for STAT recruitment. The receptor-bound STAT is phosphorylated, then dimerizes with other STAT proteins and translocates to the nucleus to mediate specific gene expression. The seven known STAT family members are constitutively expressed in a variety of tissues in an inactive form until receptor-mediated phosphorylation events occur. The expression of three members of this family, STAT1, STAT5A, and STAT5B, is upregulated during differentiation of cultured preadipocytes in a coordinate fashion with the expression of PPAR γ and C/EBP α .^{100,101} While the function of STATs during adipocyte differentiation is still unclear, gene ablation of STAT5A and STAT5B produces animals with markedly less white adi-

pose tissue compared to wild-type littermates, demonstrating a significant role for these proteins during adipogenesis.¹⁰²

2.4.4.2 Nutritional Considerations

Differentiation-dependent expression and loss-of-function studies suggest that the upregulation of specific STAT proteins is likely to be an important regulated process during adipogenesis. It should be emphasized that activation of these inducible STATs (discussed above) and the constitutively expressed STATs (i.e., STAT3 and STAT6) in the adipocyte may also function as a regulatory mechanism in mediating adipocyte gene expression before, during, and after the process of differentiation. The notion that numerous cytokines, growth factors, and hormones are known to regulate STAT activity in other cell types⁹⁹ and that these extracellular effectors have pronounced effects on gene expression in the mature adipocyte^{2,103} suggests that some of these effects may be mediated by one or more STAT proteins. For example, interferon- γ , leukemia inhibitory factor, and oncostatin-M induce significant tyrosine phosphorylation and prominent translocation of STAT3 into the nucleus following acute treatment in mature 3T3-L1 adipocytes.^{104,105} Furthermore, growth hormone stimulates a dramatic activation of STAT5A and STAT5B in fully differentiated 3T3-L1^{104,105} and 3T3-F442A¹⁰⁶ adipocytes. In contrast, interleukin-4 induces STAT6 tyrosine phosphorylation and DNA binding, but only in undifferentiated preadipocytes.¹⁰⁷

A recent study also reports that STAT3 is phosphorylated in subconfluent preadipocytes and during postconfluent mitotic clonal expansion that follows induction of differentiation, suggesting a possible role for STAT3 during proliferative phases of adipogenesis.¹⁰⁸ The *in vivo* effects of fasting and refeeding or obesity on STAT expression or activation in adipose tissue are not currently available. It would not be unexpected, however, to find that various dietary aspects of nutrition also regulate STATs. In differentiated 3T3-L1 adipocytes, acute insulin treatment results in serine phosphorylation of STAT3.^{105,109} The relevance of this post-translational event are yet to be determined since insulin does not appear to affect the expression, activation, and translocation of any STAT protein in cultured adipocytes, including STAT3.^{104,105} The function of highly regulated STAT expression and activation during physiological and pathological states of lipid and glucose homeostasis will become clearer following the completion of studies addressing the effects of nutrition and the identification of gene targets of STATs in the adipocyte.

2.5 Perspectives and General Conclusions

Over the last decade, there has been tremendous development in our understanding of the mechanisms by which nutrients direct changes in gene expres-

sion. In addition to the well-characterized hormonal signaling of the fed state imparted by insulin, it now appears that the diet contains biological molecules (fatty acids, eicosanoids, vitamins) that can activate intracellular receptors in much the same way as steroid hormones. These nuclear hormone receptors provide a direct link between the availability of nutrients and the gene expression mediating fuel storage and utilization. The discovery that nuclear receptors can modulate adipogenesis through a transcriptional mechanism has opened a new field of research in adipocyte biology and is consistent with dietary factors being important modulators of gene expression, *in vivo*. Lipid-related molecules that bind to these receptors can no longer be regarded simply as biological substrates for energy metabolism, but rather as mediators that signal for changes in gene expression important for energy homeostasis. As the link between nutrients and the regulation of gene expression has been poorly understood, these findings represent a breakthrough in the molecular understanding of the relationship between dietary intake and energy homeostasis.

Although our knowledge concerning the transcriptional control of adipocyte gene expression has advanced significantly over the last few years, there are many questions still unanswered. Currently, we position PPAR γ and C/EBP α as the most prominent transcription factors mediating adipogenesis. The direct mechanism whereby these *trans*-acting factors, independently and/or cooperatively, activate gene expression important for adipocyte function and their roles in regulating the expression of intermediary transcription factors will undoubtedly represent a focus of future investigations. The increasing list of adipocyte functions beyond the storage of triglycerides will require an evolving definition of adipocyte differentiation and, consequently, the cascade of transcriptional events mediating adipogenesis. Analysis of the mechanisms whereby hormonal and nutritional signaling impinges on the adipogenic transcription factors will provide an important link between the cellular environment and regulation of gene expression important for glucose and lipid homeostasis.

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3

Regulation of the Stearoyl-CoA Desaturase Genes by Dietary Fat: Role of Polyunsaturated Fatty Acids

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3.1 Introduction

When dietary intake of fat is insufficient for daily requirements, animals derive fatty acids mainly from *de novo* synthesis by the fatty acid synthase complex (FAS) which catalyzes the condensation of acetyl-CoA, and malonyl-CoA producing palmitate (C16:0) as the major end product.¹ Palmitate then serves as a substrate for the microsomal malonyl-CoA dependent elongase to produce stearate (C18:0). Because stearate and, to a lesser extent, palmitate are to be stored,² the end product of this pathway is usually oleate (C18:1 Δ 9) and palmitoleate (C16:1 Δ 9) which are synthesized by the stearoyl-CoA desaturase (SCD) (Fig. 3.1). This enzyme is the terminal component of a multicomponent complex which includes cytochrome b_5 and an NAD(P)H-dependent cytochrome b_5 reductase. In the presence of molecular oxygen this complex catalyzes the desaturation of methylene-interrupted fatty acyl-CoA substrates by the insertion of a double bond between carbons 9 and 10.³

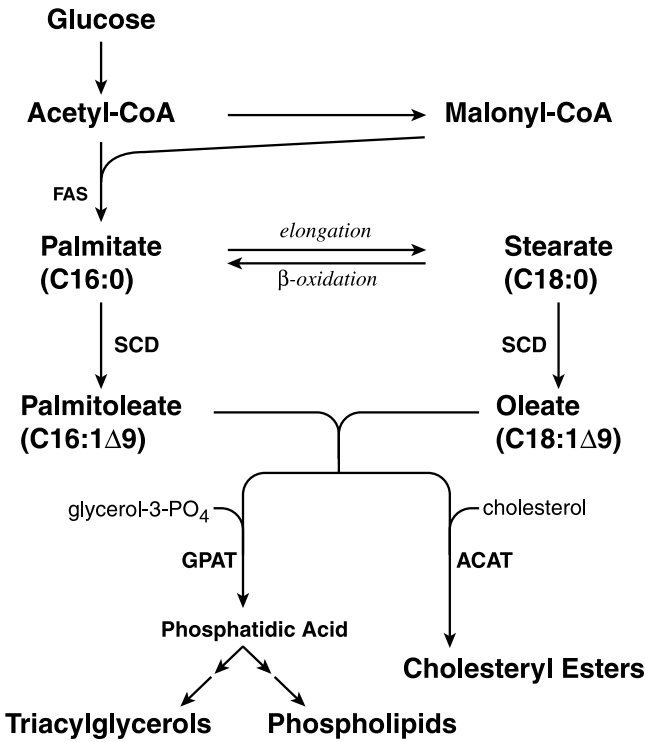


FIGURE 3.1

Pathway for synthesis of palmitoleic and oleic acids from palmitate and stearate and their incorporation into triacylglycerols, phospholipids, and cholesterol esters. The sites for the action of SCD are depicted. SCD: stearoyl-CoA desaturase; FAS: fatty acid synthase; GPAT: glycerol-3-phosphate acyltransferase; ACAT: acyl-CoA:cholesterol acyl transferase; G-3-PO₄: glycerol-3-phosphate

Although several substrates including vaccenic acid⁴ are used by the SCD, the main substrates are palmitoyl- and stearoyl-CoA which are converted to palmitoleoyl- and oleoyl-CoA, respectively.³ The overall rate of the desaturation reaction is limited by the terminal component, the SCD, and it is the SCD that shows changes in expression in response to dietary changes, hormonal imbalance, developmental processes, temperature changes, metals, alcohol, peroxisomal proliferators, and phenolic compounds.^{5–6} Changes in SCD activity in tissues are reflected in the composition of cell membrane phospholipids, cholesterol esters, and triglycerides, and therefore, regulation of SCD is of considerable physiological importance and has the potential to affect a variety of key physiological variables including insulin sensitivity, metabolic rate, adiposity, atherosclerosis, cancer, and obesity.

The mouse and rat genome contain two well-characterized structural genes (SCD1 and SCD2) that are highly homologous at the nucleotide and amino acid level and encode the same functional protein.^{7–9} Both genes are structurally similar with 5 exons and 6 introns with translated region present in every

exon. Recently, a single human SCD gene that is highly homologous to the mouse SCD1 and SCD2 genes was cloned and characterized.¹⁰ Other SCD cDNAs and genes have been isolated from different species including yeast,¹¹ ovine,¹² and hamster,¹³ and their regulation is currently being studied. Despite the fact that both mouse SCD genes are structurally similar, sharing ~87 % nucleotide sequence identity in the coding regions, their 5' flanking regions differ resulting in divergent tissue-specific gene expression. The physiological significance of having two or more mouse or rat SCD isoforms and their tissue distribution is not currently known but could be related to the substrate specificity of each SCD isoform or the means by which cells compartmentalize lipid biosynthesis for specific functions.¹⁴ Many mechanisms may exist for regulating the expression of the SCD genes by nutritional, hormonal, developmental, and environmental factors in normal and disease states. It is the goal of this chapter to discuss the recent advances of the regulation of the mouse SCD genes by dietary fat, emphasizing the role of polyunsaturated fatty acids. Several reviews provide excellent coverage of the role of polyunsaturated fatty acids in the regulation of other genes involved in lipid and carbohydrate metabolism.¹⁵⁻¹⁷

3.2 Regulation of SCD Expression by Polyunsaturated Fatty Acids

The ω -3 and ω -6 polyunsaturated fatty acids (PUFAs) are essential fatty acids that cannot be synthesized by mammals and therefore can be obtained from plant sources. These fatty acids have been shown to reduce the expression of SCD¹⁸ and many enzymes involved in lipid and carbohydrate metabolism.¹⁷ By reducing the expression of these enzymes, it is believed that PUFAs control the *de novo* synthesis of saturated, monounsaturated, and polyunsaturated fatty acids. It would, therefore, appear that in a normal healthy animal receiving adequate amounts of the essential fatty acids, *de novo* fatty acid synthesis would be suppressed. Only when the animal ingests carbohydrate over and above its energy requirements is the repressive effect of PUFAs overcome and the lipogenic enzymes induced. The inhibitory effects of PUFAs on lipogenesis may explain the results of stable isotope experiments which show that *de novo* fatty acid synthesis in humans who consume recommended American diets that are relatively rich in polyunsaturated fatty acids is minor.¹⁹ In support of this observation, when humans are fed diets that are fat free and rich in fructose there is indeed induction of lipogenesis.²⁰

The specific regulation of SCD activity by PUFAs may be central to the overall repression of lipogenesis. The SCD catalyzes the first regulatory step in the formation of long-chain unsaturated fatty acids such as (n-9)-eicosatrienoic acid (20:3 n-9).²¹ Accumulation of 20:3 n-9 is associated with reduction of ω -3 and ω -6 PUFAs of membrane phospholipids²¹ and is the hallmark

of essential fatty acid deficiency in humans.²¹ Therefore, an attractive explanation for the unique suppression of lipogenesis by ω -3 and ω -6 PUFAs is their ability to suppress the formation and accumulation of ω -9 fatty acids by inhibiting SCD gene expression in order to maintain the proper phospholipid membrane composition. If not suppressed, enrichment of membrane phospholipids with ω -9 fatty acids would occur at the expense of the essential fatty acids and would compromise several cellular functions including hormone binding, signal transduction, accelerated heat loss, increased transdermal water loss, and impaired fetal and neonatal growth.¹⁵ In the shorter term, PUFAs would inhibit the expression of the SCD first, thus limiting the net synthesis of palmitoleoyl- and oleoyl-CoA resulting in an accumulation of palmitoyl-CoA and stearoyl-CoA. These saturated fatty acyl-CoAs would then feedback inhibit acetyl-CoA carboxylase and fatty acid synthase thereby inhibiting general fatty acid biosynthesis.

The regulation of SCD by PUFAs may occur at several levels. Some evidence has been provided that some PUFAs, such as sterculic acid and thia fatty acids, directly inhibit the SCD activity.^{22,23} However, studies of liver, lymphocyte, brain, and adipocytes indicate that the effects of PUFAs on SCD activity are mainly at the level of SCD gene expression.^{24–36} Under normal dietary conditions, mouse and rat SCD2 mRNA is expressed constitutively at high levels in the brain, is not expressed in liver, and its expression in kidney, adipose, and lung tissue is slightly increased by shifting mice from a diet containing unsaturated fatty acids to a fat-free diet.⁸ The tissue distribution and expression of the mouse and rat SCD1 mRNA differ markedly from that of SCD2, being constitutive in adipose tissue, and are markedly induced in liver in response to feeding of a high-carbohydrate fat-free diet.^{7–8,24,36} The increase in SCD1 mRNA in response to feeding of a high-carbohydrate fat-free diet was due to both insulin and carbohydrate.³⁶ Studies conducted in mice showed that when the fat-free high-carbohydrate diet was supplemented with various triglycerides containing linoleic (18:2n-6), arachidonic (20:4n-6), and linolenic (18:3n-3) acids, the hepatic SCD1 mRNA expression was repressed whereas triglycerides containing saturated (i.e., 18:0 and 16:0) and monounsaturated fatty acids (16:1n-9 and 18:1n-9) had very little effect.²⁴ Similar results have been obtained with primary hepatocytes and mouse cell lines.^{27,33} Using a diabetic mouse model it was shown that induction of SCD mRNA expression by insulin could be repressed by PUFAs, showing that the mechanism of PUFA repression of the SCD gene is insulin independent.²⁶

The repression of SCD gene expression by PUFAs is not liver specific. PUFAs repress the expression of SCD mRNA in adipose tissue of both lean and obese Zucker rats.³⁰ Interestingly, the SCD mRNA content was much higher in obese rats compared to normal rats both with and without PUFA supplementation.³⁰ In the 3T3-L1 adipocyte cell line, arachidonic acid, linoleic, linolenic, and eicosapentaenoic acid (EPA) decreased SCD1 mRNA in a dose-dependent manner.²⁵ The regulation of SCD gene expression by PUFAs has also been observed in the brain, and cells of the immune

system,^{31–32,37} indicating that PUFA regulation of SCD gene expression is far more widespread than originally thought.

It is not known whether the PUFAs have to be metabolized first in order to repress SCD gene expression. Inhibiting eicosonoid synthesis did not prevent the PUFA suppression of SCD1 gene expression in adipocytes and lymphocytes, suggesting that the oxidative metabolism of arachidonic acid to eicosonoids is not involved in the arachidonic acid-mediated decrease of SCD1 mRNA expression^{28,31} Furthermore, as indicated above, the desaturase gene expression is repressed by a range of PUFAs, some of which do not give rise to eicosonoids, suggesting that repression of SCD mRNA expression is PUFA specific. The basic requirement for a dietary fatty acid to inhibit expression of lipogenic genes had been proposed to contain 18 carbons and possess at least 2 double bonds, one between position 9 and 10 and the other between 12 and 13.^{15–16} However, recently it has been demonstrated that the *trans*-10, *cis*-12 isomer of conjugated linoleic acid (CLA) also represses SCD mRNA expression in liver,³⁵ although this fatty acid does not contain a double bond at position 9. It contains instead double-bonds at positions 6 and 8 from the ω -carbon. The only double bond position that CLA and other PUFAs share is at position 6. The inhibitory effects PUFAs have on the expression of the SCD1 mRNA levels may, therefore, be related to the position and orientation of just one of the double bonds present in these fatty acids. This information could become important in determining the PUFA structure that is required to inhibit gene expression. Many mechanisms may exist for regulating the tissue-specific expression of the SCD genes by PUFAs but recent investigations show that PUFAs repress the SCD gene expression mainly at the level of gene transcription and mRNA stability.^{5,24–25,27–28,32–34,38}

3.3 Mechanisms of Polyunsaturated Fatty Acid Control of SCD Gene Expression

3.3.1 Transcriptional Control

PUFA-mediated suppression of SCD1 expression in liver and primary hepatocytes and of SCD2 expression in lymphocytes was shown to be largely due to a decrease in their rates of gene transcription.^{5,24,27–28,32–34,38} Therefore, many recent studies have focused on the hypothesis that a *cis*-acting PUFA responsive element (PUFA-RE) exists in the promoters of SCD genes to which a transcription factor binds blocking transcription. Using deletion analysis, the SCD1 and SCD2 PUFA-REs were localized to a 60-bp region in each of their promoters.³⁸ This is the only region of high sequence homology within the promoters of the two SCD genes.⁸ The PUFA-REs of the rat S14 and pyruvate kinase genes have also been mapped^{39,40} and were found to share homologous sequences.

transgenic mice that overexpress mature SREBPs.^{45–47} Thus, the SREBPs appear to play a pivotal role in the expression of lipogenic genes. The nuclear abundance of SREBP-1 and mRNA has been found to be reduced by fasting and greatly increased by refeeding a high-carbohydrate diet.⁵⁰ In addition, changes in the nuclear content of SREBP-1 resulting from starving–refeeding displayed a temporal pattern that is similar to the pattern observed for several lipogenic genes.⁵⁰ In light of these observations it was postulated that PUFAs coordinately suppress the transcription of hepatic genes by suppressing the expression of SREBP-1.⁵¹

PUFAs are potent ligand activators of a family of nuclear transcription factors termed peroxisome proliferator-activated receptors (PPARs) and have, therefore, been hypothesized to be the endogenous activators of this receptor.¹⁶ The dominant PPAR in liver is PPAR α , and fatty acid activation appears to coordinately induce genes encoding enzymes involved in fatty acid oxidation and ketogenesis.^{52,53} Both peroxisome proliferators and PUFAs^{16,54} repress lipogenic genes, such as S14 and FAS, but the direct involvement of PPARs in the PUFA suppression of these genes has not been demonstrated.^{55,56} However, one possibility is that PUFA activation of PPAR α could lead to the suppression of SREBP that is common to several lipogenic genes and in this way indirectly lead to the inhibition of lipogenic gene expression. Such a mechanism would provide a unifying explanation for how PUFAs induce genes of hepatic lipid oxidation and concomitantly suppress genes of lipogenesis. When rats were fed the fat-free diets supplemented with n-6 and n-3 PUFAs, the hepatic level of SREBP as well as FAS and SCD were dramatically reduced while as expected the expression of the acyl-CoA oxidase was increased.^{51,57} It has, therefore, been speculated that PUFAs repress SREBP-1 by a PPAR α -dependent mechanism. Consistent with this hypothesis, Xu et al.⁵¹ found that feeding rats the potent PPAR α -specific activator WY-14,643 reduced the abundance of hepatic SREBP-1 mRNA to the level comparable with that found in rats fed diets containing PUFA. Taken together, one would speculate that the PUFA-activated PPAR α might repress SCD1 gene transcription as well. Unexpectedly, studies on the effects of peroxisome proliferators on SCD1 gene expression have shown that PUFA and peroxisome proliferators had opposing effects on the SCD1 mRNA levels in mouse liver.⁵⁸ Therefore, unlike the S14, FAS and SREBP-1 genes, peroxisome proliferators induced the expression of the SCD1 gene. In addition, transient transfection experiments localized the SCD1 peroxisome proliferator response element (PPRE) to an area of the SCD1 promoter that is distinct from the PUFA-RE.⁵⁸ This indicates that different mechanisms account for the transcriptional regulation of the SCD1 gene by peroxisome proliferators and PUFAs and suggests that PUFA-repression of the SCD genes may proceed through an SREBP-independent mechanism.

PUFAs and oleic acid have been shown *in vitro* to reduce the activity of promoters with SREs by inhibiting the proteolytic maturation of SREBP.⁵⁹ These studies suggest that the mature form of SREBP participates in the repression of the SCD gene transcription by PUFA. However, oleic acid could not reduce the transcription of the SCD genes both *in vivo* and *in vitro*.^{24,27} Cotransfection of the

SCD1 promoter with expression vectors of the mature form of SREBP leads to activation of the SCD1 and SCD2 promoter activity and no repression is observed in the presence of cholesterol, consistent with the role of cholesterol in inhibiting the maturation of SREBP. However, unlike the case of cholesterol, activation of the SCD promoter by mature form of SREBP remains sensitive to PUFA repression (T. Drews and J. M. Ntambi, unpublished). Thus, SREBP maturation does not seem to exhibit the selectivity required to explain PUFA control of SCD gene transcription, suggesting that PUFA may utilize a different protein to repress SCD1 and SCD2 transcription. This protein is probably not NF-Y because cotransfection of a dominant negative form of NF-Y reduces the basal activity of the SCD promoter but the residual activity remains sensitive to PUFA repression.⁴² In addition, the SREBP or the NF-Y elements on their own do not mediate PUFA repression in a heterologous promoter context,⁵⁸ but PUFA repression is observed only when the entire 60 bp PUFA-RE is used in a heterologous promoter context.⁵⁸ Therefore, although there are indications that SREBP maturation and binding of the SREBP and NF-Y to the PUFA-RE are involved in PUFA repression, there is strong evidence for the existence of an SREBP-independent mechanism involving a putative PUFA-binding protein through which PUFA repress SCD gene expression.

3.3.2 Post-Transcriptional Control

Studies on the SCD genes in mature adipocytes and in yeast have shown that the effect of PUFA on these genes could be at the level of mRNA stability.^{28,60} When added to cultures of fully differentiated 3T3-L1 adipocytes, arachidonic acid decreased the SCD1 mRNA half-life from 8 to 4 h.²⁸ By contrast, oleic acid and stearic acid did not affect SCD1 mRNA stability. Therefore, this response is unique to PUFAs. Arachidonic acid also decreased the stability of the SCD2 mRNA transcript in adipocytes (A. Sessler and J. M. Ntambi, unpublished). Although transcriptional regulation could not be ruled out completely, changes in transcriptional rates were not detected, suggesting that transcriptional regulation does not play a significant role in PUFA suppression of SCD gene expression in mature adipocytes.²⁸ The observed reduction in enzyme activity (60%) could be completely accounted for by decreases in SCD1 mRNA levels (80%). Thus, there appears to be no additional downregulation occurring post-translationally. Therefore, in contrast to what occurs in hepatocytes, changes in mRNA stability are the major determinant of SCD1 mRNA abundance in adipocytes in response to PUFA. Interestingly, it has been recently found that changes in SREBP mRNA abundance in liver of rats fed PUFAs and the specific PPAR α activator WY-14,643 were not accompanied by changes in SREBP gene transcription, suggesting that PUFA and WY-14,643 reduced the hepatic content of SREBP-1 mRNA possibly by accelerating the rate of SREBP mRNA degradation. In this respect the regulation of SREBP mRNA expression in liver would be similar to that of SCD in adipocytes.

Destabilization of SCD mRNA in adipocytes may be regulated through mRNA sequences in the 3'-untranslated region (UTR). The mouse, rat, and human SCD cDNAs contain an unusually long 3'-UTR.^{7-9,10} The role of such a long 3'-noncoding stretch is currently unknown, though it contains several structural motifs (e.g., AUUUA) characteristic of mRNA destabilization sequences.⁶¹ Four of these sequences are clustered close to the 3'-end of the coding region. Because these AU-rich elements (ARE) play active roles in the selective degradation of several mRNAs in response to various factors, these sequences could be possible targets of PUFA effects on SCD1 and SCD2 mRNA in adipocytes. In yeast, PUFAs act through sequences in the 5'-UTRs to decrease the $\Delta 9$ -desaturase gene 1 (OLE1) mRNA stability.⁶⁰ Northern blot analysis shows that the single human SCD gene gives rise to two mRNA transcripts of 3.9 and 5.2 kb which arise as a consequence of the two polyadenylation signals indicating that the two differently expressed transcripts encode the same SCD polypeptide.¹⁰ The function of the polyadenylation is not known but could be in addition to the transcriptional control, a means by which the two transcripts differ in stability or translatability thus allowing for rapid and efficient changes in cellular environment.¹⁰

The work on SCD gene regulation by PUFA in adipocytes and SREBP in liver suggests that PUFAs regulate gene expression through different mechanisms in different tissue types, the reasons for which are not yet understood. How PUFAs may alter the stability of SREBP and SCD transcripts remains to be determined. However, it is interesting to note that significant quantities of PPAR α are located in the cytosol of some cells, suggesting that PPAR α could regulate gene expression by influencing both transcriptional and post-transcriptional events.⁵¹ The ongoing search for the possible protein mediators that destabilize SCD1 and SREBP mRNA should provide further definition to the molecular basis of PUFA regulation of lipogenic gene expression.

3.4 Conclusions and Future Direction

PUFAs can control the synthesis of monounsaturated fatty acids in liver and other tissues by regulating the expression of the SCD genes. In this way PUFAs can affect the membrane fluidity and the metabolic state of cells. The role of SREBP in mediating the transcriptional regulation of SCD gene expression by PUFA needs more study as does the possible role of PPAR α in mediating SCD and SREBP mRNA stability. Several lipogenic genes such as FAS and S14 are expressed at very low levels in humans but the SCD mRNA expression is high in several human tissues. This makes sense because some of the saturated fatty acids ingested in the diet have to be desaturated to maintain the correct saturated to monounsaturated fatty acid ratio required to preserve membrane fluidity of the cells. The regulation of the SCD gene may, therefore, be more important than that of other

lipogenic genes considering the multitude of human diseases linked to abnormal synthesis of monounsaturated fatty acids. Further studies will be required to elucidate the mechanisms by which PUFAs alter cellular monounsaturated fatty acids to determine the impact of SCD gene regulation in various human disease states.

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4

Fatty Acids, White Adipose Tissue Development, and Adipocyte Differentiation

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4.1 Introduction

In adult animals and humans dietary fat intake is now widely recognized to be associated with gain of fat body mass.^{1,2} During development, evidence also exists that a fatty diet is associated with fat body mass and obesity.^{3,4} It has been proposed that the large percentage of lipids in many foods overwhelm fat-induced satiety signals.⁵ Since changes in dietary fat fail to promote acutely fat oxidation, increase in fat stores follows.⁶ In humans under normal dietary conditions and in contrast to rodents, adipose tissue relies on the exogenous supply of fatty acids (FA) from chylomicrons for triglyceride synthesis,⁷ although *de novo* fatty acid synthesis is likely taking place in white adipose tissue (WAT) in lipoprotein lipase (LPL)-deficient patients,⁸ as has been reported in LPL-knockout mice.⁹ The available evidence indicates that WAT is the primary target of FA originating from triglyceride-rich lipoprotein particles.

Until the late 1980s, it was thought that FA were only playing the role of substrates for complex and neutral lipid synthesis. In the last 10 years, however, it has become clear that both amount and type of dietary fat were important in regulating fat pad weight through hyperplasia and/or hypertrophy. It has also become clear that FA and some of their metabolites are active as signal transducing molecules¹⁰ and are implicated in the differentiation of precursor cells into adipocytes.^{11,12} Important molecular sensors of FA have been characterized in the recent years as nuclear *trans*-acting factors of the steroid hormone super-family, termed peroxisome proliferator-activated receptors (PPARs).¹³ The properties and mechanisms of PPAR action have provided an important link not only between FA and adipocyte differentiation *in vitro* but also between high-fat intake and augmented development of adipose tissue *in vivo*. This review intends to present these various points and to discuss some nutritional issues.

4.2 WAT Development: Relationships with Amount and Type of Dietary Fats

High-fat diets were shown to induce in adult mice and rats an increase in the cellularity of various adipose depots which takes place by hypertrophy often accompanied by hyperplasia.^{14,15} Dietary fats induce adipose tissue growth independent of caloric intake.¹⁶ However, intake of diets enriched in saturated fats as compared to diets enriched in unsaturated fats has led to conflicting results. With similar caloric intake, hypertrophy of perirenal and epididymal adipose tissues was lower with high-fat diets enriched in mono- and polyunsaturated FA than with high-fat diets enriched with saturated FA.^{17–20} In another study, hyperplasia appeared to be involved: The role of dietary fats (45% calories as fat) was analyzed in 1-month-old male Sprague-Dawley rats fed for 5 to 7 weeks with either beef tallow (high saturated fat), safflower oil (high polyunsaturated fat), or corn starch. When fed *ad libitum* and compared to corn starch fed rats, dietary saturated fats induced expansion of inguinal retroperirenal fat pads through increased adipocyte number.¹⁸ Similarly, in 1-month-old male Sprague-Dawley rats fed for 12 weeks (26% calories as fat) with perilla oil (high in α -linolenic acid), beef tallow, olive oil, or safflower oil, perilla oil was shown to lower the growth of epididymal and perirenal fat pads that was associated with hypoplasia and mainly hypotrophy as compared to the other dietary fats.²¹

It has been reported recently that 5-week-old fa/fa rats but not Fa/? rats exhibit a greater reduction in body weight gain and a larger increase in heat production when the animals were fed (65% calories as fat) for 8 weeks with soybean oil (high in linoleic acid) than when they were fed with palm oil (high in palmitic acid).¹⁹ More recently,²⁰ mother rats were fed diets (45% calories as fat) containing either coconut oil resembling milk fat (high in lauric, myristic,

and palmitic acids) and termed COD or safflower oil (high in oleic and linoleic acids) and termed SOD and mated to provide Fa/Fa and Fa/fa lean offsprings as well as fa/fa obese offsprings. Suckling rats were analyzed at 17 days of age, whereas additional 28-day-old male rats were maintained after weaning on the same diet as their mothers until 12 weeks of age. Interestingly, although inguinal fat pad weight were similar in the 17-day-old pups, fat cell number was higher in the inguinal fat pad of the three genotypes in SOD pups, whereas fat cell size was higher in COD pups. The enhanced neutral lipid accumulation is consistent with the increased activities of lipoprotein lipase (LPL), fatty acid synthase (FAS), and malic enzyme (ME) in COD pups as compared to SOD pups. In the 12-week-old rats, when analyzing inguinal, retroperitoneal, and epididymal fat pads, fat cell number was nearly 2-fold higher in SOD rats than in COD rats of the three genotypes, whereas fat cell size was significantly higher in COD rats in fa/fa rats only, again consistent with increased LPL, FAS, and ME activities observed in COD rats.

Thus, despite a similar quantitative fat intake, these data indicate qualitative differences with respect to dietary fatty acid composition regardless of the genotype, where long-chain polyunsaturated FA (18:1 ω 9, C18:2 ω 6, and C18:3 ω 3) appear to favor hyperplasia through enhanced adipose cell differentiation, whereas long-chain saturated FA (C12:0, C14:0 and C16:0) appear to favor hypertrophy through enhanced triglyceride accumulation. In other words, the development of adipose tissue soon after birth and in the few weeks after birth is sensitive to fatty acid composition and amount of ingested fats. Nevertheless, even at later ages, fat depots remain able to respond to dietary fats in a site-specific manner. When 50-day old Wistar rats are fed for 4 weeks with high-fat diets (40% calories as fat) enriched with ω 3 polyunsaturated FA, i.e., eicosapentaenoic acid or docosahexaenoic acid, a lower weight gain of the internal retroperitoneal depot was observed as compared to the subcutaneous depot, consistent with a lower expression of lipid-related genes in the internal depot.²² The mechanisms for such differences remain unclear but should implicate metabolic differences among FA (oxidation for energy requirements, esterification processes for complex and neutral lipid biosynthesis, desaturation and elongation, eicosanoid and leukotriene formation). The mechanisms should also implicate differences *in vivo* between FA and FA metabolites as signal transducing molecules reported to trigger *in vitro* adipogenesis, i.e., the differentiation of precursor cells into adipocytes (*vide infra*).

4.3 Fatty Acids and Regulation of Gene Expression in Preadipose and Adipose Cells

The first line of evidence that FA were involved in the process of adipose cell differentiation was obtained after purification and characterization of the

main adipogenic component of fetal bovine serum because the latter was originally used extensively to grow and differentiate preadipose into adipose cells. This main component of serum was characterized as being arachidonic acid (AA).²³ Both the long-term adipogenic effect as assayed by glycerol-3-phosphate dehydrogenase (GPDH) activity and the short-term intracellular production of cAMP induced by AA were blocked by cyclooxygenase inhibitors such as aspirin or indomethacin, suggesting strongly that some prostanoïd(s) was involved in these responses. Prostacyclin (PGI₂), under the form of its stable analogue (carba)prostacyclin (cPGI₂), was then found to induce short- and long-term effects of AA.²⁴ The adipogenic effect of carbacyclin could be extended to the differentiation of rat and human adipose precursor cells in primary culture.²⁵ Other analogues of PGI₂, such as 6 β -PGI₁ and iloprost, although less potent than cPGI₂, were also active.²⁶ It is interesting to note that glucocorticoids which were able to increase AA mobilization and its metabolism to PGI₂ in Ob1171 preadipose cells also behaved as strong adipogenic hormones.²⁷

Antibodies directed against PGI₂ were shown to strongly diminish the adipogenic effect induced by AA in 5F medium. Thus, PGI₂ secreted into the culture medium of Ob1771 preadipocytes upon exposure to AA behaved as an autocrine/paracrine effector of adipose cell differentiation.²⁶

The second line of evidence in preadipose and adipose cells that FA were important regulators of the process of adipose cell differentiation was obtained when it was shown that long-chain FA could act as transcriptional regulators of lipid-related genes, as first reported for the gene encoding the adipocyte lipid-binding protein (ALBP), also termed adipocyte fatty acid-binding protein (a-FABP) or aP2 protein.¹⁰ The accumulation of aP2 protein was parallel to that of aP2 mRNA. The transcriptional effect of exogenous FA was observed in culture in glucose-supplemented preadipose cells as well as in glucose-free adipose cells, that is under conditions where endogenous fatty acid synthesis remained low. Long-chain FA (LCFA) (saturated, mono- and polyunsaturated) were able to activate within a few hours the aP2 gene but also the gene encoding for the acyl-CoA synthase-1 (ACS1).^{11,28} Thus the expression of two key proteins involved, respectively, in FA transport and activation was regulated at the gene level by FA. Metabolism of FA was not required as α -bromopalmitate, a non-metabolized LCFA in preadipose cells, was more potent than natural LCFA in activating the aP2 gene at a transcriptional level.²⁹ This effect of α -bromopalmitate could be extended to cultured adipose precursor cells from rat adipose tissue in a serum-free, chemically defined medium, thus excluding any specific effect of FA in cells from preadipocyte clonal lines and any synergy of FA with serum components.¹¹ The transcriptional effect of FA occurred early in preadipose cells and was confined to some but not to all lipid-related genes, i.e., to the fatty acid translocase (FAT) gene as an early marker of differentiation³⁰ and also the ACS1 and aP2 genes among intermediate markers, but the genes encoding for late markers such as GPDH and adipsin were not affected.^{10,11} In addition to transcriptional effects of

FA, a stabilizing effect of FA was also assumed in adipose cells because aP2 mRNA accumulation appeared accompanied only by a weak increase in the transcription of the aP2 gene.³¹

At odds with this observation, a destabilization effect of FA in adipose cells appears to take place. When exposed to AA, the half-life of SCD1 mRNA³² and that of GLUT4 mRNA³³ were decreased in 3T3-L1 cells by 67 and 43%, respectively. Both ω 3 (AA, linolenic acid, and eicosapentaenoic acid) and ω 6 polyunsaturated FA (linoleic acid) led to a decrease in SCD1 mRNA stability.³² The transcriptional effects of FA in adipose cells are well described for the PEPCK gene³⁴ but, in some instances, the mechanisms by which FA regulate the expression of the products of lipid-related genes in preadipose and adipose cells appear more complex than anticipated. Preadipose cells are known to express LPL constitutively. However, when preadipose and adipose cells were exposed to LCFA or α -bromopalmitate, a rapid (2 to 8 h) and dose-dependent increase (up to 6-fold) in LPL mRNA occurred, primarily due to increased transcription which is accompanied by a decrease in LPL cellular activity. Under these conditions, secretion of active LPL was nearly abolished. Removal of LCFA led to full recovery of LPL activity and secretion. Thus, FA appeared to exert their main biological effects at translational and post-translational levels to regulate secreted active LPL.³⁵ This suggests that the regulation of LPL by LCFA may be important *in vivo* with regard to the fine tuning of FA entry into preadipocytes during adipocyte formation and in adipocytes during fasting/feeding periods.

4.4 Fatty Acids and Adipocyte Differentiation

The involvement of FA as inducers of the differentiation of preadipose to adipose cells was first reported in cultured cells of Ob1771 and 3T3-F442A clonal lines.^{11,12} The triggering effect of FA took place in preadipose cells, and the adipogenic action of FA did not require their metabolism as the non-metabolized α -bromopalmitate was more effective than metabolized natural LCFA. Of note is the fact that the synthetic ω 3 AA was less efficient than the natural ω 6 AA in promoting terminal differentiation (R. Négrel, personal communication). When compared to control adipogenic conditions, a brief exposure of preadipose cells (1 to 3 days) to LCFA was sufficient to bring a maximal effect and led within 1 to 2 weeks to hyperplasia, an increase in the number of differentiated cells and to hypertrophy, an increase in triglyceride accumulation accompanied by enhanced overexpression of terminal differentiation-related genes.^{11,12}

In other words, in the appropriate hormonal milieu, a hyperplastic phenomenon could be observed *in vitro* upon short exposure of precursor cells to exogenous, unprocessed LCFA. For instance, under these conditions, the concentration of unbound α -bromopalmitate in the external milieu should be

very low.³⁶ After its uptake and intracellular traffic, it is likely that its transient increased concentration within the nucleus should remain very low as part may remain bound within the cytosol of preadipose cells by the epidermal fatty acid-binding protein (eFABP).³⁷ If this were so, this low rise in FA concentration would remain sufficient to activate directly or indirectly the expression of lipid-related genes (*vide infra*).

4.5 Molecular Sensors of Fatty Acids and Fatty Acid Metabolites in Preadipose and Adipose Cells

Like retinoic acid and fibrates, FA are amphipatic carboxylates. As retinoic acid binds specifically and activates specific nuclear receptors, it was hypothesized that fibrates and FA could act in a similar way via new members of the nuclear receptor families. PPAR α , a member of the nuclear steroid hormone receptor superfamily activated by fibrates was first cloned from mouse liver.³⁸ PPAR α as well as other PPAR isoforms were thereafter identified from cDNA libraries obtained from various organs of different species.^{30,39–47} Evidence was obtained that peroxisome proliferators and FA can activate PPAR α in transactivation assays.⁴⁰ With one exception,⁴⁸ PPAR α could not be detected in preadipose cells, in contrast to PPAR δ and trace levels of PPAR γ . Once differentiated, PPAR δ remained present in adipose cells, whereas PPAR γ was then expressed at high levels.³⁰ PPAR γ was shown to be predominantly, but not exclusively, expressed in adipose tissue; PPAR δ and PPAR α showed a more widespread tissue distribution although a higher expression was observed in lipid-synthesizing tissues (adipose tissue, liver, intestine).^{30,44}

All the PPAR isoforms are active as heterodimers with retinoic X receptor α (RXR α) by recognizing *cis*-acting sequences (DR-1, direct repeat, 1 base spacer).¹³ By direct comparison of the various PPARs expressed at low levels in transfected NIH-3T3 fibroblasts, the adipogenic potential of PPAR γ and to a lesser degree that of PPAR α was shown, whereas that of PPAR δ was not observed.⁴⁹ However, using a direct approach with 3T3-C2 fibroblasts, the critical role of this nuclear receptor in adipogenesis has been recently shown: no PPAR γ expression, and no subsequent adipocyte formation, can take place unless prior activation of PPAR δ by natural LCFA or α -bromopalmitate in the presence of a specific agonist of PPAR γ , i.e., the thiazolidinedione BRL49653. Treatment of PPAR δ -expressing fibroblasts with FA stimulated the expression of the early FAT gene. Of note is the fact that the most potent effector was (carba)prostacyclin (EC₅₀ ~ 20 nM) whereas the most specific agonist ligands of PPAR γ , i.e., BRL49653 and 15d-PGJ₂,^{50,51} were inactive.⁵² The data strongly suggest that active PPAR δ led to PPAR γ expression which, upon activation, promoted the formation of adipose cells. The key initial role in adipose cell

differentiation of PPAR δ as primary target appears consistent with recent experiments showing that a dominant-negative form of PPAR δ impairs the expression of PPAR γ and the differentiation of Ob1771 and 3T3-F442A preadipose cells (P. Grimaldi, personal communication).

The sequential promotion of adipose cell differentiation by PPAR δ and PPAR γ emphasizes their role as nuclear sensors of FA and FA metabolites. Until recently it was uncertain whether FA, fatty acid metabolites, and several exogenous substances such as fibrates were mere activators of PPARs by displacing intracellularly the natural ligands of PPARs or whether these effectors could indeed bind to the nuclear receptors. Binding of FA and FA metabolites as well as that of fibrates and other amphipathic carboxylates to PPAR α , PPAR δ and PPAR γ has been shown using various indirect approaches.^{53–55} Very recently the direct proof of FA binding to PPARs has been elegantly brought by direct binding assays and X-ray crystallography.^{56,57} PPAR α and PPAR δ bind saturated and unsaturated long-chain FA whereas PPAR γ bind most efficiently polyunsaturated FA and only weakly monounsaturated FA. The crystal structure of PPAR γ ⁵⁸ and that of PPAR δ ⁵⁷ provide amazing insight into the ability of PPARs to interact with a variety of synthetic compounds (fibrates, thiazolidinediones) and natural compounds, including FA₃ $\geq$ C14 of varying degrees of unsaturation. However, it should be pointed out that despite these remarkable observations, the nature of the FA and/or that of the FA metabolites, which are the actual ligands of PPAR δ and PPAR γ within the nucleus of preadipose and adipose cells, remains to be shown.

Among FA metabolites, circumstantial evidence favors prostacyclin as a putative ligand involved in the activation of PPAR δ in preadipose cells as

1. Prostacyclin (PGI₂) is produced by preadipose cells in which PPAR δ is expressed simultaneously as does the cell surface IP receptor which binds prostacyclin specifically.⁵⁸
2. Using authentic target cells, i.e., preadipose cells, PGI₂ and its stable analogue cPGI₂ behave as adipogenic hormones whereas other prostaglandins such as PGD₂, PGJ₂, Δ^{12} -PGJ₂ and 15d- Δ^{12-14} -PGJ₂ are inactive.⁵⁹
3. In preadipose cells (carba)prostacyclin regulates the expression of several genes including α -FABP, angiotensinogen, and the uncoupling protein UCP-2. This effect of cPGI₂ could *not* be mimicked by PGE₁, 6-keto-PGE₁, or BMY45778 despite the fact that they are all ligands of the cell surface IP receptor, altering cAMP and calcium levels. Once the cells are differentiated, the cell surface responses induced by PGI₂ or cPGI₂ disappear, but cPGI₂ remains able to regulate the expression of these genes in adipose cells.⁶⁰

4. Prostacyclin analogues, especially (carba)prostacyclin, transactivate all PPAR isoforms in the expected range of concentrations (0.025 to 10 μ M).⁴⁸
5. (Carba)prostacyclin induces adipogenesis in transfected fibroblasts expressing PPAR α or PPAR γ within the same range of concentrations as in transactivation assays.⁴⁹
6. In PPAR δ -expressing fibroblasts, (carba)prostacyclin shows the unique ability to substitute for a combination of a PPAR δ activator (α -bromopalmitate) and a PPAR γ ligand (BRL49653) for the promotion of adipose differentiation ($EC_{50} \sim 20$ nM).⁵²
7. (Carba)prostacyclin is indeed a ligand of PPAR δ .⁵³

Although of interest, these data cannot rule out that the natural ligand(s) of PPAR δ differ from prostacyclin, but it should be stressed that this prostaglandin is the only metabolite of arachidonic acid currently characterized in preadipose cells with PGE₂ and trace levels of PGE_{2 α} .⁶¹ Moreover, PGI₂ has been shown recently to upregulate via the IP receptor in preadipose cells the expression of C/EBP β and δ , which plays a key role in adipose tissue development by regulating, in turn, like PPAR δ (see above), the expression of PPAR γ .^{62–65} The above observations emphasize a role of prostacyclin via PPAR δ in the early events of adipose cell differentiation and underline that the role played by PPARs can hardly be envisioned *independently* from the site of synthesis and/or the availability of their natural ligands.

4.6 Conclusions

Taken together, current *in vitro* data suggest two complementary roles of LCFA in the development of adipose tissue (Fig. 4.1). The intensity and duration of the flux of dietary LCFA entering adipose tissue, proportionate to the amount of ingested fat, is assumed to alter transiently the intracellular concentrations of LCFA and/or their metabolites within the nucleus. In preadipose cells PPAR δ , which binds both saturated and unsaturated FA and appears as their primary target, leads to the rapid expression of PPAR γ . In addition, AA arising from both the diet and the elongation/desaturation processes of the essential linoleic acid, is the substrate entering the lipoxigenase and cyclooxygenase pathways. Among AA metabolites, prostacyclin appears unique in its ability to promote terminal differentiation of preadipose to adipose cells.^{52,59} Prostacyclin via the cell surface receptor IP triggers the expression of C/EBP β and δ critical for PPAR γ and C/EBP α expression and adipose tissue formation,^{62–65} but it could also bind directly to PPAR δ .⁵³ If that were so, among unsaturated FA, AA would specifically

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5

Acyl-CoA Synthetase 1 (ACS1): Regulation and Role in Metabolism

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5.1 ACS1: Structure and Function

The Acyl-CoA synthetase 1 (ACS1) (EC6.2.1.3)¹ gene encodes a key gate-keeper enzyme in long-chain fatty acid metabolism. ACS1 catalyzes the activation of free long-chain (C12–18) fatty acids to long-chain acyl-CoA esters, the requisite initial step in the cellular utilization of long-chain fatty acids. Both endogenous and exogenous long-chain fatty acids must be activated by ACS1 to their CoA esters before they can be utilized for triacylglycerol synthesis or for fatty acid oxidation.

ACS1 is a member of a growing family of acyl-CoA synthetases which displays a range of substrate specificities. ACS1 was the first mammalian acyl-CoA synthetase to be purified and to be cloned. ACS1 was originally purified to homogeneity from rat liver by Tanaka et al.² in 1979 from both the microsomal and mitochondrial fractions. All properties of the enzymes isolated from the mitochondrial and microsomal fractions were identical and they have, therefore, been regarded as the same enzyme. The purified rat liver ACS1 enzyme was determined to have a specific activity of 26 to 29 units/mg pro-

tein at 35°C and most efficiently catalyzes the activation of saturated fatty acids with 10 to 18 carbon atoms and unsaturated fatty acids with 16 to 20 carbon atoms. The purified enzyme absolutely requires adenosine tri-phosphate (ATP), Mg^{2+} , fatty acid, and CoA for activity; *in vitro* analysis confirmed that the enzyme catalyzes the stoichiometric conversion of ATP, fatty acid, and CoA to adenosine monophosphate (AMP), inorganic phosphate (PPi), and acyl-CoA. The reaction proceeds in two steps: first, ACS reacts with the carboxyl group of the fatty acid and with ATP to form an acyl-AMP and free PPi in the presence of Mg^{2+} ; then the ACS enzyme catalyzes the formation of acyl-CoA and free AMP from acyl-AMP and CoA.³

ACS1 has also been demonstrated to have important pharmacologic substrates. The profen (2-arylpropionate) class of non-steroidal anti-inflammatory drugs, which includes the widely used compounds ibuprofen and naproxen, are administered as racemic mixtures. The unidirectional chiral inversion from the inactive (R) to the active (S) enantiomer occurs by activation of the 2-arylpropionic acid by forming an acyl-CoA thioester intermediate, which is catalyzed by ACS1.^{4,5} The subsequent epimerization and hydrolysis steps in the activation process are not enantioselective. It has been suggested that profens might alter lipid biochemistry, e.g., as a substrate which competes with long-chain fatty acids for ACS1, thereby inhibiting β -oxidation and/or lipogenesis.⁶ The hypolipidemic xenobiotic carboxylic acid agents of the fibrate class of peroxisome proliferators (e.g., clofibrate, gemfibrozil, ciprofibrate, bezafibrate) are also substrates for ACS1 and are competitive as substrates with natural long-chain fatty acids.⁷ It is thought that the induction of peroxisome proliferation in rodent livers by these compounds requires their activation to the acyl-CoA thioester.^{7,8} Although there is some evidence for the existence of other ACS isoenzymes as activators of the profens and xenobiotic carboxylic acids,^{9,10} most laboratories have found that ACS1 accounts for all activity toward these substrates.

Further progress in characterizing the structure of ACS1 was achieved by Suzuki et al.,³ who reported the sequence of the rat ACS1 messenger RNA (mRNA), which they identified by using a polyclonal antibody raised against the purified enzyme to screen an expression recombinant DNA (cDNA) library prepared from rat liver. The rat liver ACS1 mRNA encodes a polypeptide of 699 amino acids, 78 kDa in length. The protein is organized into five regions: an NH₂ terminus, two luciferase-like regions, a linker connecting the luciferase regions, and a COOH terminus. Further studies in which ACS1 was overproduced in *E. coli* and purified to homogeneity have permitted rigorous characterization of the specific activity and substrate specificities of ACS1 *in vitro*: ACS1 has a specific activity of 26.2 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$; results confirmed that among saturated fatty acids the most preferred substrates were palmitic, myristic, pentadecanoic, and stearic acids, and the most preferred unsaturated fatty acids were palmitoleate, oleate, and linoleate.¹¹ Increasing concentrations of Mg^{2+} will increase the relative substrate preference of ACS1 for both oleic acid and arachidonic acid as well increasing the overall V_{max} of the enzyme.¹² More recently, evidence has emerged indicat-

ing that ACS1 is the enzyme that activates the branched-chain fatty acid phytanic acid in peroxisomes where it undergoes α -oxidation to pristanic acid.¹³

Mutational analysis revealed that all five regions of ACS1 were required for functional activity; deletion of any region abolished enzyme activity.¹¹ The similarities among both prokaryotic and eukaryotic acyl-CoA synthetases indicate a common ancestry; and there is a common 25-amino acid consensus sequence near the COOH terminus (DGWLHTGDIGXWXPXGXLKIIDRKK) that is common to all ACS genes.¹⁴ Site-directed mutational analysis has demonstrated that this ACS signature motif is essential for catalytic activity and appears to compose part of the fatty acid binding site since several site-specific mutations within the sequence affected substrate specificity with respect to chain length.¹⁴ This region is thought to project into the cytoplasm, at least when ACS is associated with microsomal, peroxisomal, and outer mitochondrial membranes.²

Studies of the subcellular localization of ACS1 have demonstrated that it is abundant in mitochondria and microsomes and is also found in peroxisomes.^{2,15,16} In heart, ACS activity is more than 80% associated with the mitochondrial fraction, whereas in adipose tissue ACS is predominantly associated with the microsomal fraction.¹ These tissue-specific differences in subcellular compartmental distribution undoubtedly reflect primary organ functions; e.g., the heart prefers fatty acid for fuel substrate, which it metabolizes by mitochondrial β -oxidation, whereas a major adipose tissue function is lipid synthesis and storage, with adipocyte lipogenic enzymes coordinately regulated and associated with microsomal membranes.

Another recent study has provided evidence for the association of ACS1 with adipocyte plasma membranes, where it has been proposed to play a role in facilitating fatty acid uptake.¹⁷ Several studies have given evidence that ACS1 is an integral membrane protein.¹⁵⁻¹⁷ The amino acid sequence predicted by the rat liver ACS1 cDNA contains a potential membrane spanning region.³ Biochemical studies using proteases as probes for exposed domains performed by Hesler et al. delineated the transverse plane topography of ACS1 in the mitochondrial outer membrane.¹⁶ These studies determined that ACS is a transmembrane enzyme with crucial domains on both sides of the outer membrane, and that one protease-sensitive essential domain which is involved in CoA binding is localized to the cytosolic surface of the membrane. Other studies of the peroxisomal localization of ACS1 using proteolytic enzymes also demonstrated that the enzymatic site of ACS1 is on the cytoplasmic surface in peroxisomal membranes as well.¹⁵

5.2 ACS1 Gene Expression: Tissue Distribution and Developmental Regulation

Tissue distribution of ACS1 has been studied by several groups. ACS1 is expressed most abundantly in tissues that are specialized to utilize long-chain

fatty acids to synthesize triacylglycerol or for β -oxidization of long-chain fatty acids. The enzyme is most abundant in liver, adipose tissue, heart, and muscle,¹⁸ but it is expressed in a wide range of tissues including lung, brain, intestine, kidney, adrenal, testis, pancreatic β -cells,¹⁹ and probably is responsible for the ACS activity in certain blood leukocytes, e.g., neutrophils.²⁰ ACS1 expression in lung and small intestine is only about 10% of that in liver, heart, and epididymal adipose tissue.³

Developmental expression patterns have not been well characterized, but ACS1 is not expressed in proliferating or confluent 3T3-L1 preadipocytes cultured in standard medium supplemented with fetal bovine serum.^{21,22} However, when adipocyte differentiation is induced by the standard differentiation treatment with dexamethasone and 1-methyl-3-isobutyl-xanthine (MIX), ACS mRNA is strongly induced by day 3 and reaches maximal abundance by day 5.^{21,22} Insulin-like growth factor I (IGF-I) is essential for activation of the ACS1 gene during adipose differentiation; in fact, cDNA sequences for ACS1 were first cloned by differential screening of cDNA libraries constructed from 3T3-L1 preadipocytes and from adipocytes differentiated by treatment with IGF-I in medium containing serum which had been depleted of insulin, corticosteroids, and growth factors.^{21,22}

Although preadipocytes do not synthesize large amounts of lipids as adipocytes do, the ability to activate long-chain fatty acids is still essential for other cellular functions such as membrane synthesis or protein acylation. Another enzyme that is 60% homologous to ACS1 has recently been identified in proliferating adipocytes and in intestinal epithelial cells: ACS5 consists of 683 amino acids, and it differs from ACS1 in that its substrate preference is for C16–C18 unsaturated fatty acids, although it utilizes a wide range of fatty acids similar to those that are substrates for ACS1.²³ Unlike ACS1, ACS5 mRNA expression levels do not change during adipose differentiation.²³ However, in the Caco-2 human intestinal cell line, acyl-CoA synthetase activity increased approximately 40% during the differentiation of Caco-2 cells *in vitro*, and the incorporation of palmitic acid into triacylglycerol increased nearly 2-fold.²⁴ It is not known which ACS isozyme is induced during Caco-2 cellular differentiation. Thus, cellular requirements for activated long-chain fatty acids apparently can be handled by one or more isoforms of acyl-CoA synthetase with overlapping ranges of substrate specificity, or one ACS may compensate for the absence of another under certain metabolic circumstances, e.g., during preadipocyte proliferation. However, in some circumstances a given cell type may contain only one form of ACS or may require a specific form for viability. Triacsin C, a compound produced by some bacteria, is an 11-carbon alkenyl chain with a terminal N-hydroxytriazene moiety and is a potent and specific inhibitor of ACS1 enzyme activity.²⁰ Triacsin C has been shown to inhibit the proliferation of several cultured mammalian cell lines including Vero, HeLa, and Raji (Burkitt lymphoma) cells.²⁵ Inhibition of ACS activity in blood neutrophils by triacsin C *in vitro* also prevented the induction of peroxide generation and degranulation.²⁰

5.3 The ACS Gene Family

The family of known mammalian acyl-CoA synthetases has grown rapidly. ACS2 is expressed in different tissues than ACS1; ACS2 mRNA is most abundantly expressed in brain.¹¹ Its specific activity is $7.4 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ (compared to $26.2 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for ACS1), but its substrate preferences are the same as ACS1.¹¹ ACS3 is a recently identified isozyme that preferentially utilizes laurate, myristate, arachidonate, and eicosapentaenoate.²⁶ ACS3 is most abundant in brain, but it is also detectable in lung, adrenal gland, kidney, and small intestine.²⁷ It consists of 720 amino acids and has two major forms of 79 and 80 kDA which arise from alternative translation initiation sites, i.e., the first and second in-frame AUG sites. Both have the same activities toward palmitate and myristate. The 80-kDA ACS3 isoform was found to be abundant in rat cerebrum, primarily compartmentalized to microsomes; only trace amounts of the 79-kDA polypeptide were detected.¹⁷ ACS4 has been identified as an acyl-CoA synthetase that preferentially utilizes arachidonate and eicosapentaenoate and has a low affinity for palmitate.²⁸ It is 68% homologous to ACS3 and contains 670 amino acids. Although it is detectable in a wide range of tissues, it is most abundantly expressed in steroidogenic cells of the adrenal gland, corpus luteum and stromal luteinized cells of ovary, and Leydig cells of testis in the rat. The human homologs of ACS1,^{28a} ACS2,²⁹ and ACS4³⁰ have been identified.

The multiplicity of mammalian long-chain acyl-CoA synthetases is mirrored by the system in the yeast *Saccharomyces cerevisiae*, which may be even more complex, or may predict additional isoforms for which mammalian homologs may eventually be characterized. Like mammalian ACS isozymes, these yeast ACS enzymes display a variable range of specificities: Faa1p prefers C12:0-C16:0; Faa2p is active toward a wider range of substrates and prefers C9:0-C13:0; Faa3p prefers C16 and C18 fatty acids with a cis-double bond at C-9-C-10.³¹ A unique feature of *S. cerevisiae* long-chain acyl-CoA synthetases is that only Faa1 and Faa4 have the ability to activate imported exogenous long-chain saturated and unsaturated fatty acids.^{32,33} Although functional separation of these fatty acid pools by specific mammalian ACS isozymes has been suggested, their existence has not yet been definitively demonstrated.

Genomic sequences for rat ACS1 have been partially characterized, and results revealed multiple promoters that generate several mRNA transcripts which display 5'-end heterogeneity and which are expressed in a tissue specific manner.³⁴ Three promoters (A, B, and C) span 20 kb of 5'-genomic sequence and each generates a unique exon 1 which is spliced to exon 2; exon 1 is 5'-UTR and the protein coding region begins and is completely contained within exon 2. Exon 1A is the major species of ACS1 exon 1 usage in rat liver. It is abundant in adipose tissue and is also found in heart. Exon 1B is detectable at low levels in liver isolated from animals

treated with an agent that induces liver peroxisome proliferation, but it is not found in normal liver. Form 1C ACS mRNA is the major transcript in heart. It is expressed in low levels in normal liver but is strongly induced in liver by a peroxisome proliferating agent. The C promoter contains E1 and E3 sequences that are commonly found in the 5'-flanking regions of inducible peroxisomal β -oxidation genes.³⁴

5.4 Nutritional and Hormonal Regulation of ACS1 Gene Expression

Acyl-CoA synthetase 1 was originally considered to be a constitutive enzyme because early studies found little or no change in enzyme activity measured under various nutritional conditions. Studies with pharmacologic agents sometimes produced conflicting results. The lack of conclusive evidence for physiologic regulation of ACS1 may have been due to the instability of the enzyme in membrane preparations. The isolation and characterization of ACS1 cDNA sequences have made it possible to obtain much more extensive and definitive information about the regulation of ACS1 gene expression.

Nutritional regulation of ACS1 gene expression in rat liver was demonstrated by Suzuki et al. by refeeding animals fasted for 48 h with either a high-carbohydrate or high-fat diet.³ The fasting/carbohydrate refeeding protocol has been widely used to identify and characterize genes regulated by insulin, e.g., lipogenic genes such as fatty acid synthase.³⁵ Refeeding with either high carbohydrate or high fat diets produced a 7- to 8-fold increase in ACS1 mRNA levels in liver, in contrast to unchanged levels of expression in rats refed standard chow diet, which indicates that both increased exogenous fatty acid delivery and *de novo* lipogenesis induces ACS1 gene expression.³ These results also suggested that ACS would be positively regulated by insulin. The fasting–carbohydrate refeeding protocol is widely regarded as a nutritional method for decreasing circulating insulin levels during fasting and then stimulating maximal insulin release during high carbohydrate refeeding.^{35,36} Schoonjans et al. observed that fasted neonatal rats had 37-fold lower levels of liver ACS1 mRNA compared to fed rats.³⁶ In contrast, both groups found minimal decrease in liver ACS1 mRNA levels in fasted adult rats previously fed on standard chow. They also found that during weaning there was a transient decrease in liver ACS1 mRNA expression, which they proposed might be due to either or both decreased caloric and decreased lipid intake. Lee et al. found modestly decreased ACS1 mRNA only in adipose tissue of starved adult rats,³⁷ whereas Memon et al. found dramatically decreased ACS1 mRNA expression in adipose tissue only in Syrian hamsters fasted for 24 h.³⁸ Furthermore, rats fasted for 24 h have been observed to have 18% decreased adipocyte mitochondrial ACS1 enzyme activity³⁹ and 25% decreased activity in adipocyte homogenates.⁴⁰

The first evidence for hormonal regulation of ACS1 was developed by R. M. Bell and coworkers.⁴¹ They incubated isolated rat fat cells with 400 μ U/ml of insulin for 60 min. and measured the specific activity of ACS and found an approximately 2-fold increase in ACS1 activity, which was maximal at 2 min. and stable during the 60 min. of incubation.⁴¹ The mechanism for this effect of insulin on ACS1 enzyme activity is still not known, but it is possible that regulation is mediated by phosphorylation/dephosphorylation since the ACS1 amino acid sequence contains several potential serine phosphorylation sites.

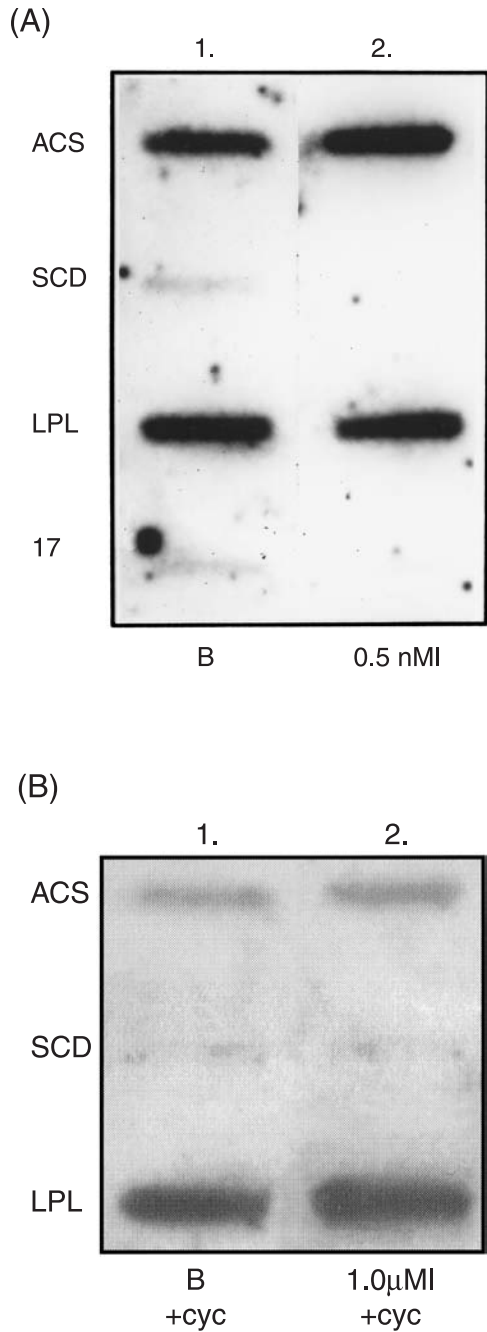
More recently, we have demonstrated that physiologically relevant subnanomolar concentrations of insulin rapidly and maximally induced a 2.4-fold increase in ACS1 gene transcription *in vitro* in cultured 3T3-L1 adipocytes, which does not require protein synthesis⁴² (Fig. 5.1). Moreover, ACS1 was more sensitive to the stimulatory action of insulin than two other lipogenic enzyme genes, lipoprotein lipase, and stearyl-CoA desaturase (Fig. 5.2). ACS1 mRNA expression in adipocytes is dependent on one or more hormones and/or growth factors found in serum; when adipocyte cultures are incubated in serum-free medium, ACS1 mRNA levels decrease significantly within 24 h.⁴² Addition of 0.5 nM insulin for 24 h resulted in at least a 2.3-fold increase in ACS1 mRNA levels. We have also demonstrated induction of ACS1 expression by insulin *in vivo* in mesenteric adipose tissue of streptozocin-diabetic mice.⁴³ Previously, Saggerson and Carpenter observed that streptozocin-diabetes caused a decline in epididymal adipose ACS1 enzyme activity that was significantly restored 2 h after administration of insulin.⁴⁴

Genes whose expression is positively regulated by insulin are very often negatively regulated by counterregulatory hormones such as corticosteroids. Fasting, the nutrient-deprived state, is associated with increased circulating levels of corticosteroids and elevated intracellular concentrations of 3',5'-cyclic monophosphate (cAMP). Therefore, we also characterized the responses of ACS1 to these agents in cultured 3T3-L1 adipocytes. The 3T3-L1 adipocyte system is widely used as an *in vitro* model of insulin action in the control of cellular processes such as lipid and carbohydrate synthesis and metabolism. Terminally differentiated 3T3-L1 adipocytes were pre-incubated in serum-free medium for 24 h followed by treatment with 7 nM dexamethasone for 24 h. Dexamethasone treatment downregulated ACS1 mRNA expression and gene transcription significantly below basal levels.⁴² The effect on ACS1 gene expression of 1-methyl-3-isobutylxanthine (MIX), which raises intracellular cAMP, was also evaluated in 3T3-L1 adipocytes. Adipocyte cultures maintained in Dulbecco's Modified Eagles (DME) media supplemented with 10% fetal bovine serum display maximal levels of ACS1 gene transcription and mRNA expression. Addition of MIX ranging in concentration from 0.05 mM to 0.5 mM for 24 h inhibited both ACS1 gene transcription and mRNA expression in a dose-dependent manner; at 0.5 mM MIX the expression of ACS1 mRNA and gene transcription were almost abolished.⁴²

Nutritionally replete states, e.g., carbohydrate feeding, are associated with increased circulating levels of triiodothyronine (T3).⁴⁵ We also characterized the effect of T3 on ACS1 gene expression in 3T3-L1 adipocytes. Adipocytes

FIGURE 5.1

(A) Effect of physiological concentration of insulin (0.5 nM) on transcription rate of the native ACS gene in 3T3-L1 adipocytes, and comparison with LPL and SCD-1. Nuclei were isolated from fully differentiated 3T3-L1 adipocytes preincubated for 24 h in serum-free medium + 0.1% BSA (slot blot 1) followed by treatment with 0.5 nM insulin for 24 h (slot blot 2), and nuclear transcription run-assays were performed. Equal aliquots of ^{32}P -labeled transcripts (5×10^6 counts/min) were hybridized with excess denatured pBluescript, pACS, pSCD-1, pLPL, and pC.17 plasmid DNA fixed to each nitrocellulose slot blot. Vector sequences (negative control) were not detectable on the autoradiographs and thus are not shown. Autoradiographs are representative of 3 separate experiments. (B) Effect of inhibition of protein synthesis on stimulation of ACS gene transcription without hormones. Adipocyte cultures were preincubated for 24 h without hormones. Cultures were then treated with 0.1 mM cycloheximide (cyc) for 1 h (slot blot 1) before μM insulin was added for 45 min (slot blot 2). Vector sequences (negative control) were not detectable. Autoradiographs are representative of 4 separate experiments. (From Kansara et al., *Am. J. Physiol.*, 270, E875, 1996. With permission.)



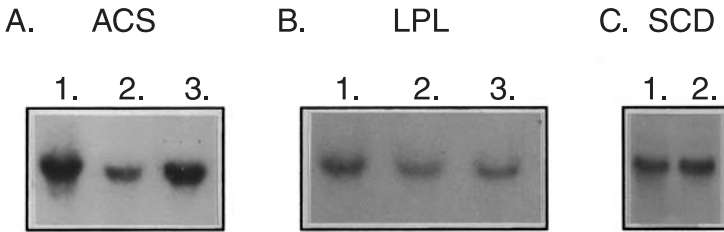
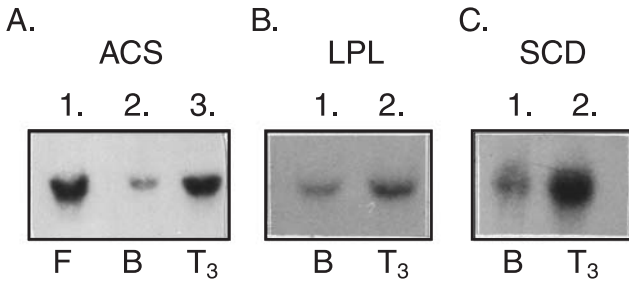


FIGURE 5.2

Effect of insulin on acyl-CoA synthetase (ACS) mRNA expression in 3T3-L1 adipocytes and comparison with lipoprotein lipase (LPL) and stearoyl-CoA desaturase (SCD-1) mRNA levels; Northern analysis. Total cellular RNA was prepared from fully differentiated 3T3-L1 adipocytes treated for 24 h with 0.5 nM insulin after 24 h of preincubation in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% bovine serum albumin (BSA) from control cultures maintained in standard medium, DMEM + 10% fetal bovine serum (FBS). The RNA, 10 μ g per lane, was subjected to denaturing agarose gel electrophoresis. Blots were probed with ^{32} P-labeled randomly primed ACS, LPL, and SCD-1 cDNA inserts excised from recombinant pBlue-script plasmids. The autoradiograph in (A) is representative of 6 independent experiments, and those in (B) and (C) of 3 independent experiments. (A) Effect of 0.5 nM insulin on ACS mRNA levels. Lane 1, DMEM + 10 FBS (F); lane 2, DMEM + 0.1% BSA (B); lane 3, DMEM + 0.1% BSA + 0.5 nM insulin (I). (B) Effect of 0.5 nM insulin on LPL mRNA abundance. Lane 1, DMEM + 10 FBS; lane 2, DMEM + 0.1% BSA; lane 3, DMEM + 0.1% BSA + 0.5 nM insulin. (C) Effect of 0.5 nM insulin on SCD-1 mRNA abundance. Lane 1, DMEM + 0.1% BSA; lane 2, DMEM + 0.1% BSA + 0.5 nM insulin. (From Kansara et al., *Am. J. Physiol.*, 270, E875, 1996. With permission.)

were pre-incubated in serum-free medium to reduce ACS1 gene expression to basal unstimulated levels. Cultures were then incubated with 10 nM T3 for 24 h. Both ACS1 gene transcription and mRNA levels were strongly induced, 2.8-fold and 2.4-fold, respectively⁴² (Fig. 5.3). We have also confirmed induction of ACS1 mRNA *in vivo* in mice 24 h after injection with T3.⁴⁶

Long-chain fatty acids are major regulators of ACS1 gene expression. Schoonjans et al. have conducted elegant studies characterizing the ACS1 gene promoter and the molecular signal transduction pathways by which ACS1 gene expression is activated by fatty acids and the related xenobiotic carboxylic acid compounds known as peroxisome proliferators and used as lipid lowering agents.⁴⁷ Treatment of cultured Fa-32 rat hepatoma cells and primary hepatocyte cultures for 48 h with 100 μ M α -linolenic acid resulted in a 9-fold induction of the C isoform of ACS1 mRNA. Fatty acids are metabolized so rapidly that the maximum possible activating effects of fatty acids on ACS1 gene expression are most accurately measured by using nonmetabolized fatty acids such as α -bromopalmitate, which at 100 μ M induced a 21-fold increase in ACS1 mRNA in cultured hepatocytes.⁴⁷ The peroxisome proliferator fenofibrate (500 μ M) induced the C isoform of ACS1 mRNA 29-fold in cultured hepatocytes, and the A-isoform also increased.⁴⁷ Regulation of ACS1 gene expression by fibrates has also been demonstrated to occur in a tissue-specific manner. Fenofibrate induced a rapid transient approximately 7-fold increase in adipose ACS1 mRNA expression that peaked at 24 h in rats fed fenofibrate (0.5% w/w, approximately 0.5 g/kg/day) for 14 days,⁴⁸ while ACS1 expression in kidney

**FIGURE 5.3**

Effect of triiodothyronine (T₃) on ACS mRNA levels and gene transcription; comparison with LPL and SCD-1. (A) Effects of T₃ on ACS, LPL, and SCD-1 mRNA abundance. Total cellular RNA was isolated from 3T3-L1 adipocytes, treated with 10 nM T₃ for 24 h, and subjected to Northern analysis. Representative autoradiographs for ACS are the result of 3 independent experiments, for LPL, of 5 independent experiments and for SCD-1, duplicate experiments. For ACS, lane 1, DMEM + 10% FBS; lane 2, DMEM + 0.1% BSA; lane 3, DMEM 0.1% BSA + 10 nM T₃; for LPL, lane 1, DMEM + 0.1% BSA; lane 2, DMEM + 0.1% BSA + 10 nM T₃; for SCD-1, lane 1, DMEM + 0.1% BSA; lane 2, DMEM + 0.1% BSA + 10 nM T₃. (B) Effects of T₃ on ACS, LPL, and SCD-1 gene transcription. Nuclei were isolated from adipocytes preincubated for 24 h in hormone-free medium (DMEM + 0.1% BSA; slot blot 1) followed by addition of 10 nM T₃ for 24 h (slot blot 2). Run-on transcription assays were performed. Vector sequences (negative control) were not detectable and hence not shown. Autoradiographs are representative of 4 independent assays of ACS and LPL gene transcription and 2 assays of SCD-1 gene transcription. (From Kansara et al., *Am. J. Physiol.*, 270, E877, 1996. With permission.)

was induced less strongly (maximum 3-fold)⁴⁹ and in heart, skeletal muscle, and intestine ACS1 gene expression was relatively insensitive to fenofibrate.⁴⁸ ACS1 enzyme activity in liver and adipose tissue also increased in a dose-dependent manner that correlated with the increase in mRNA levels.⁴⁸

After demonstrating that the induction of ACS1 gene expression by fenofibrate and fatty acids (10- and 3.5-fold, respectively) was mediated at the transcriptional level in a manner that was independent of protein synthesis, Schoonjans et al. characterized the ACS1 promoter region to identify the sequences in the ACS1 gene that mediate responsiveness of the ACS1 gene to fatty acids and fibrates.⁴⁷ Compounds that function as peroxisome proliferators in rodents are well-known to induce multiple genes involved in fatty acid metabolism, especially genes encoding enzymes for fatty acid oxidation, e.g., acyl-CoA oxidase. The basal expression of several of these enzymes is lower in mutant peroxisome proliferator-activated receptor (PPAR α)-null mice.⁵⁰ The signal transduction pathway mediating the effects of peroxisome proliferators has also been well characterized. Nuclear receptor proteins of the PPAR α class have been found to activate these fatty-acid oxidation genes by binding to a consensus sequence, the PPRE (peroxisome proliferator response element) that was initially identified in the acyl-CoA oxidase gene.⁵¹ Since long-chain fatty acids must be activated by the ACS1 enzyme before they can undergo oxidation, and since ACS gene expression is stimulated by peroxisome proliferator compounds, PPAR α would likely be involved in mediating ACS1 induction by

fibrates and fatty acids. Schoonjans et al. prepared plasmids containing sequences corresponding to the A, B, and C promoter regions placed upstream of the CAT (chloramphenicol acyl-transferase) reporter gene and transiently co-transfected the ACS1 reporter plasmids into Fa-32 hepatocytes with a PPAR α expression plasmid in the presence of 500 μ M fenofibric acid.⁴⁷ Only the C promoter CAT construct was induced by the fibrate, and was therefore further characterized by progressive 5' deletion mutagenesis to position 282 upstream of the translation initiation site. All constructs were inducible by fenofibric acid and PPAR α , indicating that the PPAR response element resided within the 282 nucleotides immediately 5' to the translation start site. Computer homology search identified a potential PPRE localized around positions -175 and -154. This sequence (TGACTGATGCCCTGAAAGACCT) contained 3 copies of a motif similar to the consensus steroid hormone receptor binding half-site TGACCT arranged in direct repeats. When this sequence was site-specifically mutated in reporter constructs, responsiveness to fenofibric acid was lost. This sequence was also demonstrated to be specifically bound by complexes of PPAR α and its obligate heterodimeric partner, the RXR (rexinoid) orphan nuclear hormone receptor.⁴⁷ Additional experiments demonstrated that the ACS1-C promoter PPRE is a lower affinity PPRE than the acyl-CoA oxidase PPRE.

It is of significant interest that co-transfection of another PPAR isoform, PPAR γ , with the ACS1 reporter plasmid in the presence of fenofibric acid yields a similar level of induction of the reporter gene. In fact, the ACS1 gene PPRE oligonucleotide bound PPAR γ -RXR heterodimers as well as it bound PPAR α -RXR heterodimers.⁴⁷ Many fatty acids have been shown to bind both the PPAR α and PPAR γ receptor isoforms, often with differences in affinity for one over the other.⁵² Generally they have higher affinity for PPAR α , but several have nearly as high affinity for PPAR γ as for PPAR α , (i.e., IC₅₀ values of 2.2 μ M or lower for PPAR γ and 1.2 μ M or lower for PPAR α); these include dihomo- γ -linolenic, arachidonic, and eicosapentanoic acid.⁵² Other fatty acids that have significantly high affinity for PPAR γ (e.g., < 6.5 μ M IC₅₀) include palmitoleic, oleic, linoleic, and α -linolenic acids; all display high affinity for PPAR α (IC₅₀ < 2.0 μ M). All of these fatty acids, with the possible exception of arachidonic acid, which has its own isoform (ACS4), are good substrates for ACS1, which prefers C10–16 saturated fatty acids and C16–20 unsaturated fatty acids. These fatty acids would also be likely to regulate ACS1 gene expression through either or both PPAR α and PPAR γ isoforms, depending on the concentration of these PPARs and their co-activators in specific tissues.

5.5 Disease State-Specific Regulation of ACS1 Gene Expression

The cytokine tumor necrosis factor- α (TNF α) is an important physiological mediator of feeding behavior and lipid metabolism as well as functioning

as a key pro-inflammatory signaling molecule released from macrophages in response to endotoxin (lipopolysaccharide [LPS]).⁵³ TNF α is also produced by adipocytes and has been implicated as a mediator of insulin resistance by inhibiting insulin signaling and by acting as a lipolytic and anti-lipogenic cytokine to oppose insulin action.⁵⁴ TNF α has also been observed to de-differentiate adipocytes *in vitro*.^{53,55} Studies in 3T3-L1 adipocytes demonstrated that incubation with 5 nM TNF α for 24 h resulted in 75 to 90% downregulation of ACS1 mRNA, with a similar decline in the rate of ACS1 gene transcription²² (Fig. 5.4). Several other lipogenic enzymes are similarly regulated by TNF α , e.g., lipoprotein lipase⁵⁶ and stearoyl-CoA desaturase.²²

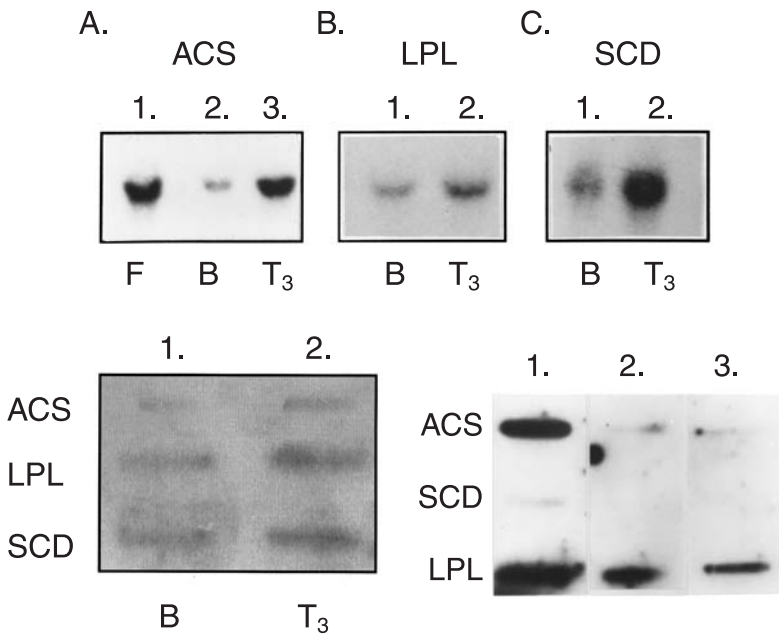


FIGURE 5.4

Effect of TNF α on the transcription rates of the ACS, SCD-1, and LPL genes. (From Weiner et al., *J. Biol. Chem.*, 266, 23527, 1991. With permission.)

The endotoxin (LPS)-stimulated release of the cytokines TNF α and interleukin-1 (IL-1) into the circulation results in increased hepatic lipid synthesis and inhibition of hepatic fatty acid oxidation.³⁸ Adipose triglyceride storage decreases and heart and muscle uptake of fatty acids decreases as well.³⁸ Studies by Memon et al. of the effects of *in vivo* administration of LPS, TNF α , and IL-1 on ACS1 mRNA expression and enzyme activity demonstrated that very low concentrations of LPS (1 to 100 μ g/100 g body weight) potently inhibited ACS1 mRNA expression in liver, adipose tissue, heart, and muscle³⁸ (Fig. 5.5). Hepatic ACS1 mRNA was decreased by 90% 4 h after LPS administration (100 μ g/100 g body weight) and was decreased in adipose tissue by

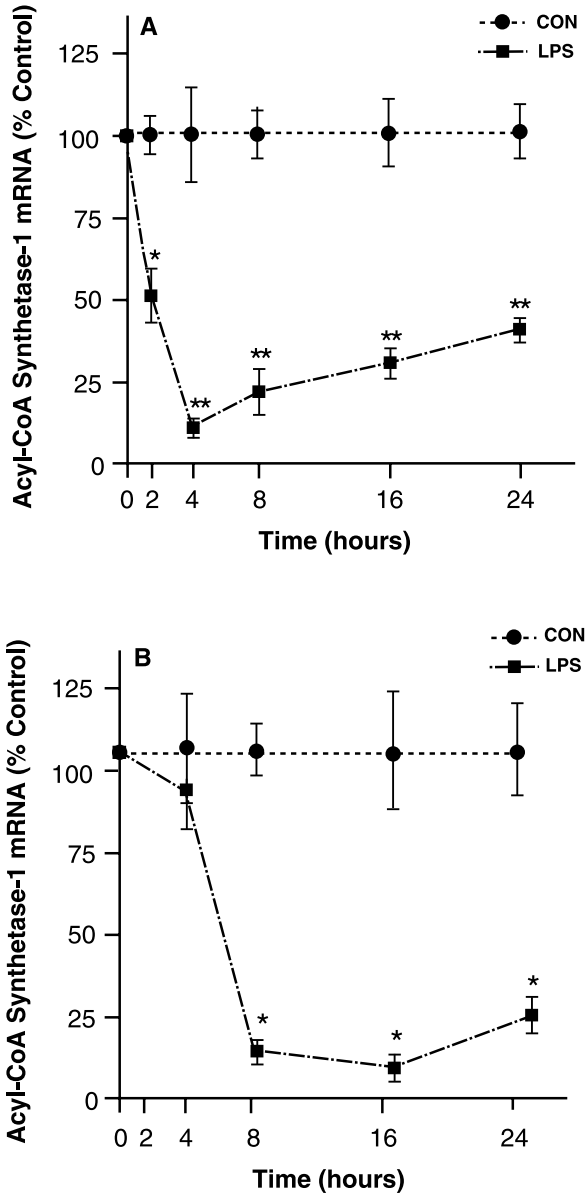


FIGURE 5.5

Time course of endotoxin (lipopolysaccharid (LPS)) effect on mRNA levels of ACS 1 in liver (A) and adipose tissue (B). Syrian hamsters were injected i.p. either with saline (controls) or LPS (100 μ g/100 g body wt.), and food was removed from both groups. At the indicated times, animals were euthanized and tissues were harvest for RNA isolation. ACS 1 mRNA levels were determined by Northern blotting. Values are means SE; $n = 5$ for each time point. (A) * $P < 0.002$; ** $P < 0.001$; (B) * $P < 0.001$. (From Memon et al., *Am. J. Physiol.*, 275, E66, 1998. With permission.)

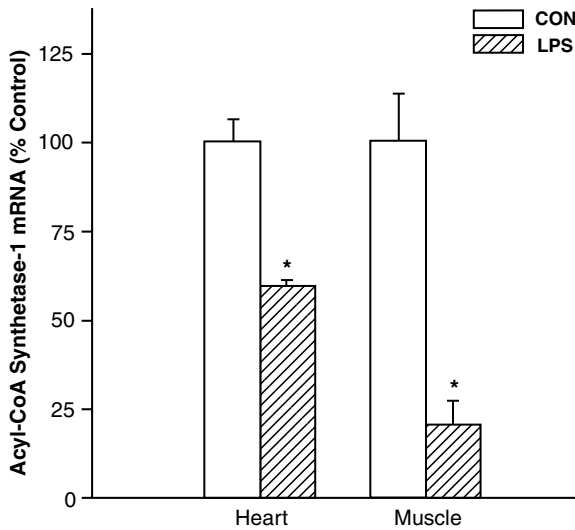


FIGURE 5.5 (CONTINUED)

(C) Effect of $\text{TNF}\alpha$ on the transcription rates of the ACS, SCD-1, and lipoprotein lipase genes. on ACS1 mRNA levels in heart and muscle. Syrian hamsters were injected i.p. either with saline (controls) or LPS (100 $\mu\text{g}/100\text{ g}$ body wt.), and food was removed from both groups. Sixteen hours later animals were euthanized and tissues were harvested for RNA isolation. ACS1 mRNA levels were determined by Northern blotting. Values are means $\pm$ SE; $n = 4$ for each group. * $P < 0.001$. (From Memon et al., *Am. J. Physiol.*, 275, E68, 1998. With permission.)

the same amount after 8 h, despite little change at 4 h; 16 h after LPS treatment ACS1 mRNA was 40% decreased in heart and 80% in muscle.³⁸ Similar results were obtained with administration of $\text{TNF}\alpha$ or IL-1 alone or in combination at concentrations that have previously been shown to mimic the metabolic effects of LPS. Administration of LPS also resulted in significant decreases in ACS1 activity in heart (38%), muscle (51%), and adipose tissue (54%) (Fig. 5.5). ACS1 activity was only 20% decreased in liver homogenates despite the very large mRNA decrease; however, LPS produced a 61% decrease in mitochondrial ACS1 activity and a 55% increase in microsomal activity. This compartmental shift or differential activation of the ACS1 enzyme is consistent with the lipogenic hepatic metabolic response to LPS because triacylglycerol synthesis is predominantly localized to microsomes and fatty acid oxidation to mitochondria. These effects appeared to be mediated primarily by interleukins-1 (IL-1).

A similar differential localization of ACS1 activity has been observed in livers of *ob/ob* mice, which are a null mutant for leptin production and are widely studied as an animal model of obesity, insulin resistance, and Type II diabetes.⁵⁷ In *ob/ob* mice 34% of hepatic ACS1 enzyme activity was associated with mitochondria and 66% with microsomes, in contrast to 61% mitochondrial association and 39% with microsomes in lean control animals⁵⁵ (Fig. 5.6). Adipose ACS1 mRNA expression was also higher in *ob/ob* animals.⁵⁷ These data are consistent with the increased hepatic production of the

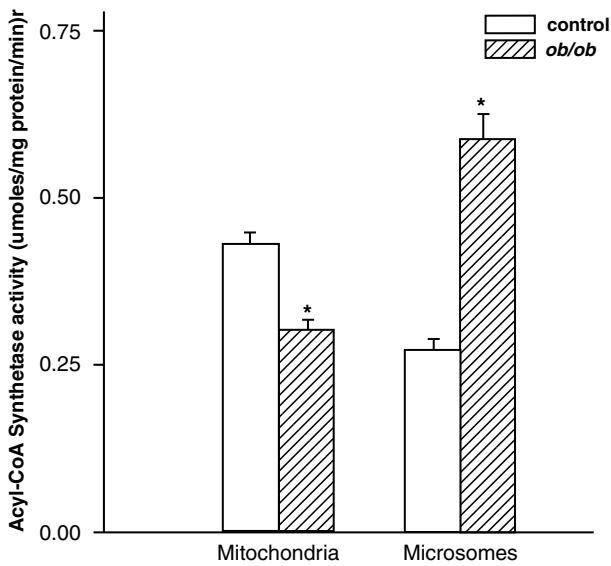


FIGURE 5.6

Mitochondrial and microsomal ACS activity in liver of *ob/ob* mice and their control littermates. Mice were killed and livers were obtained. Mitochondrial and microsomal fractions were isolated by differential centrifugation, and ACS activity was measured. Data are means SE, $n = 5$ for each group. * $P < 0.001$. (From Memon et al., *Diabetes*, 48, 126, 1999. With permission.)

triglyceride-rich lipoproteins and increased adipose lipid stores that are characteristic of the *ob/ob* phenotype.

In another leptin mutant, the hyperphagic Zucker (*fa/fa*) rat, ACS1 activity and mRNA have been shown to be 3.3- and 3.9-fold higher, respectively, in abdominal subcutaneous adipose tissue than in lean controls, and mesenteric ACS1 activity and mRNA concentrations were elevated 2.0- and 2.2-fold, respectively.⁵⁸ These data are consistent with the greatly increased adipose lipid synthesis and storage characteristic of the Zucker (*fa/fa*) rat. Liver ACS1 activity and mRNA levels were only modestly increased, but another previous study reported that in Zucker (*fa/fa*) liver acyl-CoA is much more utilized for esterification to triacylglycerol than for β -oxidation (80 vs. 20%, respectively), compared with lean control rats (40 vs. 60%).⁵⁹ These observations are also consistent with a subcompartmental shift of ACS1 enzyme activity from mitochondria to microsomes.⁵⁹ The mechanisms for the increased expression of the ACS1 gene in these animal models is unknown. Potential regulatory signals could include hyperinsulinemia, elevated free fatty-acid levels, and/or the lack of suppression by the defective leptin pathway. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is another model of obesity and insulin resistance that is characterized by visceral fat accumulation and hyperlipidemia prior to the onset of insulin resistance; at this stage, hepatic ACS activity and mRNA expression are also elevated.⁶⁰ In

another hyperphagic animal model of obesity, the VMH (ventromedial hypothalamus)-lesioned rat, ACS mRNA concentrations and enzyme activity are significantly elevated in both subcutaneous and mesenteric adipose tissue, which is consistent with any or all of these pathways as well.⁶¹

Excessive levels of free fatty acids and/or long-chain acyl-CoAs have been suggested as a major mechanism for several abnormalities that are characteristic of Type II diabetes, e.g., impaired glucose-stimulated insulin secretion, elevated basal levels of insulin, and impaired suppression of hepatic gluconeogenesis. We tested the effect of inhibiting ACS1 enzyme activity on basal and glucose-stimulated insulin secretion in the cultured β TC3 pancreatic β -cell line in the presence or absence of elevated levels of long-chain fatty acid. Treatment with 2 mM palmitic acid for 4 days induced ACS1 mRNA expression 2.4-fold and produced a 3.5-fold increase in basal insulin secretion. Treatment with the ACS1 inhibitor Triacsin C (50 μ M) for the last 24 h completely abolished the increase in insulin secretion.⁶² Treatment of the cultured β TC3 cells with Triacsin C (50 μ M) resulted in a 75 to 85% inhibition of glucose stimulated insulin secretion.⁶² In contrast, Newgard and co-workers found no effect of a lower concentration of Triacsin C (10 μ M) on glucose-stimulated insulin secretion in the INS-1 insulinoma line despite a 47% decrease in long-chain acyl-CoA and a potent inhibition of fatty acid oxidation.¹⁹

Inhibition of insulin secretion by long-chain acyl-CoA has been proposed to be mediated by effects on malonyl-CoA levels⁶³ or on intracellular Ca^{++} flux that triggers insulin release.⁶⁴ However, long-chain acyl-CoA levels must also be at a level permissive for budding and eventual exocytosis of insulin secretory vesicles.⁶⁵ It is possible that intracellular concentrations of long-chain acyl-CoA required to affect insulin secretion may vary among these regulatory mechanisms. The potential role and mechanisms of action of ACS1 in mediating metabolic defects in Type II diabetes that are associated with abnormal lipid metabolism clearly require more study.

The thiazolidinedione class of ligands that specifically activates PPAR γ has been demonstrated to induce the expression of the ACS1 gene and several other genes involved in fatty acid uptake in adipose tissue.⁴⁹ The compound BRL49653, which is a high-affinity thiazolidinedione ligand for PPAR γ , induced ACS1 mRNA 9-fold by 4 days of treatment. This coordinate induction has been proposed to lead to increased adipose fatty acid uptake, resulting in a free fatty acid “steal” from muscle that may improve insulin sensitivity and glucose metabolism.⁶⁶ However, ACS1 enzyme activity was not measured in these studies, and therefore, the ultimate effect of thiazolidinediones on ACS1 enzyme function has not been definitively characterized.

A recent study reports that the major metabolite of troglitazone, its sulfoconjugate derivative (M1) noncompetitively inhibits ACS1 enzyme in both mitochondria and microsomes, and thereby inhibits hepatic long-chain fatty acid oxidation and triglyceride synthesis.⁶⁷ Presumably the same effect would occur in adipose tissue, and if that were the case, an increase in ACS1 mRNA would be a compensatory feedback response to increased substrate accumulation due to insufficient available active enzyme. Another recent

study reports that troglitazone treatment of cultured pancreatic islets from Zucker diabetic fatty rats (ZDF) resulted in decreased fatty acid esterification and increased oxidation with a 38% decrease in ACS1 mRNA.⁶⁸ The inconsistencies among these studies are puzzling and serve to emphasize the need for further study of the regulation of tissue-specific expression of the ACS1 gene and gene product, especially in metabolic disease states, in order to understand its role in the pathophysiology of diseases that are major causes of morbidity and mortality, such as Type II diabetes, obesity, and dyslipidemias.

Last, the endproducts of the reaction catalyzed by ACS1, long-chain acyl-CoA esters, have been proposed to regulate multiple cellular processes in addition to insulin secretion, as discussed by Faergeman and Knudsen in their recent review.⁶⁹ Acyl-CoA esters have been observed *in vitro* to regulate many enzymes involved in lipid and energy metabolism. However, only acetyl-CoA carboxylase is inhibited and AMP-activated kinase is stimulated by nanomolar concentrations of long-chain acyl-CoA esters. A number of important metabolic enzymes are inhibited *in vitro* by micromolar concentrations of acyl-CoA esters, e.g., HMG CoA reductase, carnitine palmitoyltransferase I, (CPT I), long chain acyl-CoA dehydrogenase, hormone-sensitive lipase, adenine nucleotide translocase (ANT), glucokinase, glucose-6-phosphatase, and pyruvate dehydrogenase. However, it is not yet known whether these effects are physiologically relevant because intracellular concentrations of long-chain acyl-CoA under normal conditions are likely to be well under 200 nM, and probably below 10 nM. It has been proposed, however, that long-chain acyl-CoAs can be donated directly from a binding protein, e.g., acyl-CoA binding protein (ACBP) and fatty acid binding protein (FABP), since it has been observed that the acyl-CoA-ACBP complex can donate acyl-CoA to the acyl-CoA:lysophospholipid acyl transferase for β -oxidation in red blood cells.

In *E. coli*, long-chain acyl-CoA esters regulate gene transcription by preventing the binding of the *fadR* gene product to the *fadB* gene promoter, a regulon by which fatty acid biosynthesis and degradation are coordinately regulated. It is not known whether long-chain acyl-CoAs regulate transcription in a similar manner in mammals, but there is evidence that long-chain acyl-CoA esters may also be involved in regulating transcription of genes for lipid biosynthesis and metabolism in yeast. Both saturated and unsaturated long-chain acyl-CoA esters have been demonstrated to inhibit the binding of T3 to its nuclear receptor in rat liver (K_i approximately 0.45 μ M) more efficiently than the corresponding nonesterified fatty acids. Since T3 induces lipogenic enzymes, this action of long-chain acyl-CoA esters would provide negative feedback regulation when lipid synthesis and stores are sufficient.

Long-chain acyl-CoA esters have also been linked to the etiology of the insulin resistance that is a key feature of Type II diabetes and is also characteristic of obesity. For instance, high-fat feeding has been shown to induce insulin resistance in muscle, and there was a strong inverse correlation between glucose uptake and long-chain acyl-CoA concentrations in rat quadriceps muscle during a hyperinsulinemic glucose clamp study.⁷⁰ Furthermore, although hyperinsulinemia significantly suppressed circulating free fatty acid in these

high fat-fed rats, skeletal muscle intracellular fatty acid availability was not reduced, with long-chain fatty acyl-CoA esters remaining 2.3-fold above control levels.⁷¹ Further research is urgently needed to delineate the role of ACS1 and its acyl-CoA ester endproducts in the pathophysiology of insulin resistance. It has also been suggested that dysregulated ACS1 activity may be involved in triggering some of the pro-inflammatory^{20,72} and pro-oxidant⁷³ pathways that have been etiologically linked to the premature development of atherosclerosis in people with Type II diabetes.

In summary, we have described the structure of ACS1 as a member of an extensive gene family of long-chain acyl-CoA synthetases that are specialized by substrate specificity ranges and tissue and species distribution. The long-chain acyl-CoA synthetase 1 enzyme catalyzes an essential step in the activation of long-chain fatty acids for incorporation into lipid or for their metabolism. The expression of the ACS1 gene and its activity are tightly regulated by nutrients such as saturated and unsaturated long-chain fatty acids; by hormones such as insulin, T3, and corticosteroids; by cytokines such as TNF α ; and by ligands for the peroxisome proliferator-activated receptor (PPAR) family of nuclear steroid hormone receptors.

ACS1 has been demonstrated to act not only on naturally occurring long-chain fatty acids but also on pharmacologic substrates such as xenobiotic carboxylic acids, which include the peroxisome proliferator class of fibrate lipid-lowering compounds, and the profen (2-arylpropionate) class of nonsteroidal anti-inflammatory drugs such as naproxen. The ACS1 enzyme, which is membrane bound and requires ATP, CoA, and Mg²⁺, catalyzes a reaction that results in an endproduct, long-chain acyl-CoA, which itself is considered to have an important role in the regulation of multiple diverse cellular processes. These functions include coordinate feedback regulation of lipid, carbohydrate, and energy biosynthesis and metabolism; modulation of pancreatic insulin secretion and skeletal muscle sensitivity to insulin; and effects on atherogenic pro-inflammatory and pro-oxidant pathways.

Contrary to initial biochemical characterization of ACS1 as serving a constitutive cellular function, much evidence has emerged in recent years from studies utilizing recombinant technology that reveals an important and complex multifactorial regulatory role for acyl-CoA synthetase 1 and its long-chain acyl-CoA endproducts in health and disease.

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6

Nutritional Regulation of Fatty Acid Transport Protein Expression

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6.1 Introduction

The typical Western diet contains abundant triacylglycerol (TG), with average daily intakes of 90 to 100 gm.¹ The gastrointestinal tract efficiently digests the TG and absorbs the high levels of fatty acids produced, making fatty acid flux of great importance in the absorptive enterocyte. Several other cell types are also notable for high levels of fatty acid metabolism and transport, including the hepatocyte, muscle cell, and adipocyte. Virtually all other cell types, though not necessarily considered to be particularly active in lipid metabolism, must also process fatty acids for energy production, membrane phospholipid synthesis, and intracellular signal transduction. Thus, fatty acid transport into cells and within cells is a critical process that contributes to the utilization and function of this important nutrient. Perhaps not surprisingly, the intake of dietary lipid has been shown, in turn, to influence the expression of proteins involved in fatty acid transport.

Cellular proteins that are designated as fatty acid transport proteins consist of two types: transmembrane proteins and intracellular cytoplasmic proteins. It is generally thought that the former are involved in uptake and efflux of fatty acids across the plasma membrane (and, perhaps, organellar membranes, although little information in this regard is available), while the latter are involved in intracellular fatty acid transport and targeting. In this chapter, an overview of the basic biochemical and functional properties of the fatty acid transport proteins will be presented, followed by discussion of the nutritional regulation of expression of these two classes of proteins.

6.2 Mechanism of Fatty Acid Flux across the Plasma Membrane

The diverse metabolic roles of long-chain fatty acids in the maintenance of intracellular energy stores, post-translational modification of proteins, and the synthesis of membrane lipids have led to careful examination of how these lipophilic compounds traverse the plasma membrane of cells. Historically, two distinct mechanisms have been proposed: a diffusion-driven flux across the membrane and protein-mediated transport mechanisms. The diffusion mechanism holds that unbound unesterified fatty acids bind to the outer leaflet of the membrane bilayer, become protonated, and then flip to the inner leaflet.^{2,3} However, kinetic evidence has suggested that a diffusional mechanism may be too slow to accommodate the high cellular requirements for FA,⁴ and numerous other studies support a protein-mediated uptake process. For example, fatty acid uptake to cells has been shown to be rapid, temperature sensitive, and saturable, and can be inhibited by FA analogs,

TABLE 6.1

Fatty Acid Transport Proteins

	Tissue Distribution	Size	Ref.
<i>Membrane Associated</i>			
FABPpm	Liver, heart, adipose, intestine	40 kDa	10
FAT/CD36	Heart, skeletal muscle, adipose, intestine	88 kDa	15
FATP	Adipose, brain, skeletal muscle, heart	63 kDa	9
Caveolin-1	3T3-L1 adipocytes, fibroblasts, endothelial, etc.	22 kDa	30
FAR	Heart	56 kDa	32
<i>Intracellular</i>			
LFABP	Liver, small intestine	14 kDa	150, 151
IFABP	Small intestine	15 kDa	152
HFABP	Muscle, brain, mammary gland, kidney, adrenals, ovaries, testis	15 kDa	153
AFABP	Adipose, monocytes	15 kDa	154, 155
BFABP	Central nervous system	15 kDa	40, 156
KFABP	Epidermis, adipose, mammary, testis	15 kDa	41, 157
MFABP (myelin P2)	Central and peripheral nerve myelin	15 kDa	158

blocked by specific antibodies, and inhibited by proteases.⁵⁻⁹ These investigations have led to the identification of a series of membrane-associated proteins thought to be involved in FA transport across the plasma membrane (Table 6.1). It is currently thought that these high-affinity protein-mediated transport systems work in conjunction with a low-affinity diffusional process to affect fatty acid influx and/or efflux across the plasma membrane of cells. The following discussions will focus on FA transporters identified in mammalian systems.

6.2.1 FABPpm

Stremmel and Berk identified the first membrane-associated fatty acid carrier protein from a solubilized rat hepatocyte plasma membrane preparation using substrate affinity chromatography.⁶ A 40-kDa protein was purified based on its specific binding to an oleate-agarose column and was named FABPpm. This protein was also shown to reversibly bind a variety of unesterified long-chain FA. Interestingly, FABPpm was determined to be a membrane-bound form of the mitochondrial enzyme, aspartate aminotransferase (mAspAT).¹⁰ FABPpm is expressed on the plasma membrane of a variety of tissues, predominantly the liver, heart, adipose, and intestine.^{10,11}

Other than ligand-binding properties, direct evidence for the involvement of FABPpm in cellular fatty acid transport has been obtained via a series of antibody studies. Incubation of hepatocytes, myocytes, or adipocytes with anti-FABPpm (or anti-mAspAT) led to approximately 50% reductions in [^3H]-FA uptake into these cells.^{10–12} In addition, expression of FABPpm in both *Xenopus laevis* oocytes and 3T3 fibroblast cells corresponded with a marked increase in FA uptake rates for these cell systems.^{11,13} Transfection of the fibroblasts resulted in a saturable, high-affinity uptake process which was inhibited by anti-FABPpm antibodies, thus demonstrating that FABPpm plays some direct role in modulating FA flux across the plasma membrane of 3T3 fibroblasts.^{11,14}

6.2.2 Fatty Acid Translocase (FAT/CD36)

Abumrad and colleagues employed a series of reactive sulfo-*N*-succinimidyl fatty acid derivatives in identifying an 88-kDa protein involved in FA uptake in rat adipocytes, designated as fatty acid translocase (FAT).⁸ The radiolabeled protein was purified and its cDNA isolated from a rat adipocyte library, and was found to be the rat homolog of human CD36, a thrombospondin-binding protein first isolated in 1989.¹⁵ FAT/CD36 is a large glycoprotein with two putative transmembrane domains. It can bind native long-chain FA with high affinity but does not bind medium-chain FA, in accordance with the specificity of FA transport in adipocytes.^{16,17} Inspection of the amino acid sequence has highlighted a region of similarity between a 150 residue extracellular domain of FAT/CD36 and the fatty acid-binding domain of the intracellular heart fatty acid-binding protein (HFABP, see below), suggesting a potential region for FA binding to FAT.^{16,18} FAT mRNA expression is associated with cells that have a high metabolic capacity for long-chain FA utilization, with levels highest in adipose, cardiac muscle, skeletal muscle, intestine, and spleen.¹⁵ In adipocytes, FAT/CD36 expression is induced during adipocyte differentiation.¹⁹

A direct role for FAT/CD36 in FA transport across the membrane has been supported by several different experimental approaches. For example, anti-CD36 antibodies inhibit the uptake of arachidonic acid into platelets.²⁰ In addition, transfection of Ob17PY fibroblasts, which lack CD36, with the gene for CD36 results in an increased rate of long-chain FA uptake and subsequent metabolism of the FA. The magnitude of the increased uptake correlates with levels of expressed CD36 and the uptake is a saturable, high-affinity process.²¹ Diminished levels of CD36, secondary to antisense expression, resulted in diminished FA uptake rates,²² and patients with a congenital absence of CD36 displayed defective FA uptake into the heart.²³ Recently, mice lacking expression of CD36 were shown to have decreased fatty acid uptake into adipocytes, and myocytes isolated from the knockouts were found to have decreased oleate transport rates at low FA:albumin ratios.²⁴ These studies provide the most direct evidence thus far that FAT/CD36 plays an integral role in cellular fatty acid flux and metabolism.

6.2.3 Fatty Acid Transport Protein (FATP)

A third putative FA transport protein was identified from adipocytes by Schaffer and Lodish using expression cloning.⁹ A cDNA library constructed from 3T3-L1 adipocytes was transfected into COS7 fibroblast cells, which were then screened for their ability to incorporate a fluorescently labeled fatty acid analog by fluorescence-activated sorting. This approach led to the identification of a cDNA that encodes a 63-kDa protein that was named fatty acid transport protein (FATP).⁹ Hydropathy modeling of the predicted amino acid sequence of FATP identified 4 to 6 potential transmembrane regions⁹ and immunohistochemistry has demonstrated that the protein is localized to the cell surface.^{9,25} Recent evidence suggests that FATP represents a large evolutionarily conserved gene family found to be functional long-chain fatty acid transporters in such diverse organisms such as *Fugu rubripes*, *Drosophila melanogaster*, and *Caenorhabditis elegans*.²⁶ In humans there are at least six FATP homologue genes that demonstrate tissue specific expression.²⁶ FATP contains a highly conserved ATP-binding and hydrolysis domain that is essential for its FA transport activity,²⁷ and displays 40% identity with very-long chain acyl-CoA synthetase.^{19,27} Studies wherein its yeast homolog, FAT1, was disrupted, demonstrated that FAT1 has cellular acyl-CoA synthetase activity against very-long and long-chain fatty acids and its disruption results in the accumulation of very-long chain fatty acids.²⁸ Still, the role of coenzyme activation in FATP-mediated fatty acid transport across the membrane is not fully understood. FATP mRNA levels are highest in tissues with high FA requirements: brain, adipose tissue, skeletal and heart muscle.⁹ Specific involvement of FATP in FA transport has been supported by studies on stably transfected fibroblast cell lines, in which a marked increase in the specific and saturable uptake of fluorescently labeled long-chain FA⁹ was observed, indicating a role for FATP in FA flux.

6.2.4 Other Putative Transporters: Caveolin-1 and FA Receptor (FAR)

Two additional putative transporters have been identified but not well characterized. First, in studies of 3T3-L1 preadipocyte differentiation, Trigatti et al.⁷ and Gerber et al.²⁹ used a photoreactive fatty acid analog to identify proteins with high affinities for lipids. A 22-kDa plasma membrane protein was labeled by 11-m-diazirinophenoxy[11-³H]undecanoate in a specific and saturable manner. Subsequent immunohistochemical studies demonstrated that this protein was caveolin-1,³⁰ a structural component of membrane caveolae, involved in receptor-mediated transport.³¹ Second, a 56-kDa protein which exhibits high affinity for binding to fatty acids has been purified from rat cardiac sarcolemmal membranes and has been named fatty acid receptor (FAR). Initial biochemical characterization of FAR revealed it to be a single, amphiphilic polypeptide with high-affinity binding for long-chain FA.^{32,33} Additional information on this protein is not available but there is some speculation in the literature that it may, in fact, be FATP.¹⁹ Functional involvement of caveolin-1 and FAR in cellular fatty acid flux is currently uncertain.

6.3 Intracellular Fatty Acid-Binding Proteins

The intracellular fatty acid-binding proteins (FABPs) are 14 to 15-kDa proteins that were discovered in the early 1970s as abundant cytoplasmic proteins that bind long-chain fatty acids *in vitro*.^{34,35} (See Table 6.1.) FABPs from intestine (IFABP),^{34,36} liver (LFABP),^{34,35} and heart (HFABP)³⁷ were the first of the family to be well characterized. A specific FABP has also been purified from another lipid-active tissue, adipose tissue (AFABP),³⁸ as well as from several other tissues such as nervous system and skin (BFABP, KFABP).^{39–41} It is now known that the FABPs encompass a growing list of related proteins, some of which bind small hydrophobic ligands other than, or in addition to, fatty acids.^{42–44} Information on their tissue distribution, ligand-binding affinities and specificities, and developmental regulation^{18,42,45,46} has led to the rather broad hypothesis that the FABPs are important in intracellular lipid metabolism and transport. The function of the FABPs in intracellular transport and targeting has recently been reviewed.⁴⁷

Equilibrium binding analyses and structural studies clearly demonstrate that most FABPs bind long-chain fatty acids (C_{16} – C_{20}) with high affinity and a molar stoichiometry of 1:1.^{48–51} The most prominent exception is LFABP, which binds other acyl ligands as well as long-chain fatty acids, and binds fatty acids at a molar ratio of 2:1.^{52–57} Sensitive fluorimetric assays have demonstrated previously unrecognized differences in equilibrium binding affinities of various FABPs for different fatty acids. For example, lower ligand binding affinities for the adipocyte FABP relative to other FABPs have been reported.⁵⁸ Affinities of most of the FABPs are generally highest for palmitate, oleate, and stearate, and increased water solubility of polyunsaturated long-chain fatty acids is reflected by higher K_d values.^{58,59}

X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy have been used to obtain high-resolution structures for several of the FABPs. All of the proteins appear to have the same basic tertiary structure, being composed of 10 antiparallel β -strands which form a barrel-like configuration containing the ligand-binding cavity, with the barrel capped by two short α -helical segments.¹⁸ The helices and the closely appositioned β -turns are often referred to as the portal region of the FABP, where it is hypothesized that ligands enter and exit the binding cavity. Despite their similar tertiary structures and their interactions with long-chain fatty acids, abundant circumstantial evidence suggests that different FABPs may have different intracellular functions. Unlike other intracellular lipid-binding proteins such as the acyl CoenzymeA-binding protein (ACBP) and several phospholipid transport proteins, which do not exhibit singular tissue-specific forms, the FABP family has diverged to express a large number of tissue-specific homologues. Indeed, in the intestinal absorptive cell high and approximately equivalent levels of two separate FABPs are expressed,⁶⁰ further suggesting a functional specificity for different family members. Differences in ligand-binding properties, fatty acid transfer mecha-

nisms, and different patterns of tissue specificity and developmental expression provide further indication of functional diversity.^{47,58,61}

6.4 Nutritional Regulation of Fatty Acid Transport Proteins

As might be anticipated from their roles in nutrient metabolism, it is likely that both the plasma membrane and intracellular classes of fatty acid transport proteins are modulated by nutritional factors. In the case of the transmembrane transporters, that link is only beginning to be explored. More information is available for the intracellular FABPs. With the likelihood very high for functional diversity among the various intracellular FABPs, it is perhaps not surprising that they are not coordinately regulated by nutritional factors, as will be shown below.

6.4.1 Regulation of Membrane-Associated Fatty Acid Transporter Expression

Extensive studies examining the effect of diet on the levels of putative membrane transporters have not yet been published. Studies employing whole animal models, however, have provided some information regarding overall regulation of these proteins. For example, FABPpm expression is markedly elevated in the white adipose tissue of Zucker diabetic and obese (*fa/fa*) rats.⁶² These rats have lost insulin sensitivity and therefore an increased level of FABPpm mRNA in these animals indicates insulin is a negative regulator of FABPpm expression. The insulin effect in these animals appears to be tissue sensitive, as there was no difference in FABPpm levels in the liver of these rats.⁶² No information regarding the presence or absence of an insulin-responsive element (IRE) upstream of the FABPpm coding region is available to date. FABPpm levels are also affected by the FA utilization requirements of the tissue. FABPpm levels have been shown to increase in oxidative skeletal muscle during fasting, a metabolic state that necessitates high FA utilization.⁶³ In addition, studies in humans have demonstrated that the protein is induced in skeletal muscle with prolonged endurance training, again concomitant with an increased need for FA utilization.⁶⁴⁻⁶⁶

Similar to FABPpm, FATP mRNA levels can also be increased markedly by nutrient depletion. This marked increase was observed in murine adipose tissue and has been attributed to an insulin effect at the level of transcription, with insulin downregulating FATP mRNA.⁶⁷ An insulin-responsive element (IRE) for FATP has been identified, and is similar to other known insulin-responsive regulatory sequences.^{67,68} In accordance with the presence of an insulin-control element, FATP expression is elevated in the adipose tissue of the insulin-resistant Zucker diabetic and obese *fa/fa* rats.⁶² FATP expression

is also under the control of peroxisome proliferating agents, which include fatty acids, and will be discussed below.

FAT/CD36 expression is also altered in a diabetic state. For example, CD36 protein levels are increased several-fold in NOD diabetic mice⁶⁹ and CD36 mRNA levels are slightly elevated in the Zucker diabetic and obese rats, although not to the extent that the FABPpm and FATP mRNA levels are increased.⁶² Further clarification of the regulatory relationship between FAT/CD36 expression and insulin is required.¹⁹ The distribution of FAT mRNA along the gastro-colic axis is greatest in the jejunum, the main site of fatty acid absorption, and its mRNA levels are regulated by the intake of dietary fat. Specifically, high fat diets administered to rats increase FAT mRNA levels, with a diet rich in long-chain fatty acids increasing levels most dramatically.⁷⁰ The effect of dietary fats may be due directly to the action of a series of fatty acid-responsive nuclear receptors. Indeed, both fatty acids and other lipophilic compounds such as peroxisome proliferators induce FAT mRNA expression,^{71,72} and the gene appears to be regulated directly by these ligands, as will be discussed below.

6.4.2 Regulation of Intracellular FABP (IFABP) Expression

From their earliest identification approximately 30 years ago, the FABPs have been proposed as central to the cellular disposition of dietary lipid, in that tissues with high rates of fatty acid uptake, metabolism, and storage have high FABP levels.^{46,73} Particularly supportive of the relationship between diet and FABP expression is the intestinal distribution of LFABP and IFABP, from duodenum-to-colon and crypt-to-villus tip, which correlates well with the distribution of dietary lipid absorption and intracellular processing.^{74–76} It would, therefore, seem logical to examine the influence of diet, particularly the amount and type of dietary fat, on the abundance of FABP protein in various lipid-active tissues. Although a limited number of studies in rodents have examined effects of dietary fat and carbohydrate on FABP levels, primarily on the two major gastrointestinal FABPs, liver FABP and intestinal FABP, surprisingly little information which directly addresses the nutritional regulation of the FABPs is available. This could be due to the relatively modest effects of diets that have been reported, which may have dampened enthusiasm for further investigation. The abundance of the FABP protein itself in various cell types is likely to contribute to the modest scale of nutritional regulation. Several of the FABPs have been estimated to comprise as much as 6% of soluble protein.^{77–80} It would seem, therefore, that increases of manyfold would not be expected. Further, the turnover rate for the LFABP was found to be relatively slow, with a half-life of 3.1 days, thus making acute regulation by dietary influence less likely.⁸¹ It should be kept in mind, nevertheless, that owing to their great abundance even modest changes, as expressed on a percentage or “fold” basis, represent quite large changes in absolute amounts of FABP.

6.4.2.1 Liver FABP

Of all the FABPs, the nutritional regulation of LFABP levels has been the most extensively investigated. Studies for the most part have examined dietary fat level rather than fat type, and have demonstrated a positive correlation between lipid intake and liver and intestinal LFABP levels. In comparison to a chow-based 5% fat diet, high fat diets consisting of 20 to 38% vegetable oil consumed for 3 to 4 weeks increased the level of liver FABP by 40% in liver, 30% in jejunum, and 50% in the ileum.⁷³ Since the ileal abundance of LFABP in control animals is approximately one third that of jejunal LFABP, it was reasoned that the larger relative change in the distal small intestine represents an adaptive response so as to increase the ability of the intestine to absorb and process the additional lipid, whereas the already high levels of LFABP expression in proximal intestine are only modestly increased by additional dietary fat.⁷³ Studies in Zucker rats⁸² and pigs⁸³ have also found that high fat diets increase hepatic LFABP levels. Recently, Veerkamp and van Moerkerk examined the effects of diets containing 5 to 40% of energy as fat, and the results suggested a dose-dependent increase in LFABP content, with approximately 25% greater liver levels on the highest fat intake. A comparison of diets containing 10% of calories as corn oil (high n-6 PUFA), menhaden oil (high n-3 PUFA), or lard (high SFA) showed no differences in liver LFABP level.⁸⁴

Few studies have reported the regulation of LFABP mRNA levels by diet. In the adult hamster, Lin and colleagues found that a high fat diet resulted in approximately 80% and 40% increases in LFABP mRNA in liver and intestine, respectively.⁸⁵ Poirier et al.⁸⁶ also found that proximal small intestinal LFABP mRNA and protein levels were elevated following sunflower oil feeding. Interestingly, they found similar effects after infusing very small quantities of linoleic acid, the primary fatty acid found in sunflower oil. Changes in LFABP expression as a function of development indirectly suggest that the LFABP gene is induced by dietary fat content. The mRNA for LFABP in the liver and intestine increases markedly at birth, concomitant with the onset of suckling and a high-fat maternal diet.⁸⁷ A 20-fold increase in LFABP protein level between fetal and adult liver has also been shown; however, an abrupt increase in mRNA at birth was not observed.⁸⁸

In contrast to the effects of a high fat diet, fasting decreases LFABP expression. A 48-hour fast resulted in a marked decrease in liver content of LFABP, in parallel with total protein, such that the relative abundance of LFABP remained unchanged.^{81,89} A decline in hepatic LFABP and its mRNA following a 3-day fast has also been shown using immunocytochemistry and *in situ* hybridization, respectively.⁹⁰ Interestingly, fasting did not cause a relative decrease in LFABP levels in the intestine. Furthermore, it resulted in the appearance of LFABP in villus crypt cells, from which it is typically absent.^{76,91} It has been suggested that the increased expression in crypt cells could be secondary to the increased plasma concentrations of fatty acid that accompany the starved state.^{91,92}

A protocol of fasting followed by high carbohydrate refeeding, well known to cause the rapid induction of many lipogenic enzymes, resulted in little change in the cytosolic concentration of LFABP⁸¹ in accord with its relatively long half-life. Consistent with these results, a 60% sucrose diet fed for several weeks did not alter liver LFABP levels.⁷³ The fact that LFABP levels increase secondary to a high fat diet, and do not respond to a lipogenic diet, implies that LFABP expression may be regulated by exogenous fatty acid but not endogenous fatty acid.⁷³ Nevertheless, insulin, which is lipogenic, appears to be involved in the expression of LFABP, as streptozotocin-diabetic rats were found to have decreased LFABP expression.^{93,94} Effects of insulin may be indirect, given that an insulin-responsive promoter element has not been identified in the LFABP gene.

As will be shown below, the regulation of LFABP expression by lipid is likely mediated via peroxisomal proliferator activated receptor (PPAR) transcription factors. Among the ligands that are now known to bind to these nuclear hormone receptors are long-chain fatty acids, thereby providing a mechanism by which the ligand modulates expression of its binding protein. Nevertheless, intestinal isograft studies have demonstrated that LFABP expression is not solely dependent on signals related to lipid flux. Rubin et al.⁹⁵ implanted fetal proximal small intestinal isografts into the subcutaneous tissues of adult mice, where luminal signals are absent, and found appropriate proximal-distal and crypt-villus positional expression of the LFABP gene. In addition, the proximal-to-distal gradients for LFABP and IFABP were seen at day 17 of gestation in the mouse, a time when exposure of the intestinal mucosa to luminal fat is quite low.⁹⁶

6.4.2.2 Intestinal FABP

The correlation of intestinal IFABP expression with the anatomical localization of dietary lipid absorption,⁹⁷ the higher expression of IFABP in villus compared to crypt cells,⁶⁰ and the increased accumulation of IFABP mRNA at the onset of the suckling period,⁸⁷ all support a causal relationship between dietary fat and IFABP expression. Early studies of intestinal FABP showed no effect of high fat feeding on jejunal levels and a 40% increase in ileal levels in rats fed a high fat diet, with little effect or even a decrease in intestinal FABP levels in animals fed a high sucrose diet.³⁶ Fasted rats were found to have a sparing of intestinal FABP levels relative to total intestinal protein,³⁶ perhaps indicative of the need for maintaining maximal absorptive capacity. In contrast, a 16% fat diet, relative to an 8% diet, had no effect on IFABP mRNA levels in any segment of mouse small intestine.⁸⁶ Thus, there is a somewhat uncertain relationship between IFABP expression and luminal fat. Studies of the IFABP gene in intestinal isografts demonstrated the establishment of positional expression in the absence of exposure to luminal contents,⁹⁵ indicating that for IFABP, as for LFABP, expression is regulated by both nutritional and non-nutrient-related means.

Unlike the 2- to 3-fold increases of liver and intestinal LFABP expression in clofibrate-treated rats,^{73,77} intestinal IFABP levels were increased by only 25%,⁷⁷ and no peroxisome proliferator-responsive element (PPRE) has been reported in the promoter region of the IFABP gene. Thus, for IFABP, the molecular mechanisms of dietary lipid modulation, if indeed present, are not known.

6.4.2.3 Adipocyte FABP

Our understanding of the nutritional regulation of AFABP expression has come mostly from studies using cultured adipocyte cell lines, with only a modest amount of information available about the dietary regulation of AFABP expression in animal models. Rats fed a high fat diet had a 50% increase in adipose tissue AFABP content,⁸⁴ a robust effect given existing high levels of AFABP expression. The involvement of insulin was first shown by studies in streptozotocin-diabetic rats, where AFABP protein and mRNA levels were decreased, and the changes reversed by insulin administration.⁹⁸

Analysis of the promoter region of the AFABP gene in cultured cells has shown that the activation of expression by insulin is likely to occur indirectly, via CCAAT/enhancer-binding protein (C/EBP α)-stimulated transcription.^{99,100} Insulin-like growth factor I, glucocorticoid, fatty acids, and agents that increase intracellular cAMP levels also increase AFABP expression.^{101–105} Addition of oleic acid to confluent preadipocytes treated with the glucocorticoid analogue dexamethasone was shown to potentiate the increase in AFABP mRNA levels.¹⁰¹ Treatment of preadipocytes with long-chain FA results in a dramatic (>20-fold) increase in AFABP mRNA levels.¹⁰⁶ As will be discussed further, AFABP expression is also regulated by the transcription factors peroxisomal proliferator activated receptor γ (PPAR γ) and fatty acid activated receptor (FAAR, also termed PPAR δ). Thus, the lipid effects noted above are likely mediated via these nuclear receptors.

6.4.2.4 Heart FABP

Heart and skeletal muscle utilize long-chain fatty acids to provide a majority of their oxidative requirements. Studies of the regulation of HFABP expression have, therefore, focused on conditions under which mitochondrial β -oxidation is modified.⁸⁴ For instance, higher levels of HFABP are expressed in red gastrocnemius muscle than white, and expression increases following endurance training, both situations that parallel the relative mitochondrial β -oxidation levels.^{84,107–109} In a murine muscle cell line, HFABP content was increased in differentiated relative to undifferentiated cells, in parallel with the expression of creatine kinase activity and fatty acid β -oxidation.¹¹⁰

Little information is available about the regulation of HFABP expression by diet, and the available data are somewhat contradictory. Early studies by

Fournier and Rahim showed a 30% increase in heart HFABP levels following a high fat diet,¹¹¹ whereas more recently others found no change in HFABP levels in rats fed diets ranging from 5 to 40% fat.⁸⁴ It has also been reported that HFABP expression in mammary gland is increased upon feeding a high fat diet.¹¹² In contrast to results for LFABP and IFABP, fasting was reported to result in an increase in the level of HFABP expression in heart, via an increase in gene transcription rate.¹¹³

The developmental expression of HFABP may also offer some insight into its potential for nutritional regulation. HFABP mRNA levels rise rapidly in rat heart 2 days prior to birth, and continue to rise during the high-fat diet suckling period. They reach a peak at the initiation of weaning, upon transition from the maternal high fat diet, and then decline slightly into adulthood. It was noted that these changes parallel those in cardiac mitochondrial β -oxidative capacity and mitochondrial abundance.¹¹⁴

A number of investigators have reported that heart HFABP levels increase during experimental diabetes mellitus.^{113,115,116} Although it would appear that the mechanism of this increase might be the same as that observed in starvation, the increase appears to be secondary to an increase in mRNA turnover time rather than to increased transcription rate.¹¹³ Unlike HFABP expression in the heart, HFABP expression in the aorta was found to be decreased in streptozotocin-diabetic rats, and this was reversed upon insulin treatment.¹¹⁷

The mechanism(s) of nutritional regulation of HFABP expression are unknown at present. Peroxisome proliferators have little or no effect on the levels of HFABP in heart and skeletal muscles, and little information is available about the promoter region of the HFABP gene.^{84,118} The existing literature supports the suggestion that the modulation of HFABP abundance is consistent with a role in fatty acid metabolism, particularly with the capacity of tissues to oxidize fatty acids for energy. The signals that affect this regulation remain to be revealed.

6.4.2.5 Other FABPs

Minimal information is available about the nutritional regulation of other members of the FABP family. Ibrahim et al. reported an increase in the expression of KFABP (skin type) in Ob1771 adipocytes incubated with fatty acids or thiazolidinedione antidiabetic agents,¹¹⁹ and it is thus likely that the KFABP promoter region will be found to contain a PPRE. Studies relevant to the nutritional regulation of myelin P2 protein (MFABP) and BFABP are not available at present. Of interest, however, is that these two proteins are maximally expressed during cellular growth and differentiation, when neuronal cells undergo abundant membrane biogenesis, rather than in fully mature cells, so signals regulating their expression are likely to be different than those regulating expression of other FABPs that are more highly expressed in differentiated cells.

6.5 Molecular Mechanism of Fatty Acid Regulation of Transport Proteins

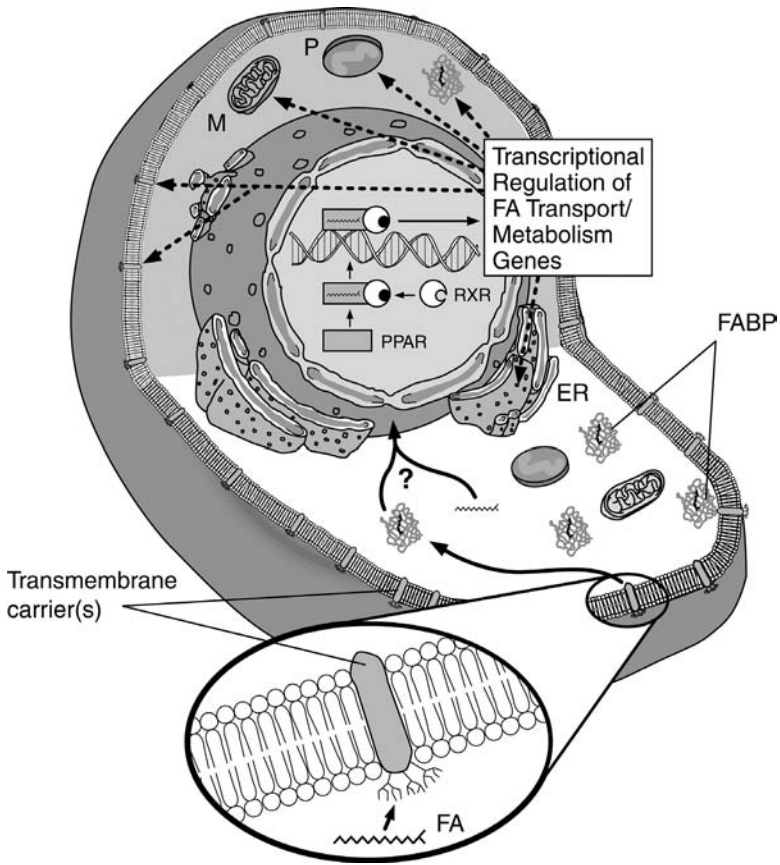
The gross nutritional regulation of fatty acid transport proteins discussed above is likely facilitated in large part by a nuclear phenomenon whereby dietary ligands affect gene transcription through binding to a class of nuclear receptors. The role that fatty acids may play in nuclear transcriptional events and, in turn, in overall cellular homeostasis is summarized schematically in Fig. 6.1.

6.5.1 Peroxisome Proliferator-Activated Receptors

Peroxisome proliferation is a complex cellular phenomenon initiated by a class of structurally diverse compounds such as fibrates, herbicides, and phthalate plasticizers that increase peroxisomal β -oxidation of fatty acids. In 1990 the effects of these peroxisomal proliferating agents was shown to be mediated via a nuclear receptor.¹²⁰ Based on sequence homologies, this receptor was concluded to belong to the steroid-hormone superfamily of nuclear receptors and was named the peroxisome proliferator-activated receptor (PPAR). Thus far, three major PPARs, α , δ (also referred to as β , NUC-1, or FAAR) and γ have been cloned from multiple species, including *Xenopus*, rodents, and humans.^{121–123} Each of the PPAR forms is coded by a separate gene and has a distinct tissue distribution. The PPAR receptors have retained the main functional domains of the other members of the steroid-hormone superfamily of receptors. Of note, they have a highly conserved DNA-binding domain and a multifunctional ligand-binding domain, which is responsible for ligand binding, dimerization with other receptors, and nuclear localization. Like other members of this superfamily, PPARs act at the nuclear level via ligand-induced binding to cognate DNA sequences on particular genes. These highly conserved DNA sequences on the target genes are referred to as peroxisome proliferator responsive elements (PPREs).^{121–123}

PPAR α was the first member of the subfamily to be cloned and is expressed mainly in tissues with high catabolic rates for fatty acid and peroxisomal metabolism (liver, kidney, heart, intestinal mucosa, and brown adipose tissue). PPAR δ is also an abundant and widely distributed protein whereas PPAR γ displays a distribution limited primarily to white adipose tissue and immune cells. The role of PPAR δ is not as well understood as the other two. The discussion here will focus on the role of PPARs in the regulation of fatty acid transport proteins and thus will concentrate on known functions of PPAR α and PPAR γ .^{121–125}

PPAR ligands include a broad array of both natural and pharmacological compounds. PPAR α binds a variety of endogenous fatty acids,

**FIGURE 6.1**

Fatty acid transport and fatty acid-induced nuclear regulation of transport protein genes. Schematic diagram of a fatty acid (FA) entering a mammalian cell through a putative transmembrane carrier, binding to the intracellular fatty acid binding proteins (FABPs) and moving to the nucleus. The mechanism(s) by which fatty acid enters the nucleus is unknown at this time. Upon entering the nucleus, the FA binds to a peroxisome proliferator-activated receptor (PPAR) that dimerizes with holo-retinoic acid X receptor (RXR). This heterodimer recognizes and binds to a peroxisome proliferator-responsive element (PPRE) on target DNA, thereby effecting transcription of a downstream coding region. In particular, the expression of membrane fatty acid transporters and FABPs as well as many genes involved in both mitochondrial (M) fatty acid metabolism and peroxisomal (P) fatty acid oxidation are mediated via PPRE elements.

although it demonstrates a preference for the C18 unsaturated fatty acids such as oleic (18:1) and α -linoleic acid (18:3), with binding affinities of approximately 5 nM, thought to be well within the physiological range.^{126,127} PPAR α is also activated by naturally occurring eicosanoids, such as leukotriene B₄, a mediator of the inflammatory response, and by 8-hydroxyeicosatetraenoic acid, a product of the lipoxygenase pathway. Synthetic ligands for PPAR α include the fibrates, a class of hypolipidemic

drugs widely prescribed for the treatment of hypertriglyceridemia. Recent competitive binding studies have demonstrated that naturally occurring fatty acids were actually more potent ligands for human PPAR α than the synthetic fibrate WY-14,643, previously thought to be the highest-affinity ligand for PPAR α .^{126,127}

As with PPAR α , PPAR γ demonstrates broad ligand specificity. Natural ligands for PPAR γ include prostaglandins A, D, and J, and certain mono- and polyunsaturated FAs, in particular, linolenic (18:2), linoleic (18:3), and arachidonic acid (20:4).¹²⁸ Synthetic activators of PPAR γ include the insulin-sensitizing thiazolidinedione drugs used as oral antidiabetic agents.

6.5.2 Mode of Action — Ligand Binding and Cofactor Proteins

In order to affect the expression of target genes, ligand bound forms of both PPAR α and PPAR γ dimerize with the 9-*cis*-retinoic acid receptor, RXR, when it is also bound by ligand. This receptor dimer is the functional unit that recognizes and binds the PPRE on target DNA, thereby affecting transcriptional activity. RXR heterodimers are required for other members of the steroid hormone superfamily to modulate gene expression (i.e., other retinoic acid receptors, the thyroid hormone receptor, and the vitamin D receptor), and these dimers represent convergence in the signaling pathways of these hormone systems.^{121–123} In addition to receptor dimerization, a series of cofactor proteins have also been shown to adjust the transcriptional activity of PPARs by interacting directly with the receptors, to either repress (corepressors) or enhance (coactivators) their activities. For example, p300, a component of the TATA-binding protein complex, has been shown to stimulate transcriptional activity of PPAR α .¹²⁹ The number of identified protein cofactors for the PPARs is growing and reflects the complexity by which this nuclear receptor system regulates transcriptional activity.

The diversity of ligands that individual PPARs are capable of binding is unlike the strict ligand specificity observed for other members of the steroid–receptor superfamily, for example, RXR, which is thought to bind only the 9-*cis* form of retinoic acid. Recent crystallographic data on the ligand binding domain of PPAR δ demonstrate that the carboxylic acid moieties of two structurally different classes of ligands for PPAR δ , an eicosenoid and a fibrate, are both oriented toward a particular region of the receptor, and this orientation stabilizes a helical conformation via a series of highly conserved hydrogen bonds.¹³⁰ This stable helix within the holo-receptor, then, is thought to allow for dimerization and/or interaction with cofactor proteins. In addition, the X-ray data show that the binding pocket of PPAR δ is relatively large and allows for binding of ligands in multiple configurations. The overall size of the binding pocket and its use of a number of stabilizing hydrophobic interactions help explain the structural diversity of PPAR ligands, as well as why PPAR δ bind FA of only certain chain lengths ($14 \leq C \leq 20$).¹³⁰

6.5.3 The Effect of PPARs on Fatty Acid Transport Proteins

6.5.3.1 Membrane Fatty Acid Transport Proteins

In general, PPAR α and its concomitant ligands have been implicated in the regulation of many genes involved in hepatic lipid metabolism and energy homeostasis.^{122,124,131} These include both mitochondrial and peroxisomal β -oxidative enzymes as well as fatty acid synthetase and lipoprotein assembly and transport proteins.^{124,132} PPAR γ has been associated primarily with lipolytic effects and is known to regulate enzymes involved in adipocyte differentiation and lipid storage and metabolism, for example, LPL and acyl-CoA synthetase, as well as the hormone leptin.^{122,124,133}

Recent molecular evidence has shown that certain putative membrane fatty acid transport proteins are also modulated in a tissue specific manner via PPARs (Table 6.2). In mice, treatment with the synthetic fibrate WY-14,643 and other PPAR α ligands resulted in increased mRNA levels of two of the three major membrane carrier proteins. FAT mRNA levels were increased in both liver and intestinal tissue by treatment with a fibrate,⁷¹ whereas FATP mRNA increased primarily in liver only.^{71,72} The observed increase in both FAT and FATP mRNA in liver was not observed in PPAR α -null mice, demonstrating that PPAR α was obligatory for the hepatic transcriptional effect.⁷¹ FABPpm mRNA levels increased only slightly in liver following treatment with the fibrate and were unaffected by treatment with any other PPAR α ligands. Transcription of FAT and FATP mRNA also increased in white adipose tissue of mice and in preadipocyte cell lines by treatment with PPAR γ ligands, but again transcription of FABPpm was unaffected.^{71,72} Thus, tissue-specific regulation of FAT and FATP expression has been demonstrated, with hepatic expression of FAT and FATP under the control of PPAR α and adipocyte expression under the control of PPAR γ .^{71,72} In contrast, it appears that FABPpm expression is not under the control of PPAR α or PPAR γ .

Cloning and sequence analysis of the murine FATP gene identified a putative PPRE in its 5' untranslated region.⁶⁸ A recent study further characterized

TABLE 6.2
Identified PPRE Sequences in Fatty Acid Transport Proteins

Protein	Species	Sequence	Proposed Function	Ref.
CONSENSUS PPRE SEQUENCE		AACT AGGTCA A AGGTCA T		121
AFABP	Mouse	CTCT GGGTGA A ATGTGC	Intracellular fatty acid binding/transport	159
LFABP	Rat	ATAT AGGCCA T AGGTCA	Intracellular fatty acid binding/transport	137
FATP	Mouse	AAGTGGGGCA A AGGGCA	Transmembrane fatty acid transport	134
FAT	Mouse	TGGCCT C TGACTT	Transmembrane fatty acid transport	a

^a P. Grimaldi and L. Teboul, personal communication.

the PPARE on FATP and found that this sequence is very similar to the consensus sequence for other identified PPAREs (Table 6.2), and that it does confer transcriptional regulation of FATP mRNA by PPAREs. Transient transfection of an FATP-luciferase reporter construct into CV-1 cells resulted in ligand-dependent upregulation of the reporter activity by both PPARE α and PPARE γ in a PPARE-mediated manner.¹³⁴ In 3T3-L1 adipocyte cells, luciferase reporter activity was increased by ligand bound PPARE γ but not by PPARE α . Further, in the 3T3-L1 cells activation of FATP expression with bound PPARE γ correlated with an increased uptake of oleate into the cells; the physiological ligand, linoleic acid, was also shown to activate FATP transcription in a PPARE-dependent manner.¹³⁴ A functional PPARE has also been identified recently in the mouse FAT promoter (P. Grimaldi and L. Teboul, personal communication).

6.5.3.2 Intracellular FABPs

It has been known for some time that the expression of LFABP was increased by a seemingly heterogeneous group of compounds that caused the proliferation of peroxisomes,^{124,135–138} and that induction of hepatic peroxisomal β -oxidation capacity correlates strongly with the increase in LFABP levels.¹³⁹ Indeed, when ethanol, which impairs mitochondrial β -oxidation and stimulates peroxisomal β -oxidation, was added to a high fat diet, LFABP was found to be expressed at levels up to 20% of soluble protein.¹⁴⁰ Dicarboxylic acids, FA metabolites that are found under conditions of impaired mitochondrial β -oxidation or with high levels of FA flux, have also been shown to increase LFABP mRNA levels in cultured hepatocytes.^{135,141} It is now known that these compounds, which include long-chain FA, bind to and activate PPAREs. In mice null for PPARE α , fasting induced a fatty liver accompanied by severe impairment of fatty acid oxidation, and LFABP expression was dramatically decreased in the PPARE α ^{-/-} fasted rats.¹⁴² The relationship between LFABP expression and peroxisome proliferation is not absolute, however, as normal LFABP levels are present in infants with Zellweger's syndrome, who have a complete absence of peroxisomal biogenesis.⁷³ Thus, LFABP expression appears to be coordinated with, but not dependent on, peroxisomal oxidative capacity.

Studies in cultured cell lines

are consistent with PPARE-mediated regulation of LFABP expression. Besnard and colleagues demonstrated that long-chain FA increases the transcription rate of the LFABP gene in rat hepatoma cells, and that 9-*cis*-retinoic acid enhances this induction.^{143–145} Increased LFABP synthesis following incubation with linoleic acid was also reported,¹⁴⁶ although others have found that oleate was effective only under conditions where β -oxidation was inhibited.^{135,141}

It is likely that the effects of long-chain fatty acids on LFABP expression are also mediated via interactions with PPARE transcription factors. PPARE γ was shown to bind the arachidonate metabolite 15-deoxy $\Delta^{12,14}$ prostaglandin J₂,^{147,136} forming a heterodimer with RXR α and thereby activating

AFABP transcription.¹⁴⁸ Indeed, both LFABP and AFABP contain functional PPRE homolog sequences in the promoter region of their genes (Table 6.2) and are upregulated by PPAR α and PPAR γ , respectively.^{137,141,148} Antidiabetic agents such as the thiazolidinediones increase AFABP mRNA levels presumably by increasing insulin sensitivity, and appear to be operating via the same mechanism of activation of nuclear hormone receptors as do long-chain fatty acids.¹¹⁹

6.6 Concluding remarks

Nutritional regulation of FA transport protein gene expression, in particular by FA itself, highlights the potential for a direct effect of diet on transcription, and demonstrates at the molecular level how diet can directly influence gene transcription, even in the absence of metabolic transformation.

The effects of the PPARs on expression of numerous fatty acid metabolizing enzymes, and on particular fatty acid transport proteins (Fig. 6.1), demonstrate how PPARs and their associated ligands are capable of coordinate regulation of FA flux and metabolism at the nuclear level. Thus, the participation of intracellular FA in their pleiotropic functions requires regulation by a complex sequence of ligand–receptor–DNA interactions which, in turn, requires not only other hormone-signaling pathways but also a series of co-repressor/activator proteins. The result of these multiple regulatory events leads, ultimately, to a particular FA availability within the cell. The coordinated regulation of the transmembrane FA transporters and the intracellular FABPs, as well as particular enzymes of FA metabolism, supports the hypothesis that cellular FA trafficking is vectorial rather than simply random or diffusion controlled, and occurs via a series of specific protein–protein and/or protein–membrane interactions.^{47,149}

The role of PPAR α in hepatocyte lipid metabolism, and the role of PPAR γ in adipocyte lipid metabolism, suggest that significant pathological conditions may be associated with aberrant function of these FA-activated transcription factors. For example, alterations in PPAR α function may play a role in hyperlipoproteinemia and alterations in PPAR γ may play a role in insulin resistance and diabetes. Therefore, the dietary availability, intracellular transport, and trafficking of their respective ligands may, by association, also play a significant role in certain pathological states. However, that both the intracellular and membrane transport proteins for FA are themselves regulated by PPARs (Fig. 6.1) and are, therefore, regulated by the availability of their FA ligands, highlights the complicated feedback regulation between dietary fats and nuclear events which may influence many metabolic conditions such as obesity and diabetes. Clearly, the involvement of fatty acids as transcriptional regulatory factors in their own homeostasis is complex, and to date may present more questions than answers. These questions include the complete

identification of regulatory proteins, protein cofactors, and the cognate DNA sequences recognized by the various effectors, as well as the role of fatty acid specificity, and potential intracellular compartmentalization of different FA. The potential functional redundancies in both intracellular and transmembrane FA transport proteins, with two or three of each type expressed simultaneously in particular cell types, respectively, must also be clearly defined.

Abbreviations

AFABP adipocyte FABP

BFABP brain FABP

cAMP cyclic adenosine monophosphate

C/EBP- α CCAAT-enhancer binding protein α

FA fatty acid

FABP fatty acid binding protein

FABPpm plasma membrane fatty acid binding protein

FAT fatty acid translocase (CD36)

FATP fatty acid transport protein

HFABP heart FABP

IFABP intestinal FABP

KFABP keratinocyte FABP

LFABP liver FABP

MFABP myelin FABP (myelin P2)

mRNA messenger RNA

NMR nuclear magnetic resonance

PPAR peroxisome proliferator-activated receptor

PPRE peroxisome proliferator response element

PUFA polyunsaturated fatty acid

RXR retinoid X receptor

SFA saturated fatty acid

TG triacylglycerol

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Alcohol and Gene Expression in the Central Nervous System

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7.1 Introduction

Alcohol is one of the most widely used chemical substances in our society. Nearly 75% of American adults use alcohol, and 15% of these adults develop life-long health problems associated with its abuse. Alcohol dependence (alcoholism) is characterized by the development of tolerance (a reduction in the effect of alcohol after single or repeated administration), withdrawal (a state of central nervous system hyperexcitability once alcohol is removed), and a maladaptive pattern of use (loss of control).¹ Why some individuals become alcoholics and others do not has been a question under active investigation by researchers for a number of years. There are clear genetic components to the multidimensional disorder of alcoholism supported by half-sibling and adoption studies.^{2–5}

Animal models also provide evidence for the role that genetics plays in the development of alcohol dependence and other drug-related disorders.⁶ However, no one animal model of alcoholism encompasses every aspect involved with the disorder, but some key advantages of using animal models are (1) the experimenter controls the genotype, i.e., reduced heterogeneity; (2) a plethora of inbred strains are available in both rats and mice for testing; and (3) the use of forward and reverse genetic techniques can be utilized.⁶ Even though these studies indicate that genetics are an important factor in determining susceptibility for the development of alcoholism, the specific genes involved and their mode of regulation have yet to be identified.

Alcohol affects a wide range of activities in the central nervous system including the activity of numerous genes expressed within this system (Fig. 7.1). Thus, understanding the genetics underlying the development of alcoholism will require not only the identification of the genes involved but also how alcohol regulates their expression. The aim of this chapter is to present and discuss findings on how alcohol (ethanol) affects the expression of genes in the central nervous system (CNS), and how this might result in the development of alcoholism. Our review will focus on ethanol's effects on gene expression *in vivo* within the central nervous system. Other reviews have recently focused on the effects of ethanol on expression in cultured cells, and the effects of ethanol outside the central nervous system. These data are reviewed elsewhere in several excellent reviews.^{7–9}

7.2 Ligand-Gated Receptors

7.2.1 NMDA Receptors

Glutamate is the primary mediator of excitatory neurotransmission in the mammalian brain, and studies have suggested a role for glutamate in ethanol

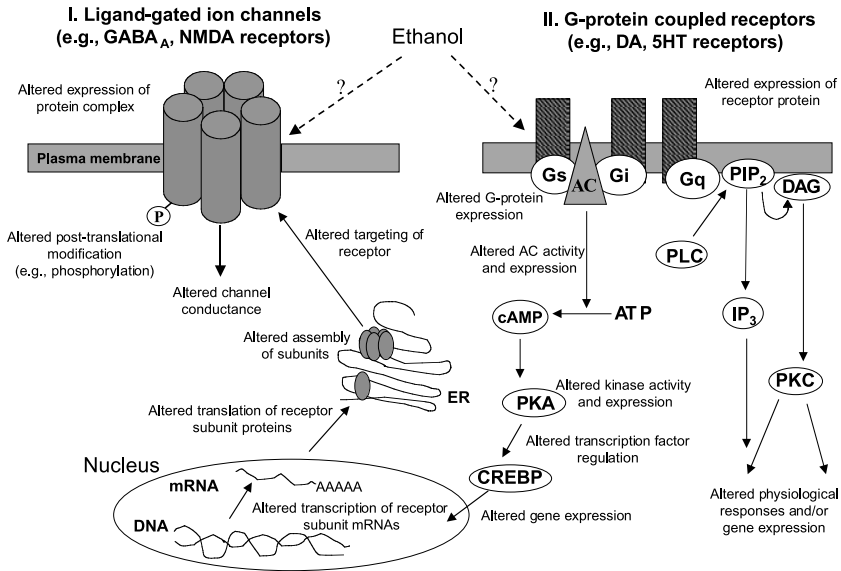


FIGURE 7.1

Potential adaptations of neurotransmitter receptors after chronic ethanol exposure in animal models of ethanol physical dependence. (I) Ligand-gated ion channels. GABA_A and NMDA receptors are two examples of ligand-gated ion channels that are affected by chronic ethanol exposure. GABA_A receptors are the primary sites of inhibitory neurotransmission, while NMDA receptors mediate excitatory neurotransmission in the mammalian brain. These receptors are thought to be constructed from four to five subunits, and modulate neuronal activity by changes in channel conductance. GABA_A receptors are permeable to Cl⁻ ions, while NMDA receptors are permeable to Ca²⁺ and monovalent cations. There is evidence to suggest several mechanisms by which chronic ethanol exposure alters these ion channels including functional alterations in channel conductance, altered transcription of receptor subunit mRNAs, altered translation of receptor subunit proteins, altered assembly of receptor subunits, altered targeting of receptor to plasma membrane, altered post-translational modifications, and altered expression of the protein complex. (II) G-protein coupled receptors. DA and 5HT receptors are two examples of G-protein coupled receptors affected by chronic ethanol exposure. There are multiple subclasses of DA receptors (e.g., D1 and D2) that are either positively or negatively coupled to AC. There also exist several subclasses of 5HT receptors (e.g., 5HT-2A and 5HT-2C) that are coupled to the phosphoinositol second messenger pathway. Chronic ethanol exposure may alter G-protein coupled receptors and their associated second messenger pathways through several mechanisms, including altered expression of G-proteins, altered AC activity and expression, altered protein kinase activity and expression (e.g., PKA and PKC), altered transcription factor regulation (e.g., CREBP), and altered expression of receptor proteins. Any of these alterations may affect a host of other physiological responses downstream of the receptor (e.g., gene expression, phosphorylation of ion channels, general metabolism, and neurotransmitter synthesis). It is not known if ethanol interacts directly or indirectly to produce these effects.

Abbreviations: γ -aminobutyric acid type A receptor (GABA_A), *N*-methyl-D-aspartate receptor (NMDA), dopamine (DA), 5-hydroxytryptamine (5-HT), adenylyl cyclase (AC), protein kinase A (PKA), protein kinase C (PKC), phospholipase C (PLC), phosphoinositolbiphosphate (PIP₂), inositoltriphosphate (IP₃), cAMP responsive element-binding protein (CREBP), diacylglycerol (DAG).

dependence.¹⁰ Glutamate-N-methyl-D-aspartic acid (NMDA) receptors are linked to a voltage-sensitive ion channel permeable to calcium and monovalent cations (e.g., Na⁺, K⁺). This receptor contains binding sites for a number of amino acids including glutamate, glycine (a co-agonist required for activation), D-serine, and L-aspartate. In addition, the NMDA receptor contains binding sites for the dissociative anesthetic phencyclidine (PCP), and dizcolipine (MK-801), which work as noncompetitive antagonists. The NMDA receptor is thought to be constructed of five subunits. There are two major classes of NMDA subunits: NR1 (ζ1 in the mouse) with eight possible splice variants, and NR2 of which there are four distinct subtypes (NR2A, -2B, -2C, and -2D; ε1 to 4 in the mouse, respectively). Both classes of NMDA receptor subunits show distinct brain regional distributions, which provide for a great degree of receptor heterogeneity and suggest functionally distinct isoforms of the receptor. Along these lines, acute ethanol has been found to inhibit NMDA receptor responses in certain brain regions (e.g., inferior colliculus and hippocampus), while having no effect in others (lateral septum).¹¹

Receptor binding studies of NMDA receptors after chronic ethanol have produced somewhat contradictory results (Table 7.1). Early studies reported both increases and decreases in the density of [³H]glutamate-binding sites after acute and chronic ethanol.^{12,13} Chronic ethanol also increases the binding density of [³H]MK-801 in certain brain regions,^{14–16} but some studies have reported no change with 4 to 8 months of ethanol exposure.¹⁷ Thus, it is unclear whether the effects of chronic ethanol are reflected consistently in changes in binding site densities of the NMDA receptor. These contradictory results have forced researchers to investigate more specific molecular mechanisms resulting from chronic ethanol exposure. Specifically, researchers have begun to focus on the effects of chronic ethanol on the expression of NMDA receptor subunits.

TABLE 7.1

Effect of Chronic *in Vivo* Ethanol Administration on Ligand and Voltage-Operated Ion Channels, Receptor Recognition Sites, and mRNA and Peptide Expression in the Brain

Receptor /Property	Alteration	Refs.
<i>Ligand-Gated Ion Channels</i>		
NMDA Receptor		
[³ H]glutamate binding ^c	Increased	12
	Decreased	13
[³ H]MK-801 binding ^{a, c}	Increased	14
		15, 16
	No change	17
NR2A mRNA and peptides ^{a, b, c, e}	Increased	18–20, 23
	No change	18, 21
NR2B mRNA and peptides ^{a, b, c}	Increased	18, 19, 21–23
	No change	18

TABLE 7.1 (CONTINUED)

Effect of Chronic *in Vivo* Ethanol Administration on Ligand and Voltage-Operated Ion Channels, Receptor Recognition Sites, and mRNA and Peptide Expression in the Brain

Receptor /Property	Alteration	Refs.
NR2C mRNA ^{a, b}	No change	18, 21
NR1 mRNA and peptides ^{a, b, c, e}	Increased	19, 20, 23
	No change	18, 20, 21
NR1 (5' insert) mRNA ^a	Decreased	21
NR1 (3' insert) mRNA ^a	No change	21
GABA_A Receptor		
GABA-mediated Cl ⁻ flux ^{a, b}	Decreased	28, 29, 40, 46
	No change	30, 33
Pentobarbital-enhanced Cl ⁻ flux ^a	Decreased	28
	No change	30
Benzodiazepine-enhanced Cl ⁻ flux ^a	Decreased	30
Inverse agonist-inhibition of Cl ⁻ flux ^a	Increased	30
Low affinity [³ H]GABA binding ^d	Decreased	35
[³ H]Flunitrazepam binding ^{a, b}	No change	37–39
[³ H]zolpidem binding ^{a, b}	Increased	48
	No change	49
[³ H]Ro 15-4513 binding ^{a, b}	Increased	54, 55
α ₁ subunit mRNA and peptides ^{a-d}	Decreased	41–45, 47, 51, 61, 65
α ₂ subunit mRNA and peptides ^a	Decreased	42–44
α ₃ subunit mRNA ^a	No change	42, 44
α ₄ subunit mRNA and peptides ^{a, c}	Increased	45, 47, 63
	No change	62
α ₅ subunit mRNA ^{a, c}	Increased	45
	No change	61
α ₆ subunit mRNA and peptides ^b	Increased	43, 51, 52
β ₁ subunit mRNA ^a	Increased	57
β ₁ subunit mRNA ^a	No change	45
β ₂ subunit mRNA and peptides ^{c, d}	Increased	47, 57
β ₂ subunit mRNA and peptides ^{a, b, c}	Increased	58, 59
	No change	45, 58, 62
β ₃ subunit mRNA and peptides ^{a, c}	Increased	47, 57
	No change	45, 62
γ ₁ subunit mRNA and peptides ^a	Increased	45, 47
γ _{2S} subunit mRNA ^a	Increased	45
γ _{2L} subunit mRNA ^a	No change	45
γ ₂ subunit peptides ^{a, c}	No change	47, 62
γ ₃ subunit mRNA ^a	No change	45
δ subunit mRNA ^a	No change	45
Glycine Receptor		
[³ H]glycine binding ^c	No change	15
<i>Voltage-Operated Channels</i>		
L-Type Calcium Channel		
[³ H]nitrendipine binding ^{a, d}	Increased	69, 70

^a Cerebral cortex, ^bcerebellum, ^chippocampus, ^dwhole brain, ^ehypothalamus

Several studies have shown that chronic ethanol exposure alters the expression of both NR1 and NR2 subunits (Table 7.1). The hippocampus seems to be a brain region where most of the changes in NMDA receptor subunit messenger RNA (mRNA) and protein are observed, probably due to the high abundance of NMDA receptors in this region. Follesa and Ticku¹⁸ found a 30% increase in both the NR2A and NR2B subunit mRNAs in rat hippocampus, but only in rats withdrawing from chronic ethanol. In addition, they also found similar increases in these subunit mRNAs in the cerebral cortex from the same rats, but found no changes in NR1 subunit mRNA at any time point or in any brain region tested (e.g., cerebral cortex, cerebellum, and hippocampus). This same group also found an upregulation of the NR1, NR2A, and NR2B subunit proteins by approximately 35% compared to both the hippocampus and cerebral cortex in ethanol-naïve rats.¹⁹ These observed increases in NMDA receptor subunit proteins returned to control values by 48 h, which parallels the disappearance of behavioral signs of withdrawal. Thus, these results suggest that alterations in NMDA receptor subunit expression following chronic ethanol could contribute to CNS hyperexcitability observed during ethanol withdrawal. Another group has reported increases compared to control values in NR1 subunit protein in the hippocampus and cerebellum (50 and 95%, respectively) as well as an upregulation of NR2A subunit protein in the hippocampus and cerebral cortex of mice (25 and 40%, respectively) with no alterations in these subunit mRNAs.²⁰ As mentioned previously the NR1 subunit may be expressed as one of eight possible alternatively spliced variants. In an attempt to delineate a role of individual NR1 splice variants, Hardy et al.²¹ reported a decrease in mRNA encoding the NR1 subunit containing a 5' insert compared to those lacking it in ethanol-dependent rats. This change persisted through 48 h of ethanol withdrawal. This 5' NR1 subunit insert is located in the N-terminal domain of the protein. The authors suggest that reduced expression of the N-terminal domain insert may result in NMDA receptors that have a greater likelihood of agonist activation, and thus increased NMDA receptor function.²¹ This result further supports the hypothesis that NMDA receptor function is increased as a consequence of chronic ethanol exposure.

Another report of mice made physically dependent on ethanol using a multiple withdrawal paradigm, which results in an exacerbation of seizure severity following repeated episodes of intoxication and withdrawal, has shown an increase in the $\epsilon 2$ subunit mRNA in the hippocampus which was greater than that in mice continuously exposed to ethanol (i.e., uninterrupted chronic ethanol exposure).²² In contrast, this same treatment paradigm resulted in a greater increase in $\epsilon 2$ subunit mRNA in the cerebral cortex of continuously exposed mice compared to mice undergoing multiple ethanol withdrawals.²² Thus, these results indicate that the type of ethanol treatment paradigm used differentially alters NMDA receptor subunits, and a previous withdrawal history influences NMDA receptor subunit gene expression. Gender is another important variable which may affect the expression of NMDA receptor subunits following chronic ethanol. Devaud and Morrow²³ reported gender-specific effects

of chronic ethanol on the expression of NMDA receptor subunits in rats. They found that males showed increases in NR1 protein in the hippocampus, whereas females showed increases in the cerebral cortex and hypothalamus. In addition, hippocampal NR2A subunit protein was only increased in males, with no changes observed in females, while NR2B subunit protein was increased similarly in the cerebral cortex of both males and females.²³

Taken together, these results suggest that ethanol regulates the expression of NMDA receptor subunit genes which may contribute to ethanol withdrawal-related CNS hyperexcitability. The majority of evidence points to an augmentation of NMDA receptor function following chronic ethanol as shown by upregulation of both NR1 and NR2 subunits in specific brain regions. This overexpression of NMDA receptor subunits might be a neuroadaptive mechanism by which the central nervous system compensates for the chronic inhibitory effects of ethanol.

7.2.2 GABA_A Receptors

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain, and thus plays an important role in regulating neuronal excitability. GABA_A receptors consist of a heterologous combination of subunits that are assembled as a pentamer and form a Cl⁻ channel. The majority of these subunits exist in variant forms (e.g., α₁₋₆, β₁₋₃, γ₁₋₃, δ, and ε) which adds another degree of complexity to the structure of the GABA_A receptor.²⁴⁻²⁶ GABA_A receptors are members of the ligand-gated ion channel superfamily of neurotransmitter receptors. The GABA_A receptor contains several recognition sites for various sedative (e.g., benzodiazepines, barbiturates, neurosteroids) and convulsant agents (e.g., β-carbolines, picrotoxin) which are allosterically coupled to a Cl⁻ channel. These agents work by allosterically modulating the actions of GABA at the GABA_A receptor.

There is a substantial amount of evidence suggesting that both acute and chronic ethanol affect the GABA_A receptor system.²⁷ It is widely established that GABA_A receptor subunit genes are sensitive to ethanol manipulations (Table 7.1). These effects on gene expression have been demonstrated at the level of both mRNA and protein regulation after chronic ethanol. Alterations in GABA_A receptor subunit expression following chronic ethanol could result in receptors with different functional and pharmacological properties from the native receptor. This hypothesis is supported by numerous functional studies of GABA_A receptor activity following chronic ethanol treatment along with studies of recombinant GABA_A receptors expressed in *Xenopus* oocytes or other expression systems.²⁸⁻³² Functional studies show that after chronic ethanol the ability of GABA to gate the associated Cl⁻ channel is reduced, although some studies show no change.^{28-30,33} In addition, while pentobarbital- and benzodiazepine-mediated ³⁶Cl⁻ flux is also reduced, the ability of benzodiazepine inverse agonists (e.g., Ro 15-4513, DMCM) to inhibit ³⁶Cl⁻ flux is enhanced.^{28,30} Recombinant expression studies of GABA_A

receptors, which examine the relationships between subunit composition and receptor function show that different combinations of subunits confer different functional and pharmacological properties to the receptor.^{24,34} These studies have also identified combinations of subunits that show differential responses to ethanol.^{31,32} In light of this evidence, changes in GABA_A receptor densities at various modulatory sites after chronic ethanol have not correlated well with measures of subunit expression and function.^{35–40} Thus, it appears that altered expression of GABA_A subunits is most likely reflected in the functional status of the receptor rather than in the total density of its modulatory binding sites. Therefore, understanding the underlying mechanisms of functional alterations will require a thorough understanding of how ethanol regulates GABA_A receptor subunit gene expression.

GABA_A receptor subunit expression is affected by chronic ethanol in several brain regions including the cerebral cortex, cerebellum, and hippocampus. In addition, subunit gene expression has been shown to be bi-directionally altered. Bi-directional expression patterns of GABA_A receptor subunits might be a unique neuroadaptive mechanism by which the central nervous system compensates for the continued presence of ethanol without affecting receptor density. In the cerebral cortex the α subunit genes appear to show the most consistent changes with chronic ethanol. The α_1 and α_2 subunit mRNAs and peptides are decreased, while α_4 subunit mRNA and peptides are increased.^{41–47} However, binding-site densities are slightly increased or unchanged for the selective α_1 subunit ligand, [³H]zolpidem, following chronic ethanol exposure in the cerebral cortex and cerebellum.^{48–50} In contrast, α_3 and α_5 subunit mRNAs show no change.^{41,42} In the cerebellum, the α_1 subunit mRNA and peptide are decreased, while the α_6 subunit mRNA peptide is increased.^{43,44,51,52} The α_6 subunit is only found in the cerebellum and is thought to confer binding to a diazepam-insensitive site labeled by [³H]Ro 15-4513, a partial inverse agonist.⁵³ Thus, an increase in the α_6 with a concomitant decrease in the α_1 subunit might explain the upregulation of diazepam-insensitive [³H]Ro 15-4513 binding-site densities in the cerebellum,^{54,55} and behavioral sensitization to benzodiazepine inverse agonist following chronic ethanol.⁵⁶

After chronic ethanol the β subunits appear to show increases in expression in the cerebral cortex and cerebellum, but some studies have reported no change. For example, in a study conducted by Mhatre and Ticku⁵⁷ they found increases in all three β subunit mRNAs (i.e., β_1 , β_2 , and β_3) in rats chronically exposed to ethanol within the cerebral cortex. In contrast, Devaud et al.⁴⁵ found no changes in any of the β subunit mRNAs following ethanol treatment. We also found no consistent changes in the cerebral cortex for β_2 subunit mRNA in C57BL/6J (B6) or DBA/2J (D2) mice chronically exposed to ethanol for a period of 72 h.⁵⁸ Although in all these studies rodents were ethanol-dependent, there were variations in blood ethanol concentrations (BECs) maintained among the animals which might explain the discrepancies. For example, in the study by Mhatre and Ticku⁵⁷ rats had BECs of about 4.0 mg/ml, whereas in our study, Reilly and Buck,⁵⁸ and the

study conducted by Devaud et al.⁴⁵ rodents had BECs between 1.0 and 2.0 mg/ml. These results suggest that the β subunit mRNAs might only be sensitive to ethanol regulation in the cerebral cortex at higher BECs, which may or may not reflect direct effects of chronic ethanol exposure. In contrast, we and others have reported increases in the β_2 subunit mRNA following chronic ethanol treatment in the cerebellum.^{58,59} Using RNase protection analysis we found a differential regulation of β_2 subunit mRNA content in the cerebellum of B6 and D2 mice. The D2 strain was more sensitive to ethanol-induced increases in β_2 subunit mRNA content in the cerebellum, showing significant increases at lower blood ethanol concentrations than B6 mice. The ethanol-induced regulation in B6 mice appeared to be more complex, with decreases in β_2 subunit mRNA content at low blood ethanol concentrations, and increases at higher concentrations. These data suggest that differences between B6 and D2 mice in the degree of physical dependence (withdrawal) on ethanol may be related to differential sensitivity to ethanol regulation of β_2 subunit expression.

The hippocampus is another region that shows changes in GABA_A receptor subunit expression after chronic ethanol, but in this region the changes observed seem to depend on duration of ethanol exposure. Decreases in subunit gene expression have been noted for the α_1 subunit mRNA and peptide after 12 weeks of chronic ethanol in rats,⁶¹ while longer durations of exposure (e.g., 40 days) produce no change in α_1 peptide content.⁶² In contrast, the α_4 subunit mRNA and peptides are found to be increased after either 40 days of continuous ethanol exposure, or after using a 60-day, chronic, intermittent ethanol exposure paradigm.^{62,63} The hippocampus has not been found to show changes in $\beta_{2/3}$ peptide content.⁶² In addition, γ_2 subunit peptide content shows no change after chronic ethanol exposure in this region.⁶²

In summary, it is clear that GABA_A receptor subunit gene expression is sensitive to ethanol manipulations. Alterations in subunit gene expression may alter the functional properties of the GABA_A receptor, which seems to be a likely mechanism by which the central nervous system adapts to the continued presence of ethanol. Further studies will be needed to determine how altered subunit gene expression affects receptor assembly, post-translational modifications, and interactions with second messenger systems.

7.2.3 Glycine Receptors

Glycine, like GABA, is an inhibitory neurotransmitter that is primarily localized in the brain stem and spinal cord where it acts on strychnine-sensitive receptors.⁶⁵ Two types of subunits have been identified for the glycine receptor: an α subunit which has three isoforms denoted (α_{1-3}) and a β subunit. Despite low levels of strychnine binding in the forebrain, more specific molecular biological techniques have shown the presence of glycine receptor α subunits in the cerebral cortex,⁶⁶ and have provided

evidence for a widespread distribution of the β subunit throughout the brain.⁶⁷ Receptor-binding studies have not shown changes in glycine receptor densities in the hippocampus of ethanol-dependent mice,¹⁵ but as we have seen for GABA_A and NMDA receptors a more specific analysis of subunit expression might indicate that the glycine receptor does contribute to ethanol withdrawal severity.

7.3 Voltage-Operated Channels

7.3.1 L-Type Calcium Channels

The L-type calcium channel is a member of the voltage-operated calcium channel family which also includes N, P, T, and Q-type channels. It is distinguished from the other voltage-operated calcium channels based on both electrophysiological and pharmacological properties. The L-type channel is activated under high voltage, and is blocked by dihydropyridines. The L-type channel consists of five subunit proteins (α_{1-2} , β , γ , and δ) of which the α_1 subunit is thought to be the major voltage sensor and pore-forming protein,⁶⁸ but the native subunit composition of the L-type channel is not known. In addition, how each subunit contributes to the functional properties of the channel has not been completely determined. However, the L-type calcium channel may represent an important target for regulation by chronic ethanol due to its effects on neurotransmitter release and second-messenger mediating functions in neurons.

Several studies have noted an upregulation of the L-type calcium channel following chronic ethanol exposure as determined by [³H]nitrendipine-binding-site densities.^{69,70} For example, Guppy and Littleton⁶⁹ found a 40% increase in [³H]nitrendipine-binding sites in ethanol-dependent rats. To further examine the time course of changes in L-type calcium channel regulation by chronic ethanol, Guppy et al.⁷⁰ measured [³H]nitrendipine-binding-site densities in mice during (i.e., up to ten days) and after the induction of ethanol physical dependence (i.e., 8 and 24 h of withdrawal). In the cerebral cortex, [³H]nitrendipine-binding site densities show a sharp increase between days 3 and 4 and remain at this level throughout the 10-day ethanol exposure. This change in receptor density is preceded by a decrease in receptor affinity, which returns to control values by 6 days of exposure.⁷⁰ In contrast, [³H]nitrendipine-binding-site densities are reduced at 8 h of withdrawal compared to ethanol-dependent mice, and return to control values by 24 h of withdrawal.⁷⁰ These observed time-dependent changes in the regulation of L-type calcium channels by chronic ethanol correlate well with the time course of the appearance of ethanol withdrawal signs in mice.

This evidence suggests that the L-type calcium channels are potential targets of ethanol regulation which may contribute to ethanol withdrawal

severity. Further studies will be needed to determine how this ethanol-induced upregulation of the L-type calcium channels occurs on a molecular level in terms of specific subunits.

7.4 G-Protein Coupled Receptors

7.4.1 Dopamine Receptors

Special interest has been raised on alterations in the dopaminergic system, since it is believed to be an important interface for multiple substances of abuse.⁷¹ Behavioral studies of rodents administered electric currents intracranially have shown that electrical stimulation in the ventral tegmental area triggers repetitive self-stimulating behavior and a dopaminergic efflux in the nucleus accumbens core and shell.^{72,73} Lesions of the pathway abolish the rewarding efficacy of multiple substances of abuse, e.g., ethanol, cocaine, and amphetamine.⁷⁴ Moreover, chronic intake of multiple substances of abuse like ethanol, cocaine, amphetamine, or opioids induces a dopamine efflux in the nucleus accumbens, which is important for the rewarding efficacy of these drugs.^{71,75–78} These findings have initiated a number of investigations on the interactions between ethanol administration or withdrawal and the activity of the limbic and forebrain dopaminergic system.⁷⁹ Molecular-cloning studies have revealed at least five distinct dopaminergic receptors. The D₁ and the D₅ receptor subtypes belong to the D₁ family, while the D₂, D₃, and D₄ subtypes belong to the D₂-like group.⁸⁰

Chronic ethanol treatment alters the expression of a number of dopaminergic receptors in a complex manner (see Table 7.2).⁸¹ Two studies showed an increase in D₁-receptor binding sites in the striatum,^{82–83} whereas three other groups failed to confirm this finding (see Table 7.2).^{84–86} In the case of D₂ receptors three studies reported a strong increase in receptor binding or D₂ mRNA expression after chronic ethanol ingestion,^{83,86,87} but these results were not replicated in independent studies (see Table 7.2).^{84,85,88} There are several differences in ethanol treatment (injection vs. free choice two-bottle regimen) and duration (7 days vs. e.g., 7 months) among the investigations that strongly affect blood ethanol levels and subsequent gene expression. The expression of important target genes also varies on the genetic background of the animals. The ethanol-preferring C57BL/6J mice displayed higher D₁ and D₂ receptor mRNA content and higher D₁ and D₂ receptor densities in the limbic forebrain than the ethanol-avoiding DBA/2J strain under non-ethanol conditions.⁸⁹ Controversial findings might also result from an age dependent decline in receptor density, especially of D₂ receptors in the forebrain regions beginning at the age of 5 months as well in ethanol-treated animals as in the controls.⁸⁷ Fewer studies have been performed for the D₃, D₄, or the D₅ dopaminergic receptors. Eravci et al.⁸⁵ reported a decrease in dopamine D₃

TABLE 7.2

Expression of G-Protein Coupled Receptors and Related Peptides in the CNS after Chronic Ethanol Treatment

Receptor/Property	Alteration	Refs.
<i>G-protein Coupled Receptors</i>		
Dopamine Receptor		
[³ H]SCH 23390 binding ^{a,e}	Increased	82, 83
	No change	84
D ₁ mRNA ^{a,c,e}	No change	85
D ₂ mRNA ^{c,e}	No change	85
	Increased	86
[³ H]spiperone binding ^{a,c,e}	Increased	83, 87
	No change	84
[³ H]YM09151 binding ^e	Decreased	88
	Increased ^o	
D ₃ mRNA ^{c,e}	Decreased	85
D ₄ mRNA ^{c,e}	No change	85
D ₅ mRNA ^{c,e}	No change	85
Serotonin Receptors		
5-HT _{1A} mRNA ^{a,b,h}	Increased	100
	Decreased	100
[³ H]8-OH-(DPAT) binding ^{a,b,h}	Increased	100
	Decreased	100–102
5-HT _{1B} mRNA ^{e,g}	Increased	100
	No change	100
[¹²⁵ I]GTI- binding ^f	Increased	100
[³ H]ketanserin binding ^{a,b,e,h}	No change	102, 104
5-HT stimulated phosphoinositide hydrolysis ^a	No change	105
Noradrenergic Receptors		
[¹²⁵ I]pindolol binding ^a	No change ^p	118, 119
Opioid Receptors		
δ-opioid receptor mRNA ^{d,e}	No change	127, 128
[³ H]H-Tyr-Tic psi[CH ₂ -NH] Phe-Phe-OH binding ^e	No change	129
μ-opioid receptor mRNA ^{d,e}	No change	127
[³ H]Tyr-D-Ala-Gly-MePhe-Gly-binding ^c	Decreased	129
prodynorphin, mRNA ^d	Increased	130
vasopressin, mRNA ^d	Decreased	130
Other G-Protein Coupled Receptors		
mACh-receptor [³ H]quinuclidinylbenzilate binding ^{a,b,e}	No change	132
	Decreased	131
Adenosine A ₁ receptor mRNA and [³ H]DPCPX binding ^e	No change	135
Adenosine A ₂ receptor mRNA and [³ H]CGS 21680 binding ^e	No change	135
neurotensin receptor [³ H]SCH23390 binding	Decreased	133
Neurotransmitter Transporters		
5-HT transporter [³ H]serotonin binding ^a	Increased	140, 141
[³ H]citalopram binding ^{a,b,h,e}	No change	102

TABLE 7.2 (CONTINUED)

Expression of G-Protein Coupled Receptors and Related Peptides in the CNS after Chronic Ethanol Treatment

Receptor/Property	Alteration	Refs.
<i>Second Messenger Systems</i>		
G-Proteins		
G _s mRNA and protein ^{a,b,c,e,j}	No change	144, 145
G ₀ mRNA and protein ^{a,b,g,j}	Decreased	144
	No change	145
G ₁₁ mRNA and protein ^{a,b,c,e}	No change	144, 145
	Increased	145
G ₁₂ mRNA and protein ^{a,b,c,e,j}	No change	144, 145
G ₁₃ mRNA and protein ^{a,b,e,f,j,k}	Increased	144
	No change	145
Adenylyl Cyclase System		
protein kinase A (PKA) protein ^l	Decreased	149
CAM-kinase protein ^l	Decreased	149

^acortex; ^bhippocampus; ^cnucleus accumbens; ^dhypothalamus; ^estriatum; ^fglobus pallidum; ^gsubstantia nigra; ^hdorsal raphe; ⁱcerebellum; ^kventral tegmental area; ^lwhole brain; ^mamygdala; ⁿparaventricular nucleus; ^ostimulation 2 weeks, decrease after 10 weeks; ^pK_d decrease

mRNA expression whereas D₄ and D₅ receptor densities remain unaltered (see Table 7.2). There is special interest on ethanol's effects on the gene expression of the D₃ and D₄ receptors since they are preferentially expressed in the mesofrontal/mesolimbic dopaminergic system.⁸⁰ These receptors display a high affinity to several clinically used atypical antipsychotic agents like clozapine or olanzapine. Clozapine administration has been shown to reduce stimulant and ethanol abuse in patients with a double diagnosis of psychotic and substance abuse disorder.^{90,91}

Overall, a significant number of studies have shown an increase in D₁ or D₂ receptors in the nucleus accumbens and cortex of rodents. Increased numbers of D₁ and D₂ receptors might also induce ethanol-seeking behavior or a relapse in humans. The behavioral relevance is supported by studies on knockout animals. Animals lacking the D₁ receptor gene consumed significantly lower amounts of ethanol than the control mice.⁹² Taken together, these studies argue for a sensitization of the dopaminergic system after chronic alcohol intake that might be important for the reinforcing properties of the drug.

7.4.2 Serotonin Receptors

Studies using knockout and transgenic animals indicate that the intake of ethanol is dramatically influenced by functional alterations in the serotonergic system.⁹³ Moreover, a number of human serotonergic genes may be associated with subtypes of alcohol dependence.^{94–96} Molecular-cloning studies have yielded at least fourteen subtypes of serotonergic receptors.⁹⁷ The serotonergic

receptors are widely expressed in the CNS on non-serotonergic and serotonergic neurons thereby influencing the activity of several other neurotransmitter systems, including the dopaminergic system.⁹⁷ There is some evidence for decreased function of the serotonergic system by ethanol.^{81,98} Chronic ethanol treatment induced a strong decrease in the density of postsynaptic 5-HT_{1A} receptors in cortex and hippocampus (see Table 7.2).^{99–102} In contrast, the number of presynaptic auto-inhibiting 5-HT_{1A} and 5-HT_{1B} receptors in the dorsal raphe was markedly increased (see Table 7.2).¹⁰⁰

Interestingly, the 5-HT_{1B} knockout mice consume greater amounts of ethanol (10% unsweetened solution) in a two-bottle choice paradigm.⁹³ Using an operant ethanol self-administration procedure the differences between the 5-HT_{1B} knockout animals and controls were only statistically significant for the groups consuming the unsweetened 10% ethanol solutions, but not for the either unsweetened or sweetened 5%, 10%, 20%, solutions.¹⁰³ In contrast to the alterations in the densities of the 5-HT_{1A} or 5-HT_{1B} receptors, the number of 5-HT_{2A} or 5-HT_{2C} receptors in the cortex or hippocampus remains unaltered after chronic ethanol treatment (see Table 7.2).^{102,104,105} Overall, these studies argue for different ethanol-induced changes in the serotonergic neurotransmission: enhanced 5-HT_{1A}- or 5-HT_{1B}-mediated inhibition of serotonergic neurons in the dorsal raphe nuclei, impaired 5-HT_{1A} postsynaptic transmission in the hippocampus and the cortex, but unaltered 5-HT_{2A} expression in all areas investigated. A gain in 5-HT₃ receptor function is also evident in *in vitro* studies,^{106–109} but no studies regarding direct effects of ethanol on 5-HT₃ receptor expression have been carried out. These alterations do not fit into a simple model of reinforcement or impairment of serotonergic neurotransmission and may be related to the inconclusive findings of treatment trials using selective serotonin reuptake inhibitors (SSRIs) for relapse prevention in alcohol-dependent patients.^{110–112}

7.4.3 Noradrenergic Receptors

In comparison to studies on the serotonergic and dopaminergic systems fewer studies have been reported on ethanol's effects on the noradrenergic system. Ethanol withdrawal induces a strong increase in the activity of the brainstem noradrenergic system¹¹³ that can be abolished by bilateral lesions of the locus coeruleus area.¹¹⁴ Moreover, α_2 -adrenergic receptor agonists like clonidine are commonly used drugs in the treatment of ethanol withdrawal symptoms.^{115,116} *In vitro* ethanol administration in NG108-15 cells induced a strong induction of α_{2B} -adrenergic receptors over 48 h and α_{2C} -adrenergic receptor expression over a 5-day treatment period.¹¹⁷ Unfortunately, no comparable *in vivo* studies on α_2 -adrenergic receptor expression have been reported. Studies on ethanol's effects on the densities of β -adrenergic receptors have shown controversial results (see Table 7.2), but a decrease of high affinity β -adrenergic binding sites is evident in some investigations.^{118,119} These data also correspond to a recently reported

finding that chronic ethanol intake reduces adenylyl cyclase activity only in intoxicated alcohol dependent patients.^{120,121} Taken together, these studies provide evidence that ethanol intoxication impairs noradrenergic neurotransmission, as indicated by reduced high affinity adrenoceptor binding sites.

7.4.4 Opioid Receptors

The endogenous opioid system has been implicated in mediating the effects of ethanol and other drugs of abuse. Ethanol withdrawal is associated with an increased endorphin release in the nucleus accumbens.¹²² Special interest has been raised on the μ -opioid receptor function, since animals lacking the μ -opioid receptor lose almost all the rewarding efficacy of morphine.^{123,124} Two clinical treatment trials with the μ -opioid receptor antagonist naltrexone have reported reduced relapses in ethanol-dependent patients.^{125,126} The expression studies provide preliminary evidence of specific alterations in the opioid system by ethanol. Chronic ethanol treatment induced a decrease of μ -opioid receptor expression in the hypothalamus,¹²⁷ the nucleus accumbens, but not in the striatum of mice (see Table 7.2). In contrast, the density and mRNA expression of the δ -opioid receptors remain unaltered after continuous ethanol administration in all brain regions investigated (see Table 7.2).^{127–129} The expression of opioid ligands is also altered by chronic ethanol administration. One week of ethanol treatment induced a 50 to 60% increase in prodynorphin mRNA expression and 60% decrease in vasopressin mRNA expression (see Table 7.2).¹³⁰

In conclusion, ethanol and ethanol withdrawal alter the expression of opioid genes, particularly the μ -opioid receptor. This might have relevance to drug-seeking behavior in animals and in humans.

7.4.5 Other G-Protein Coupled Receptors

Chronic ethanol treatment has been shown to affect a number of other G-protein receptors. Syvalahti et al.¹³¹ reported a 30% downregulation in the density of the muscarinic acetylcholine receptor (mAChR) after chronic ethanol treatment, but Rothberg et al.¹³² found no change (see Table 7.2). Special interest has focused on receptors that are colocalized with dopamine or opioid receptors, e.g., neurotensin receptors and adenosine receptors. The results of these studies are shown in Table 7.2. Chronic ethanol administration induced a significant reduction in neurotensin receptor density and binding affinity in the striatum of different mice lines.^{133,134} Adenosine receptor densities were measured in brains of rats who had life-long access to ethanol solutions as well as in rats who had no access to alcohol.¹³⁵ This study reported an age-dependent decline in the number of adenosine A₁ and adenosine A₂ receptors in striatopallidal cells that was observed in both the drug naive and in ethanol-consuming groups.¹³⁵ In

contrast, ethanol withdrawal (either single or multiple episodes) was able to induce a 20 to 50% increase in adenosine A₁ receptor densities in the cerebral cortex of standard laboratory mice.¹³⁶ Clearly, more work is needed examining ethanol's effects on gene expression of neurotensin and adenosine receptors before conclusions can be drawn about their roles in ethanol dependence.

7.5 Neurotransmitter Transporters

7.5.1 Dopamine Transporter

The dopamine transporter (DAT) is an important molecule for controlling dopaminergic turnover in the brain. Moreover, it is an important initial site of action for psychostimulants such as amphetamine and cocaine. Chronic ethanol treatment affects the function of the DAT: A single photon emission computer tomography (SPECT) study on Finish alcohol-dependent patients found a 30% decrease in the DAT while these patients were intoxicated.¹³⁷ However, another human positron emission tomography (PET) study with the radioligand 11C d-threo-methylphenidate showed no significant difference in DAT densities between alcohol-dependent patients and normal healthy volunteers.¹³⁸ The divergence among the data might be related to different blood ethanol levels that were not controlled in these studies. Further investigations will help to clarify the functional meaning of this molecule.

7.5.2 Serotonin Transporter

The serotonin transporter (5-HTT) has received attention in alcohol research since human studies have identified a promotor polymorphism in the gene that encodes this transporter.¹³⁹ This polymorphism is associated with several subtypes of alcoholism.^{94,95} Initial animal studies have indicated that chronic ethanol treatment increases [³H]serotonin uptake after chronic ethanol ingestion.^{140,141} However, human studies on ethanol's effects on 5-HTT expression have shown controversial results. A PET study with the radioligand [¹²⁵I]-citalopram showed a 30% decrease in density of 5-HTT sites in the dorsal raphe region of ethanol-dependent patients.¹⁴² Another post-mortem study in humans reported an overall increase in 5-HTT sites in the dorsal raphe region of alcohol-dependent patients as well as increased ligand binding with [¹²⁵I]-citalopram.¹⁴³ Interestingly, the binding intensity was largely influenced by the 5-HTT genotype.¹⁴³ Further imaging studies on a larger number of subjects are needed to confirm the 5-HTT as a trait marker for alcoholism.

7.6 Second Messenger Systems

7.6.1 G-Proteins

The observed changes in radioligand binding studies for several neuroceptors after chronic ethanol administration raise the question whether these alterations might be accompanied by changes in second messenger function or gene expression. Interest has focused on G-protein subunits, adenylyl cyclases, and components of the phosphoinositoldiphosphate signaling system (see Table 7.2). Two investigations argue for a shift of G-protein function toward an increased expression of several inhibitory G-protein subunits after chronic ethanol exposure (see Table 7.2).^{144,145} However, the study of Wenrich et al.¹⁴⁶ emphasizes that these alterations in G-protein subunit expression depend dramatically on the duration of ethanol treatment and the method of ethanol administration. Further investigations using more standardized ethanol treatment protocols and behavioral analyses will help to elucidate the functional meaning of these findings.

7.6.2 Adenylyl Cyclase

Interest has focused on adenylyl cyclase (AC) activity, since this enzyme can easily be measured in human platelets. One clinical study reported a significant decrease in adenylyl cyclase activity in platelets of alcohol-dependent patients after stimulation with guanine nucleotide or prostaglandinE (PGE) as compared to normal healthy volunteers, even if they were abstinent for more than a year.¹⁴⁷ The same group showed that basal activity of adenylyl cyclase was reduced in platelets of male alcohol-dependent patients.¹⁴⁸ Data on animal models support these findings. Seven days of oral ethanol administration reduced forskolin-stimulated AC activity in the cerebral cortex of mice by about 20%.¹⁴⁵ However, more recent clinical investigations show that the reduced activity of platelet adenylyl cyclase might be due to elevated blood ethanol levels, which was not controlled for in previous investigations.^{120,121}

7.7 Transcription Factors

7.7.1 cAMP Responsive Element-Binding Protein (CREBP)

The induction of different transcription factors is believed to play an important role in mediating the effects of long-term ethanol exposure. Pandey et al.¹⁵⁰ investigated the effects of ethanol on the expression of the cAMP-responsive element-binding protein (CREBP). Acute ethanol administration

or withdrawal did not alter CREBP-binding activity,¹⁵⁰ but did stimulate CREBP phosphorylation in granular cells of the cerebellum in rats.¹⁵¹ In contrast, withdrawal after chronic ethanol treatment induced a strong expression of CREBP-binding in the rat cortex.¹⁵⁰ Interestingly, the expression of the brain-derived neurotrophic factor (BDNF) was increased in a similar manner.¹⁵⁰ Since the observed changes in CREBP expression occur nearly in the same time window as withdrawal induced anxiety syndromes,¹⁵⁰ these alterations may contribute to drug-seeking behavior and ethanol craving.

7.7.2 Immediate Early Genes (IEGs)

Investigation of IEG expression has been an interesting research field, since IEGs are believed to represent neuronal activity after different tasks, e.g., after seizures or other stress related behavioral events.¹⁵² In addition, long-term changes in gene expression are often preceded by the activation of genes of the IEG group. The *c-fos* gene is one well-characterized IEG member. The activator protein 1 (AP-1) complex is formed from hetero- and homodimers of the *c-fos* protein.¹⁵³ Early studies indicated that acute ethanol administration strongly suppresses pentylenetetrazole-induced *c-fos* mRNA expression, whereas the noncompetitive NMDA receptor antagonist dizocilpine (MK-801) suppresses ethanol withdrawal-induced *c-fos* mRNA expression in rat hippocampus and cortex.^{154,155} In addition, an acute ethanol administration (2 g/kg i.p.) was able to suppress stress-induced *c-fos* mRNA expression and immunoreactivity in the hippocampus and piriform cortex of the rat (see Table 7.3).¹⁵⁶ Moreover, low doses of acute ethanol reduced some environmental induced *c-fos* expression in the cortex, the hippocampus, and hypothalamus, but also induced expression in the central nucleus of amygdala of mice, which may represent GABAergic activity (see Table 7.3).¹⁵⁷ Interestingly, the ethanol-specific induction of *c-fos* mRNA expression was 2-fold greater in the DBA/2J mouse strain, as compared to the C57BL/6 strain, which may underlie genetic differences in ethanol withdrawal severity.¹⁵⁸ Other IEGs have not been extensively studied for alterations in gene expression following ethanol exposure. Some interest has focused on the expression of a zinc finger protein termed *zif268* (also termed *egr-1*, *NGFI-A*)¹⁵⁹ and the *c-fos*-related gene *fosB*. Acute ethanol strongly suppressed the expression of *zif268* and *fosB* after a stress paradigm (see also, Table 7.3),¹⁶⁰ whereas ethanol withdrawal is able to induce an increase in expression.¹⁶¹ The IEG activation during ethanol withdrawal induces the expression of a variety of other genes like neuroreceptors and other transcription factors, which might be responsible for prolonged withdrawal syndromes or relapse.

7.7.3 Retinoic Acid Receptors (RARs)

Prenatal vitamin A-deficiency syndrome has been viewed under certain aspects as a model disease for the fetal alcohol syndrome.^{164–166} Although

TABLE 7.3
Ethanol-Induced Changes in Transcription Factors or Growth Factors Gene Expression

Gene	Alteration	Refs.
<i>Transcription Factors</i>		
CREB		
CREB protein ^a	No change	150
Immediate Early Genes (IEGs)		
c-fos ^a mRNA and protein ^{a,b,e,m,n}	Decreased	156
	Increased	157, 158, 163
NGFI-A ^a mRNA and protein ^{a,b}	Decreased	160, 162
fosB ^a protein ^b	Decreased	160
<i>Growth Factors</i>		
NGF mRNA and protein ^b	No change	175
	Decreased	174
p75 protein ⁱ	Decreased	176
trkA protein ⁱ	Decreased	176
BDNF mRNA ^b	Decreased	171
trkB mRNA ^b	Increased	172
b-FGF mRNA ^b	No change	171
neurotrophin3 mRNA ^b	No change	171

^acortex; ^bhippocampus; ^cnucleus accumbens; ^dhypothalamus; ^estriatum; ^fglobus pallidum; ^gsubstantia nigra; ^hdorsal raphe; ⁱcerebellum; ^kventral tegmental area; ^lwhole brain; ^mamygdala; ⁿparaventricular nucleus; ^ostimulation 2 weeks, decrease after 10 weeks; ^pK_d decrease; ^qacute high-dose ethanol administration.

ethanol treatment did not alter brain vitamin A levels, the embryos of the ethanol-fed mothers displayed a 70% decreased concentration of RAR β mRNA in the fetal brain on gestation day 12, which returned to control values by day 20 of gestation.¹⁶⁵ Moreover, maternal ethanol treatment resulted in increased RAR α mRNA expression in the fetal brain at gestation day 20, which was accompanied by normal RAR β and RAR γ mRNA levels. The availability of free vitamin A was reduced via increased levels of cellular retinol-binding protein (CRBP) on both dates investigated.¹⁶⁵ However, only one study regarding ethanol's effects on the retinoic acid system in adult animals has been reported. Chronic ethanol intake in the ethanol-tolerant C57BL/6J mice induced an 80 to 90% increase in RAR β receptor mRNA expression in whole brain homogenates, which was reversed 4 months after ethanol withdrawal.¹⁶⁷ Interestingly, chronic ethanol exposure induced the expression of the enzyme transglutaminase (tTG) by 40 to 50%,¹⁶⁷ an enzyme, which is involved in RAR-dependent apoptotic processes.¹⁶⁸ These data support the hypothesis that interactions between ethanol and the retinol system are partially responsible for fetal alcohol syndrome abnormalities,¹⁶⁹ but further studies are needed to clarify the role of the retinoic acid receptor system in adult animals.

7.8 Growth Factors

Interest in growth factors has risen since neurodevelopmental abnormalities are observed in fetal alcohol syndrome. The findings on the expression of the nerve growth factor (NGF) have remained controversial. Angelucci et al.¹⁷⁰ demonstrated that a single intraperitoneal ethanol injection at gestation day 15 reduced the expression of NGF and the NGF receptor (p75NGFR) in the hippocampus of a developing rat at various time points. Chronic ethanol treatment alters the expression of various growth factors and their receptors (see Table 7.3). These data suggest that chronic ethanol exposure during pregnancy, in particular, alters the expression of brain-derived neurotrophic factor (BDNF) and its receptor trkB in the rat hippocampus.^{171,172} However, studies on the expression of NGF, basic-fibroblast-growth-factor (bFGF) and neurotrophin-3 have shown unaltered expression or controversial results.^{171,173–175} Interestingly, chronic ethanol treatment in adult animals induced the expression of the NGF receptor p75 and trkA in the hippocampus.¹⁷⁶ Studies on adult animals will help to understand the relationship between altered growth factor expression and neurotransmitter function. Taken together, the changes in expression of several growth factors and their receptors might partially explain long-lasting cognitive impairments observed after fetal chronic ethanol intake.

7.9 Summary

Alcohol clearly affects the expression of numerous genes within the central nervous system. Within the last 10 years knowledge about which genes are regulated by alcohol has increased rapidly, and with the advent of gene expression micro arrays (gene chips) the next 10 years should expand this knowledge at an even greater speed by enabling the researcher to examine simultaneously the regulation of thousands of genes. For example, a recent study using microarray analysis to examine gene expression changes in the frontal cerebral cortex of human alcoholics found selective reprogramming of myelin-related genes as well as changes in cell cycle genes and several neuronal genes.¹⁷⁷ Animal models for specific alcohol responses have been an important tool of researchers for identifying which genes are regulated by alcohol in the mammalian brain. However, it remains to be determined to what extent genetic variation in the expression of these genes contributes to a predisposition to alcoholism. This will be an important area of investigation, and should provide exciting new insights into the genetic basis of alcoholism.

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Abbreviations

AP-1	activator protein 1
AC	adenylyl cyclase
bFGF	basic-fibroblast-growth-factor
BDNF	brain-derived neurotrophic factor
CAM-kinase	calmodulin dependent kinase
CREBP	cAMP responsive element binding protein
CRBP	cellular retinol binding protein
CNS	central nervous system
MK-801	dizocilpine
DA	dopamine
DAT	dopamine transporter
GABA_A	γ -aminobutyric acid type A receptor
5-HT	5-hydroxytryptamin
5-HTT	5-hydroxytryptamin transporter
IEG	immediate early gene
G_i	inhibitory G-protein
G₀	modulatory G-protein subunit
mAChR	muscarinergic acetylcholine receptor
NGF	nerve growth factor
NGFI-A	nerve growth factor inducible gene A
NMDA	N-methyl-D-aspartate receptor
PET	positron emission tomography
PGE	prostaglandin _E
PKA	protein kinase A
RAR	retinoic acid receptor
SSRI	selective serotonin reuptake inhibitor
SPECT	single photon emission tomography
G_s	stimulatory G-protein
tTG	transglutaminase
trkA	tyrosin kinase A
trkB	tyrosine kinase B
VOCC	voltage-operated calcium channel

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8

Nutrient Control of Insulin-Stimulated Glucose Transport in 3T3-L1 Adipocytes

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8.1 Introduction

It has been known for many years that nutrients control the expression of genes in bacteria and lower eucaryotes, which control hexose transport.¹⁻³ It is only more recently that the effects of nutrient availability on mammalian transport systems have been examined. These studies have been aided by the development of cell lines and culture techniques which allow long-term exposure to nutrients under defined conditions. For lack of model systems much of the earlier work focused on the effect of glucose deprivation on insulin-insensitive transport activity.⁴⁻¹⁰ Results showed that in every system glucose transport activity increased in response to glucose deprivation in a protein synthesis-dependent fashion. Conversely, raising glucose concentrations decreased transport activity in a protein synthesis-independent manner. With the development of insulin-sensitive cell lines like the murine 3T3-L1 adipocytes and rat L6 and L8 myocytes, experiments like these were

repeated. For example, van Putten and Krans¹¹ showed in the mid-1980s that glucose deprivation of 3T3-L1 adipocytes resulted in an increase in the $V_{\max}$ of glucose transport activity similar to that seen in the non-insulin-dependent cells. Together these results suggested that either the number of transport molecules were increasing at the cell surface in response to glucose deprivation or that the transporter at the cell surface was activated in some fashion.

8.2 Facilitated Glucose Transporters

Mueckler et al.¹² cloned the first mammalian transporter from human hepatoma cells. The expression of this transporter was widespread and became known as the constitutive glucose transporter, GLUT1; cloning of the insulin-sensitive glucose transporter by a number of laboratories followed within a few years.^{13–15} To date, a total of five* transporters have been cloned, one of which possesses significant fructose transport activity.¹⁶ These transport proteins have in common a structure which includes a 12-membrane spanning domain and an *N*-linked glycosylation site within the first extracellular loop. Although mutant and chimeric transporters have been designed^{17–23} these expression studies have revealed few clues in understanding the relationship of structure and function.

8.3 Alteration in Glucose Transporter Activity in Response to Glucose Stress in 3T3-L1 Adipocytes

Fig. 8.1 shows an experiment similar to that published by Van Putten and Krans¹¹ and later by others,²⁴ including our own laboratory.²⁵ In this experiment, fully differentiated 3T3-L1 adipocytes were incubated for up to 24 h in the presence of 25 mM glucose (A), the absence of glucose (B), or the absence of glucose but presence of 25 mM fructose (C). At specific times, transport activity was evaluated under both basal (open symbols) and insulin-stimulated (closed symbols) conditions. In glucose-fed cells, basal transport values remained fairly constant over the course of the experiment. Insulin stimulation was robust with a 6-fold increase in activity relative to basal values. This stimulation rate was also fairly constant over the course of the experiment. Glucose deprivation increased the basal activity and over time reduced the ability of insulin to further stimulate (see also Fig. 8.2). Interestingly, fructose when substituted for glucose resulted in the same changes (C) as did glucose deprivation.

* Three new members of the glucose transporter family have been identified since this manuscript was first submitted. These include GLUTXI,⁵⁴ GLUT8,^{55,56} and GLUT9.⁵⁷

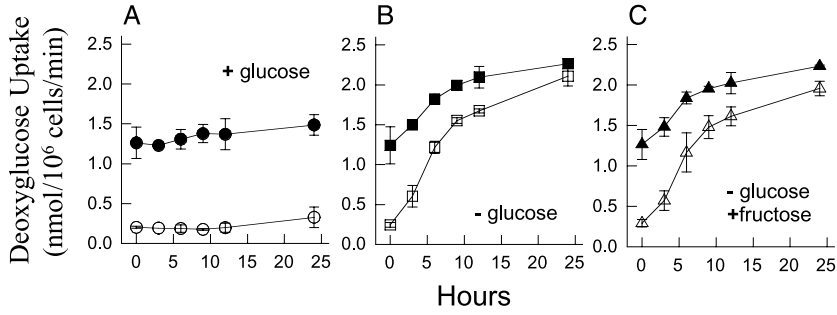


FIGURE 8.1
Effect of glucose deprivation on insulin-sensitive glucose transport activity. 3T3L1 cells were grown and differentiated according to Frost and Lane.²⁶ Cells were fed 24 h in advance of the experiment with complete medium to assure metabolic uniformity. At time 0, cells were exposed to medium that contained 25 mM glucose (+glucose), no glucose (-glucose), or no glucose but supplemented with 25 mM fructose (-glucose, +fructose). Transport activity was measured in the absence or presence of 1 μ M insulin as previously described.²⁶ Data are expressed as the mean $\pm$ S.D. of three independent experiments with each time point run in duplicate.

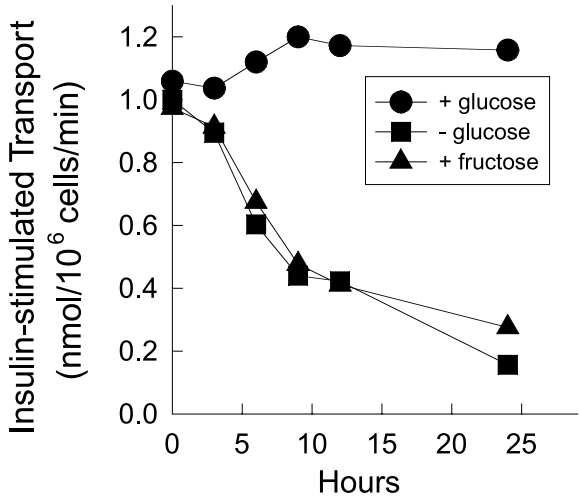


FIGURE 8.2
Change in insulin-sensitive transport activity in response to glucose deprivation. Basal transport values from Fig. 8.1 were subtracted from insulin-stimulated values and plotted against time of deprivation.

8.4 Effect of Glucose Deprivation on GLUT4 mRNA Expression

With the development of molecular probes for GLUT1 and GLUT4, a better understanding of the mechanism underlying the glucose-dependent changes in glucose transport activity has surfaced. First, it must be stated that regulation appears to be cell-type specific. In rat glial cells, Northern blot analysis revealed that glucose deprivation increases the expression of GLUT1 mRNA by 4 to 6-fold over glucose-fed controls.²⁷ This correlated with an increase in the expression of GLUT1 protein. While it was not established that the increase occurred in the plasma membrane, it is reasonable to assume such as transport activity increases coordinately with increased GLUT1. Similar results were observed in L6, L8, and BC₃H1 myocytes,^{28,29} leading to the hypothesis that GLUT1 belongs to the stress-inducible, glucose-regulated protein family.²⁸ Other explanations have also been offered to explain the increase in transport activity. Ortiz et al.³⁰ have demonstrated that lysosomal degradation of GLUT1 in murine fibroblasts is blocked in the absence of glucose which allows the accumulation of GLUT1 protein in the face of constant synthesis. In these cells, GLUT1 mRNA was unchanged by glucose deprivation. We have shown that neither the expression of GLUT1 mRNA nor GLUT1 protein is affected in 3T3-L1 adipocytes during the early stages of glucose deprivation where much of the activation occurs. This suggests the synthesis of another transport protein or the synthesis of a transport activating protein. Several investigators have identified proteins which interact with GLUT1, but thus far none of these proteins have been shown to alter transport activity or are induced by glucose deprivation.^{31–33}

In 3T3-L1 adipocytes and other insulin-sensitive cells, GLUT4 expression must be considered in addition to GLUT1 in order to understand the effects of glucose deprivation. Like GLUT1, the expression of GLUT4 appears to be regulated in a cell-specific fashion. Koivisto et al.³⁴ showed little effect of glucose deprivation on GLUT4 mRNA expression in L6 myocytes. Mayor et al.²⁹ were actually unable to detect GLUT4 in BC₃H1 cells in either the presence or absence of glucose. However, we have shown a significant decrease in GLUT4 mRNA in 3T3-L1 adipocytes in response to glucose deprivation such that by 48 h, no GLUT4 could be detected.²⁵ An experiment similar in design to this is shown in Fig. 8.3. The level of GLUT4 mRNA in glucose-fed cells was nearly constant over the course of 24 h (Fig. 8.3A). In glucose-deprived cells, we observed a decrease in GLUT4 mRNA at 15 h. By 24 h, there was little GLUT4 remaining. Note that actin mRNA expression was relatively constant over this timeframe. Fig. 8.3B shows a composite of three separate experiments, demonstrating that from 15 to 24 h there was a statistically significant decrease in GLUT4 mRNA expression. Despite the fact that glucose was removed from the cells at time zero, it is interesting that the level of GLUT4 mRNA remained stable over the first 12 h. We believe that this is

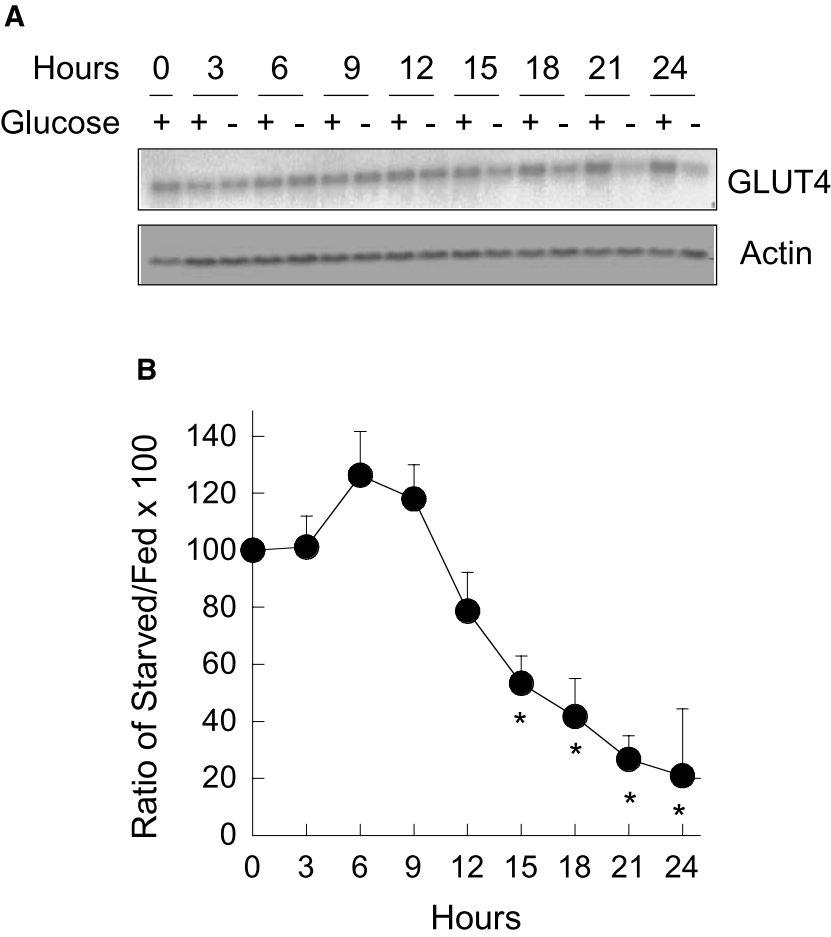


FIGURE 8.3

Time-dependent loss of GLUT4 mRNA in response to hexose deprivation. (A) Cells were treated for 24 h in advance of the start of the experiment. At time zero, cells were exposed to medium containing 25 mM glucose in the presence of 10% FBS or to glucose-free DMEM containing 10% dialyzed FBS. After the indicated time, total RNA was isolated and separated by size on a horizontal polyacrylamide gel. After transfer to nylon overnight and UV cross-linking, the blot was probed with a GLUT4 cDNA (provided by Maureen Charron) or an actin cDNA (provided by Harry Nick).²⁵ (B) Three replicates of the experiment described in (A) were used to calculate the mean $\pm$ S.D. of the starved-to-fed ratios. *, $p < .05$.

related to the provision of intracellular glucose through an endogenous source, namely, glycogen.³⁵ Removal of glucose from the medium results in a time-dependent loss of glycogen. With a $t_{1/2}$ for glycogen loss of approximately 6 h, nearly 80% of the pool is gone by 12 h and greater than 90% at 24 h. We conclude from these combined studies that glucose regulates the expression of GLUT4 mRNA. Tordjman et al. reported similar results in this same cell line.²⁴

We were intrigued by the similarity in the effect of glucose deprivation on insulin-sensitive transport activity in the presence or absence of fructose (see Fig. 8.1). Thus, we examined for the first time the effect of fructose, in a glucose-free background, on GLUT4 expression (Fig. 8.4). These results show that the presence of fructose prevents the loss of GLUT4, despite the lack of glucose. This suggests that a metabolite of glucose rather than glucose itself is responsible for regulating the expression of GLUT4. In this regard, both glucose 6-P^{36,37} and xylulose 6-P³⁸ have been implicated as potential mediators of other glucose-dependent genes, although this has not been studied with respect to GLUT4.

8.5 Effect of Glucose Deprivation on GLUT4 Protein Expression

Changes in mRNA would only have significance if the level of protein expression were, in turn, affected. To determine whether glucose deprivation leads to changes in the expression and compartmentalization of GLUT4 protein, we fractionated cells which had been exposed or not to glucose for 24 h. Shown in Fig. 8.5 is the distribution of GLUT4 in the plasma membrane (PM), the high-density microsomal fraction (HDM), or the low-density membrane fraction (LDM). The PM fraction contains little

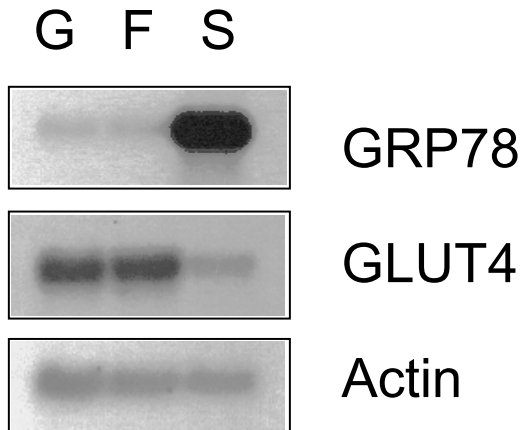


FIGURE 8.4

Effect of glucose deprivation on GLUT4 mRNA expression. Cells were fed 24 h in advance of the experiment and then 24 h in the presence of glucose (G), in the absence of glucose, but presence of 25 mM fructose (F), or in the complete absence of hexose (S). Total RNA was isolated using techniques described previously.²⁵ Northern blots were probed with cDNA for GLUT4, Actin, or GRP78 (provided by Amy Lee).

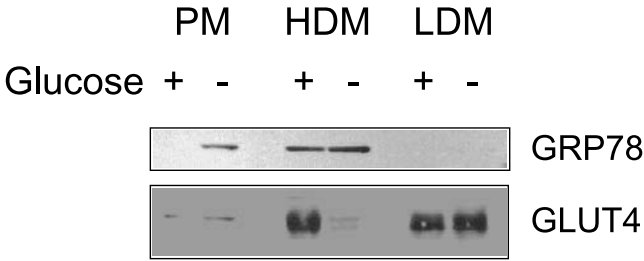


FIGURE 8.5

Distribution of GLUT4 in control and glucose-deprived cells. Cells were incubated in complete medium or glucose-free medium for 24 h. Membranes were isolated as described and characterized previously.³⁹ Proteins were separated by SDS-PAGE (7.5%) and transferred to nitrocellulose. Immunoblot analysis was performed using an antibody generated against a synthetic peptide analogous to the 12 N-terminal amino acids of GRP78 or an antibody generated against a synthetic peptide analogous to the 12 C-terminal amino acids of GLUT4. Enhanced chemiluminescence was used for final detection.

GLUT4 in control cells (glucose-fed), as noted previously.^{39,40} Likewise, there is little GLUT4 in the glucose-deprived cells. This provides evidence that the increase in basal activity observed in the absence of glucose is not contributed to by an increase in GLUT4. The HDM fraction contains both endoplasmic reticulum (ER) and Golgi membranes and thus represents the biosynthetic compartment. This is illustrated here by the presence of GRP78. The amount of GLUT4 in glucose-deprived cells in the HDM fraction relative to the controls is significantly reduced. Interestingly, there appears to be little difference in the level of GLUT4 in the LDM fraction in glucose-deprived cells relative to the fed controls. This membrane fraction contains the pool of GLUT4 which we and others have shown translocates to the plasma membrane in response to insulin.⁴⁰ The reason for this protection is not completely clear. It is possible that insufficient time had passed for the storage compartment to reflect the decrease in the synthetic pool. However, we have also shown that protein degradation, specifically that of GLUT1, is inhibited with extended glucose deprivation.⁴¹ As GLUT1 appears to resemble total protein in this instance, it is possible that GLUT4 degradation is blocked, as well. Regardless, our data show that GLUT4 protein is downregulated, which reflects the loss of GLUT4 mRNA. Tordjman et al.²⁴ came to a different conclusion in studies performed similarly. As mentioned above, these investigators showed that GLUT4 mRNA decreased over time in response to glucose deprivation. Yet, their data also suggested that GLUT4 protein did not reflect this decrease. However, these investigators examined only the PM and LDM fractions. It is thus easy to understand the reason for their conclusion. We would have concluded the same, had we not examined the biosynthetic pool, the HDM fraction, as well.

8.6 Effect of Glucose Deprivation on GLUT4 Translocation

To determine whether the lack of insulin-sensitive transport was due to a defect in translocation, we examined subcellular fractions isolated from cells exposed to medium with or without glucose for 24 h and then challenged acutely with insulin. Shown in Fig. 8.6 is the result of this experiment. As seen before in glucose-fed controls, insulin stimulated the recruitment of GLUT4 from the LDM fraction to the PM. Surprisingly, translocation of GLUT4 in the glucose-deprived cells was similar to that in control cells. Thus, despite the loss of insulin-sensitive glucose transport in glucose-deprived cells, the level of GLUT4 in the PM after exposure to insulin appears similar to glucose-fed cells. This may suggest that the transporter is not correctly inserted in the PM for functional expression. One way to test this is to label the transporter at the exofacial glucose-binding site. Experiments like this were done in the early 1990s by Kozka et al.⁴² using cell the impermeant bis-mannose photolabel, 2-*N*-(4-(1-azido-2, 2, 2-trifluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA). In fact, these investigators showed essentially the same level of GLUT4 on the cell surface in response to insulin in glucose-fed vs. glucose-deprived cells. Thus arises a dilemma. Not only is there an increase in the amount of GLUT4 on the cell surface in glucose-deprived cells in response to insulin, but also, the transporter is inserted such that the exofacial glucose-binding site is accessible to extracellular glucose. Why there is no insulin-stimulated glucose transport activity remains a mystery.

8.7 Conclusions and Future Directions

In recent reviews of this topic, questions were raised for future investigation.^{43,44} Among these were questions regarding how glucose affects transport distribution and gene expression. It seems little information has been gained in the intervening years despite solid evidence that such regulation must exist. Thus, we still have many challenges ahead. For example, is it possible that the activity of GLUT4 is regulated by a protein which does not exist in glucose-deprived cells? Or is the activity associated with transport activation in glucose-deprived cells a function which is distributed between that process and the GLUT4 transporter? Or perhaps GLUT4 substitutes for the activation process which might mean that the PM can accommodate only a limited number of transport proteins.

At the transcriptional level, one possible avenue of investigation may lie in the identification of regulatory elements in the promoter regions of GLUT4, in particular, and identification of proteins that interact with those

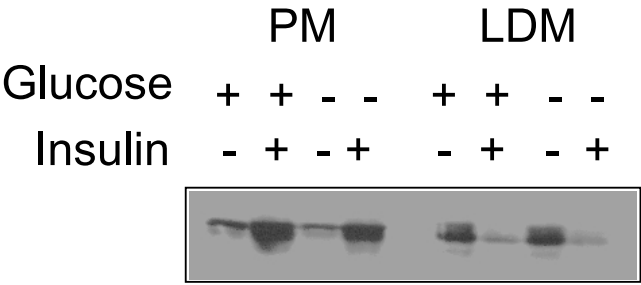


FIGURE 8.6
Translocation of GLUT4 in response to insulin. 3T3-L1 Adipocytes were incubated in Dulbecco's modified Eagle's medium (DMEM) in the presence or absence of 25 mM glucose. Cells were then washed and treated with 1 μ M insulin for 10 min in KRP. Membrane fractions were collected and proteins separated by SDS-PAGE (10%). After transfer, GLUT4 was identified by immunoblotting.

elements. Other genes like those which encode pyruvate kinase,⁴⁵ fatty acid synthase,⁴⁶ and spot14⁴⁷ appear to be controlled by glucose availability through regions, known as E-boxes, which have the canonical sequence CANN TG.⁴⁸ This motif is recognized by the transcription factors USF1 or USF2 (upstream stimulatory factors).⁴⁹⁻⁵¹ These proteins are members of the basic helix-loop-helix leucine zipper family of transcription factors, characterized by a highly conserved C-terminal domain responsible for their dimerization and DNA binding.⁵² Evaluation of the 5' promoter region of GLUT4 [see Reference 53] reveals the presence of a single non-palindromic E-box CACGTG upstream from the typical AP2 and SP1 binding sites. Studies investigating the regulatory potential of this site would be of significant interest.

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9

Prohormone Processing and Disorders of Energy Homeostasis

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9.1 Introduction

Research into the molecular causes of obesity and diabetes has grown significantly in recent years. This is due in part to the rapid increase in the prevalence of obesity and related morbidities, and most importantly because the molecular genetic tools to identify underlying causes for these diseases have now become available. The identification of the five single-gene obesity mutations in the mouse has, in particular, changed the field of obesity. Whereas in the past, obesity was perceived predominantly as a metabolic disease, the notion that obesity is a disease of the central nervous system (CNS) has now gained wide acceptance. A striking feature of the genes underlying the “big” five spontaneous single-gene mutations in mouse, *ob*, *db* (encoding leptin and leptin receptor, respectively), *A^y* (encoding agouti — antagonist for melanocortin receptors), *fat* (encoding carboxypeptidase E), and *tub* (encoding a novel neuronal protein) is that they all act in the hypothalamus and affect neuro/endocrine signaling.¹ This suggests that, at the least, a significant subgroup of obesities arises from defects in the central regulation of energy homeostasis, and not simply from defects in metabolic pathways.

Food intake behavior is controlled primarily by inhibitory neural and humoral signals (e.g., melanin stimulating hormone (MSH), leptin, or insulin) that lead to cessation of ongoing ingestion; a new meal is initiated when these signals diminish in the post-absorptive state. However, this apparently simple behavior is modulated by a multitude of internal and external stimuli whose modes of action are not well understood. Control over feeding behavior rests within the CNS. For example, the spinal cord and brainstem affect energy homeostasis via the autonomic nervous system. The hypothalamus and the limbic system receive signals indicating the metabolic state of the body (quality of ingested food, gastric extension, etc.) and integrate this information with cortical information about factors such as taste, memory, and competing desires.^{2,3}

Important neuro/endocrine signals that govern energy homeostasis involve the neuropeptides. Although these neuropeptides were originally thought only to function as hormones, it has become clear that they possess hormonal as well as neuromodulator and neurotransmitter activities.⁴ Often the same peptide can carry out several of these functions, depending upon the cell type in which it is expressed. The insights gained from the obesity mutations are, in part, responsible for the expanded research interest into the action, biosynthesis, and regulation of neuro/endocrine peptides. This renewed interest builds upon a successful half a century of research, primarily at the biochemical level, which has shown that neuro/endocrine peptides are synthesized as precursor proteins and undergo extensive processing before they are released as mature peptides upon stimulation.^{5–7} Many of the mechanisms and enzymes involved in prohormone processing have been identified and it has recently been shown that rare mutations in the genes of processing enzymes can lead to syndromes of obesity in animals and humans.^{8–9}

Interest in novel neuropeptides and peptide hormones has also increased because their receptors are attractive drug targets. In the course of the genome project, a large number of orphan receptors, particularly the G-coupled seven transmembrane spanning receptors, were discovered, suggesting the existence of currently unknown peptides. Indeed, the search for endogenous agonists for these orphan receptors has become a new paradigm for the identification of novel bioactive peptides.^{10,11}

In this brief overview, we will outline the prohormone processing pathway as it acts in most neuro/endocrine tissues and describe the major enzymes constituting this pathway. We will introduce mouse models with defects in prohormone processing and describe two human conditions in which processing defects lead to obesity syndromes. Finally, we will discuss a genetic approach for identifying factors that act downstream of the processing pathway in mediating the effects of prohormone maturation on energy homeostasis.

9.2 Processing of Neuro-Endocrine Peptides

Even with our currently limited knowledge, we are faced with a bewildering array of neuropeptides, often encoded by the same precursor molecule. We are just beginning to elucidate the mechanisms by which such a system has evolved through the identification of families of receptors (e.g., opioid,¹² neuropeptide Y (NPY) receptor family¹³), of peptides (e.g., oxytocin/vasopressin,¹⁴ opioid/orphanin family¹⁵), and of processing enzymes (subtilisin/kexin family¹⁶).

It was commonly thought that during vertebrate evolution two or three genome duplications occurred.¹⁷ Receptor duplication may have afforded protection from a lethal loss of receptor function, but once a duplicated receptor was present in an organism, it was also less restrained in conserving function and could assume a new role. For example, some neuropeptides can still bind to a number of receptor subtypes that are often expressed in a cell-specific manner (e.g., neuropeptide Y (NPY)).¹⁸ Similarly, new peptides could evolve by gene duplication, and acquisition of mutations may have made them more active toward a specific receptor subtype. In addition, once a basic peptide processing system was in place, a new way of generating novel bioactive peptides was available by the generation of new processing sites in peptide precursor proteins. The complexity observed today most likely arose through this ability to achieve diversity without compromising the function of existing peptide/receptor systems and the viability of the organism. It is less likely that there was a particular need to have the same stretch of amino acids encode a peptide-mediating pain response (beta-endorphin, β -END), as well as a peptide-regulating eating behavior (alpha-melanin stimulating hormone, α -MSH) as for example in proopiomelanocortin (POMC). If such a view is correct, then we would expect important regulatory events to occur post-translationally. The processing pathway and the enzymes involved then assume particular importance.

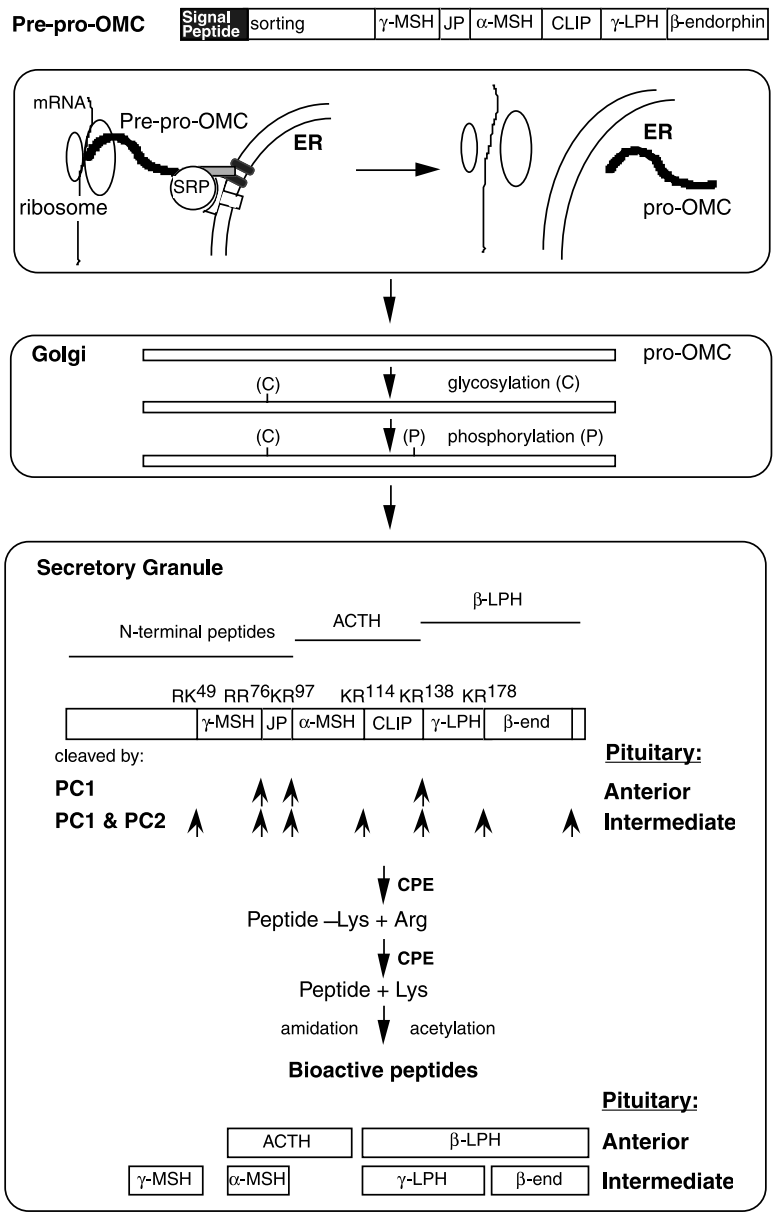


FIGURE 9.1
A schematic description of the major processing steps in the maturation of pituitary pre-proopiomelanocortin (POMC). POMC is synthesized by ribosomes, a preprohormone containing a hydrophobic signal peptide at the N-terminus. After targeting to the endoplasmic reticulum (ER) via binding to the signal recognition particle (SRP), this preprohormone associates with the ER membrane, the signal peptide traverses the membrane, and is cleaved off cotranslationally. POMC is then transported to the Golgi apparatus. The glycosylation of POMC begins in the rough ER and is completed in the Golgi apparatus with a number of modifications to the carbohydrate side chain.

9.2.1 The Prohormone Processing Pathway

Most secretory proteins are synthesized as precursors with an amino terminal signal peptide (amino acid residues)^{15–25} that is necessary for the targeting of the protein to the membrane of the endoplasmic reticulum (ER). As soon as the first few N-terminal amino acids of the hydrophobic signal peptide have been synthesized, the ribosomes become associated with the ER membrane and continue translation. These targeting, attachment, and translocation events are thought to be mediated by the signal recognition particle, its receptor, and a protein-conducting channel.¹⁹ While the protein chain is being inserted into the ER membrane the signal sequence is cleaved cotranslationally by a membrane-bound signal peptidase. After protein synthesis is finished the rest of the protein completes traversal through the membrane into the ER (Fig. 9.1).

From the ER, the proteins are transferred to the Golgi apparatus, and then are directed to their ultimate destination, the secretory vesicles or granules via the trans-Golgi network (TGN). It is also in the TGN that the proteins are sorted into a constitutive and a regulated secretory pathway. Precursor proteins in both pathways have to mature before they are released as bioactive peptides from the secretory vesicles, but maturation occurs in different compartments and is mediated by different enzymes.²⁰ In this review we are mainly concerned with the biosynthesis and action of neuro/endocrine peptides that are stored in the large dense-core vesicles (LDCV) of the regulated secretory pathway, from which they are exocytosed in response to a stimulus (secretagogue).²¹ In many cases, the maturation process is initiated by cleavage at pairs of basic amino acids (Arg-Arg, Arg-Lys, Lys-Arg, or Lys-Lys) that in precursor proteins link sequences of the bioactive peptides. This cleavage occurs in an acidic milieu and is mediated by prohormone convertases (PCs) followed by removal of C-terminal basic residues by carboxypeptidase E (CPE, also known as enkephalin convertase and carboxypeptidase H).

Polypeptide precursors can be classified by their bioactive peptide compositions: (1) several copies of the same peptide (e.g., proenkephalin A and B);

FIGURE 9.1 (CONTINUED)

Within the Golgi apparatus, POMC is sorted and packaged into the secretory granule where it is proteolytically processed to biologically active peptides that are released from the cell upon stimulation. The major endoproteolytic cleavages of POMC, catalyzed by PC1 and PC2, occur at pairs of basic amino acid residues. PC1 preferentially cleaves at Glu-Gly-Lys-Arg⁹⁷- and Glu-Phe-Lys-Arg¹³⁸-generating ACTH and β -LPH which are normally observed in the anterior pituitary. In the intermediate lobe of the pituitary the PC1 cleavage products are further processed by PC2 to generate γ -MSH, α -MSH, γ -LPH, β -MSH, and β -endorphin. Completion of POMC processing in the pituitary requires removal of Lys and Arg residues from the carboxy termini of the PC1 and PC2 cleavage products by the exopeptidase CPE. In addition to proteolytic modification, chemical modifications including amidation and acetylation take place during POMC processing, altering the biological activity of the modified peptide. PC1, prohormone convertase 1; PC2, prohormone convertase 2; ACTH, adrenal corticotropin hormone; β -LPH; β -lipotropic hormone; MSH, melanocyte-stimulating hormone; β -end, β -endorphin; CLIP, corticotropin-like intermediate lobe peptide.

(2) two or more different, yet homologous, peptides (e.g., proglucagon and provasoactive intestinal peptide (VIP)); (3) only one peptide (e.g., the pancreatic polypeptide-neuropeptide Y family, insulin, oxytocin-vasopressin precursors); (4) only one bioactive peptide released at varying lengths (e.g., cholecystokinin (CCK)-83, -58, -39, -33, -22, -8, and -5); and (5) entirely different types of bioactive peptides (e.g., proopiomelanocortin).⁵

9.2.2 Proopiomelanocortin, an Example of Prohormone Processing

An illustrative example of key features of prohormone processing is presented by proopiomelanocortin (POMC), a precursor molecule which encodes a number of different neuropeptides that are produced by differential post-translational processing (Fig. 9.1).

POMC is processed differently in various tissues. In the anterior pituitary, POMC is mainly converted to adrenal corticotropin hormone (ACTH), β -lipotropic hormone (lipotropin; β -LPH), and N-POMC. In the intermediate pituitary, ACTH is further processed to produce α -melanocyte-stimulating hormone (α -MSH) and a corticotropin-like intermediate lobe peptide (CLIP); β -LPH is further processed to β -endorphin and γ -LPH, and N-POMC yields γ -MSH and an N-terminal fragment. The major endoproteolytic cleavages of POMC occur at pairs of dibasic amino acid residues and are catalyzed by PC1 (also known as PC3) and PC2.^{20,22,23} PC1 preferentially cleaves POMC at residues Glu-Gly-Lys-Arg⁹⁷↓ and Glu-Phe-Lys-Arg¹³⁸↓ generating ACTH and β -LPH (Fig. 9.1, the amino acid numbering follows Reference 24). However, PC1 also cleaves at Lys-Asp-Lys-Arg¹⁷⁸↓ and Ala-Gln-Arg-Arg⁷⁶↓ producing β -endorphin and joining peptide (JP), respectively.

On the other hand, PC2 is able to efficiently cleave POMC at Ala-Gln-Arg-Arg⁷⁶↓, Glu-Gly-Lys-Arg⁹⁷↓, Gly-Lys-Lys-Arg¹¹⁴↓, Glu-Phe-Lys-Arg¹³⁸↓, and Lys-Asp-Lys-Arg¹⁷⁸↓ generating α -MSH and β -endorphin (Fig. 9.1). Although PC2 has a broader substrate specificity than PC1, both PC1 and PC2 appear to be required for full cleavage of POMC. Lowering the endogenous expression of PC1 without a change in PC2 levels, by expressing PC1 antisense RNA in AtT20 cells, results in secretion of large amounts of unprocessed POMC and less of the smaller peptides.²⁵ It appears that the initial steps of POMC processing in the intermediate pituitary are carried out by PC1; PC2 then catalyzes the later steps.²⁶ However, the factors which regulate the preferential sites of cleavage by PC2 have yet to be determined. This tissue-specific differential proteolytic processing of POMC is possibly directed by a distinct distribution of PC1 and PC2 mRNAs. There is evidence that PC1 mRNA is expressed in both the anterior and intermediate pituitary, while PC2 mRNA is mainly expressed in the intermediate pituitary.^{27–29}

The exopeptidase carboxypeptidase E (CPE) completes POMC maturation by removal of Lys and Arg residues from the carboxy-termini of the PC1 and PC2 cleavage products.³⁰

9.2.3 The Major Processing Enzymes

9.2.3.1 Endoproteases

In mammals, seven endoproteases currently have been identified. These include furin/PACE (paired-basic-amino-acid-cleaving enzyme, *Pcsk3*), PC1/PC3 (*Pcsk1*), PC2 (*Pcsk2*), PACE4 (*Pcsk6*), PC4 (*Pcsk4*), PC5/PC6 (*Pcsk5*), and PC7/PC8/LPC (*Pcsk7*).^{16,31,32} PC1/3, PC2, PC4, and PC5/6A are soluble forms of endoproteases, while the furin/PACE, PACE4, PC5/6B, and PC7/8 have transmembrane-spanning domains. Furin/PACE³³ and PACE4³⁴ are ubiquitously distributed, while PC1/3 and PC2 are found mainly in neural and endocrine cells.^{35,36} PC4 is primarily expressed in testis.³⁷ PC5/6 isoforms A and B are generated from a single gene by alternative splicing. PC5/6A is widely distributed in endocrine and non-endocrine tissues, and is particularly abundant in intestinal and adrenal cells, while PC5/6B is restricted to tissues including the lungs, intestines, and adrenals.^{16,38} PC7/8/LPC, which is expressed in the brain and many other tissues, appears to be a functional homologue of furin/PACE.^{39–41} PC1/3, PC2, PC4, PC5/6A are sorted to and activated in the regulated secretory pathway, while furin/PACE, PACE4, PC5/6B, and PC7/8 are involved in the processing of proproteins in the constitutive pathway.⁴²

Each endoprotease is synthesized as an inactive precursor (i.e., zymogen) and has both unique and conserved structural motifs. They commonly have an N-terminal signal peptide followed by an amino-terminal prodomain (about 80 to 90 aa); a catalytic domain with an active triad of aspartic acid, histidine, and serine residues; and a downstream domain (about 150 aa) called the P domain/homo B-domain with a conserved pentapeptide motif, RRDGL.³¹ The N-terminal prodomain appears to act as an intramolecular chaperone that assists in the folding of the zymogen within the ER. The activation of these enzymes begins with an autocatalytic cleavage of the amino-terminal prodomain at a preferred Arg-X-Lys/Arg-Arg ↓ motif.^{42,43} The P domain appears to be important for the correct folding of the endoprotease within the ER, as alteration of this domain prohibits the intramolecular cleavage of the prodomain.⁴⁴ The prodomain remains attached noncovalently even after cleavage, preventing cleavage of heterologous substrates. The cleaved convertase is then transferred to the Golgi and the trans-Golgi network (TGN), where the prodomain is completely dissociated from the catalytic domain, thereby, fully activating the enzyme.⁴³

The major convertases that function in the secretory granules of the regulated secretory pathway in neuroendocrine cells and some other tissues are PC1/PC3 and PC2. Unlike the PC1/3 activation described above, PC2 has a more complex mechanism of transport and activation. PC2 requires a neuroendocrine secretory peptide, 7B2, for its maturation. 7B2 is itself derived from a precursor protein which, when proteolytically cleaved at a multibasic site by a furin-like protease, produces a ~21 kDa N-terminal domain and a small C-terminal fragment (CT-peptide). Pro-7B2 is usually coexpressed with PC2 in most neuroendocrine cells and has chaperonin-like properties.⁴⁵ However, 7B2 does

not assist in proPC2 folding, but instead binds to completely folded PC2. The N-terminal domain of 7B2 facilitates the maturation of proPC2, and the C-terminal peptide inhibits PC2 activity. In the ER, pro7B2 binds to the catalytic domain of proPC2 through a proline-rich segment in the N-terminal fragment of the pro7B2 protein that is predicted to form a polyproline helix-like structure.⁴⁶ This facilitates the transport of the pro7B2/proPC2 complex to the Golgi apparatus. In the TGN, pro7B2 is rapidly cleaved by furin or PC3, generating its 21-kDa form. Once the 21-kDa 7B2 has been generated, the PC2 propeptide is processed intramolecularly. Upon autocatalytic processing of proPC2, the liberated inhibitory CT-peptide moves to the site previously occupied by the prodomain. The inhibitory effect of the CT-peptide is terminated by exoproteolytic removal of the two carboxyterminal lysine residues in the CT-peptide by carboxypeptidase E.^{31,47}

9.2.3.2 Carboxypeptidases

There have been eleven members of the metallo-carboxypeptidase gene family identified in mammals. Based on both function and homology, these carboxypeptidases (CPs) are generally divided into two groups. One group includes the digestive enzymes CPA (mast cell), CPA1, CPA2, CPU (plasma) and CPB (pancreas). The other group includes enzymes whose endoproteolytic activity is restricted to one or two C-terminal basic amino acids of substrate peptides and proteins. The latter group includes secretory granule CPE, plasma CPN, extracellular membrane-bound CPM, CPD, and CPZ.⁴⁸

CPN acts in plasma to remove basic residues from a variety of peptides and proteins.⁴⁹ CPM is widely expressed on plasma membranes and proposed to process peptide hormones at the cell surface.⁵⁰ CPE has previously been thought to be the only secretory granule carboxypeptidase.³⁰ However, lack of functional CPE in *Cpe^{fat}/Cpe^{fat}* mice does not cause complete elimination of mature peptides,⁸ indicating that other carboxypeptidases might compensate for the CPE defect. As a result, these additional activities were unmasked, and subsequently the CPE-like proteins, CPD^{51,52} and CPZ⁵³ were cloned. CPD is present mainly in the Golgi and/or trans-Golgi network but not in the secretory granules where the majority of propeptide processing occurs, and CPZ does not function in an acidic milieu as it exists in the secretory granule. It is, therefore, an open question whether these newly identified enzymes are responsible for the residual carboxyterminal processing in *Cpe^{fat}/Cpe^{fat}* mice.

9.2.4 Regulation of Prohormone Processing

Why do organisms possess such an elaborate system for producing neuro/endocrine peptides or peptide hormones? An attractive hypothesis is that a large pool of inactive precursors may facilitate a rapid release of peptides through enzymatic cleavage rather than undergoing *de novo* synthesis.⁵⁴ Experiments testing this hypothesis have been sparse. However, it has been

found that procholecystokinin (CCK) and progastrin accumulate in pituitary corticotrophs⁵⁵ and enkephalin precursor molecules accumulate in the adrenal medulla.⁵⁶ In the latter case, it was also shown that treatment of adrenal chromaffin cells with reserpine leads to the appearance of enkephalin pentapeptides concomitant with a decrease of the precursor molecules.⁵⁷ Overall, however, the importance of posttranslational attenuation in stimulated secretion has not yet been demonstrated. Nevertheless, cell-specific attenuation has been shown to occur and can be achieved by modulating the activity of the processing enzymes, either by their transcriptional regulation or through the action of protease inhibitors.⁵⁸

Regulation of PC1, PC2, and furin expression in several cell lines treated with activators of the cyclic AMP (cAMP) and protein kinase C (PKC) second messenger pathways shows that prohormone convertases may be differentially regulated by cAMP and PKC mechanisms. This regulation may be tissue specific, as activation of the cAMP pathway increases mRNA levels of PC1, but not PC2 or furin, in SK-N-MCIXC cells, but increases all three mRNAs in WE 4/2 cells.^{59,60} Such differential expression of the convertases should give rise to different sets of neuropeptides derived from given precursors.

Finally, the role of protease inhibitors in prohormone processing has just begun to be explored. Recently it was shown that the Kunitz protease inhibitor of the amyloid precursor protein (KPI/APP) can inhibit enkephalin processing enzyme (PTP) *in vitro* and that KPI/APP and PTP colocalize in the regulated secretory vesicles.⁶¹ A role of the C-terminal fragment of the chaperone 7B2 in the inhibition of PC2 has been proposed,⁴⁶ and an *in vivo* role for this inhibition is suggested by the proinsulin processing pattern with the accumulation of diarginyl extended des-31,32 proinsulin observed in the Cpe^{fat} mutation.⁸ However, expression of CT peptide alone has so far failed to lead to PC2 inhibition.⁶²

9.2.5 Precursor Molecules and Peptides Important in Energy Homeostasis

The emerging picture indicates a complex pattern of neurotransmitter interaction in the control of food intake and energy homeostasis in the CNS. Many of the neurotransmitters have turned out to be neuropeptides, and novel peptides are likely to be found in the coming years. In Table 9.1, we present a short listing of precursor proteins and selected bioactive peptides derived from them that are thought to play a role in energy homeostasis.

9.3 Animal Models with Defects in the Processing Pathway

Despite the undoubted success of previous research carried out on prohormone processing, the recent identification of the molecular defects in genetic

TABLE 9.1
Peptides Involved in Regulation of Food Intake

Precursors	Peptides	Food Intake	Proposed CNS Sites of Action	Ref.
POMC	α -MSH	↓	Cortex, striatum, bed nucleus of the stria terminalis, PVN and ARC	105
			PVN, SON, CEA, AP, and PBN	106
	ACTH	↓	VMH	107, 108
	β -endorphin	↑	VMH and DMH	109, 110
ProNPY	NPY	↑	ARC and PVN	111–114
ProCRH	CRH	↓	PVN and ARC	112, 115
			PVN	116
ProTRH	TRH	↓	PVN	117, 118
ProGalanin	Galanin	↑	PVN	119, 120
			PVN and AG	121
			PVN and SON	122
ProCCK	CCK-8	↓	AP	123
			PVN	124
			NTS, AP, CeA, and PVN	125, 126
ProNT	NT	↓	VMH	127, 128
			PVN	129, 130
ProMCH	MCH	↑	LHA	131, 132
			VMH and DMH	133, 134

POMC, Proopiomelanocortin; NPY, Neuropeptide Y; CRH, Corticotropin releasing hormone; TRH, Thyrotropin releasing hormone; CCK, cholecystokinin; NT, Neurotensin; MCH, Melanin-concentrating hormone; PVN, Paraventricular nucleus of the hypothalamus; LHA, Lateral hypothalamic area; SON, Supraoptic nucleus; ARC, Arcuate nucleus; DMH, Dorsomedial hypothalamic nuclei; VMH, Ventromedial hypothalamic nuclei; CEA, Central nucleus of the amygdala; AP, Area postrema; PBN, Lateral parabrachial nucleus; GAL-R1-R, galanin-R1 receptor; AG, amygdala; NTS, nucleus of the solitary tract; CeA, Central nucleus of the amygdala.

animal models has contributed new insights. Examination of peptide intermediates in null mutants for the various processing enzymes has told us which peptides are processed by what enzyme *in vivo*, as well as provided information about the temporal order of processing. Also, as in the example of the *Cpe^{fat}* mutation, a null allele has lead to the discovery of previously masked activities (e.g., carboxypeptidase D). Finally, animal models have shown for the first time that prohormone-processing defects can lead to misregulation of energy homeostasis.

9.3.1 *Cpe^{fat}*

The mutation *fat*, first described in 1990⁶³ as a late onset, moderate obesity without hyperglycemia, arose on the inbred HRS/J (HRS) strain. HRS-*fat* / *fat*

mice were chronically hyperinsulinemic from weaning, but never developed overt diabetes. The mutation has been backcrossed onto C57BLKS/J (BKS), an inbred strain whose background genes can interact with mutations like *Lep^{ob}* (*ob*) or *Lepr^{db}* (*db*) to result in hyperglycemia.⁶⁴ At early backcross generations, the *fat* mutation was not diabetogenic. However, development of maturity-onset hyperglycemia, primarily in males, has been observed in generations beyond F6.⁶⁵

The hyperinsulinemia previously described as the earliest phenotypic characteristic caused by the *fat* mutation is primarily a hyperproinsulinemia.⁸ Hyper(pro)insulinemia is demonstrable well before the development of overt obesity and is independent of the glycemic status of mutant *fat/fat* mice. Furthermore, one of the insulin-processing enzymes, CPE, is severely reduced in amount and activity in BKS-*fat/fat* mice compared to littermate controls. In BKS-*fat/fat* mice the reduction of CPE activity is associated with a reduced processing and accumulation of des-31,32 diarginyl extended proinsulin I and II. Since proinsulins and C-terminally extended insulins are less active than fully processed insulins, this reduction in biologically active insulins is thought to lead to an increased physiologic demand, producing β -cell hypersecretion.

The *fat* mutation had been previously mapped to mouse Chr 8. It was subsequently demonstrated that *fat* represents a missense mutation (Ser202Pro) in *Cpe*.⁸ This mutation renders the protein unstable, leading to its degradation in the ER.⁶⁶

The relationship between hyperproinsulinemia and the appearance of a later-developing obesity and diabetes on specific genetic backgrounds remains unclear. Hyperproinsulinemia is present in mice both with and without hyperglycemia (unpublished results) and hyperproinsulinemia in itself does not seem to be associated with gross obesity in humans.^{67,68} The observation that the CPE defect is not limited to islet tissue, but also is present in the pituitary as well as the brain (unpublished observations) suggests that the obesity and diabetes in *fat/fat* mice may develop as a result of widespread defects in the exopeptidase processing step required for full maturation of a number of neuroendocrine/endocrine prohormones in addition to proinsulin. Indeed, a number of laboratories have meanwhile confirmed such defects in the processing of gastrin,^{69,70} CCK,⁷¹⁻⁷³ POMC, enkephalin, dynorphin,⁶⁶ luteinizing hormone release hormone (LHRH) (Dr. W.C. Wetsel, pers. comm.), neurotensin, melanin concentrating hormone,⁷⁴ and substance P⁷⁵ in this model.

Like the *db* and *ob* mutants, *fat/fat* mice are infertile, indicative of defects in the neuroendocrine axis. Yet the fact that hypercorticism, a feature of BKS-*db/db* and *ob/ob* mutants, is not observed in BKS-*fat/fat* mice indicates that the CPE defect affects the neuroendocrine system at a level different from that of the defects encoded by *db* and *ob*. The low corticosterone levels found in *fat/fat* mice may be due to the defect in POMC processing and consequent lack of ACTH.

Due to the lack of CPE activity, *fat/fat* mice have secondary defects in the prohormone processing pathway as well. As described below, CPE appears

to be necessary in the activation process of PC2, so that *fat/fat* mice also have reduced PC2 activity. Such a defect would explain the presence of des-31,32-diarginylnyl extended insulins in this model. Data have also been presented suggesting that CPE acts as a receptor that targets propeptides, such as POMC, to the regulated secretory pathway. In the absence of CPE, some propeptides may be mistargeted to the constitutive pathway, where they would not undergo correct processing.⁷⁶

Considering the widespread abnormalities in prohormone processing in vital systems it seems puzzling that these mice only show an obesity syndrome but otherwise continue to be quite healthy compared to models such as *ob* and *db*. Also, since PC2 acts upstream of CPE, one might expect a similar obesity phenotype in PC2 knockout mice. However, as described below, PC2 null mice are not obese. It is possible that the obesity is the result of an interaction between the *Cpe^{fat}* mutation and the specific genetic background of BKS or HRS, respectively. Preliminary evidence indicates that this may be the case. As described further below, in genetic crosses, *fat/fat* animals can have normal body weights. And *Cpe^{fat}* made congenic on the Castaneus inbred background causes no overt obesity but the mice die prematurely between 12 and 16 weeks of age (Naggert et al., unpublished). Because obese BKS-*fat/fat* mice still produce at least a small amount of mature peptides, it has been suggested that perhaps other carboxypeptidases can substitute for the CPE defect. Indeed, the search for such activities has yielded several new candidates such as carboxypeptidase D,⁵¹ CPZ,⁵³ CPX-1,⁷⁷ and CPX-2.⁷⁸ Although CPD is not found in secretory granules, it is a good candidate for a redundant activity in *fat/fat* mice based on cellular co-localization studies with CPE.⁷⁹ It is conceivable then that in certain genetic backgrounds such redundant enzymes are less active and can no longer compensate for the CPE defect, resulting in a more severe phenotype.

9.3.2 PC2 Null Mutant

A mouse model *Pcsk2^{tm/Dfs}* lacking active (PC2^{-/-}) was generated by disrupting the third exon of the *Pcsk2* (PC2) gene which contains the C-terminal end of the prodomain and the beginning of the catalytic domain including the site for proteolytic autoactivation.⁸⁰ Homozygous null mice appeared normal at birth, but their growth rate was slightly reduced compared to wild-type mice. No dramatic disturbances in body proportions, adiposity, or major organs were observed. PC2^{-/-} mice exhibited chronic fasting hypoglycemia and a diminished rise in blood glucose levels during an intraperitoneal glucose tolerance test (GTT).

Morphologically, the α -, δ -, and γ -cells in the islets of Langerhans were hypertrophic and hyperplastic, while the β -cells were hypotrophic. Furthermore, electron microscopy studies revealed large numbers of immature-appearing secretory granules in both α - and β -cells. The proportion of proinsulin to insulin was significantly increased in the pancreas of PC2^{-/-}

mice compared with wild type (30 to 35% vs. 6%), indicating defective proinsulin processing.

PC2^{-/-} mice also had high levels of circulating and pancreatic proglucagon with no detectable mature glucagon. This is in contrast to insulin levels that were too low to be detectable. Proglucagon processing in α -cells is normally carried out by PC2.^{81,82} Because no sign of disturbed pancreatic islet morphology was observed in newborn PC2^{-/-} mice, this suggests that the α -cell hyperplasia was probably due to chronic severe glucagon deficiency rather than the result of aberrant developmental processes.

The partial processing of proinsulin in PC2^{-/-} mice may be ascribed to the normal coexpression of PC3 in β -cells. Although PC1/3 and PC2 have their preferential cleavage site in the proinsulin molecule, it has been demonstrated that PC3 can cleave at both sites, B-chain/C-peptide and C-peptide/A-chain junctions, in proinsulin to yield insulin.⁸³ PC2^{-/-} mice accumulate des-31,32 proinsulin intermediates which are the product of PC1/3 cleavage.^{84,85,47} There was also a complete absence of the major somatostatin peptide, somatostatin 14 (SS-14), in the pancreas of PC2^{-/-} mice. Instead, SS-28 was present as a major end-product of prosomatostatin processing. In addition to alterations in pancreatic hormone processing, the PC2^{-/-} mice also exhibited abnormalities in other neuro/endocrine tissues including marked reductions of enkephalins and dynorphins in the brain, and γ -MSH in the intermediate lobe of the pituitary.³¹

9.3.3 7B2 Null Mutant

7B2 null mutant mice (7B2^{-/-}) were created by a transposon-facilitated knock-out technique. Exon 3 of the *Sgne1* (7B2) gene was interrupted by random integration of a transposon and subsequent introduction of a *neo* cassette.⁸⁶

In 7B2^{-/-} mice, maturation of proPC2 was severely impaired and catalytic activity of PC2 was completely absent. The PC2-dependent processing of polypeptide hormones including proglucagon, proinsulin, and proenkephalin was significantly diminished, as was observed in PC2^{-/-} mice. Only 11% of 7B2^{-/-} mice survived to weaning and, unlike PC2^{-/-} mice, were runted, pale, and ecchymotic in appearance. 7B2^{-/-} mice were hypoglycemic with elevated circulating insulin-like material by 4 to 5 weeks of age. Morphologically, pancreatic architecture was disorganized and both β and non- β cells were hyperplastic. Striking increases (10- to 20-fold) in the levels of intact ACTH and minimal conversion of this peptide to α -MSH were observed in the pituitaries of 7B2^{-/-} mice. Circulating ACTH levels were also increased, accompanied by elevated corticosterone levels and adrenocortical hyperplasia. These phenotypes, together with others such as skin thinning and dermal atrophy with hyperkeratosis, make these mice a model for a Cushing's-like syndrome.

Compared to PC2-deficient mice, the 7B2^{-/-} mice appear to have more severe phenotypes, suggesting that 7B2 has additional *in vivo* functions apart

from activating PC2. Perhaps it acts as a chaperonin-like molecule for other, as yet, unidentified proteins in the secretory pathway.

9.3.4 The Murine Anorexia Mutation

Mice homozygous for the recessive anorexia mutation (*anx*, Chr 2) exhibit reduced food intake, a marked reduction in body weight, and an emaciated appearance. The mice have reduced stomach contents from about 5 days of age continuing until death at about 22 days. These findings suggest that these mice may be a model for anorexia, as they fail to ingest sufficient food to sustain life. However, other abnormal behaviors are observed, including head weaving, body tremors, uncoordinated gait, and hyperactivity.⁸⁷

Some observed characteristics (head weaving and body tremors) are typical of elevated serotonergic stimulation. Hyperinnervation of central serotonergic neurons in *anx* homozygotes has been demonstrated, while catecholaminergic innervation is normal, and normal laminar organization of the brain is retained.⁸⁸ Serotonergic mechanisms have also been implicated in modulating suckling behavior in newborns.⁸⁹

Although the defect in *anx* mice has not been identified at the molecular level, recent findings suggest that prohormone processing and/or trafficking may be affected.⁹⁰ One central pathway important in inducing feeding behavior involves elevated production of NPY in neurons of the hypothalamic arcuate nucleus. These NPY neurons project to the paraventricular nucleus where they synapse with neurons carrying NPY receptors. This projection is thought to mediate the powerful stimulation of hyperphagia by NPY. In *anx/anx* mice, NPY immunoreactivity accumulates in the perikarya of arcuate NPY neurons; however, decreased staining is observed in NPY terminals in the paraventricular, arcuate, and other hypothalamic nuclei compared to control littermates. This reduction in terminal NPY appears to be specific to the hypothalamus as other NPY systems in the striatum, hippocampus, and cortex were unaffected. However, the reduction in terminal NPY is not likely the sole cause of the anorexia phenotype since NPY knockout mice do not exhibit reduced food intake.⁹¹ It is probable then that *anx/anx* mice show a more generalized defect in axonal transport and/or prohormone processing of at least a subset of neuropeptides, including NPY. It is interesting to note in this context that Broberger et al. also reported a tendency toward reduced somatostatin in arcuate fibers, whereas CCK and 5-HT staining was unchanged.⁹⁰

9.4 Human Disease Associated with Processing Defects

Ultimately, we study animal models to give us insights into human conditions. The mouse, with its close genetic and physiological similarities to

humans, has contributed much to the understanding of human disease. After the first insights into the molecular causes of obesity had been gained in the mouse, mutations in the homologous genes in humans have been found to lead to similar obesity phenotypes.⁹²

9.4.1 PC1 Mutations

The first human disorder thought to be due to impaired prohormone processing, multiple endocrinopathy, was reported in 1995 at about the same time as the *fat* mutation in mouse.⁹³ The two diseases, caused by PC1 and CPE mutations, respectively, are remarkably similar in appearance. The 43-year-old-female proband was obese (89.2 kg body weight and 34.4 kg/m² body mass index (BMI) from childhood (36 kg at 3 years of age)). The proband had come to clinical attention because of infertility and hypogonadotropic hypogonadism was diagnosed. In addition to obesity, other clinical abnormalities shown in this patient included impaired glucose tolerance, post-prandial hyperglycemia, and hypocortisolism. Notably, this patient had markedly increased circulating proinsulin levels while levels of mature insulin in the plasma were barely detectable, indicating a defect in proinsulin processing.

Molecular genetic analysis showed that the proband was a compound heterozygote, having two different mutations in her PCSK1 gene which lead to a severe deficiency in active PC1/3.⁹ One mutation was a Gly483Arg missense mutation, which prevents processing of proPC1 and leads to retention of proPC1 in the ER. The other mutation was an A to C transversion at position +4 of the intron-5 donor splice site. This mutation causes skipping of exon 5, leading to a loss of 26 amino acid residues, a frameshift, and creation of a premature stop codon within the catalytic domain.

Although plasma insulin levels are low in PC2 null mice, they are, nevertheless, detectable.⁸⁴ PC1/3 deficiency, therefore, appears to cause more severe defects in proinsulin processing than PC2 deficiency. The elevated proinsulin in this human patient with PC1 mutations was accompanied by increased amounts of Des-64,65 proinsulin intermediates, as would be expected from a lack of PC1/3 which facilitates cleavage at the B-chain C-peptide junction generating Des-31,32 proinsulin. The patient was not diabetic, but had developed transient gestational diabetes during pregnancy of quadruplets (ovulation was induced with gonadotropins at the age of 30). While no elevation in proglucagon levels was observed in this patient, altered corticotropin precursor processing was reported to lead to a mild hypocortisolemia.

Unlike PC2 or 7B2 deficiency, but similar to the *Cpe^{fat}* mutation in mice, the lack of PC1/3 activity in humans leads to an obesity syndrome, consistent with a role of PC1/3 in the processing of hypothalamic neuropeptides controlling energy homeostasis and appetite. Nevertheless, the discrepancy in phenotype between the known mutations in the processing pathway requires further explanation. Specifically, the role of genetic background on expression of the runting and obesity phenotypes needs to be examined. A

PC1/3 null mutation on different mouse genetic backgrounds should help to further understand the mechanism of energy imbalance mediated by a PC1/3 defect.

9.4.2 POMC Mutations

Although not due to defects in the prohormone-processing machinery, two human cases of POMC deficiency caused by genetic defects are of interest⁹⁴ because mutations in the POMC gene also lead to early onset morbid obesity. The phenotypic attributes of one female patient were obesity (>30kg at 3 years of age), ACTH deficiency, and red hair pigmentation. Symptoms of increased appetite had been observed since 4 months of age. Direct sequencing of PCR products containing the entire coding region of the POMC gene revealed that this patient was a compound heterozygote for two different mutations in the third exon. One was a paternally transmitted transversion of G to T at nucleotide (nt) position 7013, which leads to a premature stop codon at amino acid 79.^{95,96} This truncated POMC protein is predicted to have lost bioactive POMC-derived peptides, including ACTH, α -MSH and β -endorphin. The other mutation in this patient was a one base pair (bp) deletion at nt 7133 (C) leading to a frame-shift which is predicted to disturb the structure of the receptor-binding core motif of ACTH and α -MSH (His-Phe-Arg-Trp to His-Phe-Ala-Gly) and introduces a premature stop codon at amino acid 131. Therefore, this compound heterozygous patient had a complete loss of ACTH, α -MSH, and β -endorphin, while the levels of other anterior- and pituitary-derived hormones were normal.

A second, male patient who was homozygous for a transversion of C to A at nt 3804 in exon 2 within the 5'-untranslated region developed early onset severe obesity (>40 kg at 8 years of age). This point mutation creates an extra out-of-frame initiation codon (ATG) and is predicted to interfere with normal POMC translation, leading to a complete lack of POMC protein.

Other variants of the POMC gene have been identified during screening of obese and underweight patients, but it is less clear in these cases whether the mutation is the cause for the observed obesity.⁹⁷

An in-frame insertion of 9-bp (AGC AGC GGC) or 18-bp (AGC AGC GGC AGC AGC GGC) between codons 73 and 74 of POMC, carboxy-terminal to γ -MSH, has been identified. One male patient, homozygous for the 9-bp insertion, was extremely obese at the age of 14 (BMI 32.2 kg/m², waist/hip ratio 0.97), and a female who was heterozygous for the 18-bp insertion had a BMI of 36.4 kg/m². In addition, a female obese adolescent was identified who carried two mutations in γ -LPH transmitted from her mother, an out-of-frame insertion of 6-bp (CCC GGG) within codon 176 introducing two amino acids, and a transversion of G $\rightarrow$ T at nt 7316 introducing a premature termination at codon 180. These mutations are predicted to cause loss of β -MSH and β -endorphin. The heterozygous mother was not obese (BMI 28.3 kg/m² at age 35.6). The patient also carried a missense mutation (A-7341-G, Glu-188-Gly)

transmitted from the father. Because the father was obese (BMI 30.4 kg/m², age 35.7 years) but presumably heterozygous, it is likely that mutations in other genes contributed to the obesity in the female patient (BMI 35.9 kg/m², waist/hip ratio 0.83, age 16.5 years).

9.4.3 Processing Defects in the Human Population

The discovery of rare forms of obesity syndromes caused by defects in prohormone processing prompted a search for such defects as causes for more common forms of obesity and non-insulin-dependent diabetes mellitus (NIDDM). Limited population studies have been carried out in which polymorphisms in the PCSK1 (PC1/3)^{98,99} and CPE genes¹⁰⁰ were examined in case-control studies. No significant associations with obesity or NIDDM were observed. For a microsatellite repeat polymorphism in intron 2 of the PCSK2 (PC2) gene, a higher frequency of one allele was found in Japanese NIDDM patients compared to controls.¹⁰¹ However, no difference in fasting glucose, insulin, or proinsulin levels were found when carriers of the associated allele were compared to noncarriers. Furthermore, only one mutation affecting the PC2 coding sequence was found in an NIDDM patient from this study cohort. Although mutations within the promoter region affecting expression levels cannot be excluded at this time, association with an NIDDM gene close to the PC2 gene is also a possibility.

Currently it does not appear as if mutations in prohormone processing genes can explain significant portions of obesity or Type II diabetes (NIDDM) in the general population; however, broader population-based screens are necessary to exclude these genes. In addition, the upstream control regions of these genes must be examined as well.

9.5 A Genetic Approach for Identifying Distal Effects on Energy Homeostasis

Much of our knowledge about the function of neuro/endocrine peptides stems from studies on localization, expression, observing changes in disease or healthy states, and administration of the peptides by nonphysiological means (intravenous or intracerebroventricular injections). While such studies have provided invaluable information about the possible functions of these peptides, they provide limited knowledge about their direct and indirect *in vivo* action. For example, injection of a single peptide in a single site may not reflect its *in vivo* action. It is entirely possible that the particular peptide is not released in isolation, but normally co-released with others in the same or different tissues. Additionally, the phenotypic effects of a peptide may not be due to changes in its presence or expression, but may be controlled by its

effects on downstream molecules. The *in vivo* function of many neuropeptides is still controversial.¹⁰²

A potentially powerful approach to revealing downstream factors that are important in mediating the effects of a mutation on the phenotype of a whole animal is quantitative trait locus (QTL) analysis. QTL analysis in mice makes use of the genetic variation between two inbred strains of mice. If the two genomes are mixed by carrying out backcrosses or F2 intercrosses, the new genetic combinations can give rise to measurable phenotypes. These traits can then be genetically mapped to QTLs. If only QTLs that interact epistatically with a particular mutation are considered, i.e., in the absence of the mutation no QTL can be detected, then these QTLs must represent genes in a pathway that connects the mutant gene with the observed phenotype. For example, the *fat* mutation is due to a loss of CPE activity, so that normal prohormone processing is impaired. However, because many neuropeptides are misprocessed, the etiology of the obese phenotype in *fat/fat* mice is not clear; e.g., is one peptide such as MSH critical or do multiple defects contribute to the observed obesity syndrome.

In order to determine which critical genes determine the obese/diabetic phenotype, a QTL mapping cross was carried out. A hyperglycemic BKS-*fat/fat* female was mated to a HRS-+/+ male and the resulting (BKS/HRS)F1-*fat*/+ offspring were intercrossed to obtain an F2 generation. The (BKS/HRS)F2-*fat/fat* animals were the experimental group and (BKS/HRS)F2-*fat*/+ and (BKS/HRS)F2-+/+ the controls. It was found that many of the obesity-related phenotypes such as body weight and adiposity, plasma glucose, insulin and serum lipid levels segregated in the (BKS/HRS)F2-*fat/fat* population.¹⁰³ From our preliminary analyses, several conclusions can be drawn.¹⁰⁴ First, there are one or more loci determining each of the subphenotypes that had been measured in this cross. Second, as has been found in other QTL analyses, some loci influence several phenotypes, while others affect only one specific phenotype. Third, the percentage of genetic variance explained by QTLs is generally high, so that a few major genes appear to control each trait. And fourth, importantly, the presence of these QTLs is entirely dependent on the presence of the *Cpe^{fat}* defect; they are not detected in its absence. For example, in F2-*fat/fat* mice, a QTL for adiposity on Chr 11 was detected ($F = 11.7$, $p = 0.003$), whereas no QTL was detected at this marker in F2-+/+ mice from the same cross ($F = 0.2$, $p = 0.905$). *Cpe^{fat}*, therefore, can be called a disease gene, i.e., a gene that is necessary, but not sufficient for the phenotype to develop. It has to interact with susceptibility genes, genes that are in themselves neither necessary nor sufficient to cause disease, but which in conjunction with the disease gene determine the observed phenotype.

The genetic mapping of such QTLs, followed by the construction of congenic strains to isolate the individual QTLs in a common genetic background, will eventually allow the identification of the underlying genes. Congenic strains are constructed by transferring a susceptibility allele onto a resistant strain background by repeated backcrossing to the resistant strain. It provides a convenient way of separating unlinked genes and is

particularly useful in assessing the nature and strength of the phenotypic contribution of individual genes in complex traits caused by multiple genes (Serreze et al. 1994).¹³⁵ These congenic strains can then be used in genetic fine mapping and positional (candidate) cloning of the gene determining the trait.

Extending such QTL analyses to the knockout mice for *Pcsk2* (PC2) and *Sgne1* (7B2) should shed light on the molecular reasons for the difference in phenotype compared to the *Cpe^{fat}* mice.

9.6 Conclusion

We have learned a great deal about prohormone processing during the past 50 years; however, major questions remain to be answered. Prominent among these is the question of the cell-specific and temporal regulation of neuropeptide synthesis, maturation, and secretion. New tools available, such as genetic analysis in animals and humans, and conditional inactivation of genes in intact organisms, will help to approach these questions.

Although the proximal actions of many neuropeptides and peptide hormones are known, understanding how these actions are translated into an observable phenotype in an intact organism is still in its infancy. With the identification of the melanocortin pathway over the past few years the first insights have been gained into the pathways controlling food intake and energy homeostasis. Others remain to be discovered.

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10

The Agouti Gene in Obesity: Central and Peripheral Mechanisms, and Therapeutic Implications

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10.1 Introduction

The cloning and characterization of several mouse obesity genes as well as their human homologues over the last 8 years have not only resulted in a quantum leap in our insight into the pathophysiology of obesity, but also present opportunities to identify new cellular targets for the development of novel therapeutic strategies. Although the *ob* and *db* genes have been the most widely studied of these, *agouti* was the first obesity gene cloned,¹ and in the ensuing 8 years, both central and peripheral mechanisms of agouti action have been studied extensively. This chapter focuses on these mecha-

nisms of agouti action and their application to the development of intervention strategies.

Variations in mouse coat color have long provided phenotypic markers for genotypic variation and mutations associated with disease. Mutations in the mouse coat color gene *agouti* were recognized to cause an adult-onset obesity by 1905,² and the *agouti* gene was finally cloned and characterized nearly a century later.^{1,3} The gene, mapped to mouse chromosome 2, encodes a 131-amino acid molecule with a consensus signal peptide.

Agouti is transiently expressed in neonatal skin, where it controls the relative amount and distribution of melanin produced by the hair follicle melanocytes. These melanocytes produce a black pigment, eumelanin, in response to α -melanocyte-stimulating hormone (α -MSH) stimulation, while in the absence of α -MSH they produce a default yellow pigment, pheomelanin⁴ [Fig. 10.1]. Follicular melanocytes produce eumelanin under the influence of α -MSH at the beginning of the hair growth cycle. During the mid-portion of this cycle, there is transient expression of *agouti*, which competitively antagonizes α -MSH receptor binding, resulting in a temporary synthesis of pheomelanin. As *agouti* expression ceases, eumelanin synthesis is restored under the influence of α -MSH, which is again able to bind to its receptor.⁵ This produces the characteristic pigmentation pattern of wild-type mice, a predominantly black hair shaft with a subapical yellow segment.⁴ There are two different wild-type alleles at the *agouti* locus: *agouti* (*A*) and

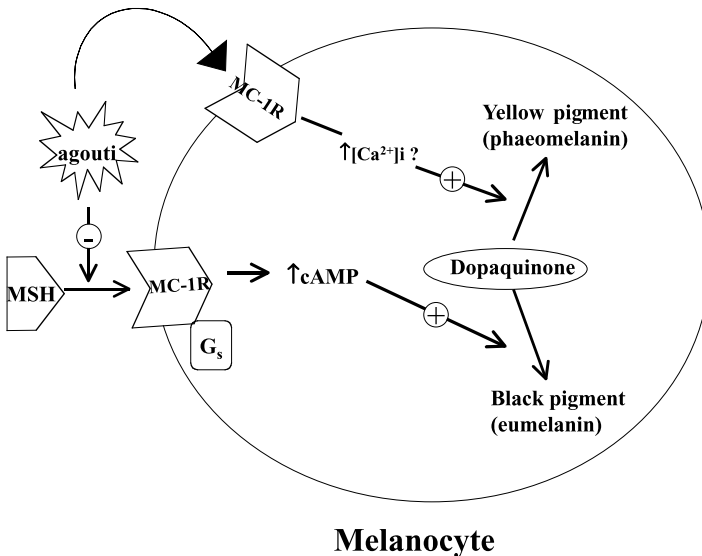


FIGURE 10.1

Agouti regulation of pigmentation. Agouti competitively antagonizes α -MSH binding to its receptor (MC1-R), thereby preventing MC1-R-G_s coupled synthesis of cAMP. Consequently, pheomelanin is synthesized. Agouti may also serve as an inverse agonist of MC1-R independently of α -MSH antagonism.

light-bellied agouti (A^w). In agouti (A) mice, yellow pigment is synthesized only during the mid-portion of the hair growth cycle, as described above, resulting in characteristic agouti hair (i.e., black hair with a subapical band of yellow). In contrast, the A^w mice have yellow hair on the ventral surface due to the expression of a ventral-specific agouti transcript, while their dorsal hairs have the characteristic agouti coloration due to the dorsal expression of the hair-cycle-specific agouti transcript noted above.^{6,7}

10.2 Agouti Mutations

At least 34 different dominant and recessive agouti alleles have been reported, resulting in a phenotypic dominance hierarchy in which phaeomelanin synthesis is generally dominant over eumelanin synthesis.⁸ A number of dominant mutations at the agouti locus result in ectopic agouti expression in virtually all tissues throughout the life of the mouse. The most dominant mutations are lethal yellow (A^y) and viable yellow (A^{vy}). These, along with sienna yellow (A^{sy}), intermediate yellow (A^{iy}), hypervariable yellow (A^{hvy}), and intracisternal A-particle yellow (A^{iapy}), are regulatory mutations in the promoter region, all of which result in ectopic expression of normal agouti transcripts. These mutations result in a uniformly yellow fur in A^y and variable coat colors in the other mutations, ranging from completely yellow to various degrees of yellow and black mottling to a black coat color, referred to as pseudoagouti, which closely resembles wild-type agouti.^{9–13} In addition, mice carrying these dominant mutations develop a pleiotropic syndrome which includes obesity, insulin resistance, hyperinsulinemia, hyperphagia, increased linear growth, and decreased thermogenesis.^{5,9,14} In general, the degree of agouti overexpression is correlated to the degree of mutant phenotype observed in terms of both coat color and metabolic derangements.¹⁵ This produces a spectrum from the yellow mice with the most pronounced obesity, to mottled mice that frequently exhibit a more attenuated syndrome, to pseudoagouti mice that exhibit minimal agouti expression (detectable via reverse transcriptase polymerase chain reaction (RT-PCR), but not via northern blot), a wild-type coat color and no apparent metabolic derangements.^{11,12,15}

Although the mechanism of somatic reversion to the lean phenotype in pseudoagouti animals is not fully defined, the detection of agouti transcripts indicates that epigenetic inactivation of regulatory sequences is responsible for the somatic reversion.¹³ Differences in the methylation status of the long terminal repeat (LTR) within the intracisternal A particle (IAP) responsible for ectopic agouti expression (see molecular characterization below) are associated with differential phenotypic expression, as the IAP LTR is methylated in pseudoagouti, but not yellow, mice.^{9,15} In addition, expression is influenced by maternal genotype and epigenetic phenotype at the agouti

locus^{15–17}, and pseudoagouti dams produce pseudoagouti offspring more frequently than do dams with yellow phenotypes.¹⁷

Each of the dominant agouti mutations results in ubiquitous expression of normal agouti protein.^{1,15,18} The concept that this ectopic expression is sufficient to induce the pleiotropic disease syndrome was confirmed in transgenic mice overexpressing agouti under the control of the promoters of one of two ubiquitously expressed genes, β -actin or phosphoglycerate kinase.¹⁹ Analysis of several lines of transgenic mice expressing agouti under the control of either promoter demonstrates agouti expression in every tissue examined, yellow fur, maturity-onset obesity, hyperinsulinemia, and marked increases in plasma leptin.^{19–21} Thus, ectopic expression of agouti is directly responsible for the metabolic derangements observed, as the transgenic animals recapitulate the phenotype of dominant agouti mutants.

10.3 Yellow Mouse Characteristics

Dominant agouti mutants exhibit maturity-onset obesity. Increases in body weight are first observed around 4 weeks of age, and peak between 8 and 17 months.^{5,22} Body fat approaches 25 to 26% of body fat in adult yellow mice, or approximately 4-fold higher than wild-type mice.²³ This increase in adiposity is characterized by adipocyte hypertrophy without hyperplasia, and adipose tissue transplantation studies demonstrate that this is largely due to host characteristics.^{24,25} In addition to their adiposity, yellow mice exhibit an overall increase in anabolic characteristics, with increased linear growth, muscle mass, and fat-free dry weight.^{26–29}

Although yellow obese mice do exhibit hyperphagia, this is not the primary cause of their obesity, as they exhibit a 4-fold increase in body fat but only a 30% increase in food intake. Accordingly, obesity in these mice is primarily due to increased metabolic efficiency, with a preferential partitioning of food energy into fat storage.³⁰ Yellow mice also exhibit increases in the expression and activity of lipogenic enzymes in both liver and adipose tissue.^{31,32} Further, lipolysis is reduced in adipose tissue of yellow mice compared to wild-type mice, and the lipolytic response to epinephrine, theophylline, and the β -agonists LY79771 and LY104119 is suppressed,^{33–35} while the response to dibutyryl cyclic AMP is normal. However, cAMP responses to stimulation with LY79771 or LY104119 are impaired, suggesting a defect in generation or maintenance of cAMP.^{34,35} Although one early report demonstrated a defect in adaptive thermogenesis in yellow obese mice,³⁶ there is no difference in brown adipose tissue thermogenic enzyme activity or thermogenic response to a β -adrenergic agonist in yellow vs. wild-type mice.^{35,37} Moreover, weight changes in *A^{vy}* mice subjected to energy restriction are not accompanied by changes in thermogenesis.³⁵ Accordingly, decreased thermogenesis does not account for the increased

metabolic efficiency characteristic of these animals. Instead, the observed coordinated augmentation of lipogenesis and inhibition of lipolysis may contribute to this increase in metabolic efficiency.

Early experiments demonstrated that parabiosis between yellow mice and normal littermates failed to affect body weight or composition in either group, demonstrating that the primary defect in yellow mouse obesity is not in a circulating factor that could be shared in the circulation of parabiotic partners.³⁸ Further, although both hypophysectomy and adrenalectomy attenuate obesity in yellow mice, differences in body composition persist.^{39–41} Thus, neither pituitary- nor adrenal-mediated endocrine pathways have a primary role in yellow mouse obesity. In contrast, given the role of insulin in promoting nutrient partitioning into adipose tissue, hyperinsulinemia may contribute to the enhanced metabolic efficiency of yellow mice. Elevations in insulin are first apparent at approximately 6 weeks of age and peaks at approximately 6 months of age,^{42,43} and there is a positive correlation between weight gain and insulinemia and between insulin levels and lipogenic enzyme activity in obese yellow mice.³² Moreover, pancreatic β -cell hyperplasia precedes the development of obesity in yellow mice. Finally, studies in transgenic mice demonstrate that excess weight gain is, in part, dependent upon insulin.⁴⁴ Nonetheless, the relationship among insulin resistance, hyperinsulinemia, and obesity is complex and yellow mouse obesity is clearly not solely due to hyperinsulinemia. Indeed, hyperinsulinemia induced in lean littermates failed to produce a comparable degree of weight gain to that found in agouti transgenic mice.⁴⁴ Thus, while hyperinsulinemia may promote the increased metabolic efficiency characteristic of obese yellow mice, it is not sufficient to produce the obesity observed. Instead, agouti and insulin appear to exhibit a synergistic interaction, discussed subsequently in this chapter, which results in the development of obesity.

10.4 Molecular Characterization of Agouti

Agouti was the first obesity gene to be cloned and characterized.^{1,3} The gene has three coding exons (exons 2, 3 and 4). The utilization of different promoters and alternative splicing of the first (non-coding) exon results in ventral-specific and hair-cycle-specific transcripts, discussed earlier in this chapter, that vary in size from 0.7 to 0.8 kb despite their identical coding sequences.^{6,7} The murine agouti gene is located on chromosome 2 and encodes a 131-amino acid protein.^{1,3} Agouti protein contains a 22-amino acid signal sequence, a highly basic central region in which 16 of the 29 amino acids are lysine or arginine residues, a poly-proline-rich region that follows the basic region, and a cysteine-rich carboxyl-terminal region (Fig. 10.2). Asn 39 is a putative N-linked glycosylation site, consistent with the purified protein having a larger apparent mass than predicted (18.5 vs. 11.8 kDa)^{3,45}(Fig. 10.2).

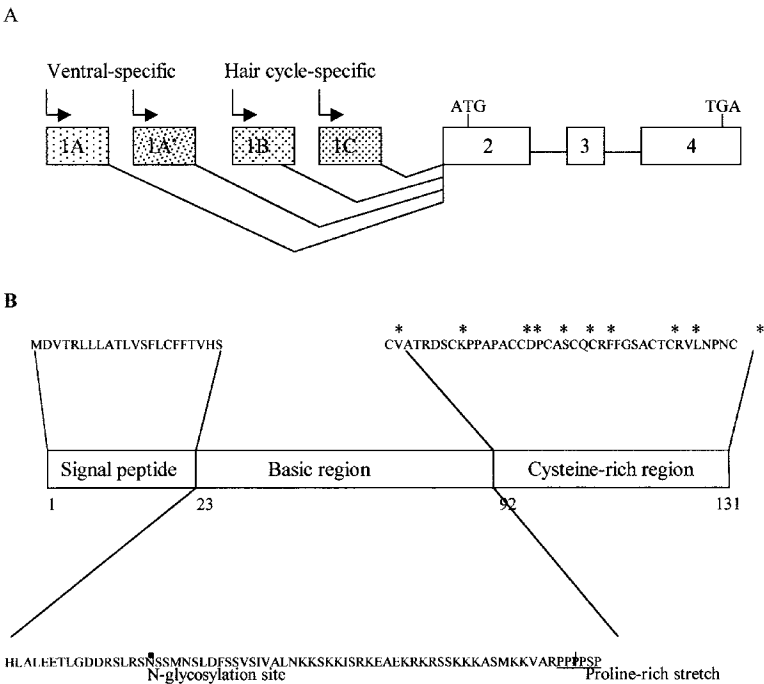


FIGURE 10.2
Genomic and protein structure of agouti. (A) Genomic structure of the mouse agouti gene. Alternative splicing of the first non-coding exon gives rise to ventral-specific (1A and 1A') and hair cycle-specific (1B and 1C) transcripts. The start and stop codons have been indicated in exons 2 and 4. (B) Agouti protein structure. • Putative N-glycosylation site. *Cysteine residues. Underline: Proline-rich stretch.

All 10 cysteine residues in the carboxyl terminal region are involved in forming disulfide bonds.⁴⁵ There is a strong spatial homology between this pattern of cysteine residues in agouti and those found in the venom of primitive hunting spiders and cone snails (plectoxins and conotoxins), toxins which primarily target calcium channels.^{45,46} It is noteworthy that the carboxyl-terminal region of agouti retains biological activity *in vitro*, while other constructs do not.^{45,47}

Lethal yellow (*A^y*) homozygotes die around the time of implantation, while heterozygotes express a larger than expected 1.1-kb agouti transcript in adult tissues. This increase in size is due to the replacement of novel sequences at the 5' untranslated region of the *A^y* agouti transcript, while exons 2 to 4 contain the 131-amino acid open reading frame identical to wild-type agouti transcripts.^{1,3} These novel 5' untranslated sequences correspond to the upstream untranslated region of a gene called *Raly* (*Ribonucleoprotein Associated with Lethal Yellow*), a ubiquitously expressed heterogeneous nuclear ribonucleoprotein which normally maps 280 kb proximal to agouti on mouse chromosome 2.^{18,48} This chimeric *Raly*/agouti transcript results from a 170-kb

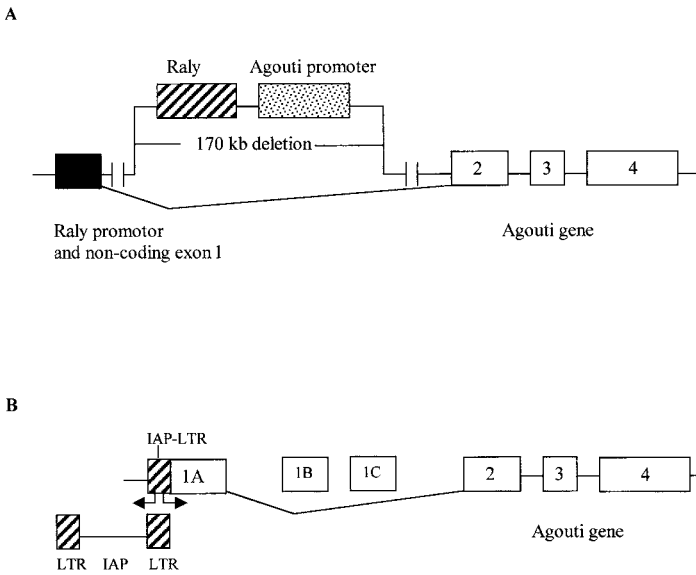


FIGURE 10.3

Proposed models for agouti dominant mutations. (A) The A^y mutation results from the 170 kb deletion which removes agouti promoter and most of the Raly gene except the first non-coding exon. (B) The A^{vy} mutation results from the insertion of an IAP element in exon 1A. IAP: intracisternal A particle. LTR: long terminal repeats.

deletion that removes all but the first non-coding exon of Raly, thereby resulting in splicing of the Raly upstream non-coding region to the agouti coding exons and ubiquitous expression of agouti driven by the Raly promoter⁴⁸ (Fig. 10.3). The deletion of Raly is responsible for the embryonic lethality of the homozygote.

In contrast, homozygotes of the other dominant agouti mutations are viable. Ubiquitous expression of agouti in A^{vy} mice results from insertion of an intracisternal A particle (IAP) into exon 1 of agouti, producing a chimeric agouti in which the normal splicing pattern, and hence the normal size, is preserved (Fig. 10.3). The promoter/enhancer within the long terminal repeat (LTR) of the IAP, which has bi-directional promoter activity, is responsible for the ectopic expression of normal agouti protein.⁴⁹ Similarly, molecular analysis of A^{iy} , A^{hvy} , and A^{iapv} demonstrates that they also result from insertion of an IAP upstream of the coding region, resulting in ectopic expression of normal agouti protein.^{49,50}

The human homologue of agouti, also referred to as agouti signaling protein (ASIP), maps to chromosome 20 and encodes a 132-amino acid protein which is 80% identical to murine agouti protein.^{51,52} Although the 3' and 5' untranslated regions of human and murine agouti are not similar, the nucleotide sequence of the open reading frames are 85% identical between the two species.^{51,52} The amino terminus signal sequence, central basic domain, and cysteine-rich carboxyl terminal regions are also highly conserved between the

mouse and human proteins.^{51,52} ASIP is expressed primarily in adipose tissue and pancreas;^{51,53} it is also found in heart, ovary, and testis and, to a lesser extent in foreskin, kidney, and liver.^{51,52} Although its function in humans has not yet been established, human agouti protein retains full functionality when expressed in transgenic mice under the control of the β -actin promoter.⁵²

10.5 Agouti Signaling: Role of Melanocortin Receptors

Although the genetic defect in the agouti yellow mouse syndrome clearly involves ectopic expression of the agouti gene, the actual mechanism of the yellow obese syndrome is not clear. Both central and peripheral effects of agouti have been explored extensively, and available evidence suggests central effects on appetite regulation, mediated by melanocortin receptors, as well as peripheral effects on adipocytes and pancreatic islets, which will be discussed in a later section of this chapter. The initial observation that agouti modulation of pigmentation is mediated by antagonism of α -MSH binding to its melanocortin receptor (melanocortin 1 receptor (MC1-R))^{45,54,55} has led to the proposal, now widely accepted, that melanocortin receptor antagonism may be responsible for many of the metabolic effects of agouti (Fig. 10.1). However, caution is warranted in extrapolating this observation, as a growing body of evidence demonstrates that agouti also exerts effects on melanocytes independent of α -MSH.^{55–60} Indeed, agouti has been proposed to act as an inverse agonist for MC1-R, as it appears to antagonize the constitutive activity of the receptor in the absence of α -MSH.⁶⁰ In addition, agouti stimulates Ca^{2+} signaling in several cell types via a mechanism that is dependent upon melanocortin receptors but is not mediated by their antagonism⁶¹ (Fig. 10.4).

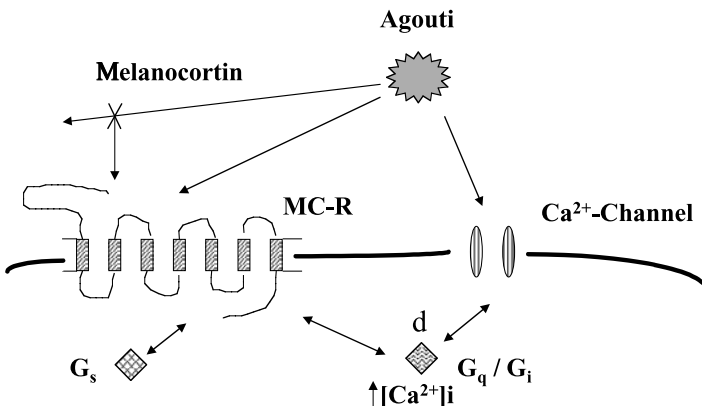


FIGURE 10.4

Agouti stimulation of Ca^{2+} influx is dependent upon the presence of melanocortin receptors, but is not mediated by melanocortin receptor antagonism.

MC1-R belongs to the melanocortin receptor family of 7-transmembrane G-coupled receptors which includes five known members.⁶² MC1-R, whose ligand is α -MSH, is primarily expressed in melanocytes and melanoma cells and is involved in the regulation of pigmentation.^{63,64} MC2-R, the adrenocorticotropin (ACTH) receptor, is expressed in the adrenal cortex, adipose tissue, and skin.⁶⁵⁻⁶⁹ MC3-R is expressed in the hypothalamus and limbic system,⁷⁰ and a high degree of expression has been noted in the dorsomedial part of the ventromedial nucleus of the hypothalamus and the arcuate nucleus; expression is also found in the septum, hippocampus, thalamus, and midbrain.⁷¹ MC3-R expression is also detectable via RT-PCR in human stomach, duodenum, and rat pancreas.⁷⁰ MC4-R expression is widely distributed throughout the brain, with a high degree of expression in hypothalamic centers involved in appetite and body weight regulation.⁷²⁻⁷⁴ MC5-R is a widely distributed receptor found in skeletal muscle and several endocrine and exocrine organs. Although its function is not fully understood, it appears to be involved in thermoregulation and immunomodulation.^{2,75,76}

The five melanocortin receptors exhibit 39 to 61% amino acid homology with one another, with MC3-R, MC4-R and MC5-R more closely related to one another (55 to 61%) than to MC1-R and MC2-R (43 to 46%). MC1-R and MC2-R exhibit the least homology (39%) in this receptor family. Each of the melanocortin receptors couples to activation of adenylyl cyclase, although MC3-R is also coupled to G_q with a consequent stimulation of inositol 1, 3, 4-phosphate production.⁷⁷

The HEK-293 human embryonic kidney cell line does not express melanocortin receptors and, accordingly, transfection of these cells with each melanocortin receptor has served as a useful tool to study the agouti-melanocortin receptor interaction.^{61,78} Lu et al.⁷⁸ used this approach to demonstrate that agouti is a high affinity antagonist of MC1-R, MC2-R, and MC4-R but exerts little or no effect on MC5-R. Of the receptors antagonized by agouti, loss or gain of function mutations at MC1-R predictably affects pigmentation without causing significant metabolic derangements, while loss of MC2-R function results in familial glucocorticoid deficiency.^{2,14,62} Thus, only MC4-R remains as a target for contributing to the agouti-induced obesity syndrome.

Indeed, several studies now suggest that MC4-R may play a role in human obesity. In the Quebec Family Study, Chagnon et al.⁷⁹ demonstrated a significant association between restriction fragment length polymorphisms (RFLP) of MC4-R and obesity-related phenotypes. Moreover, two recent reports describe a severely obese child and an adult who were heterozygous for a deletion or insertion-induced frameshift mutation, respectively, in the MC4-R gene.^{80,81} In addition, three allelic variants have been identified in obese humans by polymorphism analysis.⁸² One variant from an extremely obese individual was cloned and found to be severely impaired in ligand binding and signaling, raising the possibility that it may contribute to the development of obesity.⁸² Moreover, several mutations, including base deletion-induced frameshift, base substitution (both

resulting in truncated MC4-R), missense and haploid sufficiency, have been identified in extremely obese patients.^{83,84}

MC4-R is abundantly expressed in the paraventricular nucleus, dorsomedial nucleus, and ventromedial nucleus, major hypothalamic regions involved in the regulation of feeding, energy homeostasis, and body weight.^{85–88} Huszar et al.⁸⁹ reported that MC4-R knockout mice exhibit adult-onset obesity associated with hyperphagia, hyperinsulinemia, and insulin resistance, recapitulating many of the metabolic features of dominant agouti mutants. Further intracerebroventricular administration of MTII, a potent MC4-R antagonist, caused a dose-dependent suppression of food intake in four murine models of obesity and hyperphagia.⁹⁰ This effect of MC4-R in controlling feeding behavior was confirmed with the demonstration that MC4-R-deficient mice failed to respond to the anorectic actions of MTII.⁹¹ Moreover, food restriction results in selective upregulation of MC4-R density in rat hypothalamus, while diet-induced obesity leads to a downregulation of MC4-R, further suggesting an important role for MC4-R in the control of feeding behavior.⁹²

Although agouti is not normally expressed in the brain, an agouti homologue called Agouti Related Protein (AGRP) is expressed in the brain and serves as an endogenous antagonist of α -MSH at MC4-R.⁹³ AGRP is abundantly expressed in the arcuate nucleus, where its expression is markedly elevated in obese (*ob/ob*) mice.⁹³ Ubiquitous expression of AGRP in transgenic mice recapitulates the phenotype of obesity found in MC4-R knockout mice.^{94,95} These transgenic mice do not exhibit a yellow coat color, suggesting that AGRP does not antagonize MC1-R.⁹⁴ AGRP exhibits 25% homology to agouti, with the highest degree of identity found in the cysteine-rich carboxyl terminus.⁹³ Since the carboxyl terminus of agouti is responsible for its antagonistic role at MC4-R, this homology with AGRP predicts a similar function for this domain, and this function has been verified.^{96–98} Further, intracerebroventricular administration of a c-terminal fragment of AGRP increased food intake in rats, an effect that was mimicked by treatment with SHU9119, a potent MC4-R antagonist.⁹⁸

10.6 Role of POMC-Derived Peptides

The endogenous ligand for MC4-R is α -MSH, a peptide cleaved from pro-opiomelanocortin (POMC); sequential cleavage of this precursor also generates the melanocortin peptides β -MSH, γ -MSH, ACTH and β -endorphin.⁸⁵ Expression of the *pomc* gene in the hypothalamus and brainstem produces melanocortigenic neurotransmitters, and expression in the pituitary gland produces melanocortin hormones.² α -MSH binds to MC4-R with a high affinity and appears to act as a strong agonist in food intake and body weight regulation. Yaswen et al.⁹⁹ recently generated *pomc* knockout mice lacking all POMC-derived peptides. These mice develop hyperphagia and obesity with

a time course and severity comparable to the aforementioned MC4-R knock-out mice.⁸⁹ Interestingly, however, loss of α -MSH produced a much more subtle effect on pigmentation than was expected based on the yellow coat color seen both in agouti mutants and loss of function mutations at the MC1-R locus. The authors suggest that the failure of ligand deprivation to produce the same phenotype as loss of receptor function or receptor antagonism results from either other ligands acting at MC1-R or, more likely, some degree of basal activity of the MC1-receptor even in the absence of ligand.

To confirm the role of α -MSH in the control of body weight regulation, a stable agonist, [Ac-Cys⁴, D-Phe⁷, Cys¹⁰] α -MSH, was administered daily via intraperitoneal injection. MSH administration resulted in marked weight loss: 38% of the knockout-induced weight gain within 1 week and 46% within 2 weeks in the mutant mice but was without significant effect in wild-type littermates. These data clearly implicate POMC peptides in body weight regulation. However, several cautionary notes are warranted in extending this conclusion. First, the observation that loss of α -MSH produces a different effect on pigmentation than loss or antagonism of receptor function suggests the salutary effects of MSH on weight loss found in POMC-deficient animals may be attenuated in POMC-replete animals, even in the presence of agouti, AGRP, or other potential melanocortin receptor antagonists. Indeed, twice-daily administration of comparable doses of a stable MSH analogue (norleucine⁴, D-phenylalanine⁷, α -MSH) failed to alter any aspect of the obese phenotype (body weight, fat weight, core temperature, adipocyte lipid metabolism, circulating glucose, and insulin) of either dominant agouti mutant mice (*A^y*) or transgenic mice ubiquitously expressing agouti.¹⁰⁰ Thus, the effects of MSH administration in *pomc* knockouts may not necessarily extrapolate to other models. Nonetheless, abundant evidence now clearly implicates POMC peptides and MC4-R in agouti and AGRP-induced obesity.

10.7 Agouti Interactions with Mahogany and Mahoganoid

The mouse mutations *mahogany* (*mg*) and *mahoganoid* (*md*) are negative modifiers of agouti,¹⁰¹ as mutations at *mg* or *md* suppress the effects of *A^y* on both coat color and obesity.^{101,102} These effects are specific for agouti, as mutations of mahogany did not suppress weight gain in other models of obesity.^{102,103} Mahogany acts along the agouti-melanocortin pathway, and mutations at *mg* do not reverse obesity in MC4-R knockout mice, although *mg* does reverse diet-induced obesity.¹⁰³ Further, *mg* mice are resistant to diet-induced obesity and eat significantly more than normal mice without gaining weight, presumably due to increases in metabolic rate and spontaneous motor activity.¹⁰²

The gene encoding *mg* encodes a 1428 amino acid, single-transmembrane-domain protein that is expressed in several tissues, including the hypothalamus.^{103,104} The cytoplasmic tail of mahogany is short and contains

no previously defined signaling domain,^{103,104} while the extracellular domain is an orthologue of human attractin.^{104,105} It has been suggested that mahogany may serve as a low affinity receptor for agouti, thereby reducing its local concentration,^{103,104} and mahogany has been demonstrated to bind recombinant agouti protein, but not AGRP,¹⁰⁶ possibly by interactions between agouti and the C-type lectin domain and/or glycosaminoglycan side chains of mahogany.¹⁰⁴

10.8 Agouti–Leptin Interactions

Recent data indicate that there is significant cross-talk between the leptin and agouti-ASIP-melanocortin signaling pathways. The leptin receptor is expressed in AGRP- and POMC-producing neurons in the hypothalamic arcuate nucleus,^{107–109} and leptin has been demonstrated to regulate AGRP expression.^{93,94,107,110} Further, *pomc* expression is significantly reduced in leptin-deficient *ob/ob* mice and leptin resistant *db/db* mice and is upregulated by leptin treatment.^{111–113} Moreover, central administration of the MC4-R antagonist SHU9119 completely inhibits the anorexic response to leptin in mice, suggesting that MC4-R may, in part, also mediate leptin action.¹¹⁴ However, obese MC4-R deficient mice do not respond the anorexic effects of leptin, while non-obese MC4-R-deficient mice do.¹¹⁵ These data demonstrate that, although MC4-R plays a role in leptin signaling, it is clearly not an exclusive target of leptin action.

Plasma leptin levels are elevated in obese yellow mice, and agouti protein directly stimulates leptin expression and secretion in both 3T3-L1 adipocytes and human adipose tissue.²¹ The marked elevations of circulating leptin accompanied by persistent obesity in dominant agouti mutants has led to the suggestion that these animals are leptin resistant. However, Boston et al.¹¹⁶ studied the independent and interactive effects of agouti and leptin in mice by comparing a dominant agouti mutant (lethal yellow, A^y/a), leptin deficient (lep^{ob}/lep^{ob}) and double mutant (yellow/leptin-deficient; $A^y/a\ lep^{ob}/lep^{ob}$) mice with controls (C57BL/6J). The presence of the A^y allele produced similar degrees of weight gain in both the wild-type and leptin-deficient lep^{ob}/lep^{ob} mice, demonstrating that the effects of defective POMC signaling and leptin deficiency are independent and additive. To further evaluate the relationship between MC4-R signaling and leptin action, the effects of leptin administration on weight loss, food intake, and serum insulin were compared in the four mouse genotypes.¹¹⁶ A^y mice exhibited significant resistance to leptin action relative to the lep^{ob}/lep^{ob} mice, as indicated by an inability of leptin to induce weight loss, suppress food intake, or lower serum insulin. However, this leptin resistance is likely to result from receptor desensitization secondary to increased circulating leptin, as removal of leptin from the A^y/a mice in the double mutant lethal yellow/leptin-deficient model ($A^y/a\ lep^{ob}/lep^{ob}$) completely restored the

responsiveness of these animals to exogenous leptin. These data indicate that agouti-induced obesity is independent of leptin action, although elevated leptin in dominant agouti mutants is likely to serve as a counterregulatory mechanism to limit the degree of agouti-induced obesity.

10.9 Peripheral Actions of Agouti

An accumulating body of evidence indicates that, in addition to the aforementioned central effects of agouti, peripheral actions of agouti are likely to contribute to agouti-induced obesity and strongly suggest a role of agouti signaling in adipocytes and pancreatic β -cells. Although hyperphagia in obese yellow mice appears to be mediated by agouti antagonism of MC4-R, as discussed above, these mice also exhibit marked increases in the efficiency of energy utilization, with preferential energy partitioning into adipose tissue stores. Moreover, although there has been considerable focus on central MC4-R antagonism, MC4-R mRNA has also been detected in human adipose tissue and skeletal muscle.⁷⁹ Moreover, transgenic mice that selectively express agouti in adipose tissue under the control of the aP2 promoter develop mild obesity, especially if concomitant hyperinsulinemia is induced, while their non-transgenic littermates do not.⁴⁴⁻⁴⁶ Thus, agouti expression in adipose tissue results in an increase in energy partitioning into adipose tissue, leading to accelerated accretion of adipose tissue mass. This demonstrates that agouti-induced obesity includes a significant peripheral component. Recent data indicate that these effects are mediated by modulation of intracellular Ca^{2+} .

10.10 Role of Calcium

The C-terminal region of the agouti protein retains full functional activity relative to the intact protein in an *in vitro* assay system,⁴⁵ as discussed previously. Further, the agouti C-terminus bears a striking spatial homology in both number and spacing of cysteine residues to spider and snail venoms (ω -conotoxins, plectoxins) which target Ca^{2+} channels.⁴⁶ Accordingly, the C-terminus may form a three-dimensional structure that is functionally similar to these venoms and may thereby serve to modulate Ca^{2+} transport. Indeed, we have reported that A^{vy} mice exhibit increases in both steady-state intracellular Ca^{2+} and Ca^{2+} influx in several tissues.^{61,117} This increase in intracellular $(\text{Ca}^{2+})_i$ was closely correlated with both the degree of ectopic agouti expression and body weight,¹¹⁷ suggesting the possibility of a causal mechanism between intracellular Ca^{2+} and obesity in these animals. Since A^{vy} mice exhibit elevated rates of adipocyte lipogenesis and increased adipocyte size

relative to lean controls,^{38,118} the links among agouti, intracellular Ca^{2+} , and regulatory enzymes in lipid metabolism have been explored further.

Recombinant agouti protein directly increased Ca^{2+} influx and steady-state intracellular Ca^{2+} in a variety of cell types, including both murine and human adipocytes.^{61,117} This regulation occurs in response to physiologically meaningful concentrations of agouti (EC_{50} of 18 to 62 nM, depending upon cell type) and, although studies in HEK-293 cells demonstrate the dependence of this effect upon the presence of intact melanocortin receptors (MCRs), it is not dependent upon MCR antagonism.⁶¹ The role of these increases in Ca^{2+} in lipogenesis has been explored using fatty acid synthase (FAS), as this multifunctional enzyme is highly regulated by nutrients and hormones and is a key enzyme in *de novo* lipogenesis. FAS expression and activity are markedly increased in *A^{vy}* relative to control mice,³¹ and nanomolar concentrations of agouti protein stimulate ~two-fold increases in FAS gene expression and activity and triglyceride accumulation in 3T3-L1 adipocytes³¹ as well as in human adipocytes, similar to the maximal increases stimulated by insulin. These increases are mediated by a distinct agouti/ Ca^{2+} response sequence in the FAS promoter.¹¹⁹ This sequence maps to the –435 to –415 region of the FAS promoter and is upstream of the insulin response element, which maps to –67 to –52, consistent with the observed additive effects of agouti and insulin on FAS gene transcription.¹¹⁹ Further, recent evidence indicates that agouti exerts a regulatory effect on human FAS expression *in vivo*, and there is a strong correlation between agouti expression and FAS expression in adipose tissue obtained from normal volunteers.¹²⁰ This agouti modulation of FAS transcription appears to be mediated via intracellular Ca^{2+} , as it can be inhibited by Ca^{2+} antagonism^{14,31} and can be mimicked in the absence of agouti by either receptor- or voltage-mediated Ca^{2+} channel activation.¹²¹

In addition to activating lipogenesis, recent data also indicate that increasing intracellular Ca^{2+} may also contribute to increased triglyceride stores by inhibiting lipolysis. Increasing Ca^{2+} influx with either arginine vasopressin or epidermal growth factor was reported to inhibit lipolysis in rat adipocytes in a Ca^{2+} dose-responsive fashion.¹²² Further, we have shown that the agouti gene product similarly inhibits lipolysis in human adipocytes via a Ca^{2+} -dependent mechanism.¹²³ This inhibition can also be mimicked in the absence of agouti by either receptor- or voltage-mediated Ca^{2+} channel activation.¹²³ The anti-lipolytic effect of intracellular Ca^{2+} is due to a direct activation of phosphodiesterase 3B, resulting in a decrease in cAMP and, consequently, reduced ability of agonists to stimulate phosphorylation and activation of hormone-sensitive lipase¹²⁴. Thus, agouti regulation of adipocyte intracellular Ca^{2+} appears to promote triglyceride storage in human adipocytes by exerting a coordinated control of lipogenesis and lipolysis, serving to simultaneously stimulate the former and inhibit the latter.

However, it is important to note that agouti interaction with insulin is required for the full expression of agouti-induced obesity. Agouti and insulin exert independent, additive effects on FAS transcription and lipogenesis.¹¹⁹ Since increased intracellular Ca^{2+} is the proximate signal for insulin release,

and agouti regulates Ca^{2+} in several cell types⁶¹ (Fig. 10.4), it is reasonable to speculate that agouti may stimulate insulin release as well. Indeed, we have recently found that agouti is expressed in human pancreas and stimulates Ca^{2+} signaling in rat, hamster, and human pancreatic β cells.⁵³ Further, hyperplasia of β cells precedes the development of obesity in agouti mutant mice, suggesting that hyperinsulinemia may be a direct effect of agouti acting on the pancreas and that the combination of this hyperinsulinemia and agouti-stimulated adipocyte Ca^{2+} influx may lead to obesity. In support of this concept, transgenic mice expressing agouti at high levels in adipose tissue under the control of the aP2 promoter become obese if they are also hyperinsulinemic as a result of either exogenous insulin or a high sucrose diet, while hyperinsulinemia was without effect in non-transgenic littermate controls.^{44,125,126} Since humans exhibit a similar pattern of adipocyte agouti expression,⁵¹ similar agouti/insulin/ Ca^{2+} interactions may result in excessive adipocyte triglyceride storage.

Taken together, these data indicate that regulation of adipocyte intracellular Ca^{2+} , possibly coupled with pancreatic Ca^{2+} and insulin release, may be an important target for the development of therapeutic strategies for the prevention and treatment of obesity¹⁴ (Fig. 10.5). To evaluate this hypothesis, agouti-expressing transgenic mice were treated with high doses of a Ca^{2+} channel antagonist, nifedipine. This treatment resulted in an 18% reduction in fat pad mass and completely normalized the agouti-induced hyperinsulinemia over

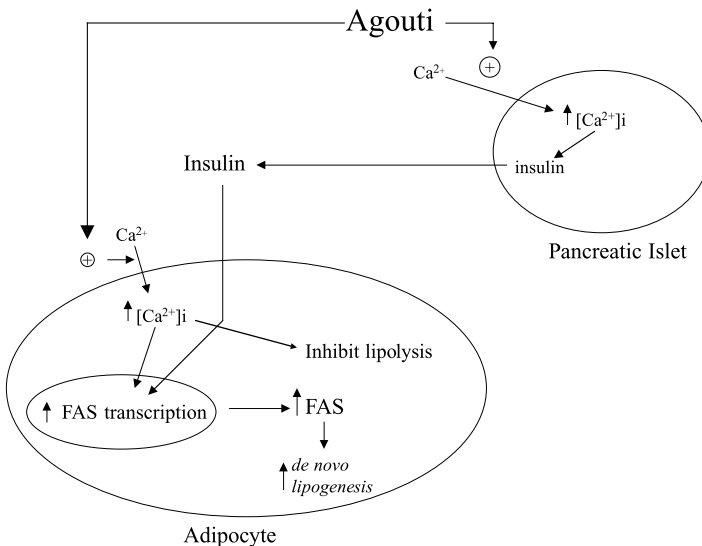


FIGURE 10.5

Agouti modulation of adiposity. Agouti stimulates Ca^{2+} influx in pancreatic β -cells, resulting in increased insulin release. Agouti stimulates Ca^{2+} influx in adipocytes, resulting in increased fatty acid synthase (FAS) transcription and activity and inhibition of lipolysis. The agouti-induced insulinemia augments the effects of agouti on adipocytes by independently increasing FAS transcription and inhibiting lipolysis.

a 4-week treatment period in the transgenic mice, but was without effect in the non-transgenic littermate controls.²⁰ Thus, adipocyte and/or pancreatic β -cell (Ca^{2+})_i appear to be reasonable therapeutic targets for the treatment and/or prevention of obesity.

We recently extended this concept by demonstrating that human adipocytes express a sulfonylurea receptor (SUR) which exerts a regulatory effect on the Ca^{2+} channel and, consequently, modulates adipocyte lipid accumulation.^{121,127} Compounds acting on the pancreatic SUR to increase (e.g., glibenclamide) or decrease (e.g., diazoxide) intracellular Ca^{2+} (indirectly, via a K^+ -ATP channel) cause corresponding increases and decreases in weight gain, although these effects have previously been attributed to the effects of these compounds on circulating insulin. However, the identification of SUR expression in human adipocytes¹²¹ suggests that it may modulate adipocyte Ca^{2+} flux and thereby regulate lipid metabolism. Indeed, the SUR agonist glibenclamide increases human adipocyte intracellular Ca^{2+} and thereby causes marked increases in lipogenic enzyme activity and inhibition of lipolysis. Moreover, inhibition of the adipocyte SUR-regulated Ca^{2+} channel with diazoxide completely prevented each of these effects. Accordingly, the adipocyte SUR may represent a new target for the development of therapeutic interventions in obesity.¹²¹ In support of this concept, diazoxide has been demonstrated to exert significant anti-obesity effects in both obese Zucker rats and hyperinsulinemic obese adults.^{128–130} Although this effect was attributed to actions on pancreatic β -cell insulin release, we subsequently found diazoxide treatment to significantly suppress adipose tissue fatty acid synthase and lipoprotein lipase in obese Zucker rats.¹²⁷

10.11 Modulation of Intracellular Calcium by Dietary Calcium: Implications for Obesity

We recently reported that 1,25-dihydroxy-vitamin D [$1,25-(\text{OH})_2\text{-D}$] stimulates Ca^{2+} influx, resulting in significant, sustained dose-responsive increases in steady-state intracellular Ca^{2+} in primary cultures of human adipocytes.¹³¹ Moreover, $1,25-(\text{OH})_2\text{-D}$ treatment of human adipocytes resulted in a coordinated activation of FAS and inhibition of lipolysis, similar to the action of agouti on these cells.¹³¹ Consequently, suppression of $1,25-(\text{OH})_2\text{-D}$ with high calcium diets would be anticipated to reduce adipocyte intracellular Ca^{2+} , inhibit FAS, and activate lipolysis, thereby exerting an anti-obesity effect.

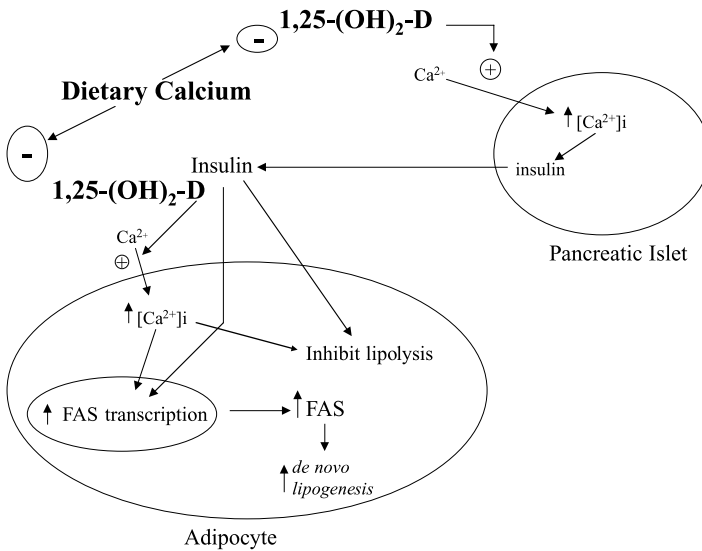
This concept was confirmed in transgenic mice expressing agouti in adipose tissue under the control of the aP2 promoter. Mice placed on low calcium (0.4%)/high fat/high sucrose diets for 6 weeks exhibited marked increases in adipocyte lipogenesis, inhibited lipolysis, and accelerated increases in body weight and adipose tissue mass. However, high calcium (1.2%) diets reduced lipogenesis by 51% and stimulated lipolysis 3 to 5-fold, resulting in 26 to 39%

reductions in body weight and adipose tissue mass, respectively.¹³¹ The magnitude of these effects depended upon the source of dietary calcium, with dairy sources of calcium exerting significantly greater effects than calcium carbonate.

Consistent with this finding, 12 months of yogurt supplementation, sufficient to raise daily calcium intake from approximately 400 to 1000 mg/day, resulted in a 4.9 kg reduction in body fat in obese African Americans without an accompanying reduction in caloric intake.¹³¹ The relevance of this finding at the population level was assessed via analysis of the National Health and Nutrition Examination Survey (NHANES III); odds ratios for percent body fat as a function of calcium intake were estimated by logistic regression, with age, race/ethnicity, activity level, and caloric intake as covariates. The odds of being in the highest quartile of body fat were reduced from 1.0 for the first quartile of calcium intake to 0.75, 0.40, and 0.16 for the second, third, and fourth quartiles of calcium intake, respectively, for women.¹³¹ The regression model for males similarly demonstrated a significant inverse relationship between dietary calcium and body fat, although the same simple dose-response relationship found in women was not evident.¹³¹

These data have significant implications for the prevention or attenuation of diet-induced obesity but do not directly address the issue of whether high calcium diets will exert any effect on established obesity. Accordingly, a follow-up study was conducted to determine whether increasing dietary calcium will reduce metabolic efficiency and accelerate fat loss secondary to caloric restriction following dietary induction of obesity.¹³² Administration of the same low-calcium/high-fat/high-sucrose diet to aP2-agouti transgenic mice resulted in a ~100% increase in adipocyte intracellular Ca^{2+} and a corresponding weight gain of 29% and a 2-fold increase in total fat pad mass, demonstrating that dysregulation of adipocyte intracellular Ca^{2+} is associated with increased adiposity in aP2-agouti transgenic mice. The animals were then placed on energy restriction (70% of an *ad libitum* fed control group) for an additional 6 weeks. Energy restriction on the low-calcium diet failed to reduce intracellular Ca^{2+} and only reduced body weight and fat pad mass by 11 and 8%, respectively. In contrast, energy restriction in conjunction with high-calcium (1.2%) diets normalized intracellular calcium and resulted in 19 to 29% reductions in body weight and 42 to 69% decreases in fat pad mass, depending upon the calcium source (calcium carbonate vs. dairy). In addition, the high-calcium diets caused marked reductions in FAS expression and activity (35 to 81%), 2 to 3-fold increases in lipolysis and increases in core temperature (0.48 to 0.67° C) and uncoupling protein-2 expression.¹³² These data demonstrate that high-calcium diets suppress adipocyte intracellular Ca^{2+} by suppressing 1,25-(OH)₂-D and thereby shifts the partitioning of dietary energy from energy storage to energy expenditure (Fig. 10.6).

Collectively, these data from agouti modulation of adipocyte Ca^{2+} flux, Ca^{2+} -channel antagonism experiments, adipocyte sulfonylurea receptor studies, and dietary calcium modulation of adipocyte lipid metabolism all demonstrate that adipocyte Ca^{2+} signaling is an attractive target for the development of obesity interventions.

**FIGURE 10.6**

Dietary calcium modulation of adiposity. 1,25-dihydroxy-vitamin D [1,25-(OH)₂-D] stimulates Ca²⁺ influx in both pancreatic β-cells and adipocytes, resulting in increased insulin release, increased FAS transcription and activity, and reduced lipolysis. Increasing dietary calcium suppresses 1,25-(OH)₂-D release, thereby removing a stimulus for Ca²⁺ influx. Consequently, insulin release is reduced, FAS transcription and activity are reduced, and lipolysis is activated.

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11

Dietary Fats and APC-Driven Intestinal Tumorigenesis

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11.1 Introduction

Epidemiological evidence clearly links dietary fat intake with colorectal cancer risk.^{1,2} As fat intake increases, the risk for colorectal cancer increases; however, the risk associated with individual fatty acids is less defined. This chapter is particularly interested in addressing what is known about the impact of dietary fatty acid composition on intestinal tumorigenesis.

11.2 Polyunsaturated Fatty Acid Metabolism

There are two major families of dietary polyunsaturated fatty acids (PUFAs): the n-3 and n-6 families. N-6 PUFAs are derived from the parent compound linoleic acid (LA, 18:2 n-6) (Fig. 11.1). LA is the major PUFA in the diet and accounts for ~6 to 7% of caloric intake. Following consumption of LA, it is converted to γ -linolenic (GLA, 18:3 n-6) acid via the Δ -6 desaturase, and subsequently converted to arachidonic acid (AA, 20:4 n-6) through the intermediate dihomo- γ -linolenic acid (DGLA, 20:3 n-6) via successive steps involving an elongase and the Δ -5 desaturase. AA is arguably the most important PUFA associated with membrane phospholipids.³ In parallel (Fig. 11.1), PUFAs of the n-3 family are derived from the parent fatty acid α -linolenic acid (ALA, 18:3 n-3), the metabolic precursor of the more highly unsaturated n-3 PUFAs found in fish oils, e.g., stearidonic acid (SDA, 18:4 n-3), eicosapentaenoic acid (EPA, 20:5 n-3), and docosahexaenoic acid (DHA, 22:6 n-3). Utilizing the same enzymatic machinery as LA, ALA is metabolized to EPA. Unlike AA

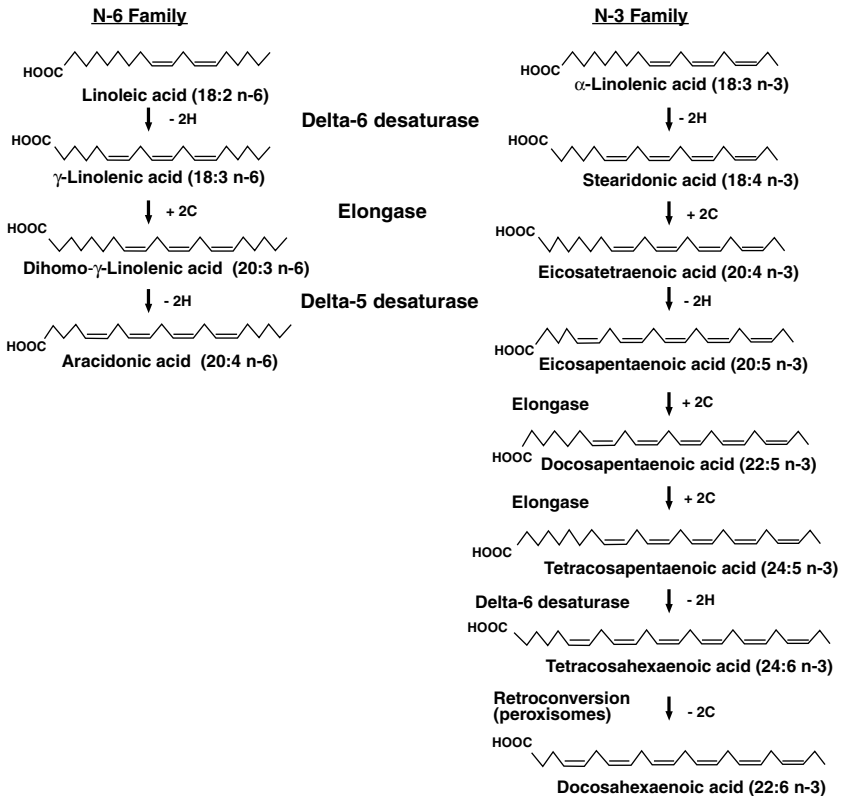


FIGURE 11.1
Metabolic pathways of n-6 and n-3 fatty acids.

which is poorly metabolized to longer more unsaturated fatty acids,⁴⁻⁶ EPA is converted to DHA via a set of unique reactions previously attributed to a putative Δ -4 desaturase.^{7,8} EPA undergoes two elongation steps to 24:5 n-3 at the endoplasmic reticulum and is subsequently desaturated to 24:6 n-3 via Δ -6 desaturase. Following the removal of two carbons via peroxisomal oxidation, 24:6 n-3 is retroconverted to DHA. PUFAs from the n-3 and n-6 families are not metabolically interconvertible, and as such, consumption of one family of fatty acids can attenuate the metabolism of the other.

It is the competition between these two fatty acid families that defines the role of dietary PUFAs and the risk of intestinal tumorigenesis. Indeed, establishing an appropriate mix of dietary n-3 and n-6 PUFAs in an effort to reduce chronic diseases has been a concern for a number of years. Dietary n-6 PUFAs appear to be pro-tumorigenic and it has been suggested that these effects are mediated via the AA cascade, in particular, via prostaglandins. N-3 PUFAs are potent antagonists to AA and its metabolism, suggesting a possible mechanism for their antitumorigenic effects. A direct comparison of each PUFA in the metabolic sequence could explicitly establish if their impact on tumorigenesis is mediated through the metabolism of AA. Certainly, determining the efficacy of these fatty acids is complicated by the fact that typical dietary intakes of LA and ALA (~15g/d and ~2g/d, respectively)⁹ are far higher than those of AA (~200mg/d)^{10,11} and EPA/DHA (~200mg/d),⁹ but the highly unsaturated fatty acids are more biologically potent than their precursors because they bypass the initial Δ -6 desaturase step, the rate-limiting step in the metabolic pathways of n-3 and n-6 PUFAs.

11.3 Cyclooxygenases

Following release of AA from membrane phospholipids by a variety of phospholipase A₂s,¹²⁻¹⁴ AA is enzymatically oxidized to prostaglandins (PGs) by a two-step process involving cyclooxygenases (also referred to as prostaglandin H synthases) (Fig. 11.2). Two known isozymes of cyclooxygenase (COX), COX-1 and COX-2, catalyze the committed step in PG biosynthesis (Table 11.1); however, a third form has been proposed.¹⁵ These bifunctional enzymes exist as homodimers. They contain an epidermal growth factor domain, a membrane-binding domain, and two enzymatic activities with distinct active sites.¹⁶ The initial activity, cyclooxygenase, oxidizes arachidonic acid through a free radical-generated mechanism to the endoperoxide intermediate PGG₂, which is subsequently reduced to PGH₂ by the protein-associated peroxidase which is located at the interface of the dimer.¹⁷ PGH₂ is the common precursor for all the prostaglandins, prostacyclin, and thromboxanes. The COX enzymes are localized within the outer leaflet of the endoplasmic reticulum and the outer and inner membranes of the nuclear membrane.¹⁸

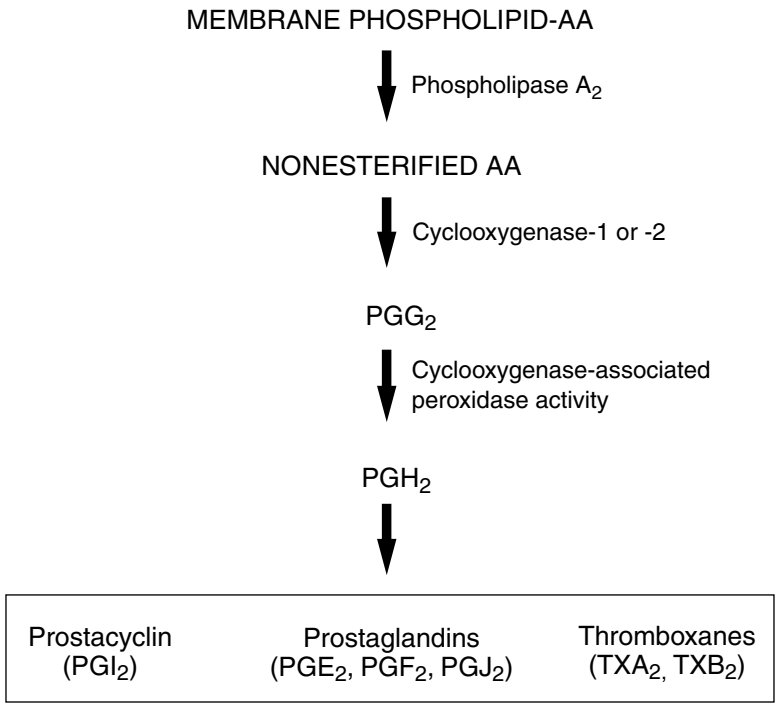


FIGURE 11.2
Metabolic pathway of cyclooxygenase-derived eicosanoids.

COX-1 is constitutively expressed in most tissues and appears to be important in housekeeping processes that modulate basic cellular functions, but has also been shown to be induced.^{19,20} COX-2 is not normally expressed in most tissues and is considered to be the inducible form of the enzyme. Its expression is upregulated in monocytes/macrophages, fibroblasts, and endothelial cells in response to cytokines, growth factors, mitogens, and tumor promoters; however, basal expression has been observed in the macula densa of the kidney, the brain, testes, and tracheal epithelium (as reviewed in References 21 and 22) Importantly, COX-2 expression has been shown to be elevated in a number of cancers, including small intestinal, pancreatic, gastric, colorectal, and lung,^{23–31} and that induction of COX-2 expression appears to play an important role in maintaining tumor integrity^{32,33} and may contribute to the metastatic process.^{34,35}

11.4 Intestinal Tumorigenesis and APC

Colorectal cancer, the third most common form of malignancy in both men and women, was attributed with over 57,000 deaths in 1995.³⁶ The disease

TABLE 11.1

Comparison of Cyclooxygenase-1 and -2. (See Refs. 18, 21, 142, 146–149)

	COX-1	COX-2
Temporal pattern of expression	Primarily constitutive, induction in some cell types	Primarily inducible in presence of cytokines, growth factors, tumor promoters (constitutively expressed in tumors, testes, trachea, kidney, brain)
Prominent tissue/cell type	Ubiquitous	Inflammatory cells, tumors, kidney, brain
Gene size	22 kb	8.3 kb
mRNA size	2.8 kb	4.5 kb
Protein size	~70 kDa	~70 kDa
Amino acid homology to COX-1	—	75%
Amino acid sequence identity with COX-1	—	63%
Structural forms	Homodimer	Homodimer
Heme-containing	Yes	Yes
Peroxidase activity	Yes	Yes
Catalytic mechanism involves free radicals	Yes	Yes
Peroxide activated	Yes	Yes
Inhibited by NSAIDs	Yes	Yes
Substrate specificity	AA>DGLA>>>LA,GLA,EPA > ALA	AA>DGLA>LA>ALA>GLA > EPA
K _m for AA as substrate	~5 μM	~5 μM
Product profile (substrate: arachidonic acid)	PGG ₂ , PGH ₂ , 11-HETE, 15-HETE	PGG ₂ , PGH ₂ , 11-HETE, 15-HETE, 15(R)-HETE (in presence of aspirin)
Subcellular localization	ER and nuclear envelope	Nuclear envelope, along with ER
Primary mode of action	Extracellular: paracrine, autocrine	Paracrine, autocrine, intracrine (nuclear)

progresses through defined pathologic stages that range from the earliest proliferation of neoplastic epithelium, referred to as an aberrant crypt focus (ACF),³⁷ to benign adenomas (or polyps), to metastatic cancer.³⁸ This morphologic progression of events has been associated with specific genetic and epigenetic abnormalities which can be used to model intestinal tumorigenesis and test the effects of dietary or pharmacologic intervention.^{39,40} In most individuals, colorectal cancer occurs spontaneously (i.e., sporadic form) but a relatively small percentage of cases occur in individuals predisposed to the disease by inherited defects in either the adenomatous polyposis coli (*APC*) gene, where the syndrome is referred to as familial adenomatous polyposis (FAP), or in a DNA mismatch repair gene which results in hereditary non-polyposis colorectal cancer (HNPCC).⁴¹ Mutations of the *APC* gene initiate formation of essentially all tumors in FAP patients, are present in most early cases of sporadic colorectal cancer, and may also contribute to tumorigenesis

in HNPCC. *APC* is considered a tumor suppressor gene because oncogenic mutations result in loss of the full-length APC protein through truncation of the transcripts and/or genetic deletion.

Animal models used to study colorectal cancer fall into two categories: those that spontaneously develop intestinal tumors as a result of predisposing inheritable mutations, and those in which neoplasia is induced by chemical carcinogens (Table 11.2). The most commonly used genetic models carry a germline mutation in the murine *Apc* gene and develop intestinal tumors with spontaneous loss of the wild-type allele, whereas chemically induced tumors in mice and rats more often result from mutations in other genes, although dysregulation of β -catenin (a downstream protein regulated by *Apc*) still may have a seminal role.⁴² A major advantage of the genetic models is the reproducible loss of *Apc* gene function, as occurs early in the development of most human colorectal cancers. A potential disadvantage is the propensity of these mice to develop more small intestinal than colorectal tumors. The chemically induced tumors are restricted to the colon of treated rodents and occur with formation of ACF, as is the case in humans. Whether a model is stronger for its molecular or anatomic characteristics is debatable, but we have chosen to use a genetic mouse model for our studies.

The *Apc*^{Min/+} model was derived from a C57BL/6J male mouse which had been exposed to the mutagen ethyl nitrosourea.⁴³ Intestinal tumorigenesis in this strain has been well characterized and widely used to study the molecular and genetic pathogenesis of this disease as well as methods of chemotherapeutic and preventative intervention. *Apc*^{Min/+} mice (available through Jackson Laboratories, Bar Harbor, Maine) carry a germline mutation in *Apc* that truncates the protein at 850 amino acids. The mutation is homozygous lethal *in utero* but heterozygotes develop adenomas throughout the intestinal tract. By approximately 60 to 80 days of age each mouse will harbor an average of 40 small intestinal tumors and up to 3 or 4 colonic polyps.⁴⁴ These tumors rarely, if ever, progress to invasive cancer presumably because of the restricted life span of the *Apc*^{Min/+} mouse (~130 days). Aging *Apc*^{Min/+} mice become anemic due to low-grade hemorrhage through the ulcerated surface of intestinal tumors.

To date, five other strains of *Apc*^{+/-} mice have been developed by targeted mutation of the gene at different locations, resulting in variable truncation of

TABLE 11.2
Rodent Models of Intestinal Tumorigenesis in Humans

	Genetic (<i>Apc</i>) Models	Carcinogen-Induced Models
Earliest genetic defect	<i>Apc</i>	β -catenin & Ras >> <i>Apc</i>
Tumor location	Small intestine > Colon	Colon
Tumor multiplicity	30+ /mouse (< 4 colonic)	< 1 to 3 /animal
Adherent crypt foci	No	Yes
Neoplastic progression	No	Yes — to invasive cancer

the encoded protein.⁴⁵ The incidence of intestinal tumors varies in these strains of mice with the germline mutation, and may progress to invasive carcinomas, but the phenotypes essentially duplicate the original $Apc^{Min/+}$ mutant. The only significant exception is the Apc^{1638T} mouse which survives as a homozygous mutant and does not develop intestinal tumors.⁴⁶ The $Apc^{Min/+}$ mouse phenotype is affected by at least one modifier gene.⁴³ Modifier of Min, (Mom)-1, is mutated in C57BL/6J mice and is believed to encode a secretory phospholipase (sPLA₂). When $Apc^{Min/+}$ mice on this background are crossed with AKR mice, which have wild-type Mom-1 alleles, the F1 progeny have a significantly reduced incidence of intestinal tumorigenesis and longer life span, attributed in large part (but not exclusively) to the effects of wild-type Mom-1. The biologic basis for this effect on intestinal tumorigenesis is unclear and the human sPLA₂ analog has not been found to be mutated in colorectal cancer.

Normally, Apc expression is upregulated as cells emerge from the small intestinal crypt or migrate up the colonic crypt to the mucosal surface. The wild-type protein downregulates the Wnt signaling pathway, contributes to microtubule function, and may affect other cell processes through interactions with less well-characterized proteins (reviewed by Nathke⁴⁷ and Polakis⁴⁸). While the Apc protein may have multiple functions in a normal cell, loss of control in the Wnt signaling pathway appears to be most significant with respect to intestinal tumorigenesis.⁴⁶ As depicted in Fig. 11.3, a

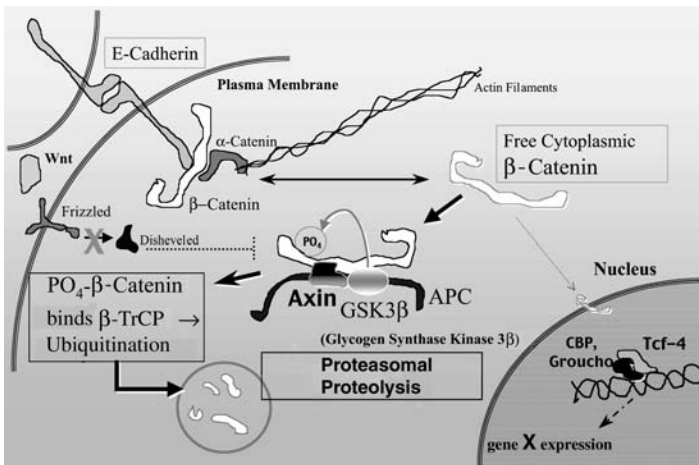
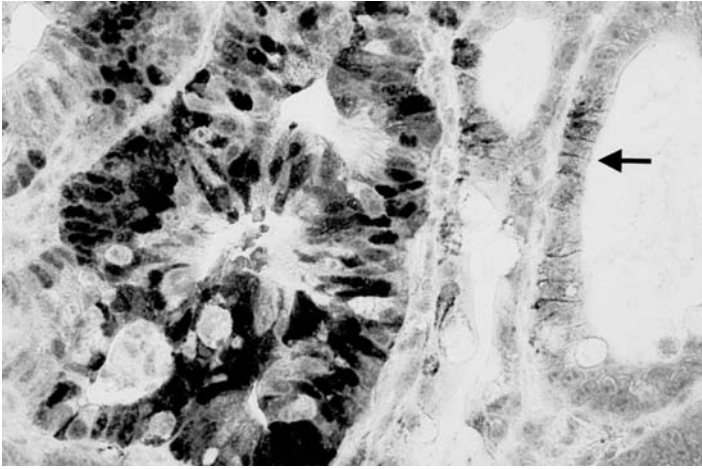


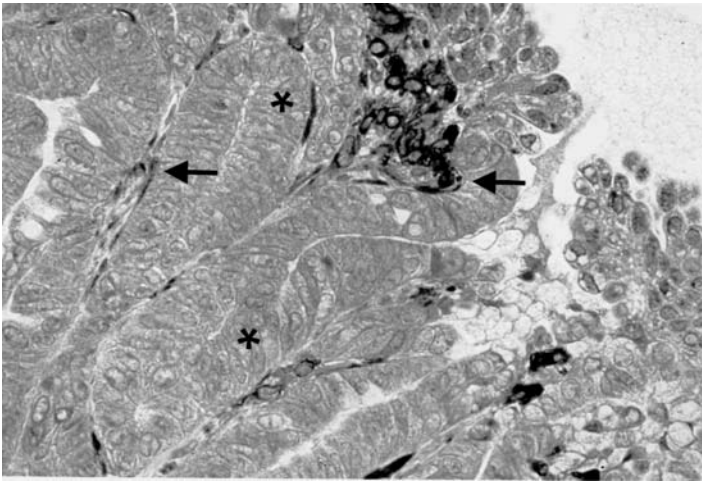
FIGURE 11.3

APC and the Wnt-signaling pathway. The tumor suppressor protein APC is a critical partner in controlling β -catenin nuclear localization and expression of downstream genes. APC acts in concert with axin to facilitate glycogen synthase kinase-3 β -mediated phosphorylation of β -catenin, ear-marking it for ubiquitination. Inactivation of APC or stimulation of the Wnt-signaling pathway results in post-translational stabilization of β -catenin and subsequent nuclear localization. Interaction of β -catenin with the nuclear transcription factor TCF transforms epithelial cells from a normal to a neoplastic phenotype.⁴⁸

**FIGURE 11.4**

Photomicrograph of an $Apc^{Min/+}$ mouse small intestinal tumor immunohistochemically stained for β -catenin (intensely dark stained regions). β -catenin accumulates in the cytosol and nucleus of neoplastic epithelial cells (left portion of figure), but is restricted to cell membranes in non-neoplastic epithelial cells (arrow) as an integral component of adherens junctions.

Wnt signal inhibits the otherwise normal (default) destruction of free cytoplasmic β -catenin by inactivating axin.⁴⁹ As a result, β -catenin migrates into the nucleus (Fig. 11.4) where it initiates transcription of target genes with members of the T-cell factor/lymphoid enhancer factor (Tcf/Lef) nuclear transcription family. It is now clear that tumorigenesis is associated with the dysregulation of intracellular levels of β -catenin. In the absence of a Wnt signal, Apc, GSK-3 β , axin, and β -catenin form a cytoplasmic complex which promotes phosphorylation of β -catenin, thereby targeting it for ubiquitination and subsequent proteasomal proteolysis.⁴⁸ Truncation or loss of Apc cripples formation of this cytoplasmic complex and the Wnt signal becomes constitutive with accumulation of β -catenin in the cytoplasm and nucleus of neoplastic cells. Therefore, with loss of full-length Apc the normal maturation processes associated with upregulation of the wild-type protein and migration of cells out of the crypt are lacking. The pivotal role of β -catenin in intestinal tumorigenesis is reflected by frequent mutations in this protein in tumors that contain wild-type Apc.^{50,51} Such mutations result in loss of phosphorylation sites critical to β -catenin proteolysis and have the same effect as loss of full-length Apc on expression of Wnt-signaling targets which include upregulation of c-Myc, cyclin D1, matrix metalloproteinase-7 (MPP-7, matrilysin), PPAR γ , CD44, and perhaps COX-2.⁴⁹ Although loss of carboxyl-terminus binding sites for EB1, DLG, and microtubules alone is not sufficient for intestinal tumorigenesis,⁴⁶ tumorigenic APC (or β -catenin) mutations that result in upregulation of Wnt/ β -catenin signaling contribute to the abnormal cell migration, differentiation, apoptosis, and proliferation that characterize neoplastic cell behavior.

**FIGURE 11.5**

Histologic section of a small intestinal tumor from an $Apc^{Min/+}$ mouse immunohistochemically stained for COX-2. Only non-neoplastic stromal mesenchymal cells express the enzyme (arrows), whereas the neoplastic epithelial cells are negative (asterisk). (Original magnification, 400 $\times$)

$Apc^{Min/+}$ mice spontaneously develop tumors throughout the intestinal tract, but the induction of COX-2 expression is localized in the stroma of the tumors, not the epithelial cells and not the adjacent normal mucosa^{52–54} (Fig. 11.5). Similarly, COX-2 expression in human colorectal tumors is also observed in both adenomas and carcinomas, with apparent preferential localization in the interstitial cells of early lesions.^{55–58} Regression of intestinal tumors following treatment with sulindac, a non-selective inhibitor of COX-1 and COX-2, occurs in both FAP patients and $Apc^{Min/+}$ mice, and removal of treatment results in tumor regrowth.^{44,52,59,60} Therefore, as a model for intestinal tumorigenesis, the $Apc^{Min/+}$ mouse possesses a number of characteristics that are similarly observed in human tumors.⁴³

11.5 Dietary Polyunsaturated Fatty Acids and Tumorigenesis

Western style diets characterized by high intakes of energy, fat, meat, refined grains, and sugar combined with low intakes of fiber, calcium, and fruits and vegetables have been strongly linked to an increased risk of colorectal cancer.^{2,61,62} Similar correlations have been observed in animal models with *Apc* gene defects. Increasing beef and fat content positively affects intestinal tumor load and some dietary fibers appear to be protective.^{63–66} The feeding of a Western style diet has been shown to increase intestinal tumorigenesis in the Apc^{1638} mouse model⁶⁷ and enhance hyperproliferation of epithelial cells (in various tissues) in null mice (wild type for *Apc*).^{68,69}

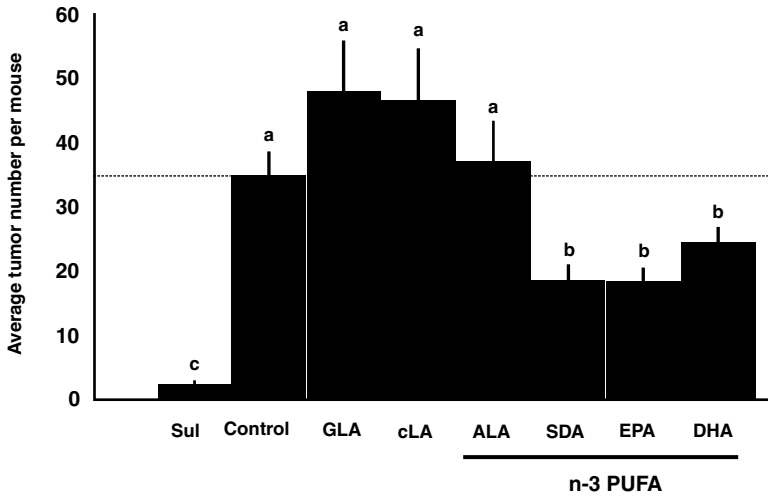
Among the components of the diet, the amount and type of dietary fat consumed is of particular importance.^{1,70–72} Certainly, a number of PUFAs have proved to be very promising as antitumorigenic lipids in both chemically induced tumor models and models containing germline mutations.^{73–77} In an effort to systematically evaluate the efficacy of a variety of dietary PUFAs on intestinal tumorigenesis *in vivo*, we fed $Apc^{Min/+}$ mice selectively tailored diets containing individual fatty acid ethyl esters (1.5 to 3%, w/w). The strength of this dietary design allowed us to directly compare different fatty acids from different families.⁷⁸ The fatty acids evaluated included GLA and AA from the n-6 family, ALA, SDA, EPA and DHA from the n-3 family, and conjugated linoleic acid (CLA). CLA refers collectively to a number of positional and geometric isomers of LA whose double bonds are in conjugation (typically in the 9 and 11 or 10 and 12 positions).²⁰

11.5.1 N-3 Fatty Acids

Recent studies have reported that not only is the amount of fat in the diet particularly important to intestinal tumorigenesis, but the type of dietary fat may even be of greater importance. Epidemiological studies indicate that consumption of fish oil-derived n-3 PUFAs (in particular, EPA and DHA) correlate with a reduced risk of colorectal cancer.^{70–72} However, it has been unclear how EPA and DHA compare to other n-3 PUFAs, i.e., ALA and SDA.

The four major n-3 PUFAs, ALA, SDA, EPA and DHA, were evaluated side by side, with diets controlled for macro- and micronutrient composition (including fatty acid composition), with identical levels of n-6 and n-3 PUFAs. Figure 11.6 summarizes the results of supplementing fatty acid ethyl esters (3% wt/wt) to the diets of $Apc^{Min/+}$ mice on intestinal tumor load. When mice were fed diets containing EPA, tumor multiplicity was reduced by 50% as compared to oleic acid controls.⁷⁸ Similarly, Paulsen et al. reported that diets containing n-3 PUFA-enriched fish oil concentrate (0, 0.4, 1.25, and 2.5%, w/w) reduced tumor multiplicity in female $Apc^{Min/+}$ mice in an dose-dependent manner, while the results in male mice were less consistent.⁷⁴ However, the authors point out an important flaw in the design of the study when they replaced corn oil (rich in pro-tumorigenic n-6 PUFAs) with the fish oil concentrate. In our study, not all n-3 fatty acids had the same effect. DHA was not as effective as EPA, reducing tumor number by only 30% ($P = 0.15$), and thus, it appears that EPA (vs. DHA) has the greater of the two effects on reducing tumor load. In the only other study examining the efficacy of dietary DHA in a similar animal model, DHA resulted in fewer tumors in female $Apc^{\Delta716}$ knockout mice, but not in their male counterparts.⁷⁷ DHA could be acting via retroconversion to EPA,^{7,78,79} thus accounting for its less-pronounced efficacy compared to EPA; however, independent effects of DHA cannot be ruled out.

When ALA was used as the n-3 fatty acid, it failed to reduce tumor number (Fig. 11.6). This is important because ALA is the major source of n-3

**FIGURE 11.6**

Effect of dietary fatty acids on tumor number. $Apc^{Min/+}$ mice were fed a variety of fatty acid ethyl esters (3%, w/w) for approximately 7 weeks and tumor number was determined following macroscopic evaluation.⁷⁸ Bars represent means $\pm$ SEM. Superscripts indicate a significant difference among groups by Fisher's least significant difference multiple comparison method at $P < 0.05$. Sulindac (Sul) at a dose of 400 ppm was used as a positive control. ALA, α -linolenic acid; cLA, conjugated linoleic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ -linolenic acid; SDA, stearidonic acid.

fatty acids in the U.S. diet. While we observed no effect with ALA, others have demonstrated protective effects with perilla oil (a source of ALA) on chemically induced colorectal tumors in rats;^{80,81} however, these studies replaced safflower oil which is rich in the tumor-promoting PUFA LA.⁸² One possible explanation for our lack of efficacy is an insufficient conversion of ALA to EPA because of the Δ -6 desaturase step, the rate-limiting step in the metabolic pathway (Fig. 11.1). However, the levels of EPA in intestinal phospholipids were 10 times higher with ALA than in control animals (3.2 mol vs. 0.3 mol%) (Table 11.3) and equivalent to those observed with the DHA-fed animals (2.8 mol%), suggesting that modification of phospholipid levels of EPA alone is not sufficient to reduce tumor multiplicity in this model.⁷⁸ Nevertheless, it appears that the Δ -6 desaturase is a limiting step for efficacy of n-3 fatty acids. This explanation is bolstered by the fact that SDA, the immediate product of Δ -6 desaturase activity, was as effective as EPA in reducing overall tumor number in this animal model.⁷⁸ Therefore, bypassing this rate-limiting step appears to be critical. These data suggest that ALA's ability to reduce tumor load is limited compared to its metabolic derivatives.

The ability of SDA to reduce tumor load could be related to its conversion to EPA rather than DHA given the fact that (1) the antitumorigenic effects of SDA were identical to EPA; (2) dietary SDA had no effect on tissue DHA levels compared to controls (5.3 vs. 5.0 mol%, respectively);⁷⁸ and (3) dietary

TABLE 11.3
Fatty Acid Composition of *Apc^{Min/+}* Mouse Small Intestinal Phospholipids after Consumption of Diets Supplemented with Various Fatty Acid Ethyl Esters for 7 Weeks

Fatty Acid	Control	Dietary Groups					
		CLA	GLA	ALA	SDA	EPA	DHA
		(mol/100 mol total fatty acids)					
18:2 (n-6)	21.2 ± 0.4 ^e	22.6 ± 0.7 ^d	16.0 ± 0.4 ^f	28.2 ± 0.4 ^b	29.7 ± 0.4 ^a	21.0 ± 0.4 ^e	27.3 ± 0.4 ^b
20:3 (n-6)	1.4 ± 0.1 ^b	1.4 ± 0.1 ^b	2.7 ± 0.1 ^a	1.4 ± 0.0 ^b	1.2 ± 0.0 ^b	0.7 ± 0.0 ^c	0.7 ± 0.0 ^c
20:4 (n-6)	19.8 ± 0.5 ^b	18.4 ± 0.3 ^b	25.3 ± 0.7 ^a	9.2 ± 0.2 ^d	5.9 ± 0.1 ^f	7.6 ± 0.1 ^e	7.0 ± 0.2 ^{df}
20:5 (n-3)	0.3 ± 0.0 ^e	0.4 ± 0.1 ^{de}	<0.1 ^e	3.2 ± 0.1 ^c	4.7 ± 0.2 ^b	12.9 ± 0.6 ^a	2.8 ± 0.1 ^c
22:6 (n-3)	5.0 ± 0.2 ^{cd}	5.0 ± 0.1 ^{bcd}	3.7 ± 0.2 ^f	5.6 ± 0.2 ^b	5.3 ± 0.2 ^{bc}	4.3 ± 0.2 ^{ef}	10.8 ± 0.1 ^a

Note: Values are means ± SEM. Superscripts within each row indicate a significant difference among groups by Fisher’s least significant difference multiple comparison method at $P < 0.05$. Abbreviations: ALA, alpha-linolenic acid; CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, g-linolenic acid; ND, none detected; SDA, stearidonic acid.⁷⁸

DHA had only a modest impact on tumor number. In addition to SDA's ability to be efficiently converted to EPA, it has the added benefit of driving down tissue AA content by inhibiting the Δ -6 desaturase (as indicated by a 40% increase in tissue LA levels) and through EPA's competition with AA for incorporation into membrane phospholipids.

In summary, these studies clearly indicate that dietary n-3 PUFAs are antitumorigenic if they contain 4 or more double bonds, and SDA's equivalent impact on tumorigenesis as that of EPA suggests it possesses "pro-EPA" activity. What is not known is the mechanism of their action. Highly unsaturated n-3 PUFAs are 2.5 to 5 times more effective than ALA in modifying tissue AA levels and eicosanoid biosynthesis.⁸³ Are they acting as antagonists of AA, as inhibitors of prostaglandin biosynthesis, or as independent mediators? Some of these questions will be addressed later.

11.5.2 Conjugated Linoleic Acid (CLA)

CLA, predominantly as 9(Z),11(E)-18:2 (n-7), occurs naturally in small amounts in cooked meats and dairy products⁸⁴ and is potentially antitumorigenic in chemically induced rat mammary tumors and in murine skin tumors at relatively low dietary levels ($\geq 0.5\%$, w/w).^{85,86} Compared to studies investigating the efficacy of CLA on mammary tumorigenesis, evidence for protection against colorectal cancer is less definitive. To date, gavage treatment with CLA has been shown to result in fewer chemically induced colonic ACF and gastric neoplasms in mice,^{87,88} and CLA treatment reduced proliferation of human colon tumor cells *in vitro*.^{89,90} In contrast to these encouraging results, supplementation of CLA to diets of Apc^{Min/+} mice failed to reduce tumor multiplicity or alter average tumor size, even at dietary levels as high as 3% (w/w) (Fig. 11.6).⁷⁸ It has been suggested that CLA may exert its antitumorigenic effect by inhibiting the conversion of LA to AA and ultimately to eicosanoids.⁹¹⁻⁹⁴ Banni et al. showed that CLA at a level of 1% (w/w) in the diet maximally lowered GLA, DGLA, and AA levels as a percentage of total lipid in normal mammary tissue.⁹¹ Others have detected conjugated AA derivatives of CLA in hepatic lipids of rats following intragastric CLA administration.⁹⁵ However, we did not observe any alterations in the small intestinal phospholipid content of LA or its derivatives nor did we detect conjugated AA and, concomitantly, did not observe any differences in prostaglandin formation (Fig. 11.7).⁷⁸

11.5.3 Gamma-Linolenic Acid (GLA)

GLA, the Δ 6-desaturase product of LA, is found predominantly in only a few dietary sources (evening primrose oil, borage oil, blackcurrant seed oil, and spirulina).⁹⁶ By bypassing the Δ 6-desaturase reaction, dietary GLA is rapidly metabolized to dihomo- γ -linolenic acid (DGLA, 20:3 n-6) and

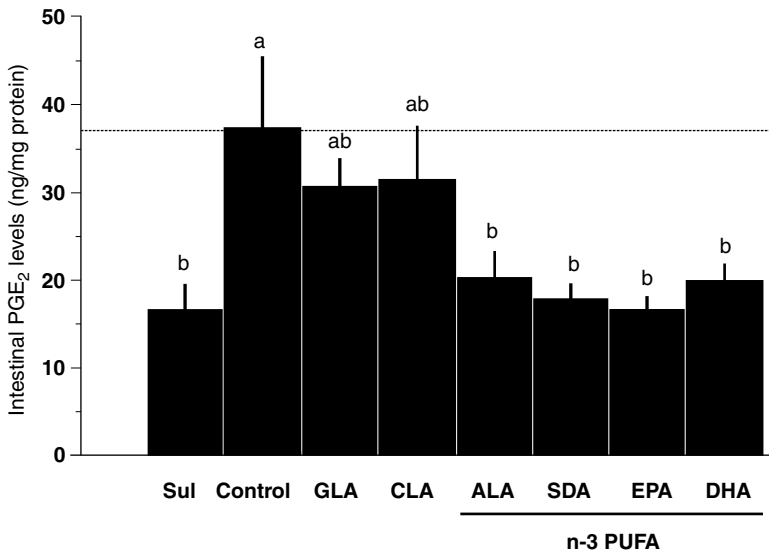


FIGURE 11.7

Effect of dietary fatty acids on intestinal PGE₂ levels. *Apc*^{Min/+} mice were fed a variety of fatty acid ethyl esters (3%, w/w) for approximately 7 weeks and PGE₂ levels were determined in normal appearing mucosa.⁷⁸ Bars represent means ±SEM. Superscripts indicate a significant difference among groups by Fisher's least significant difference multiple comparison method at *P* < 0.05. Sulindac (Sul) at a dose of 400 ppm was used as a positive control. ALA, α-linolenic acid; cLA, conjugated linoleic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; SDA, stearidonic acid.

results in significant increases in tissue AA content as compared to control animals (25.3 vs. 19.8 mol%, respectively) (Table 11.3). To date, the efficacy of dietary GLA on intestinal tumorigenesis has not been determined and the actual antitumorigenicity of GLA has been only narrowly investigated thus far. Arterial GLA injections have been shown to inhibit growth of implanted hepatoma cells in rats,⁹⁷ and intra-tumoral injections of lithium-GLA resulted in smaller tumor volumes in mice implanted with pancreatic tumor cells.⁹⁸ Dietary GLA has demonstrated potential efficacy as an anti-tumorigenic agent only in 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary tumors in rats, where a diet containing evening primrose oil (20 g/100 g) resulted in a lower tumor incidence than did one containing corn oil.⁹⁹ Similar results were observed in nude mice bearing breast carcinoma xenografts.¹⁰⁰ A preventive role for dietary GLA in gastrointestinal tumorigenesis has yet to be established, although GLA treatment has been shown to limit the metastatic potential of human colon cancer cell lines and to block cell cycle progression *in vitro*.^{101,102} However, the use of GLA at a dietary level of 3% (w/w) did not alter intestinal tumor load in *Apc*^{Min/+} mice. In fact, GLA-treated mice had the highest tumor load of all dietary treatments (Fig. 11.6).

11.6 Mechanisms of Action

11.6.1 Arachidonic Acid vs. N-3 Fatty Acids

Studies in humans and in animal models overwhelmingly indicate a protective effect of n-3 fatty acids and the mechanism is largely thought to be related to a reduction in eicosanoid biosynthesis. In particular, PGE₂ has been widely implicated as a key mediator of tumorigenesis.^{103–110} Prostaglandin biosynthesis is controlled by the availability of free AA and the activities of the two cyclooxygenase isozymes. Levels of free AA are influenced by the content of AA in cellular phospholipids and the activity of the various phospholipases responsible for its release. Cyclooxygenase activity is primarily controlled at the transcriptional level.

While it is clear that highly unsaturated n-3 fatty acids can potentially reduce tumor multiplicity in Apc^{Min/+} mice, it is less clear whether this effect is related to AA and/or its metabolism. Dietary AA (1 to 1.5%, w/w) has no effect on tumor load in Apc^{Min/+} mice despite the fact that tissue AA content is increased by ~70% and PGE₂ formation doubles^{44,73} (Table 11.4), implicating a lack of AA involvement. However, if the levels of PGE₂ formed and the amounts of AA in tissues were to exceed a threshold where maximal pro-neoplastic effects occur, augmentation of these levels would have little additional impact. Therefore, if n-3 fatty acids act by antagonizing AA and thus reducing AA signaling below the putative threshold, adding AA to the n-3 PUFA-containing diets should reverse the regression/loss of tumors. This hypothesis is based on our previous work that demonstrates dietary AA has the ability to completely abrogate the biochemical effects of EPA *in vivo*.^{5,10,111} When Apc^{Min/+} mice are fed diets containing EPA, tumor multiplicity is reduced by 50% and concomitant supplementation of AA rescued the tumors, annulling much of the effects of EPA (Table 11.4). These results suggest that the antitumorigenic effect of EPA and other n-3 fatty acids are, at least in part, via the antagonism of AA.^{10,73}

11.6.2 Δ -6 Desaturase

It has been demonstrated that desaturation of ALA to SDA is critical to the anti-neoplastic efficacy of n-3 PUFAs. An extension of this reasoning would suggest that the pro-tumorigenic effect of n-6 PUFAs would also be dependent upon this same metabolic step. Indeed, selectively inhibiting Δ -6 desaturase¹¹² should significantly reduce tumor load in Apc^{Min/+} mice. When Apc^{Min/+} mice were treated with SC-26196, a selective inhibitor of Δ -6 desaturase, tumor number was significantly reduced by ~40% (Fig. 11.8). This reduction in tumors by SC-26196 is reversed when AA is added to the diet to bypass this inhibition (Fig. 11.8). Collectively, these results affirm the importance of AA and the Δ -6 desaturase step as key mediators influencing tumorigenesis and that the Δ -6 desaturase is an important regulatory step in the antitumorigenic properties of n-3 fatty acids.

TABLE 11.4
Tumor Load, Phospholipid Fatty Acid Composition, and PGE₂ Formation from Intestines of Apc^{Min/+} Mice Whose Diets Were Supplemented with Arachidonic Acid and/or Eicosapentaenoic Acid

	Dietary Groups		
	Control	AA	AA + EPA
Tumors			
Tumor #/mouse	68 ± 9 ^a	48 ± 9 ^a	48 ± 6 ^a
Selected fatty acids (mole%)			
18:2 n-6	23.7 ± 0.7 ^b	15.3 ± 0.4 ^c	17.3 ± 0.3 ^c
20:4 n-6	16.7 ± 0.2 ^c	28.2 ± 0.7 ^a	25.1 ± 0.7 ^b
20:5 n-3	0.3 ± 0.0 ^c	0.2 ± 0.1 ^c	1.0 ± 0.0 ^b
Prostaglandins (ng/mg total protein)			
PGE ₂	22.5 ± 7.6 ^{bc}	53.6 ± 7.4 ^a	28.0 ± 5.0 ^b

Note: Apc^{Min/+} mice were fed diet supplemented with the ethyl esters of arachidonic acid (AA), eicosapentaenoic acid (EPA) or oleic acid (Control) at a level of 1.5% (w/w) for approximately 8 weeks. Data are presented as means ± SEM. Superscripts within each row indicate a significant difference among groups by Fisher's least significant difference multiple comparison method at P < 0.05.

Linoleic Acid

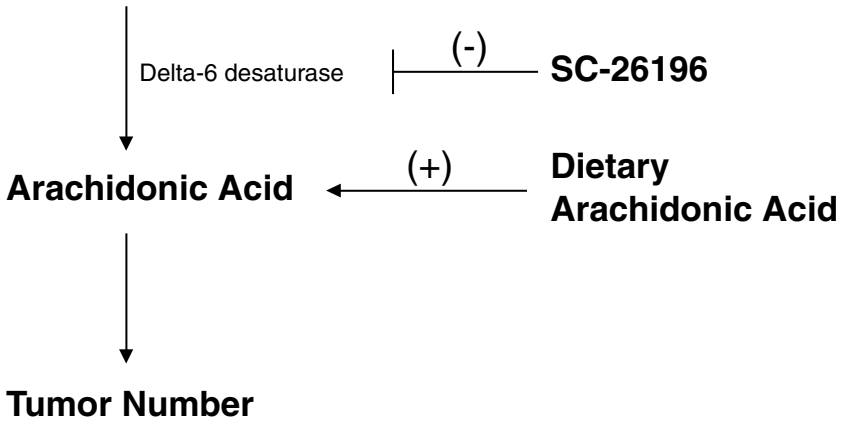


FIGURE 11.8

Effect of SC-26196 and/or dietary arachidonic acid (AA) on intestinal tumor number. *Apc*^{Min/+} mice were fed diets supplemented with the Δ -6 desaturase inhibitor SC-26196 (100 mpk) and/or the ethyl ester of arachidonate (3%, w/w) for approximately 7 weeks and tumor number was determined following macroscopic evaluation. Tumor number was reduced by ~40% with SC-26196, and concomitant administration of arachidonic acid prevented tumor reduction.

GLA also bypasses the Δ -6 desaturase step, and it has been suggested that any antitumorigenic effects of GLA occur because of its conversion and modest accumulation of its metabolic derivative DGLA.¹¹³ DGLA competes with AA for cyclooxygenase and 15-lipoxygenase (LOX) activity to produce PGE₁ and 15-hydroxyeicosatrienoic acid (15-HETrE), respectively.^{114–116} These compounds reportedly have anti-proliferative properties. (See Reference 115 for review.) However, the inability of GLA to have an effect on tumorigenesis may be confounded by the fact that feeding GLA results in higher AA concentrations in intestinal phospholipids and does not significantly reduce AA-derived prostaglandins.⁷⁸ In addition, like PGE₂, the actions of PGE₁ are mediated via the G-protein-coupled EP receptors,^{117,118} and recent evidence indicates that the EP1 receptor significantly contributes to tumorigenesis in this mouse model.^{119,120}

11.6.3 Prostaglandins

The ability of dietary fatty acids to inhibit intestinal tumorigenesis has been explained by their effects on prostaglandin biosynthesis. This hypothesis was developed largely on the strength of epidemiological evidence demonstrating an inverse relationship between colorectal cancer mortality and the use of nonsteroidal antiinflammatory drugs (NSAIDs), both selective and nonselective inhibitors of the cyclooxygenase isozymes. NSAIDs reduce chemically induced and genetically predetermined colorectal tumors in experimental

animal models, as well as the risk of human colorectal cancer.^{44,52,121–126} Aspirin is the most widely used NSAID and many epidemiological studies report a 40 to 50% reduction in the incidence of colorectal cancer with regular sustained aspirin use.^{125,126} Another NSAID, sulindac, is very effective in reducing colonic tumor number and size in FAP patients.^{59,60}

A variety of NSAIDs, eg., sulindac, indomethacin, and piroxicam, reduce intestinal tumor load by 90 to 95% and the number of pre-existing tumors by 80 to 90% in $Apc^{Min/+}$ mice.^{44,52,122,127,128} While NSAIDs like sulindac, indomethacin, and piroxicam are very effective in reducing tumor number in $Apc^{Min/+}$ mice, aspirin may not be as effective as other NSAIDs in this model.^{127,129,130} and this effect may be due to its reduced ability to inhibit COX-2 derived prostaglandins.¹³¹ There is compelling evidence for involvement of COX-2 in tumorigenesis. Cross-breeding $Apc^{\Delta 716}$ knockout mice with COX-2 knockout mice reduced tumor load by 86%.¹³² Administration of the selective COX-2 inhibitors nimesulide and MF-tricyclic reduced intestinal tumor number by 50% in $Apc^{Min/+}$ mice¹³³ and 60% in $Apc^{\Delta 716}$ knockout mice.¹³² However, differences in the ability to inhibit COX-2 may only provide a partial explanation because the efficacy of selective COX-2 inhibitors provides no advantage vs. less selective NSAIDs in $Apc^{Min/+}$ mice. Crossing COX-1 knockout mice with $Apc^{Min/+}$ mice results in 80 to 90% fewer tumors,¹³⁴ and may account for the efficacy observed with the nonselective inhibitors. Two recent articles discuss the importance of cyclooxygenase expression, in particular COX-2, by nontumor cells in the maintenance of tumor integrity and growth.^{33,135} A number of studies also suggest prostaglandin-independent mechanisms may exist.^{136–141}

The most recent data strongly implicate prostaglandin involvement in the reduction of intestinal tumorigenesis by dietary n-3 fatty acids. Intestinal prostaglandin levels are significantly lowered when n-3 fatty acids are supplemented to the diets of $Apc^{Min/+}$ mice regardless of unsaturation. However, lowering prostaglandin levels does not always equate with a lower tumor number. For example, ALA significantly attenuates PGE_2 levels, but does not alter tumor multiplicity, suggesting that prostaglandins play a partial role in tumorigenesis and the anti-neoplastic effects of n-3 PUFAs. Furthermore, regression analysis reveals a significant relationship between PGE_2 levels and tumor number.⁷⁸ It is possible that the degree of unsaturation associated with the n-3 fatty acids could differentially impact the expression, or more importantly, the activity of cyclooxygenase.^{76,142,143} Intestinal tumors in $Apc^{Min/+}$ mice express both isozymes of COX where COX-2 is primarily expressed in the stroma and not the epithelial cells (Fig. 11.5).⁵⁴ Dietary fish oils (rich in EPA and DHA) reduce COX-2 expression in an azoxymethane (AOM)-induced rat colonic tumor model⁷⁶ and immunoreactive COX-1 and -2 protein levels in DMBA-induced mammary tumors,¹⁴³ whereas oils rich in n-6 fatty acids increase COX-2 expression.⁷⁶

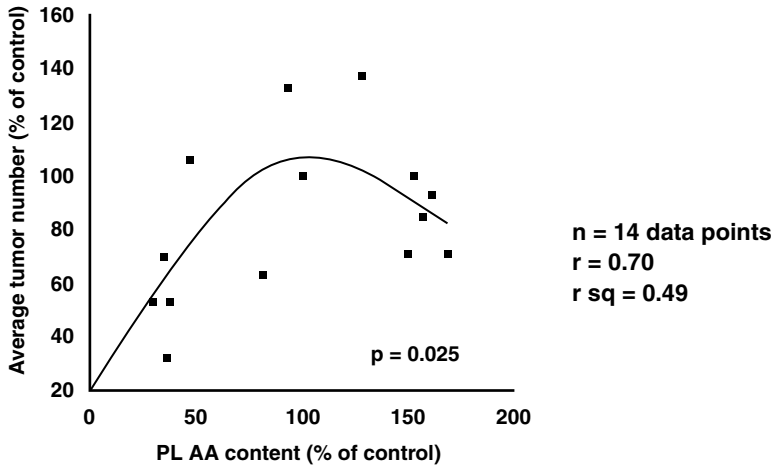
More importantly, prostaglandin involvement in intestinal tumorigenesis was recently demonstrated in a study using prostaglandin E receptor (EP)

knockout mice and an EP receptor antagonist.¹¹⁹ Watanabe et al. treated C57BL/6J mice with the colon carcinogen azoxymethane (AOM) and observed significantly fewer early neoplastic lesions (ACF) in EP1 receptor knockout mice compared to wild-type controls. Similarly, AOM-treated wild-type mice dose-dependently developed fewer ACF following administration of ONO-8711, an EP1-receptor antagonist. They also showed that ONO-8711 treatment resulted in 44% fewer tumors in *Apc*^{Min/+} mice,¹¹⁹ confirming the importance of EP1 and PGE₂ to *Apc*-mediated tumorigenesis. These results were replicated using another selective EP1 antagonist (ONO-8713) in an AOM-treated mouse model.¹²⁰ The EP1 receptor acts through a phospholipase C-mediated signaling pathway resulting in the potential activation of protein kinase C following the release of diacylglycerol. Overexpression of protein kinase C β _{II} results in downregulation of glycogen synthase kinase 3 β (GSK-3 β), an elevation in cellular β -catenin levels, and proliferation of colonic epithelium.¹⁴⁴ The *Apc* gene product acts in concert with GSK-3 β to regulate the Wnt/ β -catenin signaling pathway.⁴⁸ Loss of full-length *Apc* protein, as occurs in the *Apc*^{Min/+} mouse model, disables the cell's ability to downregulate β -catenin and, as a result, free (not bound to E-cadherin) β -catenin increases in the cytoplasm and moves into the nucleus where it acts in conjunction with the nuclear transcription factors Tcf/Lef to induce expression of target genes.⁴⁸ Treatment with NSAIDs, inhibitors of prostaglandin biosynthesis, may reduce tumor loads in this model, at least in part, by attenuating β -catenin levels,^{130,145} and n-3 PUFAs may also modulate this signaling pathway via reductions in AA and PKC activation.¹⁰²

11.7 Summary and Conclusions

While numerous studies provide compelling evidence that dietary n-3 PUFAs abrogate intestinal tumorigenesis, the data presented here clearly establish that these effects are the result of their ability, at least in part, to antagonize arachidonic acid metabolism. That is not to say that augmenting arachidonic acid and its metabolism promotes tumorigenesis, as this is clearly not the case.^{44,78,127} There appears to be a threshold for tissue AA levels where curtailing these levels results in an attenuation of tumorigenesis (Fig. 11.9), but we also present exceptions to this premise (i.e., ALA diet). Those dietary fatty acids that possess the greatest ability to minimize AA, its metabolism, and tumorigenesis have two things in common: (1) They are n-3 PUFAs, and (2) they possess at least 4 double bonds. Furthermore, the most recent data establish PGE₂ as a likely candidate involved in mediating much of the effects of dietary PUFAs.

It has recently been proposed that COX-2-derived PGE₂ is important for activating downstream growth factors, such as vascular endothelial growth factor (VEGF), and that the initial upregulation of COX-2 by tumor stromal

**FIGURE 11.9**

Intestinal tumor number as plotted against arachidonic acid (AA) content of intestinal phospholipids. *Apc*^{Min/+} mice were fed a variety of diets containing different fatty acid compositions and tumor load was correlated with intestinal AA content.^{44,71,78,127}

cells inaugurates this signaling pathway.^{33,135} Thus, attenuating PGE₂ formation, by reducing tissue AA content, downregulating COX-2 expression, or inhibiting COX-2 activity (n-3 PUFAs are reported to do all three) could reduce angiogenesis and result in tumor regression.

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12

Body Weight Regulation, Uncoupling Proteins, and Energy Metabolism

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12.1 A Brief History of Discovery of Uncoupling Proteins

12.1.1 UCP1 as a Brown Fat-Specific Modulator of Heat Production

The rich and varied history of brown adipose tissue as an anatomically discreet tissue type includes early speculations in the 17th century that it was part of the thymus. A century later it was thought to be an endocrine organ involved in blood formation or a form of fat acting as a reservoir for certain nutrients.¹ It was only in 1961 that brown adipose tissue was proposed to be thermogenic.^{2,3} Since that time, an immense body of work has shown that brown adipose tissue is uniquely capable of responding to various environmental stimuli to generate heat from stored metabolic energy. In response to sympathetic nervous system activation, brown adipose tissue undergoes an orchestrated hyperplastic and hypertrophic expansion, increased blood flow, and recruitment of lipid and carbohydrate fuels for oxidative metabolism.^{4,5} A unique and critical element of this thermogenic machine was recognition of the presence of the brown-fat specific mitochondrial uncoupling protein (UCP), originally called thermogenin.⁶ This mitochondrial protein allows controlled proton leakage for the purpose of heat generation at the expense of coupled ATP production (Fig. 12.1). The cloning of the brown fat UCP from rodents provided the opportunity to investigate the molecular mechanism of thermogenic uncoupling in mitochondria and regulation of the UCP gene by hormonal stimulation.^{7–9}

12.1.2 Overview of Defects in Thermogenesis in Rodent Models of Obesity

From the earliest studies of the obese mouse (now called leptin-deficient C57BL/6J *Lep^{ob}*), there was evidence that these mice were not only obese, hyperglycemic, and hyperinsulinemic, but that they exhibited extreme sensitivity to the cold.¹⁰ Histologically, brown adipose tissue in these obese animals appears inactive in that it is infiltrated by white adipocytes and does not possess the rich density of mitochondria expressing UCP1 as normally seen in lean animals. The blunted capacity for adrenergic stimulation of lipolysis in adipose tissue of these animals (described below) probably also hinders the activation of UCP1 function by free fatty acids. Other monogenic obesity models and hypothalamic lesioning studies in rodents indicated a complex set of neural and endocrine abnormalities, culminating in the loss of homeostatic mechanisms controlling both food intake and metabolic efficiency.¹¹

The suggestive role for brown fat and thermogenesis in body weight regulation was strengthened further by the generation of mice lacking brown fat as a consequence of targeted expression of diphtheria toxin in brown adipocytes.¹² These animals became obese and somewhat hyperphagic. Since the adipocyte-derived hormone leptin (the product of the *ob* locus¹³) regulates food intake, metabolic rate, and thermogenesis in brown fat,^{14–17} the obesity

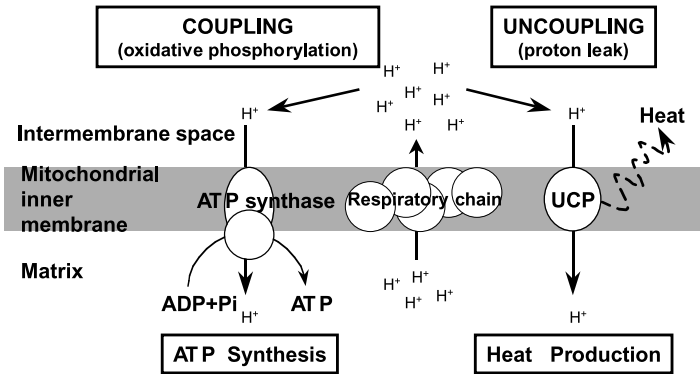


FIGURE 12.1

Schematic model of mitochondrial uncoupling. This simple cartoon compares the relationship between mitochondrial respiration that is coupled to the production of ATP via the ATP synthase vs. the UCP-dependent transfer of protons across the inner membrane down the electrochemical gradient, whose action yields heat as a result of a need for increased fuel consumption to maintain ATP levels.

in BAT-deficient mice may be a result of the inability of leptin to modulate brown fat thermogenesis. However, the metabolic capacity of brown fat may not be restricted to its expression of UCP1, since specific targeted disruption of the UCP1 gene clearly showed that this UCP is responsible for thermal regulation by brown fat, but the animals are not obese.¹⁸

12.1.3 Identity of UCP Homologs and Genetic Linkage with Body Weight Disorders

Sporadic observations that the brown fat UCP could be detected (primarily when using antisera to measure protein levels) in other tissues such as muscle,¹⁹ led Ricquier and colleagues to search for homologues of the UCP. In 1997, Fleury et al.²⁰ reported the cloning of UCP2. In addition to significant homology (59%) with the brown fat UCP (now named UCP1) and the ability to uncouple respiration as efficiently as UCP1 in model systems, we found that UCP2 was broadly expressed in many tissues. This led to the hypothesis that UCP2 was the long-sought explanation for the relative inefficiency of oxidative respiration seen in most cell types. We also noted that the UCP2 gene resides in a chromosomal location on distal mouse chromosome 7 that is coincident with a quantitative trait locus (QTL) linkage to hyperinsulinemia and high plasma leptin levels (reflective of body fat stores). In addition, we showed that the expression of UCP2 was specifically elevated in white adipose tissue in strains of mice that are relatively resistant to the development of diet-induced obesity and diabetes, but not in obesity-prone mice.^{20,21}

Other groups subsequently reported the discovery of UCP3, expressed predominantly in skeletal muscle and brown fat, and to a lesser extent in heart.^{22,23} The structural homology between these UCPs and basic features

about their regulation and expression in various rodent models and human populations have been recently reviewed.^{24–26} The UCP3 gene is also located 8 to 10 kb 5' to the UCP2 gene in both the mouse and human genomes.^{21,27} While this close linkage relationship means that either or both of these UCPs could be related to this QTL, we could not find evidence for changes in expression of UCP3 in the mouse models that originally defined this QTL.²¹ It should be noted that increased expression of UCP2 in brown fat of UCP1^{-/-} animals was observed,¹⁸ but it is not yet clear whether this increase is related to the maintenance of normal body weight in these animals.

12.2 Overview of the β_3 AR and Effects of Selective Agonists

12.2.1 Unusual Thermogenic Properties of Atypical β AR Ligands

The study of lipolysis and thermogenesis in white and brown adipose tissue by the β -adrenergic receptor (β AR) has witnessed episodic confusion and controversy as the number of adrenergic receptor subtypes has grown by molecular cloning, and new pharmacologic tools have become available. For many years it was generally accepted that a single β AR subtype controlled adipocyte metabolism.^{28,29} This view began to erode as ever more selective sympathomimetic agents were developed that could discriminate between β_1 AR and β_2 AR. However, the adipocyte β AR could not be defined clearly as being one or the other of these two subtypes or a combination thereof (especially true in rodents), and it was postulated that an atypical β AR existed in this cell type, which was either a new receptor or a modified state of β_1 AR or β_2 AR. The most significant evidence for a new receptor subtype appeared when Arch and colleagues reported³⁰ that a series of new β -adrenergic ligands, which were clearly not classical β_1 AR or β_2 AR ligands, had the remarkable ability to reverse the severe obesity and diabetes of the C57BL/6J *Lep^{ob}* (*ob/ob*) mouse. These novel compounds increased oxygen consumption, eliminated the classic cold-intolerance of these animals and, importantly, reduced their excess adipose tissue stores.

12.2.2 The Third β AR is a Fat-Specific Gene

Additional pharmacological studies and molecular cloning eventually led to the identification of the β_3 AR in 1989/1990 as a G protein-coupled receptor (GPCR) expressed predominantly in adipose tissue³¹ and the target of the agents developed by Arch, Cawthorne and colleagues.³⁰ We now know that all three β AR subtypes: β_1 AR, β_2 AR, and β_3 AR, are expressed in white and brown adipocytes,^{32–35} and together they mediate the effects of noradrenaline-stimulated lipolysis and thermogenesis. The β_3 AR is expressed in adipocytes as a

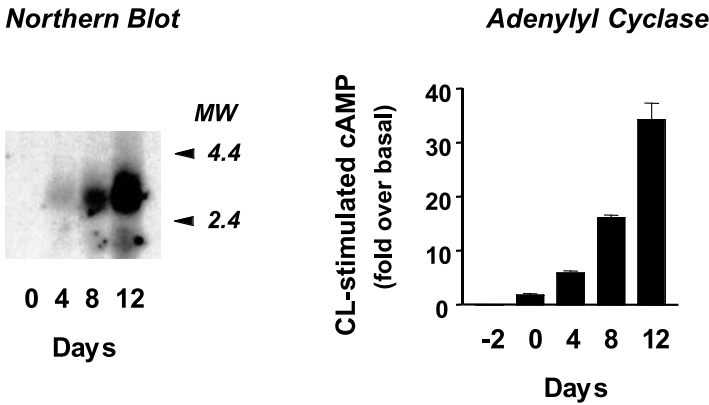


FIGURE 12.2

Differentiation-dependent expression and activity of the β_3 AR in 3T3-F442A adipocytes. As adipogenic cell lines such as 3T3-F442A differentiate, the expression and functional activity of the β_3 AR appear. Left: Northern blot analysis of β_3 AR mRNA levels. Right: Cyclic AMP production in cells differentiated for the indicated periods.

function of differentiation³⁶ (Fig. 12.2). Similar to certain other adipocyte-specific genes, the β_3 AR requires the expression of C/EBP α for induction and maintenance of expression (Dixon et al.³⁷), similar to certain other adipocyte-specific genes.^{38a,38b}

Many studies have now documented the potent anti-obesity and anti-hyperglycemic properties of selective β_3 AR agonists in a variety of animal models.^{30,39–42} These observations have fueled intense investigation of these drugs as potential obesity and/or diabetes therapies for humans. The presence of β_3 AR in human adipose tissue has been much debated. Part of the difficulty stems from the fact that agonists like BRL37344 and CL316,243 were used to assess functional β_3 AR in fat samples from humans as well as non-human primates. The problem here is that these compounds are, *at best*, weak partial agonists for the human β_3 AR,³¹ so that many studies with negative results need to be carefully viewed in this light. There is now clear evidence of β_3 AR in human adipose tissue, but the levels found in human fat cells are much lower than are observed in rodents,^{43,44} and are most evident in brown adipocytes found clustered among white fat cells particularly within intra-abdominal depots. As a result of this visceral location, it has been difficult at best to quantify the relative abundance of these brown adipocytes among individuals and in response to drug treatments. The ability of newer compounds that are full agonists and antagonists for the human β_3 AR⁴⁵ to directly modulate lipolysis in monkey or human fat cells should help to clarify these issues.

12.2.3 Impaired Adipose Tissue Adrenergic Signaling in Obesity

It was known for many years that obese C57BL/6J *Lep^{ob}* and C57KsJ *Lep^{db}* mice exhibited a marked inability to effectively mobilize triglycerides from

white adipose tissue.^{46–49} These animals are also unable to recruit brown adipose tissue for thermogenesis in response to cold temperature-induced adrenergic stimulation,^{49,50} indicating that adrenergic mechanisms regulating metabolism in both white and brown fat are affected in obesity. While defects in sympathetic outflow have been shown to be associated with obesity,^{51–53} other experiments also clearly indicated that there was impaired β -adrenergic receptor (β AR) function at the level of the adipocyte itself, independent of the availability of catecholamines.⁴⁸

Because activation of adenylyl cyclase by catecholamines and the expected elevation in intracellular cAMP are depressed in C57BL/6J *Lep^{ob}* animals, in the late 1970s and early 1980s several investigators tried to determine the nature of the molecular defect in adipocytes from obese animals. The components of the adrenergic signal transduction pathway (at least those which were known at the time, which did *not* include β_3 AR) were examined, and were not different between lean and obese animals despite a severe blunting of the β -adrenergic response, adenylyl cyclase itself, and other downstream effectors of the lipolytic process.^{48,54,55} Collectively, these results led to the conclusion that the signal transduction mechanism of the β -adrenergic receptor(s) must be defective. It is now apparent that earlier β AR radioligand-binding results in adipose tissue were misleading because the classical β AR radioligands such as cyanopindolol exhibit a 20- to 50-fold weaker affinity for the β_3 AR than for β_1 AR and β_2 AR.^{31,56} Therefore, β_3 AR levels were essentially undetected, and estimates of β_1 AR and β_2 AR were distorted. In 1989/1990 the first β_3 AR clone was isolated from a human genomic DNA library.⁵⁷

Figure 12.3 shows the impaired ability of white adipose tissue from genetically obese (C57BL/6J *Lep^{ob}*) mice to stimulate adenylyl cyclase activity in response to β -agonist stimulation, and the dramatic decrease in expression of the newly discovered β_3 AR, as well as of the β_1 AR. Although these adenylyl cyclase data are similar to many previous reports (e.g., Reference 46), a unique aspect of our method of analysis was the use of epinephrine and the very large number of data points. This allowed us to perform complex nonlinear curve-fitting routines⁵⁸ to dissect the contributions of the individual β AR subtypes to the adenylyl cyclase response. In normal lean animals, the high- and low-affinity populations correspond to $\{\beta_1\text{AR} + \beta_2\text{AR}\}$ and $\beta_3\text{AR}$, respectively. By contrast, there is only one population of sites in the C57BL/6J *Lep^{ob}* mouse, and this corresponds to the high-affinity population. Through a series of pharmacologic analyses we showed that these reductions in β AR expression (Fig. 12.3) correspond functionally to the impaired stimulation of cAMP production, and appear to be largely responsible for the defects in catecholamine-stimulated lipolysis observed in the C57BL/6J *Lep^{ob}* mouse.³³ Similar findings of depressed β_3 AR mRNA levels in the Zucker fatty (*fa/fa*) rat were reported by Muzzin et al.,⁵⁹ but the relationship to changes in the function of the receptor was not examined in that study. We have now extended our original findings to several other mouse models of obesity. We find significant deficits in the expression and function

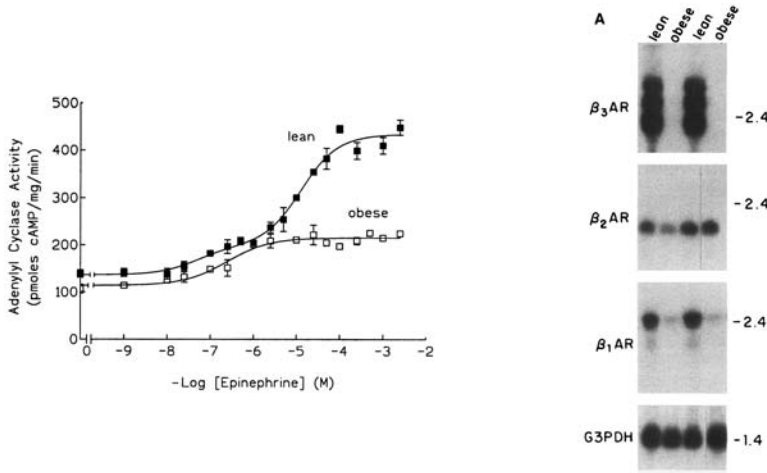


FIGURE 12.3

β -agonist-stimulated adenylyl cyclase and expression of β AR subtypes in white adipose tissue (WAT) of C57BL/6J (lean) and *Lep^{ob}* (obese) mice. Left: Dose-response curves of stimulation of cAMP production by epinephrine in WAT plasma membranes. Right: Northern blot of β_1 -, β_2 -, and β_3 AR mRNA levels in WAT.³³ (Adapted from Collins et al., *Mol. Endocrinol.*, 8, 518, 1994. With permission.)

of adipocyte β ARs in essentially every model of obesity that we have examined, including obesity induced by high-fat feeding in non-mutant mice.⁶⁰

In considering the molecular basis for this dramatic impairment of β AR expression, an important connection may exist between the abnormal endocrine profiles that are observed in these models of obesity and the expression and function of individual β AR subtypes. For example, both the C57BL/6J *Lep^{ob}* and C57BL/6J *Lep^{R^{db}}* mice exhibit many endocrine abnormalities, including hyperinsulinemia, hypercorticism, infertility, and some evidence for perinatal hypothyroidism.^{10,61–65} While glucocorticoids appear essential for the development of obesity in C57BL/6J *Lep^{ob}* and C57BL/6J *Lep^{R^{db}}* mice,^{66–68} adrenalectomy does not completely restore body composition and circulating insulin levels to those of lean controls. Most of these endocrine patterns, including the corticosteroid axis, are normalized when *ob/ob* mice are administered recombinant leptin (reviewed in Reference 69). However, our results with two other mouse mutants, *tubby (tub/tub)* and *fat (fat/fat)*, which have an intact leptin signaling system, as well as the diet-induced obese B/6J mouse, clearly show that there is reduced expression and function of adipocyte β_3 AR and β_1 AR in these animals,⁶⁰ despite their completely normal corticosteroid levels.^{70–72} As in human obesity, all of these animal models of obesity are associated with hyperinsulinemia and insulin resistance. Therefore, considering these data together, it is evident that hyperinsulinemia is the single most common feature associated with both congenital and dietary obesity. Because of this strong association, we presently hypothesize that hyperinsulinemia contributes, either directly or indirectly,

to the inhibition of adipocyte β AR expression and function in obesity, impairing catecholamine-stimulated lipolysis and exaggerating the excessive lipid storage in the adipocyte.

12.2.4 Selective β_3 AR Agonists as Potential Thermogenic and Antiobesity Agents

Since the first reports by Arch and colleagues that atypical β -adrenergic ligands had thermogenic and weight-reducing properties in C57BL/6J *Lep^{ob}* mice,³⁰ there has been great interest in trying to understand their biochemical and physiological effects and to develop such compounds as therapeutic agents. In most species studied, including some studies in non-human primates, β_3 AR-agonist treatment is associated with increased density of brown adipocytes expressing UCP1 within typical white adipose depots.^{40–42,73,74} From our studies in various inbred strains of mice, the relative success of β_3 -agonists as an anti-obesity therapy appears to parallel the extent of this expansion of brown adipocytes.⁴¹ Others reported similar effects of cold-exposure as well as acute β_3 -agonist stimulation in a series of recombinant inbred strains.⁷⁵ Importantly, in our studies we have observed that the beneficial effects of β_3 AR agonists to decrease adipose tissue mass and improve glycemic control in mouse models of obesity and diabetes can persist, even after many weeks of chronic treatment.⁴¹ This apparent lack of desensitization is rather unusual, particularly since tachyphylaxis is a hallmark of most receptor systems. Perhaps β_3 AR activation and stimulation of downstream effectors can continue because the β_3 AR is neither a target for phosphorylation⁷⁶ nor does it bind β -arrestin (Cao et al.⁷⁷), an accessory protein involved in G protein-coupled receptor desensitization.⁷⁸

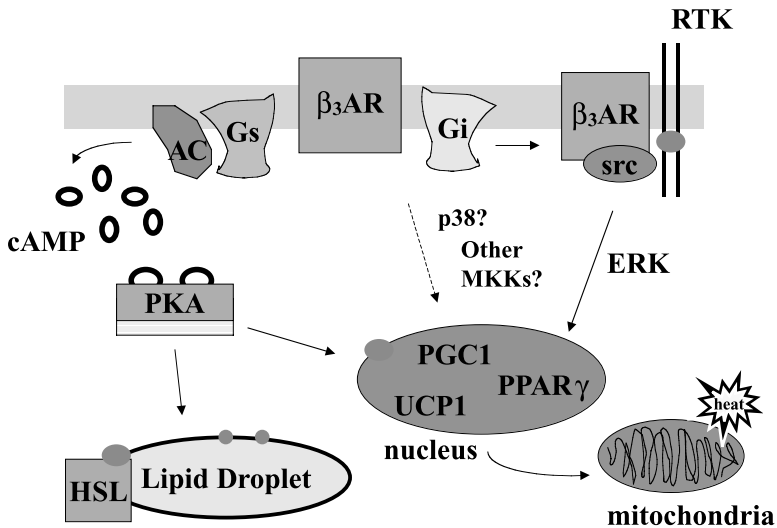
One criticism frequently leveled against studies of the β_3 AR and the effects of β_3 AR agonists is that this phenomenon is peculiar to rodents. This view rests essentially on two arguments: first, that clearly detectable brown adipose tissue in adult humans is absent, and second, that initial studies employing β_3 AR agonists such as BRL37344 in humans or non-human primates showed no effect for stimulating cAMP production or lipolysis. In fact, the situation is not so simple. To begin with, the problem with these initial pharmacologic studies is that these compounds are, *at best*, weak partial agonists at the human β_3 AR.³¹ Thus, negative results are not at all surprising. Regarding the presence of brown fat in humans, several studies of adult dogs and non-human primates provide an important comparison for consideration. Dogs and monkeys are essentially like humans in that, as newborns, there are discrete depots of brown fat that disappear with growth into adulthood. However, upon treatment with selective β_3 AR agonists, each has been shown to exhibit increased metabolic rate, decreased fat mass, and the appearance of brown adipocytes scattered within the so-called typical white adipose depots.^{42,73,74,79} Thus, in each case, whether rodent, canine, or primate, there is a link between efficacy and thermogenesis. Nevertheless, the

mechanisms responsible for these effects of selective β_3 AR agonists *in vivo* are still incomplete. This is an area of very active research, with the obvious question remaining as to whether there will be similar anti-diabetic/anti-obesity effects in humans. Preliminary studies suggest that these effects are at least possible in humans.⁸⁰ Whether there will be genetic predispositions to efficacy or complicating endocrine factors such as hyperinsulinemia that could impact therapeutic response to a β_3 AR agonist will need to be examined.

12.2.5 Novel Signaling Properties of the β_3 AR

Long before the discovery of the β_3 AR and its recognition as a unique, adipocyte-specific receptor controlling lipolysis and thermogenesis, Rodbell made the observation that there was an unusual, biphasic stimulation of cAMP production in adipocytes in response to the β AR agonist isoproterenol.⁸¹ Depending upon the concentration of GTP in the assay, isoproterenol could either stimulate or inhibit adenylyl cyclase activity in adipocyte plasma membranes. Murayama and Ui⁸² showed that this inhibitory phase could be relieved by pretreatment of adipocytes with pertussis toxin (PTX). With the cloning of the β_3 AR gene and the development of highly selective β_3 AR agonists,^{30,83} it was postulated that this novel adipocyte-specific β AR may be responsible for the biphasic adenylyl cyclase response in adipocytes.⁸⁴ In fact, we previously noted that despite the relatively high level of expression of the β_3 AR in adipocytes, the efficiency of coupling of the β_3 AR to stimulation of adenylyl cyclase is low.³³ However, until recently there has been no clear biochemical demonstration of physical coupling of the β_3 AR to G_i , other than comparative functional experiments in the presence or absence of PTX.⁸⁵ Nor had there been any indication of what additional second messenger pathway may be activated as a consequence of this putative coupling of β_3 AR to G_i . We recently reported that the β_3 AR is simultaneously coupled to both G_s and G_i , with the consequent activation of the cAMP-dependent kinase (PKA) and mitogen-activated protein kinase (MAP) (ERK1/2) pathways, respectively.⁸⁵ Physical coupling of the β_3 AR to G_s and G_i was demonstrated in cultured adipocytes using a photolabeling technique that relies upon the ability of agonist-activated receptor to trigger $G\alpha$ subunit dissociation and binding of GTP.⁸⁷ We showed that the restraining effect of G_i on cAMP production, and the dependence of MAP kinase signaling on G_i are indicated by the sensitivity to PTX.

More recent work in our lab now shows that novel sequence elements within the β_3 AR itself are responsible for the direct recruitment to the receptor of SH3 domain-containing signaling molecules such as c-Src, and this interaction is required to trigger the ERK cascade.^{77,88} This realization that the β_3 AR in adipocytes is coupled to multiple signaling pathways has important implications for understanding the unique pharmacological properties of agonists at this receptor. Indeed, it is known that the adipogenic transcription factor PPAR γ can be phosphorylated by ERK on Ser 112 and this modification

**FIGURE 12.4**

Hypothesis for the metabolic consequences of multiple signal transduction pathways elicited by the β_3 AR in adipocytes. The β_3 AR is simultaneously coupled to G_s and G_i , leading to stimulation of the protein kinase A (PKA) and the extracellular signal regulated kinase (ERK), respectively.⁷⁶ ERK activation by the β_3 AR is, like other G_i -coupled receptors, dependent upon the recruitment of a receptor tyrosine kinase (RTK) and activated Src. Potential phosphorylation targets of the PKA and ERK pathways include hormone-sensitive lipase (HSL), perilipin (integral membrane protein of the lipid droplet), and nuclear proteins such as UCP1, PPAR γ , PPAR γ -coactivator 1 (PGC1). It is also possible that other MAP kinases (e.g., p38 MKKs) could be regulated by β ARs.

serves to dampen the transcriptional capacity of peroxisome proliferator-activated receptors- γ (PPAR γ).^{89,90} Furthermore, the possibility that the combined activation of both the PKA and MAP kinase pathways could underlie the β_3 AR agonist-dependent appearance of thermogenically active brown adipocytes in white fat depots needs to be explored, although this is admittedly only a speculation at this point (Fig. 12.4).

12.3 UCP2 and UCP3: Links to Resting Metabolic Rate, Fuel Metabolism, or Signal Transduction

12.3.1 Regulation of UCP2 and UCP3 Expression by Dietary Manipulations

The discovery of UCP2 and UCP3, exhibiting significant sequence and domain structural homology to UCP1 and capacity to uncouple mitochondrial respiration (at least in the yeast expression system),²⁰ immediately led to the notion that they might play a role in metabolic rate and fuel utilization. As described above, our initial observations that mRNA levels of UCP2 were

differentially regulated in response to high-fat feeding in obesity-resistant and obesity-prone strains of mice supported this idea since this dietary upregulation occurred in at least two obesity-resistant strains. If UCP2 is an uncoupler of oxidative respiration, this would be consistent with increased expenditure of metabolic energy.^{20,21}

The mechanism responsible for this upregulation of UCP2 gene expression was postulated to be due to increased fatty acids. In support of this idea we and others reported that ligands for the PPAR γ and PPAR α could increase UCP2 expression in adipocyte cell cultures.^{91,92} (A follow-up study in human fat cells made similar conclusions.⁹³) Not long after these original studies, several groups reported that fasting and/or starvation paradigms in rodents led to substantial increases in expression of both UCP2 and UCP3 in skeletal muscle and adipose tissue.^{94,95} These observations initially led to a debate over the need to preserve body temperature vs. fuel stores. However, these studies of Dulloo and colleagues were pivotal in ultimately persuading the majority of investigators to conclude that, at least in peripheral tissues, these novel UCPs must be participating in the metabolic adaptations required during the fasted state, which requires a switch from predominantly glucose to predominantly fatty acids as a fuel source. They showed that blockading the fasting-induced rise in free fatty acids completely prevented the increase in UCP2 and UCP3 mRNA.⁹⁶⁻⁹⁸ The molecular mechanisms regulating the UCP2 and UCP3 genes are currently under investigation, and yet the larger question that remains to be answered is the true physiologic role of these UCPs.

12.3.2 Modulators of UCP2/3 Activity

The uncoupling activity of UCP1 in brown adipocytes is inhibited by guanine nucleotides and stimulated by fatty acids, although there is still debate over the exact molecular mechanism whereby fatty acids stimulate UCP1 proton passage across the inner membrane (discussed in Reference 99). From studies in isolated brown fat mitochondria, Bouillaud and colleagues recently reported that the uncoupling activity of UCP1 is enhanced by retinoic acid^{99,100} in a manner that is roughly equipotent to that of fatty acids. In addition, their comparative studies in yeast mitochondria expressing individual UCPs confirmed the effect of retinoids on UCP1, and they also showed that certain retinoids and analogs enhance the uncoupling activity of UCP2. However, unlike UCP1, there was no inhibition of UCP2 or UCP3 by GDP. Using a different approach, Garlid and colleagues compared the activity of recombinant UCP1, UCP2, UCP3 reconstituted in liposomes of defined composition.¹⁰¹ They reported that proton flux via UCP2 and UCP3 could be inhibited by nucleotides, but at much higher concentrations (50 to 100 times) than for UCP1. Because of the magnitude of these differences, even after accounting for likely rightward shifts in the dose-response common in isolated systems, the physiologic significance of these nucleotide effects on UCP2 and UCP3 is

unclear. Although unresolved at this time, the possibility remains that UCP2 and/or UCP3 are involved in the transport of lipids or other substrates across the mitochondrial inner membrane. When we consider the fact that it is many years after the discovery of UCP1 and the mechanism of uncoupling by UCP1 is still debated, perhaps we should not be surprised that efforts to determine the functions of UCP2 and UCP3 at the physiologic and molecular level are as yet unresolved.

12.3.3 Tissue Distribution of UCP2 Implicates a Role in Immune Function

At least at the mRNA level, the UCP2 gene is expressed in an enormous assortment of tissues and cell types. Some of these tissues, such as skeletal muscle, adipose tissue, and even certain brain regions such as hypothalamus, would be consistent with hypotheses that UCP2 has a role in energy expenditure and fuel metabolism. By contrast, the relative abundance of transcripts for UCP2 in tissues such as lung, spleen and intestine^{20,99} immediately raised the question as to why there would be uncoupling in such tissues. It was then shown that in liver the expression of UCP2 was largely confined to resident Kupffer cells and not hepatocytes¹⁰² (however, it appears that under certain circumstances hepatocytes can express UCP2).^{103,104} The expression of UCP2 in Kupffer cells, which are phagocytes that serve to clear antigen entering the body from the gut, further raised the possibility that UCP2 might serve some function in macrophages, and macrophages are quite abundant in spleen, intestine, and lung. Since the major activity of macrophages involves the elimination of microorganisms/antigens by generation of reactive oxygen species (ROS), one could speculate on a role for UCP2 in this process.

An intriguing connection between mitochondrial uncoupling and ROS was proposed by Casteilla and colleagues,¹⁰⁴ based on their observation that inhibition of uncoupling activity of UCP1 in brown fat mitochondria by GDP was associated with a rise in H_2O_2 production; a similar effect was observed in spleen and thymus mitochondria (rich sources of UCP2) but not in hepatocytes (lacking UCP2). From these results the authors made the hypothesis that UCPs and mitochondrial uncoupling may indeed modulate the production of ROS. This idea has obvious implications for inflammation, apoptosis, and aging. Diehl and colleagues provided additional evidence linking ROS production and UCP2 expression from studies in which they showed coordinate changes in ROS levels and UCP2 expression in hepatocytes of regenerating rat liver.¹⁰⁴ The accumulation of ROS in hepatocytes was immediately followed by a significant increase in UCP2 expression, with the peak of UCP2 expression coinciding with the maximal decline in ROS production, leading to speculation of a cause and effect relationship. In addition, they showed that pretreatment of cultured hepatocytes with neutralizing anti-TNF α antibody inhibited ROS production and

diminished UCP2 mRNA levels. Hence, these data implicated TNF α in the regulation of both ROS and UCP2 expression, and also hinted at a role for UCP2 in decreasing ROS production in support of the original hypothesis of Negre-Salvayre.

The relationship between TNF α or other cytokines and UCPs is still relatively unclear. For example, several studies have shown that treatment of mice and rats with LPS acutely increases UCP2 mRNA expression in liver, adipose tissue, and skeletal muscle, and that the mechanism is TNF α dependent.^{103,106} A single treatment of rats with TNF α directly also was associated with a significant increase in UCP2 and UCP3 mRNA levels in skeletal muscle.¹⁰⁷ In contrast, treatment of cultured human adipose tissue with TNF α was reported to provoke a 2-fold decrease in UCP2 mRNA levels.⁹³

Thus, while the relationship between cytokines, ROS, and UCPs is intriguing, there is a considerable amount of additional work required to clarify the link, if any, that exists. In the hope of understanding the physiologic role of the UCP2 gene, we recently generated mice with a targeted disruption of UCP2. Of the phenotypes exhibited by these mice, one interesting outcome is that they are completely resistant to certain infectious agents, and we find that UCP2 modulates the production of cytokines and ROS in macrophages.¹⁰⁸ This important observation will need to be explored in greater detail to understand the role of UCP2 in mechanisms of ROS sensing and signal transduction, and the possibility that pharmacologic modulation of UCP2 might play a role in septic and parasitic challenges, immunosuppression, as well as atherosclerosis.

12.3.4 Future Directions for UCP Research

The discovery of the novel UCPs (UCP2/UCP3) in 1997 led to an explosive reinvestigation of thermogenesis, the role of controlled wasting of metabolic energy, and the possibility that these new gene products might be genetically linked to certain metabolic disorders, or that the UCPs might be targets for therapeutic interventions for obesity, Type II diabetes, etc. However, after 2 to 3 years of research the situation is less clear. For a while there was much excitement surrounding the discovery of UCP3, since it is predominantly found in skeletal muscle, a major locus of metabolic fuel consumption and heat generation. Recently, the first reports describing the phenotypes of mice with targeted disruption of the UCP3 gene were, at the very least, disappointing from the perspective of metabolic control of body weight and glucose homeostasis.^{109,110} Nevertheless, the studies of Dulloo and colleagues^{96–98} indicate distinct changes in expression of UCP2 and UCP3 in response to shifts in metabolic fuel usage (carbohydrate vs. lipids), and should clearly point the way for us to try to decipher their meaning.

Other interesting developments in the study of UCPs, particularly UCP2 (because of its broad tissue distribution) may involve apoptosis. Participation of mitochondria in apoptosis is now widely accepted and supported by a

number of studies describing mitochondrial alterations during apoptosis, such as the production of ROS, collapse of the inner mitochondrial membrane potential, opening of the mitochondrial permeability transition pore (MPTP), and the depletion of ATP (reviewed in Reference 111). The first direct hints that UCP2 may be specifically involved in apoptosis were obtained by microarray screening of changes in gene expression in apoptosis-resistant and apoptosis-sensitive clonal sublines of a B-cell mouse lymphoma.¹¹² Expression of UCP2 was significantly upregulated early in apoptosis-sensitive but not in apoptosis-resistant cells. Future studies in this area should be very exciting and illuminating, learning whether UCPs are involved in the process of apoptosis either directly or indirectly.

The role of UCPs in the brain, particularly UCP2, which displays extensive expression in the brain¹¹³ including the hypothalamus, amygdala, and cerebellum, is at this point completely unknown. We can only speculate on the possibility that UCP2, if serving an uncoupling function in neurons, could shift relative production of ATP and hence impact ATP-sensitive ion channels and neurotransmission. In addition, if there is indeed any involvement of UCPs in apoptosis, ROS, etc., then alterations in UCP2 levels in the brain could possibly be involved in certain forms of neurodegeneration and dementia. The study of the novel UCPs over the past few years has certainly been an example of “the more we know, the less we understand.” After this period of challenges and confusion, we can only expect future studies to shed more light, if not necessarily more heat!

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13

Vitamin A and Gene Expression

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Keywords: *retinoid receptors, orphan receptors, retinoid binding proteins, retinoid metabolism, transcriptional regulation*

13.1 Introduction

The fundamental observation (reviewed in Ref. 40) that vitamin A deficiency leads to a profound change in the differentiation of epithelial tissues has led investigators to suggest^{204,231} and eventually prove^{62,167} that the action of the vitamin is similar to that of steroid hormones, i.e., that retinoic acid activates gene expression through nuclear receptors in a variety of tissues. It should be noted that this concept has also included mesenchymal tissues and, in fact, the most well-known clinical application of retinoic acid is in differentiation therapy of acute promyelocytic leukemic cells to mature granulocytes.^{33,41,224,225} Further, this differentiation concept has guided efforts to prevent epithelial carcinogenesis by dietary as well as topical application of retinoids^{32,75,91,205} and continues to generate considerable enthusiasm as a viable approach to controlling malignant progression.^{73,74,90,146}

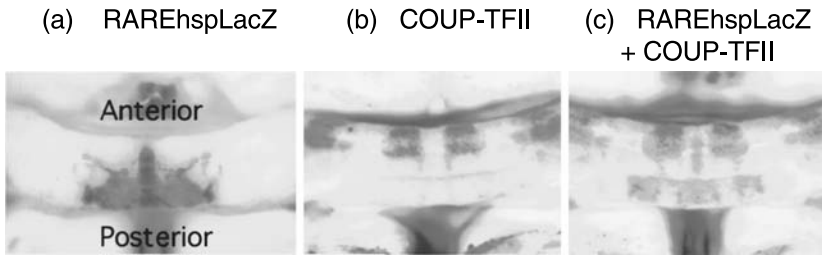
These considerations explain the tremendous interest in this family of compounds from fields as diverse as dermatology, nutrition, epidemiology, carcinogenesis, toxicology and embryogenesis.³⁹

In this review we will mainly concern ourselves with vitamin A homeostasis, plasma and cellular retinoid binding proteins, metabolism and, finally and mostly importantly, recent advances in gene activation processes which involve the RARs and the RXRs and their cognate receptors.

13.2 Aspects of Vitamin A Homeostasis

Vitamin A and its derivatives regulate fundamental physiological functions, including growth, reproduction, vision, and epithelial differentiation.^{18,39,68,161,230}

Once absorbed from dietary sources, vitamin A, i.e., retinol, is carried in the blood stream bound to serum retinol-binding protein (holo-RBP complex), which eventually delivers it to the target cells.⁶⁴ In the cytoplasm of these cells, retinol is mainly bound to cellular retinol-binding proteins (CRBPs, types I and II) to form the retinol-CRBP (holo-CRBP) complex.¹⁶² The subsequent fate of retinol is finely controlled by the vitamin A status of the animal; depending on reserves and peripheral demand, retinol can either be stored as retinyl esters or pushed into the oxidation pathway to eventually generate retinoic acid.^{57,132,153,196} The esterification reaction is catalyzed by lecithin:retinol acyltransferase (LRAT)^{132,185} and/or acyl-CoA:retinol acyltransferase (ARAT)¹⁹ and yields highly hydrophobic retinyl esters, which are then stored as depot resources for eventual utilization, depending on homeostatic control. The first reaction in the utilization-oxidation pathway is catalyzed by the cytosolic alcohol dehydrogenases (ADHs) and/or the microsomal short-

**FIGURE 13.1**

Comparison of RAREhspLacZ and COUP-TF-II distribution in postnatal day 0.3 mouse cerebellum.

chain dehydrogenase/reductase (SDR)^{17,50} and results in the formation of retinal, which in turn is further irreversibly oxidized to retinoic acid (RA) by members of the aldehyde dehydrogenase (AHD) family.¹³⁵ Retinoic acid, the physiologically active retinoid that interacts with two families of nuclear receptors (RARs and RXRs), can also be oxidized to more polar metabolites, 4-hydroxy and 4-oxo-RA by cytochrome P450s.²²⁷

A complex homeostatic mechanism controls the fate of intracellular retinol. Under vitamin A deficiency conditions, retinol is pushed to the oxidation pathway in order to overcome the reduction of RA levels associated with this condition. Conversely, any accumulation of RA prevents retinol from being oxidized and causes its sequestration into the esterification pathway. LRAT has been shown to play an important role in this autoregulative system described in human keratinocytes; in fact, in these cells, RA treatment induces LRAT activity and causes a 50% reduction in the conversion of retinol to RA.

Recently, particular attention has been focused on the role played by CRBP in vitamin A homeostasis. Even though this cellular protein had been shown to be involved in the esterification of retinol with long-chain fatty acids by LRAT, in the hydrolysis of retinyl esters to retinol, and finally in the oxidation of retinol to retinal, its actual physiological function *in vivo* remained unclear. Two independent groups have used different molecular approaches in an attempt to elucidate the function of CRBP, i.e., the generation of transgenic mice either expressing high levels of hCRBP-I or a disrupted form of the mouse CRBP-I gene.

Troen et al.²¹⁵ tested whether high expression of hCRBP-I could induce a phenotype resembling vitamin A toxicity as a result of increased production of RA, or alternatively a phenotype of vitamin A deficiency at the target tissue level, as a result of an increased sequestration of vitamin A in storage cells because of large amounts of CRBP in them. Neither phenotype occurs in tissues overexpressing hCRBP-I, thus demonstrating that excess CRBP-I does not interfere with normal retinol metabolism and function.

In contrast, a biochemical phenotype is obtained when the mCRBP-I gene is knocked out.⁶¹ The mutant mice are clearly borderline for vitamin A content. Even though healthy and fertile, they showed reduced liver retinyl

palmitate levels, due to a decreased capacity to esterify incoming retinol and faster turnover of the retinoid pool. When kept on a vitamin A-deficient diet, the CRBP-I-null mice completely exhausted their liver retinyl esters store and developed abnormalities typical of a postnatal hypovitaminosis A syndrome within a very short time compared to wild type mice. So, clearly, CRBP-I is necessary for maintenance of normal homeostatic functions for this important vitamin and its absence precipitates the vitamin A-deficiency syndrome.

Other intracellular proteins fundamental in the retinoid signaling pathways are the cellular RA-binding proteins I and II (CRABP-I and CRABP-II).^{56,152,163} These two isoforms show differential expression patterns in cells and tissues, thus suggesting that they may play distinct functions. CRABP-I is ubiquitously expressed in adult tissues, whereas CRABP-II localizes mainly in skin and female reproductive apparatus (uterus and ovaries).

CRABP-I, the better characterized isoform, has been proposed to modulate cellular response to retinoids; in fact, it may control the homeostasis of free intracellular RA available to the nuclear receptors by facilitating its catabolism and/or sequestering it.

On the other hand, little is known about the biological function(s) of CRABP-II. Even though different investigators have credited this protein with a nuclear function, no clear evidence for this role has been given until recently. Two interesting papers have been published, both indicating a nuclear function for CRABP-II. Delva et al.⁴² have shown that CRABP-II is present both in the cytoplasm and the nucleus and that it interacts *in vivo* and *in vitro* with RARs (RAR α and RXR α) and participates with the DR5-bound nuclear receptor complex to transactivate RA target genes. Further, its positive transcriptional control is RAR/RXR heterodimer-dependent as it cannot bind by itself to the RARE. CRABP-II is, therefore, a unique coactivator, in the sense that it binds the ligand although it has no structural homology with other currently known co-regulators.

In order to clarify a functional difference between CRABP-I and II in regulating the transcriptional activities of RA, Dong et al.⁴⁷ over-expressed both isoforms in COS-7 cells and showed that CRABP-II, but not CRABP-I, can markedly enhance RAR-mediated transcriptional activation of a reporter gene. As the two isoforms have almost the same affinity for the ligand (there is only a 2-fold difference in the K_d values for complex formation between RA and CRABP-I and II), a differential mechanism of delivery of RA to RAR could be the alternative explanation for the observed functional differences between the CRABPs. In this regard CRABP-I (donor) was shown to transfer RA (ligand) to RAR (acceptor) through a mechanism which requires prior dissociation of the ligand/donor (RA/CRABP-I) complex, followed by formation of the ligand/acceptor (RA/RAR) complex. In contrast, when CRABP-II was the donor, RA was shown to be transferred from the donor to the acceptor by a process which requires direct interactions of the two proteins. Therefore, the authors concluded that CRABP-II enhances the transcriptional activity of RAR by directly interacting with the receptor, thus

facilitating the formation of active RAR-RA complexes. In contrast to the work of Delva et al.,⁴² Dong and collaborators⁴⁸ could neither detect nor isolate a stable CRABP-II-RAR complex using several approaches. This failure to isolate the complex led them to hypothesize that the CRABP-II-RAR interactions are unstable and that the actual complex is a short-lived intermediate.

13.3 RBP

Thus far, the only function attributed to RBP is to transport retinol in the circulation from the vitamin A stores in the liver to peripheral tissues.^{64,202} Retinol-RBP complexes are secreted by the liver into the blood stream where they circulate in a 1:1 molar complex with another protein, transthyretin (TTR). The formation of the RBP-TTR complex in the plasma is required for preventing glomerular filtration and renal catabolism of RBP.⁶⁴ Based on its very early expression during embryogenesis²⁰⁶ and its highly regulated levels in the serum, RBP is also thought to be fundamental in maintaining the retinoid signaling pathways in the body.

For a better understanding of the physiological function of RBP, RBP^{-/-} mice were generated.¹⁷⁴ Unexpectedly, these mice were found to be viable and fertile; nevertheless, they showed reduced retinol plasma levels and, during the first months of life, a markedly impaired retinal function not caused by any developmental retinal defect. When fed a vitamin A sufficient diet, RBP^{-/-} mice acquired normal vision by 5 months, thus showing that RBP-independent pathway(s) for acquiring eye retinol must exist. The authors also postulate that the main responsible factor for the impaired retinal function is the absence of circulating retinol-RBP complexes in the plasma, rather than the low retinol levels; nevertheless, if this is the case, why do the RBP^{-/-} mice show insufficient retinol levels in the eyes but appear normal in other tissue? A possible explanation could be the expression of a receptor for RBP in the retinal pigmented epithelium (RPE), which has been long suggested and might indeed play an important role in retinol delivery to the retina, but not to other tissues.¹³ However, if this were the case it still remains unclear why dietary retinol re-establishes retinal function in the knockout animals.

The data from these studies, however, clearly demonstrate an RBP-independent mechanism for the accumulation of eye retinol. Finally, it is also noteworthy that by 5 months of age the liver retinol levels found in the RBP^{-/-} mice are higher than those in the control counterparts; however, the hepatic retinoid stores in the knockout animals cannot be mobilized, as their liver retinol levels do not fall after a short exposure to a vitamin A-deficient diet. Taken together, these data clearly suggest that the major physiological role for RBP is to insure, under inadequate vitamin A intake, availability of retinol to maintain normal cellular functions.

13.4 Gene Transcription Regulated by the Retinoic Acid Receptors and Their Interactions with the Orphan Nuclear Receptors

Retinoic acid (RA) is the transcriptionally active derivative of vitamin A (retinol) and is required for both embryonic development and adult function.^{49,121,143} Its transcriptional activity is mediated through specific receptors of the nuclear receptor superfamily. Like other ligands of this family, RA is lipophilic and can diffuse a significant distance from its site of synthesis. In the embryo, the creation of spatial gradients of transcriptional activation may be important for the function of RA in regulating pattern formation.^{49,124} Unlike other ligands of the nuclear receptor family, however, RA is not a paracrine hormone acting at a distance from its site of synthesis but is synthesized in the local area of its action and hence is analogous to a growth factor.¹³⁶ As a result, local control of synthesis and catabolism of this receptor ligand provides an important level of regulation of RA-mediated transcription.¹²¹

A second tier of control occurs at the level of the receptor itself. Since the discovery of the RA receptors as nuclear receptor family members it has become increasingly evident that the RA receptors heterodimerize with many partners of the same superfamily. These interactions influence the level of transcriptional activation of the RA receptors, or convert the heterodimeric complex to a transcriptional repressor. Competition between nuclear receptors for shared response elements adds an additional level of interaction. The activity of RA can thus be regulated by the complement of nuclear receptors present when RA enters the nucleus and binds to its receptor. A subtle change in the ratios of the nuclear receptor may alter the transcriptional outcome.

This segment of the review will describe the nuclear receptors that interact with the RA signaling pathway, with an emphasis on the orphan receptors and their role in embryonic development.

13.4.1 Retinoic Acid Receptors and Their Response Elements

13.4.1.1 Characteristics of the Retinoic Acid Response Elements

The response elements present in the promoter or enhancers of RA-responsive genes have been extensively investigated. Two classes of RA receptors exist: the RARs (α , β , γ), activated by the all-*trans* or 9-*cis* isomers of RA, and the RXRs (α , β , γ) activated only by 9-*cis* RA.¹²⁶ These receptors bind to DNA as dimers which is reflected in the paired nature of the DNA response elements, termed RARE and RXRE, respectively, for RAR and RXR. The nucleotide sequence archetype upon which the RARE and RXREs are based has been described in accordance with a direct repeat (DR) 1 to 5 rule. This pattern was first suggested by Umesono et al.²¹⁸ The response elements were based on a direct repeat of the sequence AGGTCA, separated by between 3

and 5 nucleotides. The original 3-4-5 rule was extended to include a separation of 1 or 2 nucleotides according to the following framework: RAREs were DR2 and DR5, the RXREs DR1, and the response elements for vitamin D and thyroid hormone DR3 and DR4, respectively. This guideline worked well to describe several of the strongest RA-response elements, including the DR5 type that is the most prevalent of the simple direct repeats.

The DR1-5 rule is only a framework, however, and a series of additional factors also influences the effectiveness of each response element. The three critical factors are the nature of the spacing sequences, the type of 5' flanking sequence, and the actual sequence of the repeated motif (based on PuG(G/T)TCA rather than simply AGGTCA). The repeated motif can also vary in its orientation and can be palindromic or a mixture of direct repeat and palindromic.¹²⁷ Hence the DR1-5 rule, although a useful foundation for the description of response elements, is not necessarily predictive for what will be a response element for the RA receptor dimers. Numerous modifications on this theme create a multitude of response elements that may react variably to a particular RA receptor dimer. A list of genes containing the DR1, 2, 5 or complex response elements is given in Table 13.1.

13.4.1.2 Receptor Binding to Response Elements

The differences between the two classes of RA receptors (RAR and RXR) go beyond their ligand specificities; the two receptor classes can perform quite separate functions. The RARs are ligand-regulated receptors that function similarly to the vitamin D and thyroid hormone receptors; once ligand bound they become transcriptional activators. The RXRs can also act in a ligand-activated manner; however, the RXRs perform a second essential function as accessory factors for many members of the nuclear receptor family. RXRs can heterodimerize to other nuclear receptors and, without requiring a 9-*cis* RA ligand, increase the affinity of these receptors for their respective response elements. Although the number of permutations of receptors appears limitless, there are some principles which, although not always rigidly adhered to, allow some grasp of what combinations are likely to regulate transcription.

1. The RAR/RXR heterodimers bind much more efficiently to any of the DR motifs than do the RAR/RAR homodimers.^{97,112} Work with the combinational null mutants of RAR and RXR suggests that the RAR/RXR heterodimers are, indeed, an important functional unit activating RA-mediated transcription *in vivo*.^{87,184}
2. The RXR/RXR homodimer is an effective unit for the activation of the DR-1 type response element.^{97,128} In this case, although the RAR/RXR heterodimer binds with greater affinity to the DR1 element, it is incapable of activating transcription.¹¹² This RAR/RXR heterodimer will block RXR-dependent transcription at DR1 by virtue of the RAR half of the heterodimer sitting at the upstream

TABLE 13.1

RAREs	
Gene	Ref.
<i>DR1</i>	
CRBP-II	128
CRABP-II ^a	55
Phosphoenolpyruvate carboxykinase	44, 195
PTH/PTH-related peptide receptor ^b	86
Apolipoprotein A-11	221
Lactoferrin	108
<i>DR2</i>	
CRABP-II ^a	55
CRBP-I	77
Hoxb1+	130
Alpha-fetoprotein	118
<i>DR5</i>	
RAR alpha 2	113
RAR beta 2	209
RAR gamma 2	111
Alcohol dehydrogenase 3	51
Hoxa-1 (3')	105
Hepatocyte Nuclear Factor-3 alpha	79
Tissue-type plasminogen activator	22
MGP ^b	23
Hoxb4	66
Hoxd4	142
17 Beta-hydroxysteroid dehydrogenase type 1	168
ADP-ribosyl cyclase (CD38)	94
Sonic hedgehog	30
Beta 1-adrenergic receptor	7
<i>Complex, Palindromic, or Unusual Direct Repeat</i>	
Medium-chain acyl-coenzyme A dehydrogenase	176
Oxytocin	178
Laminin B1	220
Growth hormone	228
Uncoupling protein	175
H2Kb	80
Zif268	211
Oct3/4 (repression)	170
Stat1	101
Cholesteryl ester transfer	81
Transglutaminase type II	148
Glutathione S-transferase-pi	121
Dopamine D2 receptor	188
Gonadotropin-releasing hormone	36

TABLE 13.1 (CONTINUED)

RAREs		
	Gene	Ref.
	Alpha 1 collagen	138
	Apolipoprotein C-III	106
	Complement factor H	147

- ^a The CRABP-II promoter contains both DR1 and DR2 elements
- ^b Parathyroid hormone/parathyroid hormone-related protein receptor
- ^c Matrix Gla protein

end of the direct repeat and allosterically blocking the binding of the 9-*cis* RA ligand to RXR.¹⁰³

3. The RXR receptors can heterodimerize with a variety of other nuclear receptor partners.^{96, 97} This places the RXR in a key regulatory role since this class of receptor is the only type that will heterodimerize with the vitamin D receptor, thyroid hormone receptor, and RARs, creating heterodimers of high affinity for DR3, DR4, and DR5 (or DR2), respectively.
 - a. On binding of the RXR-containing heterodimer to DR3, DR4, or DR5 elements, RXR is then situated at the upstream end of the repeated sequence and its partner at the downstream end.^{104, 243}
 - b. In the case of RXR heterodimers of the vitamin D and thyroid hormone receptors and, for the most part, RAR, the RXR becomes a “silent” partner and does not require the 9-*cis* RA ligand. The very formation of the heterodimer prevents RXR from binding to 9-*cis* RA.^{59, 103}
 - c. Exceptions exist to the above rule when RXR heterodimerizes with partners outside the vitamin D receptor, thyroid hormone receptor, and RAR groups. The PPAR⁹⁸ and farnesoid X receptors (FXR) heterodimers⁵⁸ with RXR are responsive to RXR ligands. RXR heterodimers with orphan receptors such as the liver X receptor (LXR)²²⁹ or Nur77 (NGFI-B)¹⁶⁶ are also 9-*cis* RA responsive.
 - d. Although 9-*cis* RA does not activate the unliganded RAR/RXR heterodimer, if RAR is bound to its all-*trans* RA ligand then 9-*cis* RA can bind to RXR and potentiate transcription. The likely explanation for this phenomenon is that allosteric inhibition that normally prevents 9-*cis* RA from binding to RXR is released when all-*trans* RA binds to RAR.²²⁶ In some¹¹⁴ but not all²¹² circumstances this is also true for heterodimers of RXR and vitamin D receptors acting on the DR3 element. Likewise the heterodimerization of the thyroid hormone receptor and RXR has been reported to be augmented by 9-*cis* RA.⁸⁵ It is of note in regard to these receptor-activating roles of 9-*cis* RA that a splicing variant of RXR exists that can only perform the ligand-independent functions of RXR.⁶⁰

It is evident from its heterodimerization properties that RXR is a pivotal element in the action of many nuclear receptors. Although, as described above, 9-*cis* RA is not always required for heterodimer activation, this RXR ligand may be necessary for all RXR signaling. 9-*cis* RA can initiate RXR-mediated signaling by inducing the release of RXR receptors from the tetrameric complexes which exist in solution.⁹² Monomeric RXR is then available to heterodimerize, for instance, with the vitamin D receptor.⁴⁶ The importance of 9-*cis* RA in activating RXR heterodimers has clearly been demonstrated in the embryo. In *xenopus* the RXRs are known to be essential for the developmental functions of thyroid hormone receptors.¹⁷³ In the chick embryo, liganded RXR can mediate retinoid signal transduction.¹²²

The differing roles of RAR and RXR mean that the availability and ratios of their ligands (9-*cis* and all-*trans* RA) as well as the receptors themselves will control which response element is active or repressed.¹²⁷ For instance, under circumstances of high 9-*cis* RA the RXR-RXR homodimer will be stabilized and will activate DR1 elements. High all-*trans* RA or high RAR will promote RAR-RXR heterodimerization and the activation of DR2 or DR5 elements. Low ligand concentrations can result in active repression because unliganded RAR can repress promoter activity.¹⁰

13.4.1.3 The Role of Nuclear Receptor Cofactors

The intermediaries between the RA receptor heterodimers and the basal transcriptional machinery are a set of co-activators and co-repressors.³¹ Co-activators include the closely related CREB-binding protein (CBP) and P300⁶³ that possesses histone acetyltransferase activity resulting in acetylation of the histones, destabilizing the nucleosomes and allowing access of the transcriptional machinery to the DNA. The co-repressors include SMRT (silencing mediator for RARs and TRs) and N-CoR (nuclear receptor co-repressor) which act by promoting chromatin deacetylation and nucleosome reassembly. SMRT or N-CoR binds to the unliganded nuclear receptor heterodimer and hence, when the RARs are not bound to ligand, they repress transcription. When the ligand is bound to the receptors, the co-repressors are released, allowing transcription. An example of these co-repressors in action is evident in the RA receptor fusion proteins which result from the t(15;17) chromosomal translocation of acute promyelocytic leukemia.^{67,116} These proteins consist of a fusion of RAR α and promyelocytic leukemia (PML) protein or RAR α and promyelocytic leukemia zinc-finger (PLZF) protein. Proliferation of leukemic cells with the PML-RAR alpha protein can be inhibited by RA, whereas cells with the PLZF-RAR alpha protein are resistant to RA. The crucial difference between these proteins is the ability of RA to release histone deacetylase activity from these fusion proteins while it is only the PML-RAR α protein from which RA can induce histone deacetylase release.

13.4.2 Orphan Receptors and Their Interactions with RA Signaling

The orphan nuclear receptors are a default classification of nuclear receptors, characterized only by their lack of an identifiable high affinity ligand. Several of these receptors, with relatively restricted domains of expression, have a modulatory influence on the RA receptors. The RAR α , RXR α , and RXR β receptors have a very broad range of distribution in the embryo and this extensive range, together with a level complementary function, probably accounts for the relative minor phenotype apparent in the single receptor null mutant mice.⁸⁸ For instance, null mutations of the RXRs have shown that there is functional redundancy among the three RXRs (α , β , and γ) and that RXR α , during development, can perform most of the functions of the RXRs.¹⁸¹ Because of this rather diffuse nature of the RA receptors, interactions with localized nuclear receptors are likely to be a critical factor in determining the specificity of RA transcriptional regulation. We describe below 5 groups of orphan receptors: COUP-TF-I/II; Nur77/Nurr1; ROR/RZR; PPAR α , β , and γ and HNF-4 α , β , and γ , which may refine the action of nuclear receptors, including the RARs and RXRs, in the embryo and adult.

The COUP-TFs are the best characterized of the orphan receptors which modulate nuclear receptor signaling and can inhibit RA-mediated transcription. They can modulate the action of other nuclear receptors either by competing with their hormone response elements, by heterodimerizing and sequestering the receptors, or by directly inhibiting transcription. For the remaining orphan nuclear receptors, cross-talk via heterodimerization with RXR is known for the PPARs⁸⁹ and Nur77/Nurr1.¹⁶⁶ The PPAR/RXR heterodimer contrasts with the RAR/RXR heterodimer in that the former responds to the ligand of both partners, whereas RAR/RXR is non-responsive to 9-*cis* RA. The difference in responsiveness of the heterodimers to RXR ligand has suggested that this may act as a 9-*cis* RA switch in cells that express PPAR. The ratio of RAR to PPAR, and hence the proportion of RAR/RXR to PPAR/RXR heterodimers, would determine whether the RXR signaling pathway is active.⁴⁵ The HNF-4 receptors, in contrast to the other orphan receptors described above, only form homodimers and do not bind to RXR.⁸² However, HNF-4 can compete with other nuclear receptors for the DR1 element¹⁵⁶ and HNF-4 can also activate RAREs, as in the case of the complex RARE in the medium chain acyl-CoA dehydrogenase gene.²⁵ The RORs can function as monomers, binding to one half of the direct repeat and ROR- α signaling can be blocked by competitive binding of RAR/RXR heterodimers.²¹³

All five classes of receptors can play an essential role in the developing embryo. The first three, COUP-TF-I/II, Nur77/Nurr1, ROR/RZR, are involved in CNS development while PPAR and HNF-4 are, respectively, vital for adipocyte and hepatocyte development. The primary response elements of COUP-TF, PPAR, and HNF-4 are based on the DR1 element, but a certain amount of specificity is resolved by the sequence of the direct repeat element

itself, in addition to the identity of the spacer nucleotide.¹⁵¹ Another element providing specificity to the PPARs is the 5'-extension of the direct repeat, which is patterned on a consensus of A(A/T)CT,⁸³ creating the PPREs. A 5'-extension also lends specificity to the DR1 modification that constitutes the RORE. It is the carboxy-terminal end of the ROR¹³³ and PPAR⁷⁶ that likely interacts with the 5'-extensions of their respective response elements.

13.4.2.1 COUP-TFs

The unique feature of the chick ovalbumin upstream promotor-transcription factors I and II (COUP-TF-I/Ear-3, COUP-TF-II/Arp-1) amongst the nuclear receptors is that these receptors are generally dominant repressors of both basal transcription and transactivation.²¹⁶ They will inhibit several nuclear receptors including the RAR, RXR, vitamin D, and thyroid hormone receptors. This inhibitory action is not absolute and the COUP-TFs can also activate gene transcription. COUP-TF-I and II may act as homodimers or heterodimerize with each other or other nuclear receptors such as ear-2 (COUP-TF-III),⁴ a more distant member of the COUP family. The COUP-TFs bind with greatest affinity to the DR1 type response element⁸⁴ and can be grouped with the PPAR and HNF-4 nuclear receptors which bind to variations of this element. The COUP-TFs, however, can also bind to direct repeat or palindromes with spacers greater than 1 and this provides one mode of transcriptional repression for the COUP-TFs: direct competition with the DR3, 4, and 5 response elements of vitamin D, thyroid hormone, or RA receptors. COUP-TF-I has also been shown to bind to the estrogen response element.¹⁰⁰

The inhibitory actions of the COUP-TFs have been extensively described. RXR-mediated activation has been shown to be completely repressed by COUP-TF-I⁹⁶ and this negative regulator can also block the induction of PCC7 differentiation by RA.¹⁵⁵ COUP-TF-II can also strongly inhibit transcriptional activation by PPAR/RXR¹²⁹ and HNF-4.¹⁵⁶ Four routes have been described by which the COUP-TFs interfere with nuclear receptor induced transcription.

1. Direct competition of the COUP-TF receptor and nuclear receptors for the HRE. This occurs, for instance, in the regulation of Oct 3 and 4 by COUP-TF-1 and II, where the COUP-TFs are able to displace RAR:RXR heterodimer binding to the Oct 3 and 4 RAREs.¹⁶ COUP-TFs can also bind to the direct repeats DR3, DR4, and DR5, the HREs for the vitamin D receptor, thyroid hormone receptor and RAR, respectively, and hence the inhibitory action of the COUP-TFs is quite comprehensive for the nuclear receptors.
2. Sequestering of RXR by COUP-TFs. The COUP-TFs are able to effectively heterodimerize with RXR when bound to DNA (although not when free in solution),³⁷ preventing RXR from forming stimulatory heterodimers with other nuclear receptors.

3. Active repression. Active gene repression by COUP-TF-I is mediated by the transcriptional co-repressors N-CoR and SMRT.¹⁹⁷
4. Transrepression. A second region of the COUP-TFs has transrepressor activity, acting independently of DNA binding. This route has been suggested as the main inhibitory mechanism employed by COUP-TF-II.¹

Other genes transcriptionally repressed by the COUP-TFs include those for the Purkinje cell-protein-2 in the cerebellum,² CYP17,⁹ alpha-fetoprotein,¹¹⁹ and aromatase.²⁴⁴ COUP-TF-I and II can repress transcriptional activation by the estrogen receptor,^{99,154} while COUP-TF-I can repress the activation of angiotensinogen by HNF-4.²⁴⁰

Although transcriptional repression is a well-characterized role for the COUPs, their action is by no means restricted to this. Transactivation has been reported for the COUP-TFs, including activation of the genes for arrestin¹²³ and vHNF-1,¹⁷² phosphoenolpyruvate carboxykinase,²¹⁰ NGFI-A,¹⁷¹ and cholesterol 7alpha-hydroxylase.²⁰⁸ COUP-TF-I has been described to generally promote RARE activation by RA in lung cancer cells, an effect that is counteracted by nur77.²³³ Curiously, COUP-TF-I may also promote RA induced by RAR beta expression in cancer cells via a DR8 element.¹¹⁵

The action of the COUP-TFs as transcriptional modulators renders them essential for development. The overexpression of the COUP-TFs (in *xenopus*),²¹⁹ as well as the null mutations in mouse, results in drastic effects on embryonic development. The overexpression of COUP-TF-I in *xenopus* leads to general malformations in the brain and loss of the eyes.¹⁹³ COUP-TF-II is required for angiogenesis and heart development.¹⁶⁵ While both COUP-TF-I and II are present in the CNS, COUP-TF-I exhibits the highest levels of expression. In the developing brain, null mutations of COUP-TF-I lead to loss of neurons that normally form the subplate layer of the cortex. Loss of this layer may explain the failure of neurons in the thalamus to innervate their correct target in the cortex of this mutant. Loss of these projections, in turn, leads to cell death of the neurons of cortical layer IV in their target.²⁴⁶ Abnormalities also occur in the development of the ganglia of the IXth cranial nerve (glossopharyngeal), as well as aberrations in the development of axonal connections between ganglion IX and the hindbrain. COUP-TF-I regulates a number of neurotransmitters including the glutamate receptor subunit KA2³⁵ as well as the N-methyl-d-aspartate (NMDA) receptor channel subunit NR2C.¹⁶⁹ In the developing embryo, a feedback pathway exists between RA and the COUP-TFs, and RA regulates the expression of both COUP-TF-I and II.²¹ The embryonic signaling factor sonic hedgehog has been reported to activate COUP-TF-II gene transcription.¹⁰² The COUP gene promoter contains a sonic hedgehog response element, independent of the Gl_i transcription factor generally associated with sonic hedgehog signaling.

An example of embryonic patterning generated by COUP-TF-II's interaction with RA may be evident in the cerebellum. The cerebellum is a

region of the hindbrain in which several genes reveal a segmented pattern in the form of parasagittal stripes running along the anterior to posterior length (or partial length) of the cerebellum.^{69,71,158} The developing cerebellum is believed to be a region of the CNS where RA signaling occurs^{236,237,239} and we have shown (unpublished observation) that a RAREhspLacZ reporter transgene in a transgenic mouse line delineates a subset of these stripes. This mouse line was originally generated by Dr. J. Rossant¹⁸² and the transgene construct consists of three copies of the DR5 RARE from RAR beta upstream of the promoter from the mouse heat-inducible hsp68 gene. The result of this transgene is that mice respond to endogenous retinoic acid by synthesizing the lacZ reporter gene and they can be used to identify regions of endogenous retinoic acid synthesis and retinoic acid signal transduction.

The stripes of transgene expression are predominantly in a midline structure of the cerebellum known as the vermis and are localized to the posterior half (Fig. 13.1). A number of genes are expressed in the developing cerebellum in a similar or complementary pattern in the cerebellar Purkinje cells and these are believed to set up the postnatal cerebellar parasagittal domains which form part of the intrinsic organization of the adult cerebellum.^{5,141} The RAREhspLacZ reporter transgene is also localized to stripes in the Purkinje cells suggesting that RA signaling may play a role in the set up of these stripes. It was noted in this study of RAREhspLacZ distribution that the midline stripe at postnatal day 0.5 extended to a greater extent into the anterior cerebellum than did the lacZ positive regions on either side of the stripe, which appear to be skewed into horizontal curves with upturned edges (Fig. 13.1a). This pattern was very reminiscent, but reciprocal, of that of COUP-TF-II in the same region (Fig. 13.1b).²³⁸ The most anterior regions of RAREhspLacZ expression lie immediately posterior to the two large COUP-TF-II patches that are situated on either side of the midline and the RAREhspLacZ response curves around the posterior edge of COUP-TF-II. This close juxtaposition is evident when the cerebellum was double labeled for both RAREhspLacZ and COUP-TF-II (Fig. 13.1c). In more posterior cerebellar regions there are also hints of alternating stripes of RAREhspLacZ and COUP-TF-II, although the more diffuse RAREhspLacZ stripes make this more difficult to discern. Since COUP-TF-II is a potent repressor of RA-mediated transcriptional activation, and is expressed in the same cell type as the RAREhspLacZ response, this suggests that COUP-TF-II may play a role in patterning RAREhspLacZ expression by inhibiting the lacZ response in those domains in which it is expressed.

13.4.2.2 ROR/RZR

The ROR/RZRs are a group of related orphan receptors that bind as monomers and hence do not require a direct or palindromic repeat as their response element but bind to a single hexameric motif. The two or four 5' nucleotides flanking the hexameric half-sites are crucial for specificity.²⁴ This

type of response element is present in the promoter of a number of genes including cellular retinol binding protein, RAR beta, 5-lipoxygenase, bone sialoprotein, and Purkinje cell protein-2.¹⁹² Four splicing variants of RORs alpha exist: alpha1, alpha2, alpha 3, and RZRalpha. Although ROR alpha is widely expressed, in the developing brain its strongest expression is in the cerebellar Purkinje cells from embryonic day 15 onward.¹⁴⁹ A mutation in ROR alpha creates the staggerer mutant mouse (sg/sg) in which Purkinje cells fail to develop correctly, resulting in tremor and body imbalance. The animals are also small in size and generally die within 3 to 4 weeks. Although ROR alpha is distributed evenly throughout the cerebellar Purkinje cells, the cells are disrupted in the staggerer mouse in a parasagittal fashion, as indicated by the distribution of calbindin.¹⁵⁰ Pcp-2, one of the genes downstream of ROR alpha,¹³¹ is also distributed parasagittally and is approximately complementary to RAREhsplacZ in the Purkinje cells. ROR alpha functionally interacts with the RA receptors to regulate PCP-2¹³¹ suggesting that RA may play some role in determining ROR alpha's parasagittal signaling.

Of the other two members of this orphan receptor group, RZR/ROR gamma is highly expressed in skeletal muscle⁷² while RZR/ROR beta is specific to the brain. RZR/ROR beta is present in a number of regions generally known to be involved in receiving and processing sensory input, such as the dorsal horn of the spinal cord, layers IV and V of the sensory cortex, and the thalamus.¹⁹⁰ RZR/ROR beta is also expressed in several regions involved in the regulation of circadian rhythms, including the retina, suprachiasmatic nuclei, and the pineal gland. It had been suggested that melatonin was a ligand for RZR/ROR beta;¹⁵ however, this now seems to be in doubt.¹⁴ Nevertheless, the light/dark cyclic changes in RZR/ROR beta in the pineal gland and the retina suggest that this gene plays some role in circadian timing.³ That RZR/ROR beta has an essential role in development is demonstrated by the phenotype of the null mutants which exhibit ataxia, retinal degeneration, and male infertility.³

13.4.2.3 *NUR77 and NURR1*

Nur77 and Nurr1 are two related orphan nuclear receptors. Both receptors bind as monomers to a non-repeating response element, but can also heterodimerize with RXR to activate gene transcription via a DR5 element. This heterodimerization creates an overlap with the RA signaling pathway and is promoted by the 9-*cis* RA RXR ligand.¹⁶⁶ Nur77 has also been reported to enhance ligand-independent transactivation of RAREs and reduce their responsiveness to RA.²³³ Nur77 and Nurr1 themselves can heterodimerize and these dimers enhance transcription.¹²⁵ A third member of this group also exists and is termed neuron-derived orphan receptor (NOR-1).¹⁶⁰

Nur77 (NGFI-B) was the first of this group to be identified, recognized as a gene induced by NGF following the neural differentiation of the PC12 pheochromocytoma cell line.¹⁴⁰ The induction of Nur77 by NGF is very rapid, placing it in the category of immediate early genes, i.e., rapidly inducible by growth factors or membrane depolarization.²⁴² Nurr-1 is also quickly

induced by membrane depolarization in PC12 cells, but is unresponsive to NGF.¹⁰⁷ In the brain Nurr77 can be induced by events such as seizures,²³⁴ ischemia,¹¹⁷ and stress,²¹⁷ while in the immune system, Nur77 is likely important for apoptotic cell death and negative selection of T-cells.^{120,232}

In the CNS, Nur77 is widely distributed whereas Nurr-1 is more localized in its expression pattern and is present in several sensory regions of the brain associated with the limbic system as well as the cerebellum in the internal granular cell and Purkinje cell layers.¹⁸⁹ A role for Nurr-1 in the differentiation of midbrain dopaminergic neurons was first suggested from its expression in that neuronal cell type. These dopaminergic cells are the neurons progressively lost in Parkinson's disease and in the null mutant of Nurr-1 these neurons fail to be born.²⁴⁵ Nurr-1 is specific only for the dopaminergic neurons of the midbrain and is absent, for instance, in dopaminergic neurons of the hypothalamus⁶ and these neurons are not lost in the Nurr-1 null mutant.²⁹ Nurr-1 transcriptionally activates the dopamine transporter gene¹⁸⁶ as well as the tyrosine hydroxylase gene required for dopamine synthesis¹⁸⁷ and Nurr-1 can induce a midbrain dopaminergic phenotype in neural stem cells.²²² Nurr-1 hence plays a determining role in the development of the midbrain dopaminergic neurons.

13.4.2.4 HNF-4

Hepatocyte nuclear factor-4 (HNF-4) is an orphan receptor first recognized for its role in liver development and adult hepatic function.¹⁹⁸ The actions of HNF-4 lie within a network of two other hepatic transcription factors, HNF-1 and HNF-3, which are not nuclear receptors. Together they regulate each other and coordinately regulate genes required for hepatic differentiation and metabolism.⁵⁴ The role of HNF-4 in development, however, is more fundamental; it is expressed as early as embryonic day 4.5 in primary endoderm⁵² and its null mutation results in the failure of gastrulation.³⁴ At embryonic day 8.5 HNF-4 is expressed in the liver diverticulum and the hindgut and later is present in the kidney, pancreas, stomach, and intestine.⁵² In the liver, HNF-4 promotes the determination of the hepatic phenotype from its progenitor cells²⁰³ and induces the expression of many hepatic genes.⁵³ In the adult, HNF-4 regulates a series of genes involved in glucose, cholesterol, and fatty acid metabolism. These orphan receptors are not specific to the liver and involvement of HNF-4 alpha in glucose metabolism in the pancreas is demonstrated by the development of a form of non-insulin-dependent diabetes mellitus in HNF-4 alpha mutations.²³⁵

HNF-4 binds as a homodimer⁸² to DR1 elements as well as the PPRE.⁷⁰ As described for the PPAR, the sequences surrounding the DR1 element, as well as the precise sequence of the DR1, can be important for HNF-4 binding and activation.^{151,207} HNF-4-regulated genes in the liver include apolipoprotein A-II,¹⁷⁷ alpha1-microglobulin,¹⁸³ hepatocyte growth factor-like protein,²²³ and steroid 15alpha-hydroxylase.²⁴¹ The suppression of HNF-4-regulated transcriptional activation by the COUP-TFs has been reported in a number of circumstances. COUP-TI and II antagonize the effects of HNF-4 on

apolipoprotein CIII¹³⁹ and the cytochrome P450 CYP3A1,¹⁵⁹ while COUP-TF-I represses HNF-4 activation of both ornithine transcarbamylase⁹³ and sex hormone-binding globulin.

13.4.2.5 PPAR

The peroxisome proliferator-activated receptors (PPARs alpha, beta, and gamma) can act as receptors for polyunsaturated fatty acids and several eicosanoids. These are natural ligands and hence the PPARs do not fit under the strict heading of orphan receptors; however, the relatively low affinity that these ligands have for the receptors suggests that the genuine ligands may remain to be identified. The PPARs are known to regulate glucose and lipid homeostasis and can also guide adipocyte differentiation.^{43,191} It has been suggested that the function of PPAR alpha may be related to fatty acid metabolism while PPAR gamma may be more important for adipogenesis. That PPAR gamma is essential for glucose homeostasis was demonstrated by the finding that dominant negative mutations in this receptor are associated with insulin resistance and type 2 diabetes.¹² Because the PPAR/RXR heterodimer is reponsive to ligands for both receptor partners,¹⁹⁴ antidiabetic activity is found for both PPAR gamma¹¹⁰ and RXR agonists.¹⁴⁵

During embryonic development, the null mutant of this same receptor, PPAR gamma, is lethal at embryonic day 10 due to inadequate placental vascularization. Correction of the placental maldevelopment does not protect against embryonic death due to later hemorrhages and lipodystrophy.¹¹ Null mutants of PPAR alpha, in contrast, develop relatively normally¹⁰⁹ although the mice do exhibit abnormalities in lipid homeostasis.³⁸ In the embryo, PPAR alpha, as well as PPAR gamma, is expressed in the CNS at embryonic day 13.5 and is gone by 18.5.²⁰ Of all the receptors, PPAR gamma exhibits the most restricted distribution and at embryonic day 18.5 it is localized to and very strongly expressed in the brown adipose tissue. PPAR alpha is expressed at embryonic day 18.5 in the liver, digestive tract mucosa, epidermis, and brown adipose tissue while PPAR beta is relatively ubiquitous.²⁰

The PPAR response element is similar to the DR1 repeat but is distinguished by a characteristic upstream flanking sequence.^{27,164} The PPARs are similar to the nuclear receptors RAR, thyroid hormone and vitamin D receptors, in that they bind with high affinity to their response element as a heterodimer with RXR. They differ from these other two RXR partners in that the PPAR binds to the 5' half-site of the response element and RXR occupies the 3' half-site.⁷⁸ RXR/PPAR heterodimers have also been described as activating the estrogen response element.¹⁵⁷ The RXR/PPAR heterodimer regulates numerous genes involved in glucose and lipid homeostasis and RXR/PPAR-responsive PPREs are present in the genes for hepatic phosphoenolpyruvate carboxykinase,²⁶ CYP4A6,¹⁴⁴ phosphoenolpyruvate carboxykinase,²¹⁴ malic enzyme²⁸ and acyl-CoA oxidase.⁸⁹ The PPRE of HMG-CoA synthase is a site acted upon by a number of nuclear receptors members, and is activated by PPAR and RXR, repressed by HNF-4,¹⁸⁰ and repressed or activated by COUP-TF.¹⁷⁹

13.5 Discussion

Traditionally, hormones were identified on the basis of their physiological effects, i.e., they were purified, characterized chemically, and their physiological effects determined. The identification of their specific receptor(s) followed, through ligand–receptor interaction studies, utilizing radioactive ligands. Finally, the gene and its chromosomal location were characterized. A more recent approach, termed “reverse endocrinology,”⁹⁵ has permitted the identification of orphan receptors first, and subsequently, their specific ligands. In this manner, new synthetic ligands can be tested in reporter gene activation assays to eventually open up possibilities and suggest selective ligands as new drugs in endocrine and related diseases. The potential of this approach is realized if one considers that many of these pathways interact at the transcription level.

Cross-talk between the RA, thyroid hormone, and vitamin D receptors has been extensively investigated. Recently it has been shown that these interactions can even extend to the estrogen receptors.²⁰¹ This review has described several of the orphan nuclear receptors that interact with the RA receptors and hence modulate the RA signaling pathway. New orphan nuclear receptors are still being discovered and the list of receptors that modify RA signaling is likely to expand. In an organism as simple as the nematode worm more than 200 nuclear receptor genes are estimated, this family being the most prevalent of the transcription factors.²⁰⁰ It seems likely that there will be an equivalent diversity in mammals. It has been noted that several orphan receptors are relatively specific in the control of restricted cell types, and these nuclear receptors impart identity to the cell lineage, e.g., HNF-4 for hepatocytes, PPAR gamma for adipocytes, RAR for neurons,^{65,134} and Nurr-1 for dopaminergic neurons. If the variety of nuclear receptors is comparable to that found in the nematode then it may be expected that other such cell lineage regulatory receptors exist, and perhaps one role for the nuclear receptors during embryonic development is to initiate the identity of distinct cell lineages. Several metabolic enzymes are induced as part of this cell-specific differentiation program and they continue to be transcriptionally regulated by the same nuclear receptors in the adult. For this reason the orphan receptors, such as PPAR are HNF-4, are considered essential metabolic regulators.¹⁹⁹

As with all ligand/receptor signaling events, at each progressive step of the RA signaling pathway there is an increase in the number of elements that contribute to the final signaling consequence (transcriptional modulation). In series, these steps include

1. The receptor ligand, RA, that can be synthesized and catabolized locally to create a concentration differential of ligand resulting in a gradation of transcriptional activation. Such is the case in the

developing retina where differential synthesis or catabolism of RA creates zones of differing RA transactivation.¹³⁷

2. The individual RAR and RXR receptors that distinguish the transcriptional activity of all-*trans* and 9-*cis* RA as well as determine which DNA response element (DR1, DR2, or DR5) will be activated.
3. The nuclear receptor combinations, present at any given time, that will determine which heterodimers will form and which set of receptor species will be transcriptionally active. For instance, the high expression of COUP-TF-II would favor heterodimerization with RXR to create a transcriptional repressor. Alternatively, a high proportion of PPAR to RAR would favor the formation of PPAR/RXR, rather than RAR/RXR heterodimers, allowing PPAR ligand or 9-*cis* RA signaling to occur instead of the all-*trans* RA RAR ligand.⁴⁵ Because PPAR/RXR is responsive to the ligands of both heterodimeric partners, this provides a dual switch that is at its peak potency only when the cell is exposed to both ligands.
4. The RAREs, which bring together the nuclear receptors, co-activators and polymerases, resulting in transcription. The RAREs can range from the relatively simple direct repeats to HREs with complex series of overlapping direct repeats (e.g., glutathione S-transferase with seven repeat retinoic acid response element (RARE) consensus half-sites¹²¹ or combinations of direct and palindromic repeats).

The modulators of RA-mediated transcription that act at these steps provide the necessary levels of refinement for RA transcriptional activation of each RA responsive gene. RA is known to be able to regulate transcription with broad brushstrokes, for instance, inducing neural gene expression in stem cells,⁸ or precisely modulating, either up or down, the expression of single genes, as in the case of *hoxb-1*.¹³⁰ The combination of modulating factors which act on the receptors and response elements allows RA to act either as a broad regulator or fine manipulator of transcription.

Abbreviations

RA	retinoic acid
RAR	retinoic acid receptors
RXRs	9- <i>cis</i> -retinoic acid receptors
RBP	retinol binding protein
CRBP	cellular retinol binding protein
CRABP	cellular retinoic acid binding protein

RARE	retinoic acid response element
ThR	thyroid hormone receptor
COUP-TF	chicken ovalbumin upstream promoter-transcription factor
CYP	cytochrome P450
RXRE	RXR response element
MGP	matrix Gla protein
PTH	parathyroid hormone
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator-activated receptor response element
N-CoR	nuclear receptor corepressor
SMRT	silencing mediator for RARs and TRs
CBP	CREB-binding protein
PML	promyelocytic leukemia
PLZF	promyelocytic leukemia zinc-finger
HNF	Hepatocyte nuclear factor-4
RORE	ROR response element
NMDA	N-methyl-d-aspartate
Pcp-2	Purkinje cell protein-2.

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14

Vitamin A and Mitochondrial Gene Expression

Helen B. Everts and Carolyn D. Berdanier

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14.1 Introduction

Mitochondria are the central integrators of intermediary metabolism. Key metabolic pathways located in this organelle are the β -oxidation pathway, the TCA cycle (tricarboxylic acid cycle, Krebs cycle, citric acid cycle), and oxidative phosphorylation (OXPHOS). Mitochondria contain their own DNA (mtDNA). This deoxyribonucleic acid (DNA) encodes 13 of the 76 subunits of OXPHOS. The size of the mtDNA can vary from ~16 kb in mammals to over 300 kb in flowering plants. It is believed that originally the mtDNA contained all of the genetic information for the components needed for OXPHOS, but over time most of these genes were transferred to the nucleus.

The mtDNA has been completely sequenced and mapped for a wide variety of species including man, mouse, and rat. Shown in Fig. 14.1 is the human mt genome with each of the structural genes, the transfer ribonucleic acid (tRNAs), and the ribosomes indicated. This DNA encodes 13

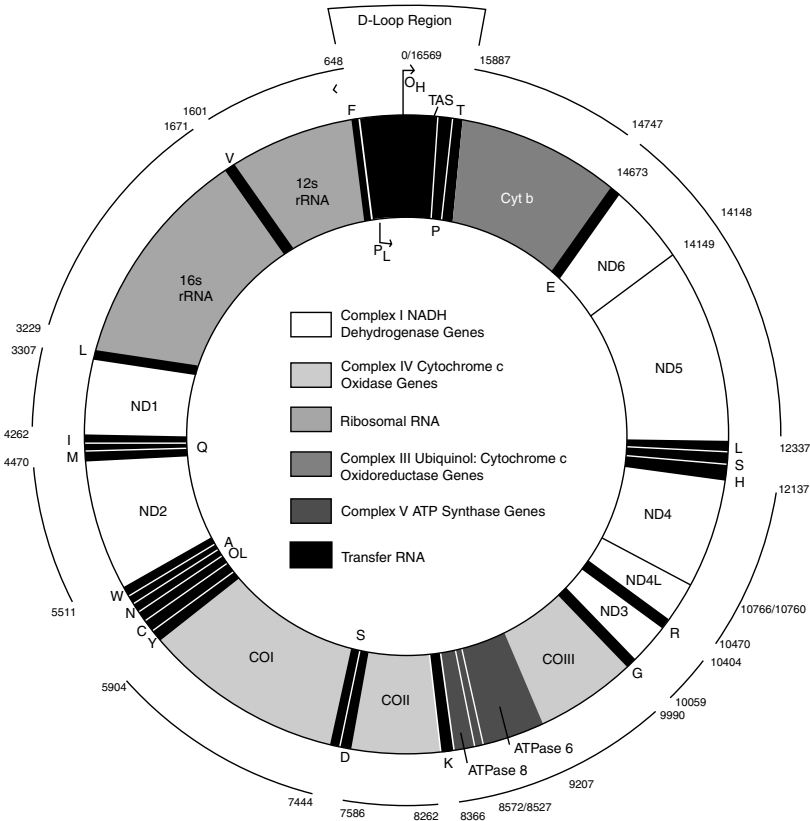


FIGURE 14.1

The human mitochondrial genome. ND1-6 = Complex I, NADH dehydrogenase genes; Cyt b = cytochrome b, complex III, ubiquinol; Cytochrome-c oxidoreductase gene; COI-III = complex IV, Cytochrome-c oxidase genes; ATPase 6 and 8 = Complex V, ATP synthase genes; rRNA = ribosomal RNA; tRNA genes are listed by their single letter amino acid name. The displacement loop (D-loop) region is indicated. P_H and P_L = promoters for the heavy and light strands; O_H and O_L = origins of replication for the heavy and light strands, respectively.

polypeptides, 22 transfer RNAs, and 2 ribosomal RNAs. Also shown is the D-loop which is thought to be the promoter region for most of the genome. The regulation of its expression is only now being investigated. Some of the steroid hormones, thyroid hormone, insulin, and retinoic acid probably play as important a role in the expression of the mt genome as occurs in the expression of the nuclear genome. Evidence is only now accumulating on the roles these compounds play in mt gene expression. In Chapter 13 of this volume is a review of the role of retinoic acid in nuclear gene expression.¹ In this chapter, the role of retinoic acid in mt gene expression will be described.

FIGURE 14.2
Schematic representation of the four respiratory complexes and the F_1F_0 ATPase (complex 5). Each of the complexes have a number of subunits. Those encoded by the mitochondrial genome are shown as black; the nuclear encoded units are shaded. Note that the F_1 portion of the ATPase projects out into the matrix.

requirement. Electron microscopy studies have shown clusters of mitochondria near the ribosomes in cells undergoing high rates of protein synthesis. Clusters of mitochondria have also been found in muscle near the contracting fibers. In each instance it is supposed that these mitochondria are providing the needed ATP to support the local process. In the former, the process is protein biosynthesis; in the latter, it is muscle work.

Mitochondrial function depends not only on the metabolic fuels needed for the support of the TCA cycle and OXPHOS and on the proteins encoded by the nuclear genome and imported into the mitochondrial compartment, but also by the transcription and translation of the mt genome. This coordination of gene expression in these two organelles is of particular interest to those interested in OXPHOS.

14.3 Oxidative Phosphorylation and the Mitochondrial Genome

The mechanism of OXPHOS has been extensively reviewed.^{2–21} Electrons shuttled into the mitochondrial compartment by the nicotinamide dinucleotide (NAD)-linked and flavinadenine dinucleotide (FAD)-linked shuttles are transported through inner membrane-bound respiratory chain enzyme complexes and joined to oxygen to form water. This process is linked to the formation of ATP by the F_1F_0 ATPase. The coupled processes for water and ATP synthesis are called OXPHOS.

Mitchell in 1961 proposed that the sequential reactions of the respiratory chain generate an electrochemical gradient of H^+ ions across the inner mitochondrial membrane.⁸ The energy of this gradient is captured by the F_0 portion of the F_1F_0 ATPase and transmitted to the F_1 portion that uses it to synthesize ATP. The newly synthesized ATP is released in the matrix only to be captured by the adenine nucleotide translocase and translocated to the cytosol. Translocation does not account for all of the ATP produced by the F_1F_0 ATPase. Some of the newly synthesized ATP is used directly by reactions in the mitochondria. In addition, OXPHOS is not very efficient in this energy capture. Most of the energy is released as heat. The proportion of the energy released as heat vs. that used for ATP synthesis varies with the physiological and nutritional state of the animal. Under a variety of conditions (stress, cold, overeating) cells will release proteins called uncoupling proteins (UCPs). These proteins serve to dissipate the proton gradient generated by the respiratory chain such that little of the energy is converted to the high energy bond of ATP. Heat production is thus increased when UCPs are produced. The actions of the UCPs with respect to energy metabolism are reviewed by Collins et al. in this volume.¹⁹

In addition to those conditions that stimulate UCP production, there are dietary conditions that affect the fluidity of the inner mitochondrial membrane.

Animals fed diets containing hydrogenated coconut oil have less fluid membranes than animals fed diets containing unsaturated fatty acids, i.e., corn oil or fish oil.²⁰ Changing the fluidity of the inner membrane changes the environment in which the ATPase functions. The ATPase must be able to rotate within the membrane^{9,13,18} and the more fluid the membrane, the greater the efficiency of OXPHOS.

There are four complexes that make up the respiratory chain and one complex, the F_1F_0 ATPase, for ATP synthesis. These are as follows: nicotinamide dinucleotide, reduced (NADH)-ubiquinone (complex I) is the site of entry for NADH protons into the chain. NADH is oxidized, ubiquinone reduced and four protons are pumped from the mitochondrial matrix to the intermembrane space. In mammals there are at least 42 subunits in this complex, seven of which are encoded on mtDNA (ND1, 2, 3, 4, 4L, 5, and 6). Succinate:ubiquinone oxidoreductase (complex II) is the site of entry for $FADH_2$ protons into the chain via ubiquinone. It contains 4 subunits, all of which are nuclear encoded in mammals. No protons are pumped at this site. Ubiquinone-cytochrome-c oxidoreductase or the bc_1 /cytochrome-c reductase (complex III) accepts electrons from ubiquinone. In this reaction, ubiquinone is oxidized, electrons are transferred to cytochrome b then c_1 and four protons are pumped into the intermitochondrial space. This complex contains 11 subunits in mammals, one of which (cytochrome b) is encoded by mtDNA. Cytochrome-c oxidase (complex IV) accepts electrons through the soluble cytochrome-c and transfers them to oxygen. This process pumps four protons into the intermitochondrial space. In mammals, this complex contains 13 subunits, three of which (COX I, II, III) are encoded on mtDNA.

The pumping of protons into the intermembrane space creates a protomotive force that consists of a proton gradient and a membrane potential. This protomotive force is then used by F_1F_0 ATPase, the ATP synthase or complex V to form ATP. If mutations occur in any one of the mt genes that encode any of these proteins then oxidative phosphorylation will be compromised. If the compromise is of sufficient magnitude then noticeable clinical conditions will develop. Mitochondrial diseases due to mutations in the mt DNA have been reviewed extensively in the clinical literature.²²⁻⁹¹ Point mutations, deletions, and duplications of this mitochondrial DNA (mtDNA) result in a variety of diseases. These diseases are degenerative in nature, maternally transmitted,⁸⁰ and all can be explained by an ATP shortfall in one or more tissues. Some of the diseases are devastating and are of early onset whereas others may take decades to develop clinical symptoms. Included in this list are diabetes, Alzheimer's disease, Parkinson's disease, and a variety of diseases involving the central nervous system, muscles, and the vital organs.

Leber's hereditary optic neuropathy (LHON) was one of the first diseases to be associated with mtDNA mutations.^{22,23} LHON is a degenerative disease of the optic nerve that results in blindness between the ages of 15 and 35. This disease can be the result of a single point mutation or a combination of point mutations in one of several protein coding genes of mtDNA. The majority of these are in subunits of NADH dehydrogenase complex I-ubiquinone. In

general terms, the single point mutations are in triplets that encode evolutionarily conserved amino acids in or near the active site of the enzyme, while mutations that need to be combined occur outside the active site and are less detrimental to enzyme function.

Mutations in the highly conserved leucine (nucleotide 8993) of the ATPase 6 gene can result in either neuropathy, ataxia, and retinitis pigmentosa (NARP)²⁴ or the more serious maternally inherited Leigh's syndrome (MILS).^{25–28} Patients with this mutation are highly variable in the severity of retinitis pigmentosa and neurological manifestations. In extreme cases, severe infantile lactate acidosis and death before the age of one can occur.²⁷ This mutation is in the proton channel of subunit a of the F_0 ATPase, near R210. Studies in lymphoblast mitochondria²⁹ and digitonized fibroblasts and leukocytes²⁷ from these patients revealed that ATP synthesis is reduced up to 50%. Oligomycin sensitive ATPase activity is also reduced.²⁹ In addition, subunit-b protein levels were reduced and subunit-c protein levels increased in heart and muscle, suggesting alterations in F_0 -complex assembly with this mutation.²⁷ Through the technology of transferring mutant mitochondria into cells depleted of mtDNA and the formation of cybrids^{92,93} it was shown that the T8993G mutation in the ATPase 6 gene is associated with decreased state 3 respiration and the ADP:O ratio.⁸¹ Also, a similar mutation in *E. coli*⁹⁴ resulted in a profound inhibition of proton translocation through F_1F_0 ATP synthase, reduced OXPHOS activity, and altered F_1F_0 ATPase assembly or stability. A recent study found that the T8993G mutation altered catalysis rather than enzyme assembly or stability.⁹⁵

The mutation at 8993 has also been found in patients with hypertrophic cardiomyopathy.³⁰ In these patients, point mutations were seen in most of the protein coding genes, as well as in tRNA and rRNA genes. Mutations in the tRNA for lysine have been associated with myoclonic epilepsy and ragged-red fiber disease (MERRF).^{31,32} Mutations in the tRNA for leucine (UUR) have been shown to result in several different diseases including MERRF, mitochondrial encephalomyopathy with lactic acidosis and strokelike episodes (MELAS),^{33,34} progressive kidney disease,³⁵ and maternally inherited diabetes and deafness (MIDD).^{36,37}

A number of mutations in the mt genome can phenotype as diabetes mellitus.^{36–71,86} In some cases the diabetes feature is secondary to more serious defects in CNS function or the neuromuscular system. In other instances the diabetes phenotype is the primary feature. In all cases the clinical condition of aberrant glucose homeostasis can be explained by the failure of the mitochondria to appropriately complete the oxidation of glucose to CO_2 and water with the production of ATP. Critical cells such as the pancreatic islet β cells thus experience an ATP production shortfall with the result of not only impaired glucose metabolism but also impaired insulin synthesis and release. These are the hallmarks of the diabetic state. Individuals with mt mutations typically have elevated blood lactate levels and redox states, again indicating aberrant mitochondrial metabolism that can be correlated with aberrant glucose metabolism.

Mitochondrial disease can also result from depletion, deletions, and duplications of the mitochondrial genome.^{24,60,72-89} The classic example of this is seen in patients with Kearns-Sayre syndrome (KSS).⁷⁵ This is a multiorgan disease of the eye, heart, and/or brain.

Through this review of mitochondrial diseases it is seen that one disease can be the result of several independent mtDNA mutations and one mtDNA mutation can cause several different diseases. All of these diseases relate to the need for ATP generated by OXPHOS. An error or mutation in any of the genes that encode components of this system results in an ATP synthesis shortfall. In turn, this depends on where in the DNA sequence the mutation occurs. Mutations distal to the active site of the translation product will be less serious in terms of the function of that product than mutation in the DNA at or near the active site. ATP synthesis can be mildly, modestly, or severely affected depending on the mutation site. Those tissues that are highly dependent on ATP, i.e., neural cells, will have their functional capacity seriously impaired whereas adipose cells or liver cells having the same mutation burden might be more able to accommodate this mutation in the heteroplasmic state. Thus, the severity of the symptoms are related to the location of the mutation, the percent of the mutated DNA, and the dependence of each of the affected cell types on its ATP supply.

In addition, the multiplicity of disease states is attributed to the unique character of mtDNA. There are thousands of mitochondria within a cell and each mitochondrion contains 8 to 10 copies of mtDNA. Both mutant and wild type mtDNA can exist within a given cell. This phenomenon is called heteroplasmy. When a cell contains all mutant or all wild type mtDNA it is a homeoplasmic cell. It should be noted that individual cell heteroplasmy, may not be reflected by mean tissue heteroplasmy making the link between percent heteroplasmy and phenotypic expression complicated. Also, in dividing cells, selection occurs against cells with a high percent of mutant mtDNA.^{72-77,79} In addition, the proportion of mutant mtDNAs can change over time such that a heteroplasmic mutation drifts towards homeoplasmy.⁷⁹ This is more of a problem in terminally differentiated cells (i.e., neurons) where rates of cell division cannot select against high percent mutant mtDNA cells and overcome this problem. Also, the inheritance of mutant vs. wild type mtDNA is random.^{77,78} Thus, a pedigree can occur with family members containing various degrees of heteroplasmy and various phenotypes.

In addition to heteroplasmic mutations, homeoplasmic mutations have also been associated with mitochondrial disease. Diabetes has been reported as a homoplasmic mutation in two locations in the BHE/Cdb rat.^{50,51} The diabetes is quite mild and results from a reduction (~20%) in ATP synthesis efficiency due to two mutations in the ATPase 6 gene. One of these is in the portion of the molecule that forms the proton channel while the other is in the hinge region. The latter has effects on the mobility of the subunit within the inner mitochondrial membrane whereas the former affects the ATPase function with respect to the proton channel.

Typically, heteroplasmic mutations are in highly conserved nucleotides in the active site of the enzyme or tRNA. These mutations would be lethal if homeoplasmic. In contrast, homeoplasmic mutations usually occur in less conserved nucleotides outside of the active site of the enzyme or tRNA. They are thought to result in milder OXPHOS defects and a late onset of disease. One example of this is the tRNA^{Gln} A4336G mutation associated with late-onset Alzheimer's disease and Parkinson's disease.^{82,97} This nucleotide is outside the active site of the tRNA^{Gln} and is only moderately conserved throughout evolution.

Another feature of mitochondrial genetics is the threshold effect: Clinical disease occurs when mitochondrial energy output falls below some minimum threshold level. This effect has been extended to the theory that a threshold level of mutant mtDNA must exist in a cell before it can affect OXPHOS and therefore clinical disease, or severity of disease. A classical example of this is the 8993 mutation in the ATPase 6 gene.^{25–27,29,78} When this mutation is present in less than 90% of mtDNA it phenotypes as NARP, but when present at greater than 90% mutant mtDNA the much more serious maternally inherited Leigh's syndrome (MILS) develops. Recently, this theory was supported by evidence that a threshold exists between % wild-type ND5 mtDNA and rotenone (complex I)-sensitive respiration.⁹⁶ In addition, it was found that (1) % wild-type ND5 DNA directly correlated to % wild-type ND5 mRNA suggesting that no compensatory upregulation of transcription or mRNA stability occurred; (2) 60 % of the normal level of wild-type mRNA was adequate to maintain normal ND5 protein synthesis; and (3) ND5 protein synthesis is near rate-limiting for complex I-dependent respiration. The results of this study suggested that the threshold effects occurred at the level of protein synthesis. It also suggested that the regulation of ND5 gene expression is an important factor in the regulation of complex I-dependent respiration.

It has also been suggested that degrees of heteroplasmy can be different between tissues leading to different phenotypes of the same genotype. This is one explanation given for the various phenotypes seen with the 3243 mutation in the tRNA^{Leu(UUR)} gene. While it has been argued that other factors besides the tRNA^{Leu(UUR)} mutation^{98,99} contribute to the different phenotypes, extensive data point to this mutation as the sole cause of the disease in these patients.^{54,56} Defects in mitochondrial protein synthesis and respiration occur in cybrids with mitochondria from patients carrying this mutation.^{32,92,93} This mutation can be suppressed by a mutation in the anticodon of tRNA^{Leu(CUN)} that enables it to decode UUR leucine.⁹⁶ In addition, when cybrids were compared between patients with the tRNA^{Leu(UUR)} mutation genotype and MIDD vs. those with progressive kidney disease phenotypes, there was no difference in mitochondrial function, suggesting that haplotype played no role in determining phenotype.⁶⁹ Also, they found differences in the % heteroplasmy between the two distinct phenotypes. Tissue-specific threshold effects were also seen in mice who had the *Tfam* gene disrupted in heart and muscle.⁹⁶ (*Tfam* is the gene for mitochondrial transcription factor A and affects mtDNA and mtRNA levels (discussed later in depth).) This disruption

produced levels of mtRNA and mtDNA that were 29 and 26% of the normal level in heart and 66 and 60% in the skeletal muscle. Only the heart had decreased ATPase-8 protein levels, and decreased respiration that lead to dilated cardiomyopathy and atrioventricular conduction blocks. Skeletal muscle had normal ATPase-8 protein levels, normal respiration, and normal morphology. This again highlights the importance of mitochondrial gene expression in the regulation of respiration and expression of mitochondrial disease.

Tissue-specific effects can also be due to different biochemical thresholds and ATP needs. Rossignol et al.⁸⁷ have shown in isolated mitochondria that different complexes within different tissues have different thresholds. For example, 60% of ATP synthase activity needs to be inhibited in brain mitochondria for respiration to be affected. But more inhibition of ATP synthase needs to occur in kidney, even more in liver, more in muscle, and yet more in heart. In contrast, inhibition of complex IV affects muscle = heart > liver > kidney = brain. Thus, the same mutation would have a very different effect on respiration in different tissues, resulting in different phenotypes. *In vivo*, different tissues can also have different thresholds due to their different respiration needs.^{22,32,33,44,56,72,76–78} In this respect, brain (and optic nerve) > skeletal muscle > cardiac muscle > kidney > liver.

Thus, it is clear that mitochondrial genetics is much more complicated than nuclear genetics. The phenotypic expression of a mitochondrial mutant genotype depends on the degree of cellular heteroplasmy, tissue-specific heteroplasmy, and threshold effects due to tissue–enzyme complex interactions and tissue needs for ATP. Environmental factors, such as diet, can also play a role by altering mitochondrial mutation rate and mitochondrial gene expression. In part, nutrients can affect mitochondrial gene expression and in part can affect the environment in which the gene product functions. An example of the latter is the effect of dietary fat on the composition and fluidity of the inner mitochondrial membrane.²⁰ In this example, a saturated dietary fat diet reduces inner membrane fluidity and this reduction impairs the movement of the F_1F_0 ATPase, reducing its efficiency in trapping the energy generated by the respiratory chain into the high energy bond of ATP. Hence, such saturated fat diets reduce OXPHOS efficiency. An example of the former is the effect retinoic acid has on mtDNA transcription.

14.4 Retinoic Acid (RA) and Mitochondrial Gene Expression

The discovery of the nuclear retinoic acid receptors, RAR and RXR, that were similar to the nuclear receptors for the steroid hormones suggested that retinoic acid could have a role in gene expression. These receptor proteins bind both retinoic acid and DNA thus playing an active role in regulating nuclear gene expression. This role is described in Chapter 13.¹

Three genes for each of the receptors, RAR and RXR, α , β , γ have been found.^{100–103} Each gene has several isoforms that arise from either different promoter usage or alternative splicing.^{101–107} The differences between the receptors occur in their N-terminal region.^{104,106} In addition, they have different expression patterns in both the embryo and the adult. Their synthesis and activation are regulated differently by vitamin A.^{107,108} In vitamin A-deficient rats, mRNA for RAR- β is decreased with no effect on mRNA for either RAR α or RAR γ in lung, liver, and intestine. In the testes, RAR α_2 expression was higher in retinol-deficient rats than retinol-sufficient rats, and this expression increased with RA feeding. RA also increased RAR α_2 expression in the embryo. In addition, RA upregulates RAR α_1 mRNA in the adult testes, but not lung, liver, intestine. RAR γ_2 , RAR β_1 , and RAR β_2 were also upregulated by RA in adult lung, liver, intestine but not testes. Embryonic tissue had a different response: RA upregulated embryonic RAR α , RAR β_1 , and RAR β_2 but had no effect on RAR γ_2 .^{105,108} The complex distribution and regulation of these receptor isoforms have led to the hypothesis that each isoform has a specific function. Studies with isoform specific null mutant mice have found that while all of the signs of vitamin A deficiency are also seen in the combination of RAR/RXR single or double null mutant mice, some redundancy does occur.^{105,108–114} In some cells single-receptor isoform mutations had little or no apparent effect. This argues against the hypothesis that each receptor isoform has a unique function. These studies suggest that while we still don't know the exact function of each isoform, it is clear that RAR/RXRs play a critical role in the function of vitamin A.

Countless genes have been shown to be regulated by retinoic acid. Studies with RXR $\alpha^{-/-}$ knockout mice have revealed that several genes involved in energy production are regulated during embryonic heart development and that RXR α is essential for energy production in the embryonic heart.¹¹⁵ Mitochondrial gene expression and mitochondrial function are abnormal in these knockout mice.

Mitochondrially encoded genes have been shown to be regulated by retinoic acid. These include subunit 5 NADH dehydrogenase,¹¹⁶ subunit I cytochrome c oxidase,¹¹⁷ and 16srRNA¹¹⁵ and the ATPase 6 gene.¹¹⁸ We have found retinoic acid receptors in the mitochondrial compartment using western blot analysis (Fig. 14.3). Antibodies against RAR β , γ -1, and γ -2 bound proteins isolated from the mitochondrial compartment.¹¹⁹ However, no such reaction was found with RAR α antibodies. The presence of the RARs in the mitochondrial compartment suggests that retinoic acid could have transcriptional effects with respect to mtDNA and, indeed, we have found this to be the case.

Other nutrients and hormones in addition to retinoic acid may also be involved in regulating the transcription of mtDNA. These are thought to exert some "local tuning" to the transcription process. Included in this list are thyroid hormone, insulin, vitamin D, and the glucocorticoids. All of these compounds act via their cognate receptors and all (except for the insulin receptor) of these receptors belong to a family called the steroid superfamily of receptors. A discussion of mitochondrial gene transcription follows.

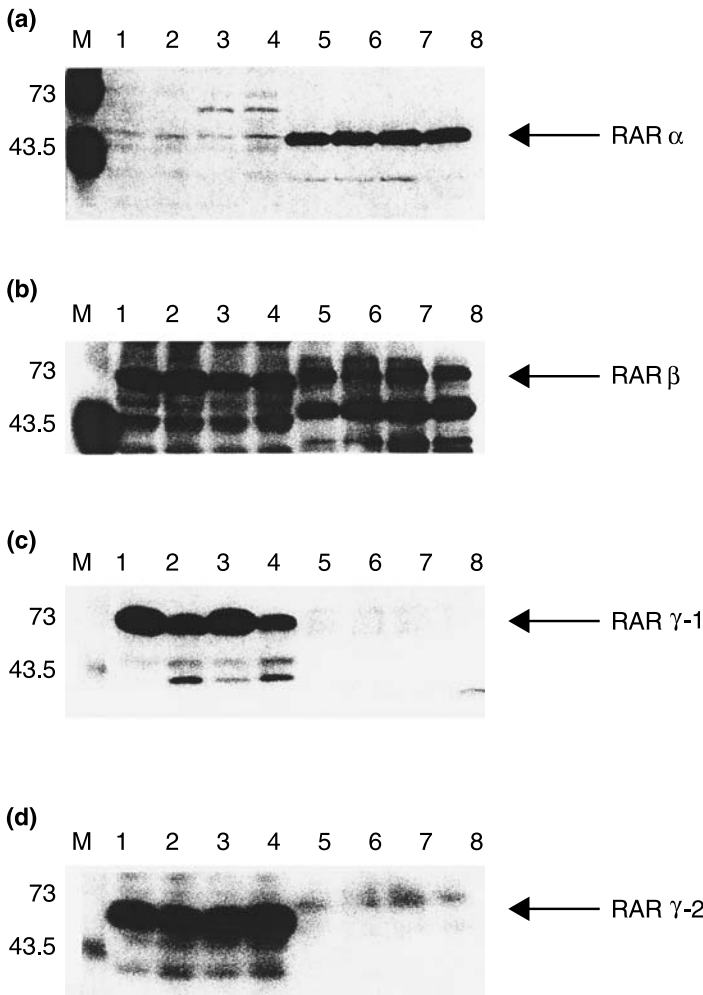


FIGURE 14.3
Western blot analysis of the retinoic acid receptors in the nucleus and the mitochondrial compartment. Cell fractions were prepared from the livers of BHE/Cdb and Sprague Dawley rats. Lanes 1, 2, 5, 6 were from BHE/Cdb rats; lanes 3, 4, 7, 8 were from Sprague Dawley rats; lanes 1 to 4 were proteins from the mitochondria; lanes 5 to 8 were proteins from the nucleus. The proteins were separated on a 10% SDS-PAGE gel. The blot was probed with a goat polyclonal IgG to Lamin B or a rabbit polyclonal antiserum to m-mtTFA.²

14.5 Mitochondrial Gene Transcription

Mechanisms for mitochondrial gene expression and its regulation have been studied and some aspects recently reviewed.¹²⁰⁻¹⁷⁷ These mechanisms

more closely resemble those of prokaryotes than do those of eukaryotes, as suggested by the endosymbiotic hypothesis of mitochondrial origin. In animals, the mtDNA exists as a double-stranded, closed circular molecule of approximately 16 Kb. These strands can be separated through a denaturing cesium chloride gradient into a light strand and a heavy strand. The heavy strand is the main coding strand and codes for 2 rRNAs, 14 tRNAs, and 12 structural genes. The light strand codes for 8 tRNAs and 1 structural gene. These genes are arranged as polycistrons, with tRNA genes interlaced between coding genes.

Early studies revealed that transcription is symmetrical¹²² and is initiated within the displacement loop (D-loop, regulatory region), with the light strand moving clockwise and the heavy strand moving counterclockwise.^{123–125} The light strand has a longer half life and is present in about 2 1/2 times greater quantity. The light strand is transcribed as one polycistron and the heavy strand as two.^{126,127} One heavy strand polycistron encodes all of the heavy strand, while the other encodes the two rRNAs. Two distinct promoter sequences, one for the light strand (LSP) and one for the heavy strand (HSP), have been defined by deletion analysis, site-specific mutagenesis, and linker-scanning mutagenesis in an *in vitro* system.^{127–134} In humans, both the LSP and the HSP contain regions near the start site containing specific nucleotides required for transcription initiation, as well as upstream regulatory regions. In the mouse, the LSP contains three domains. The first domain consists of nucleotides –10 to +9 (relative to transcription initiation) that are required for accurate transcription initiation. The second domain (nt –11 to –29) facilitates the formation of the preinitiation complex. The third domain (nt –30 to –88) affects transcriptional efficiency. In contrast, specific nucleotides are not required near or at the transcription start sites for heavy strand transcription initiation, although the start sites do make transcription more efficient.

There is an essential element upstream of the start sites in the mouse HSP. Promotor sequences are not highly conserved among species, but the mouse RNA polymerase is active on the rat mtDNA. This suggests that the rat promotor may be similar to the mouse. An additional difference between mouse and human promoters is that the two heavy strand transcription start sites are close together (near the D-loop/tRNA^{Phe} border) in the mouse. In humans, the main start site is found in this location, with a minor start site found between the tRNA^{Phe} and 12S genes. The main start site is thought to result in transcription of the 2 rRNA genes, while the minor one initiates transcription of the whole heavy strand. In addition, the light strand promoter also primes replication of the heavy strand¹³⁴ forming a RNA-DNA primer that is cleaved by the Rnase MRP (mitochondrial RNA processing) at the origin of heavy strand replication.¹³⁶ There are three blocks of sequences conserved among a number of species.¹³⁷ These sequences are thought to be involved in this cleavage.

At present, only two transcription factors have been identified and well studied. These are the mitochondrial transcription factor A (mtTFA) that is

involved in initiation and the mitochondrial transcription termination factor (mTERF) that is involved in termination of the heavy strand.^{137–140} The mTERF is active after the ribosomal RNAs are formed. *In vitro* studies have shown that mtTFA binds to both promoter regions as well as to a region between two of the conserved sequence blocks. The mtTFA condenses, unwinds, and bends mtDNA.^{141,142} In addition, *in vitro*, more mtTFA is needed to promote transcription of the heavy strand than the light strand, suggesting that low levels of mtTFA may result in the activation of the light strand and be involved in replication. This suggestion is supported by the observation that mtTFA protein levels have been correlated with mtDNA copy number.^{85,143}

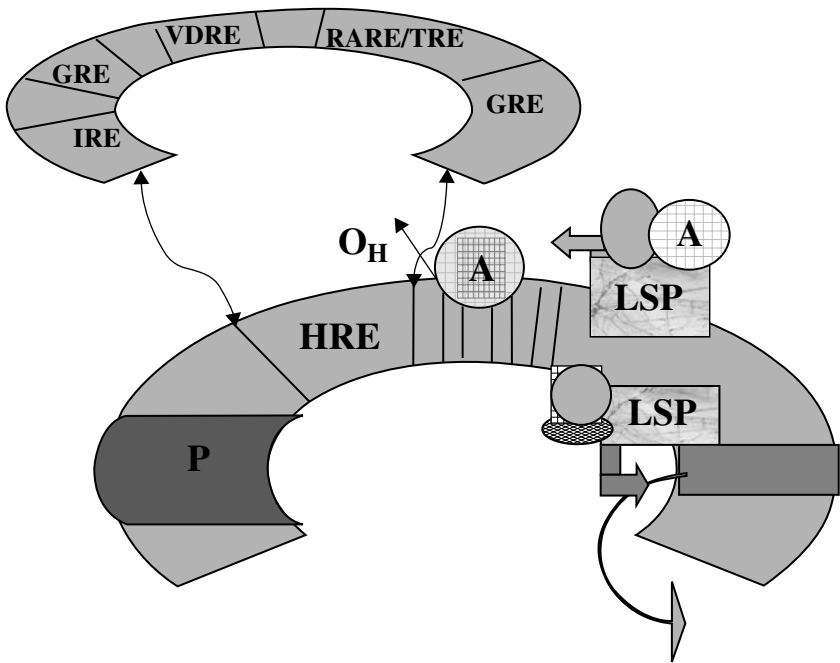
Transgenic technology has provided further evidence for mtTFA's role in transcription and replication. First, overexpression of mtTFA in HeLa cells and in isolated liver mitochondria was shown to increase mitochondrial transcription.¹⁴³ Second, heterozygous *m-mtTFA* (also called *Tfam*) knockout mice were shown to have reduced mtDNA copy number in heart, kidney, and liver; reduced mitochondrial transcription; and reduced respiratory chain function in the heart.¹⁴⁴ The kidney, liver, and skeletal muscle were variable in this respect. Interestingly, the protein levels of mitochondrially encoded cytochrome-c oxidase subunit II and ATP synthase subunit 8 were normal in all tissues investigated. Using knockout mice it was found that if the gene for the mtTFA was completely absent, this absence was lethal. Thus, there are no homozygous knockout mice. These data, in combination with the *in vitro* data, support the idea that mtTFA is essential for mitochondrial replication, primed from the light strand promoter; but is not solely responsible for regulating heavy strand transcription. It is likely that mitochondrial transcription depends on the presence of other transcription factors as well.

Because genes occur in both genomes, a mechanism for the coordinated regulation of OXPHOS genes has been thought to exist. The nuclear respiratory factors (NRF) 1 and 2 and the general transcription factor Sp1 were shown to coordinate transcription by simultaneously regulating mtTFA, as well as several nuclear-encoded OXPHOS genes in some situations.^{119,145–150} This is thought to be the case during rapid proliferation. But several researchers failed to find coordinated transcription in other situations, specifically in various thyroid hormone states.^{150–156} In addition, mitochondrial gene expression can be regulated by growth and development in a tissue-specific manner. In the heart, regulation occurs at the level of transcription.^{151,155,156} In rat heart, mitochondrial transcripts increased between 1 day and 3 months of age, then decreased between 3 months and 18 months of age. But in the liver, mRNA stability¹⁵⁷ and translational efficiency¹⁵⁸ were shown to be regulated in response to growth. They found that in neonates the half-lives of mt-mRNA were much longer than in adult liver and that translational efficiency peaked 1 hour after birth. This response to growth was also seen in nuclear-encoded OXPHOS genes.^{159,160} The mechanism for this posttranscriptional regulation has been explained for the nuclear encoded F₁ATPase β subunit.^{161,162} The 3' untranslated region (UTR) of this gene contains a translational enhancer that functionally resembles an internal ribosome entry site.

During fetal development a protein (3' β FBP) binds this enhancer and inhibits translation. Within 1 hour after birth this protein no longer binds the 3' UTR, unmasking the enhancer. This produces a spike in β F₁ATPase protein levels. In the adult, this protein is present again and translation of this gene is once again inhibited. This protein has yet to be purified and characterized. It is clear that it is regulated by development, but it would be interesting to learn exactly which developmental signals are used. It is also unknown what mechanism causes the same effects on mitochondrially encoded F₀ATPase genes. Does masking also occur in the mitochondria? If so, is the same protein responsible? It has been suggested that the import of the F₁ATPase β subunit itself triggers the response of mitochondrial translation. But this has yet to be proven experimentally. This form of posttranscription regulation has also been shown to occur in cancer cells, which take on the same characterization as fetal liver.¹⁶³

In addition to regulation during mitochondrial proliferation and differentiation, mitochondrial transcription may also be directly regulated by hormone receptors.¹¹⁹ This has been called mitochondrial local tuning. Figure 14.4 shows where putative glucocorticoid (GRE), vitamin D (VDRE), thyroid hormone (TRE), and retinoic acid (RARE) response elements have been found in the D-loop.^{129–131,137,164–168} The glucocorticoid receptor (GR) and a variant of the thyroid receptor (TR) have been shown to bind this region of mtDNA *in vitro*.^{164,165} It was originally thought that thyroid hormone only increased mitochondrial transcription by increasing mtTFA.¹⁷⁰ Recently, Enriquez et al.¹⁵⁰ showed in isolated mitochondria that thyroid hormone directly increased the transcription of all mRNAs encoded on the heavy strand, without increasing mitochondrial rRNAs. They were unable to prove that this occurred by binding at the putative TRE. They did show protein-DNA interactions near the transcription start sites, but not at termination sites, that were affected by thyroid hormone. In addition, it was recently demonstrated that the variant form of thyroid receptor (p43) is rapidly imported into mitochondria, binds three putative TREs, and increases mitochondrial transcription in a thyroid hormone-dependent fashion.¹⁷⁰ Two of these putative TREs are located within the D-loop and were shown to independently increase mitochondrial transcription in the presence of p43 and thyroid hormone in a nuclear chloramphenicol acyltransferase (CAT) assay. One of these TREs is a direct repeat with two spaces. This element has also been shown to act as a RARE in other genes.¹⁶⁸ Thus, it is possible that retinoic acid bound to its receptor could also directly regulate mitochondrial transcription.

We have found that mitochondrial ATPase 6 gene expression is enhanced by retinoic acid *in vitro* using primary cell cultures. Using BHE/Cdb rats having a mutation in the ATPase 6 gene, we have also found that ATPase 6 gene product is increased by dietary supplements of vitamin A. Improvements in OXPHOS have also been observed in these rats in a dose-dependent manner.¹¹⁹ These improvements correlated with the increase in ATPase 6 gene product. It should be noted, however, that normal Sprague Dawley rats did

**FIGURE 14.4**

Putative nuclear hormone receptor response elements (HRE) found in mitochondrial D-loop (mtDNA promoter). (a) Overview of mitochondrial D-loop, highlighting the location of the putative nuclear hormone receptor response elements. O_H = origin of heavy strand replication, LSP and HSP = light and heavy strand promoters, respectively. (b) Species sequence comparison of putative glucocorticoid (GRE), vitamin D (VDRE), retinoic acid (RARE), and thyroid hormone (TRE) response elements, based on sequence alignments from Wong et al.⁷ Mo = mouse, bov = bovine, hu = human, Xe = Xenopus.

not have this same response nor did rats that had not been depleted then repleted with vitamin A. Clearly, the retinoic acid effect on mt gene expression was genotype specific.

These studies suggest that the regulation of mitochondrial gene expression is a complicated tissue-specific process occurring at the levels of transcription, mRNA stability, and translation. There is a degree of coordination in the regulation of mitochondrial and nuclear OXPHOS gene expression. But, as there are many mitochondria within a given cell, direct regulation of mitochondrial gene expression by thyroid hormone, retinoic acid, and other factors may ultimately be found to be as important to this genome as they are to the nuclear genome.

The exact mechanism for hormone receptor action within mitochondria can only be speculative at this time. While mitochondria do not contain histones per se they do contain histone-like proteins.¹⁷¹⁻¹⁷⁴ Like histones, these proteins are rich in lysine. Since this is the site of acetylation for histones, these histone-like proteins may be acetylated during transcription activation by a similar mechanism as in the nucleus.¹⁷⁵ In addition, transcription activation could

occur by direct interactions with the basic transcription machinery, namely, mitochondrial polymerase and/or mtTFA. Direct interactions between activators and RNA polymerase also activate transcription in prokaryotes.^{176,177} Also, mitochondrial footprinting found that protein–DNA interactions near the transcription start sites were altered by thyroid hormone.¹¹⁸ No known coactivators have been found within mitochondria yet, but that does not rule out their presence. Thus, the most likely mechanism would be that liganded hormone receptors bind their respective response elements and directly, or via mitochondrial specific coactivators, interact with mitochondrial polymerase and/or mtTFA. Additionally, the acetylation or phosphorylation of histone-like proteins and/or mtTFA is also a possibility.

14.6 Conclusions

Although the mt genome has been completely sequenced and mapped for several decades and although we are now accumulating information about diseases that result when this genome mutates, we have only primitive knowledge about the control of its transcription. Two transcription factors are known and several members of the steroid superfamily are suspected to play a role in this process. Current research is now focused on the possible role for the fat-soluble vitamins in mt gene expression.

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15

Vitamin D and Gene Expression

Anthony W. Norman and Elaine D. Collins

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15.1 Background on Vitamin D

In the last two decades, a new concept concerning the mode-of-action of the fat-soluble vitamin D has emerged. The cornerstone of this concept is that in terms of its availability, metabolism, and mechanism of action, it is more accurate to consider vitamin D a steroid hormone than a vitamin in the clas-

sical sense. Synthesis in the skin on exposure to ultraviolet light obviates the dietary necessity for vitamin D which is a classical part of the definition of a vitamin. The chemical transformations through which the various derivatives of vitamin D are produced are of the same kind as those that characterize the metabolism of other steroid hormones, such as the glucocorticoid and sex steroids. The close regulation of the renal production of $1\alpha,25\text{-dihydroxyvitamin D}_3$ [$1\alpha,25(\text{OH})_2\text{D}_3$], the most potent of the naturally occurring derivatives of vitamin D, is very suggestive of its hormonal nature. The most compelling argument for the considerations of vitamin D as a steroid (pro-) hormone is the presence in classical target tissues, such as intestine, kidney, and bone of a specific, high-affinity nuclear receptor [VDR_{nuc}] for its active metabolite, $1\alpha,25(\text{OH})_2\text{D}_3$.

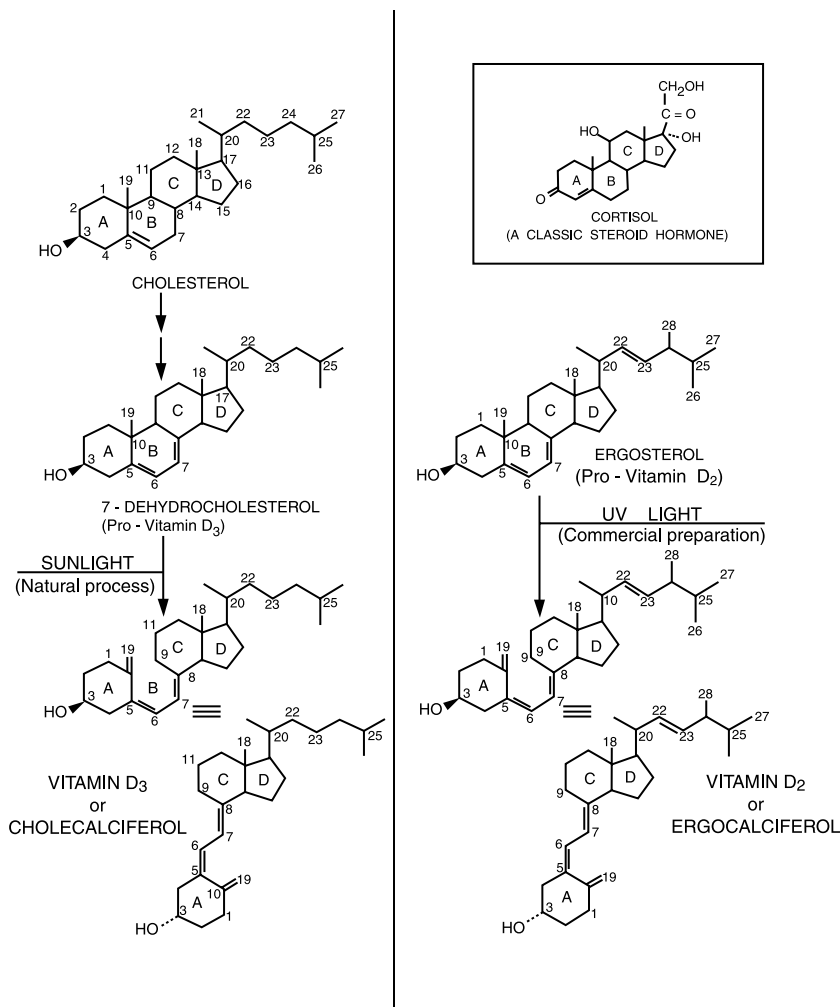
The most thoroughly studied target tissue for $1\alpha,25(\text{OH})_2\text{D}_3$ is the intestine, which depends on the hormone for adequate absorption of dietary calcium.^{1,2} In the intestinal mucosa, the steroid–receptor complex induces the synthesis of a specific calcium-binding protein (mol wt = 28,000), the precise role of which in calcium absorption has yet to be elucidated.³

In addition to the endocrine actions of $1\alpha,25(\text{OH})_2\text{D}_3$ in the classical target tissues of vitamin D, in which the actions of the hormone contribute to the body's maintenance of calcium homeostasis, the VDR_{nuc} has been identified in tissues not previously recognized as targets for vitamin D, such as the pancreas, pituitary, and brain^{4,5} as well as the hemopoietic⁶ and the immune systems.⁷ Many different cell types representing these systems respond to $1\alpha,25(\text{OH})_2\text{D}_3$, presumably through the specific receptors that have been identified in these cells, with changes in their patterns of growth and differentiation. These interactions may be of a more paracrine or autocrine nature as indicated, e.g., by the ability of activated macrophages to convert 25OHD_3 to $1\alpha,25(\text{OH})_2\text{D}_3$.

The actions of $1,25(\text{OH})_2\text{D}_3$ alluded to above appear to involve changes in gene expression in the target cells.^{8,10} In addition, in the intestine¹¹ and bone osteoblast cells,¹² components of calcium transport have been identified that respond very rapidly to $1\alpha,25(\text{OH})_2\text{D}_3$ and are thought to be mediated by nongenomic mechanisms (see later discussion).

15.2 Chemistry of Vitamin D and Related Compounds

The molecular structure of vitamin D is closely allied to that of classical steroid hormones (see Fig. 15.1). Technically, vitamin D is a seco-steroid. Secosteroids are those in which one of the rings of the cyclopentanoperhydrophenanthrene ring structure of classic steroids has undergone fission by breakage of a carbon–carbon bond; in the instance of vitamin D, this is the 9,10 carbon bond of ring B.

**FIGURE 15.1**

Structural relationship of vitamin D₃ (cholecalciferol) and vitamin D₂ (ergocalciferol) with their respective provitamins (cholesterol and ergosterol), and a classic steroid hormone, cortisol (see insert box). The two structural representations at the bottom for both vitamin D₃ and vitamin D₂ are equivalent; they are simply different ways of depicting the same molecule. It is to be emphasized that vitamin D₃ is the naturally occurring form of the vitamin; it is produced from 7-dehydrocholesterol, which is present in the skin, by the action of sunlight. Vitamin D₂ (which is equivalently potent to vitamin D₃ in humans and many mammals, but not birds) is produced commercially by the irradiation of the plant sterol ergosterol with ultraviolet light.

There is a family of vitamin D-related steroids that differ in the precise structure of the side chain attached to carbon-17. The naturally occurring form of vitamin D is that which has the side chain structure identical to that of cholesterol; this is known as vitamin D₃ or cholecalciferol. Vitamin D₂, or

ergocalciferol, is not a naturally occurring form of the vitamin. Collectively, vitamin D₃ plus vitamin D₂ are the calciferols or simply vitamin D.

In the United States, the principal form of vitamin D supplementation of food was vitamin D₂ in the interval 1930–1965; since 1965 the predominant form of vitamin D supplementation has been vitamin D₃. One International Unit (IU) of vitamin D₃ is equivalent to 25 ng or 65 pmoles of the compound. In the United States the Recommended Dietary Allowance (RDA), or more recently the Reference Daily Intake* of vitamin D (either D₂ or D₃) for an adult is 200 IU, and for pregnant or lactating women or children less than 4 years of age it is 400 IU (set in 1994 by the FDA).

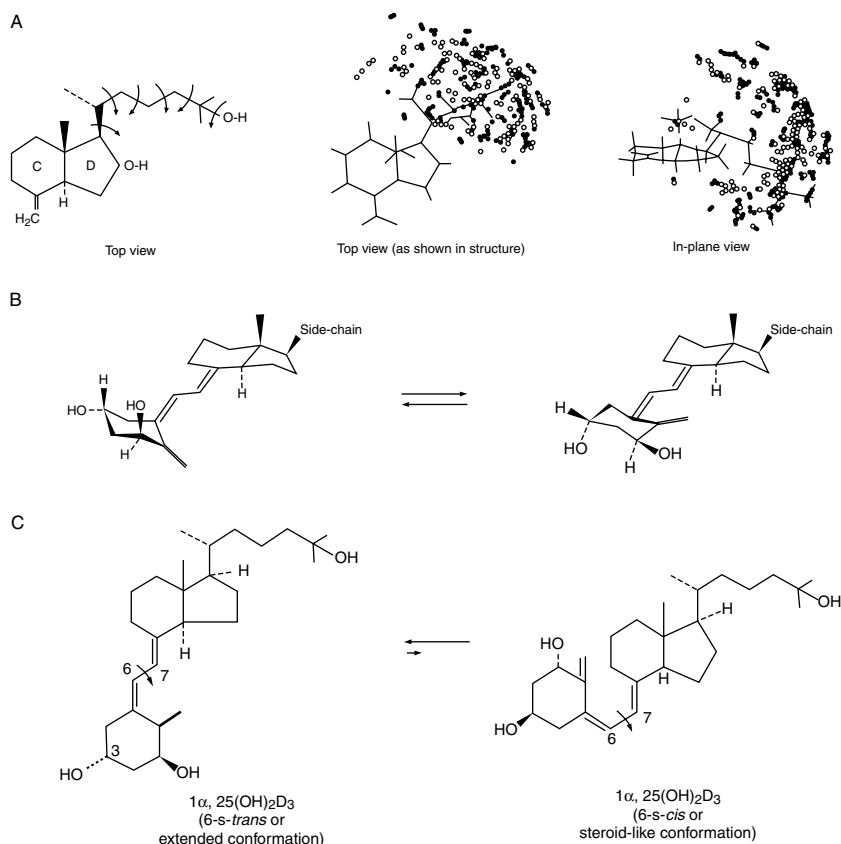
Although the chemical structure of vitamin D was determined in the 1930s, it was not until the 1975–1995 era that the unique structural aspects of the molecule became apparent. In contrast to other steroid hormones the vitamin D molecule has three structural features which contribute to the extreme conformational flexibility of this seco-steroid molecule; these include the presence of (i) an 8-carbon side chain, (ii) the broken B ring which “unlocks” the A-ring so that it (iii) can undergo chair–chair interchange many times per second. Figure 15.2 and its legend provide a detailed consideration of the conformational flexibility of vitamin D molecules.

15.3 Metabolism of Vitamin D

Vitamin D₃ is normally produced by exposure to sunlight of the precursor, 7-dehydrocholesterol, present in the skin. Vitamin D₂ is produced synthetically via ultraviolet irradiation of the sterol ergosterol. The chief structural prerequisite of a sterol to be classified as a provitamin D is its ability to be converted upon ultraviolet irradiation to a vitamin D; thus, it is mandatory that it have in its B ring a Δ^{5-7} conjugated double-bond system. In the skin, the principal ultraviolet irradiation product is previtamin D₃. The resulting vitamin D₃ is then transported into the general circulatory system by the vitamin D-binding protein.

A formal definition of a vitamin is that it is a trace dietary constituent required to effect normal functioning of a physiological process. Emphasis here is on *trace* and the fact that the vitamin must be supplied in the diet; this implies that the body is unable to synthesize it. Thus, cholecalciferol is only a vitamin when the animal does not have access to sunlight or ultraviolet light. Under normal physiological circumstances, all mammals, including man, can generate via ultraviolet photolysis adequate quantities of

* The U.S. Recommended Dietary Allowance (U.S. RDA) set in 1989 describes nutrient standards; the amounts listed represent recommendations for daily intake averaged over a 3- to 4-day interval. Effective in 1993, the U.S. RDA terminology underwent a name change to Reference Daily Intake (RDI). These RDI recommendations are used by the Food and Drug Administration (FDA) to define nutrient content guidelines for food labels.

**FIGURE 15.2**

Conformational flexibility of vitamin D molecules using $1\alpha,25(\text{OH})_2$ -vitamin D_3 [$1\alpha,25(\text{OH})_2\text{D}_3$] as an example. (A) Illustrates the dynamic single bond rotation of the cholesterol-like side chain of $1\alpha,25(\text{OH})_2\text{D}_3$ (i.e., 360° rotations about the five single carbon bonds and the oxygen, as indicated by the curved arrows). The dots indicate the position in three-dimensional space of the 25-hydroxyl group for some 394 readily identifiable side-chain conformations which have been determined from energy minimization calculations. Two orientations of the C/D/side chain are presented: a top view and an in-plane view. (B) Depicts the rapid (thousands of times per second) chair-chair interconversion of the A-ring of the secosteroid which effectively equilibrates the 1α -hydroxyl between the axial and equatorial orientations. (C) Illustrates the 360° rotational freedom about the 6,7 carbon-carbon bond of the seco- β -ring which generates conformations ranging from the more steroid-like (6-s-*cis*) conformation, to the open and extended (6-s-*trans*) conformation $1\alpha,25(\text{OH})_2\text{D}_3$.

vitamin D to meet the Recommended Dietary Allowance (RDA). It is largely through an historical accident that calciferol has been classified as a vitamin rather than as a steroid hormone. Chemists have certainly appreciated the strong structural similarity between vitamin D and other steroids but this correlation had not been widely acknowledged in the biological, clinical, or nutritional sciences until 1965–1970.

Since 1964 a totally new era in the field of vitamin D has opened with the discovery of the metabolism of vitamin D. Altogether, some 37 metabolites of vitamin D₃ have been isolated and chemically characterized (see Fig. 15.3). It is now recognized that there is an endocrine system for processing the prohormone, vitamin D, into its hormonally active daughter metabolite(s) (see Fig. 15.3).

The primary source of circulating dihydroxylated metabolites of vitamin D is the kidney, which is the vitamin D endocrine gland. In the kidney mitochondrion, 25OHD₃, the major circulating form of vitamin D, is converted to either 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃] or 24R,25(OH)₂-vitamin D₃ [24R,25(OH)₂D₃]. The predominant dihydroxy-



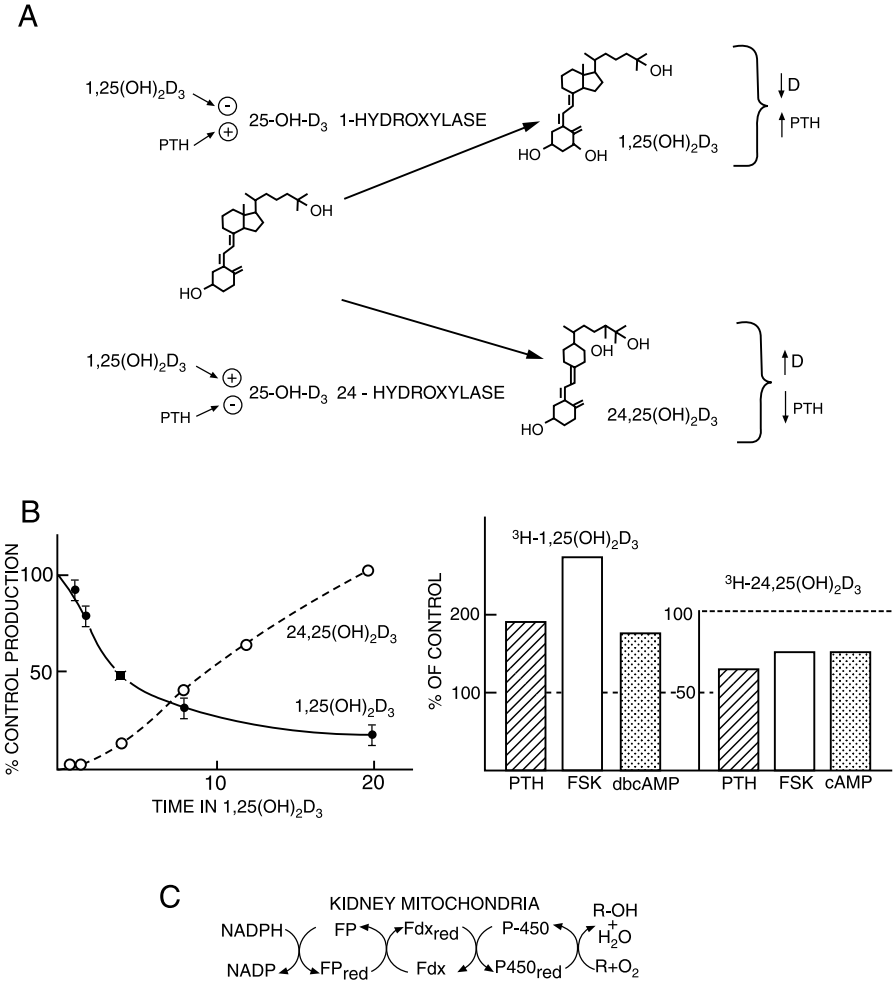


FIGURE 15.4 Regulation of vitamin D metabolism. (A) Conversion of 25OHD₃ to 1 α ,25(OH)₂D₃ is downregulated by 1 α ,25(OH)₂D₃ and upregulated by PTH. The two hormones have the opposite effects on 24R,25(OH)₂D₃ production. (B) Time course of the effect of 1 α ,25(OH)₂D₃ on its own production (solid symbols and solid line) and on that of 24R,25(OH)₂D₃ (open symbols and dashed line) in cultured chick kidney cells is shown on the left. On the right the data supporting the involvement of the cAMP intracellular signaling cascade in the regulation of 1 α ,25(OH)₂D₃ and 24R,25(OH)₂D₃ production (FSK, forskolin; dbcAMP, dibutyryl cAMP) are summarized. (C) Electron transport system supporting the reduction of O₂ to supply the hydroxyl group to be incorporated into 25OHD₃. FP, ferredoxin reductase, a flavoprotein; Fdx, ferredoxin; p-450, Cytochrome P 450. (See References 13, 129.)

lated product formed will depend on the vitamin D and parathyroid hormone (PTH) status of the individual.

In the vitamin D-deficient state, the enzymatic activity of the 1-hydroxylase is high and of the 24-hydroxylase low (see Fig. 15.4). This is because

$1\alpha,25(\text{OH})_2\text{D}_3$ represses the 1-hydroxylase and induces the 24-hydroxylase activity, probably through effects on the synthesis of specific proteins. Conversely, parathyroid hormone, elevated by the hypocalcemia resulting from vitamin D deficiency, increases 1-hydroxylase activity and decreases 24-hydroxylase activity. As had been shown in cell culture, cAMP mediates this effect of PTH, but protein kinase C may also be involved in 25OHD_3 metabolisms, because 12-*O*-tetradecanoylphorbol-13 acetate (TPA) exerts effects opposite to those of PTH. Thus, the absence of $1,25(\text{OH})_2\text{D}_3$ and the presence of PTH combine to keep $24\text{R},25(\text{OH})_2\text{D}_3$ production low. In the vitamin D-replete state, the opposite set of effects occurs, resulting in lowered $1,25(\text{OH})_2\text{D}_3$ and elevated $24\text{R},25(\text{OH})_2\text{D}_3$ production (see the References 13–15).

Both the 1- and 24-hydroxylases are classical mitochondrial mixed-function oxidases involving the transfer of electrons from NADPH through a flavoprotein (renal ferredoxin reductase) and an iron sulfur protein (renal ferredoxin)¹⁶ to cytochrome P-450.¹⁷ All three components are located in or adjacent to the inner mitochondrial membrane. Exactly how, on a molecular level, $1\alpha,25(\text{OH})_2\text{D}_3$ and PTH exert their effects on these enzyme systems is currently under study.¹³

15.4 Vitamin D Endocrine System (Generation of Biological Responses)

The molecule vitamin D itself has no intrinsic biological activity. All biological responses attributed to vitamin D are now known to arise only as a consequence of the metabolism of this seco-steroid into its biologically active daughter metabolites, namely, $1\alpha,25(\text{OH})_2\text{D}_3$ and $24\text{R},25(\text{OH})_2\text{D}_3$.¹⁸

Figure 15.5 summarizes the scope of the vitamin D endocrine system. The steroid hormone $1\alpha,25(\text{OH})_2\text{D}_3$ is produced only in accord with strict physiological signals dictated by the calcium demand of the organism; a bimodal mode of regulation has been suggested (see discussion above). Thus, under normal physiological circumstances, both renal dihydroxylated metabolites are secreted and are circulated in the plasma. There is evidence of a “short feedback loop” for both of these metabolites to modulate and/or reduce the secretion of PTH. There is also some evidence that other endocrine modulators such as estrogens, androgens, growth hormone, prolactin, and insulin may affect the renal production of $1\alpha,25(\text{OH})_2\text{D}_3$ (see Fig. 15.5). Thus, the kidney is clearly an endocrine gland, in the classic sense, which is capable of producing in a physiologically regulated manner appropriate amounts of $1\alpha,25(\text{OH})_2\text{D}_3$.

The plasma compartment contains a specific protein termed the vitamin D-binding protein (DBP), which is utilized to transport vitamin D seco-sterols. DBP is similar in function to the corticosteroid-binding globulin (CBG), which carries glucocorticoids, and the steroid hormone-binding globulin

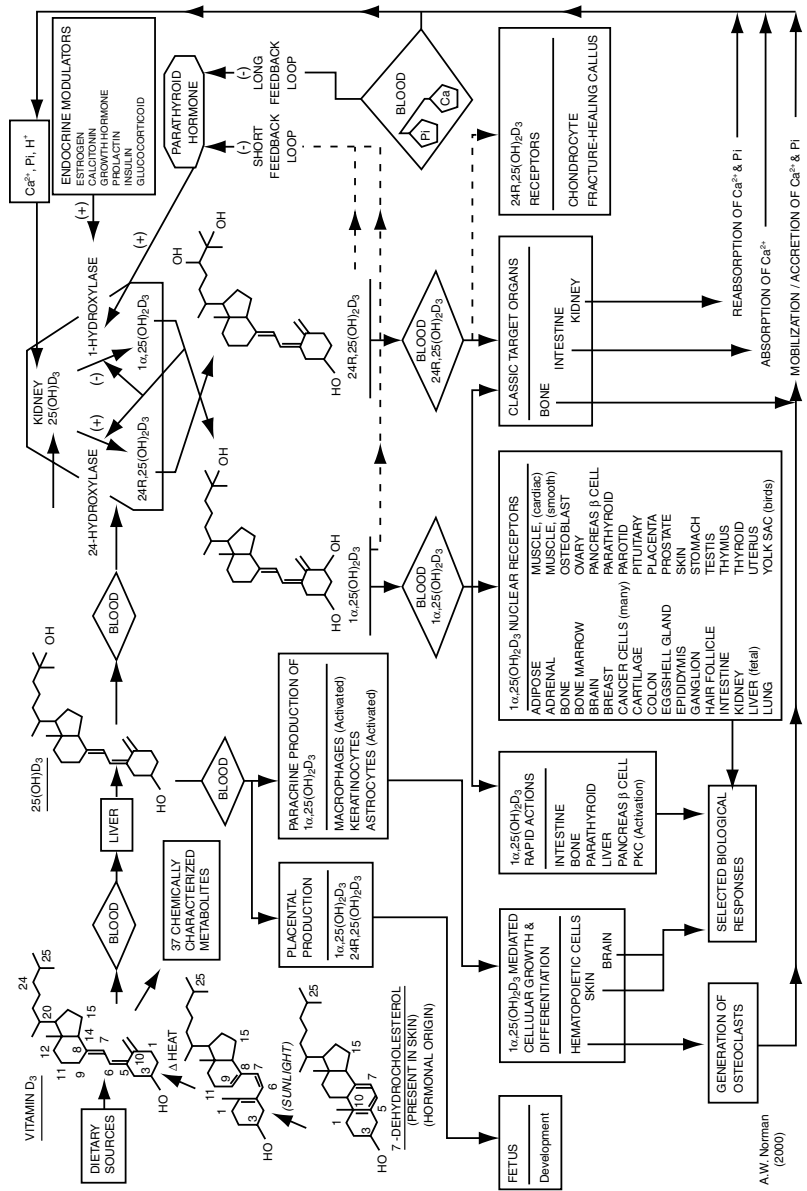


FIGURE 15.5 Summary of the vitamin D endocrine system. In addition to production of 1 α ,25(OH)₂D₃ and 24R,25(OH)₂D₃ by the endocrine gland function of the kidney, small amounts of 1 α ,25(OH)₂D₃ are also produced in a paracrine fashion and by the placenta during pregnancy. Target organs and cells for 1 α ,25(OH)₂D₃ by definition contain nuclear receptors for 1 α ,25(OH)₂D₃ (VDR_{nuc}). Also, 1 α ,25(OH)₂D₃ generates biological effects involving rapid signal transduction pathways utilizing a putative membrane receptor (see text). The precise biological roles of 24R,25(OH)₂D₃ are not yet defined although it is believed to function in bone and cartilage.

(SHBG), which transports estrogens or androgens. DBP is a slightly acidic ($pI = 5.2$) monomeric glycoprotein of 53,000 D which is synthesized and secreted by the liver as a major plasma constituent. From analysis of the cloned cDNA, it has been determined that DBP is structurally homologous to albumin and α -fetoprotein; these three plasma proteins are members of the same multi-gene family which likely is derived from the duplication of a common ancestral gene. DBP, originally called group-specific component (Gc), was initially studied electrophoretically as a polymorphic marker in the α -globulin region of human serum. (See References 19,20.)

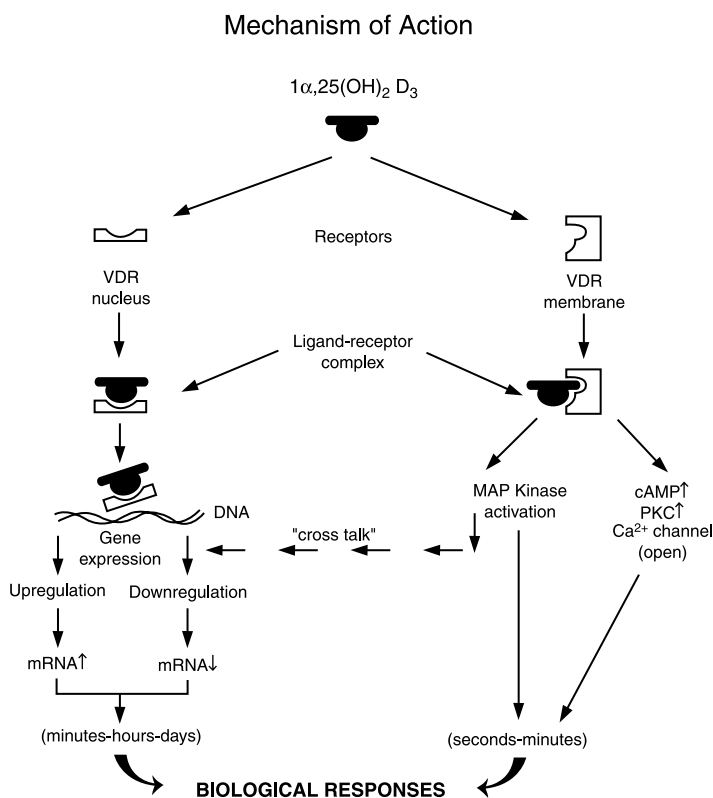
15.5 Signal Transduction Pathways Utilized by $1\alpha,25(\text{OH})_2\text{D}_3$ to Generate Biological Response

$1\alpha,25(\text{OH})_2\text{D}_3$ is believed to mediate biological responses by interaction with its nuclear receptor, VDR_{nuc} , and also via interaction with a putative membrane receptor, VDR_{mem} , which is located on the surface of the cell of appropriate target cells; a schematic diagram of this concept is presented in Fig. 15.6. These topics will be discussed separately.

15.5.1 Nuclear Receptor For $1\alpha,25(\text{OH})_2\text{D}_3$

The nuclear responses to $1\alpha,25(\text{OH})_2\text{D}_3$ are generated in a manner homologous to that of classical steroid hormones, e.g., glucocorticoids, progesterone, estradiol, testosterone, and aldosterone. In the general model, the hormone is produced in an endocrine gland in response to a physiological stimulus and then circulates in the blood bound to a protein carrier (the vitamin D-binding protein or DBP), which delivers it to target tissues where the hormone enters the cell and interacts with a specific, high-affinity intracellular receptor(s). The receptor–hormone complex then localizes in the nucleus, undergoes some type of “activation” perhaps involving phosphorylation^{21,25} and binds to a hormone response element (HRE) on the DNA to modulate the expression of hormone-sensitive genes. The modulation of gene transcription results in either the induction or the repression of specific mRNAs, ultimately resulting in changes in protein expression needed to produce the required biological response. High affinity receptors for $1\alpha,25(\text{OH})_2\text{D}_3$ have been identified in at least 34 target tissues (see Fig. 15.5). A more detailed discussion of the nuclear VDR is presented below.

Identification of vitamin D-regulated transcription for specific gene products is typically supported by one or several observations. Of these, the majority of the reports present data suggesting vitamin D-dependent modulation of mRNA levels following treatment of animals, tissues, or cells with $1\alpha,25(\text{OH})_2\text{D}_3$. Although these types of analyses are commonly accepted as

**FIGURE 15.6**

Pathways for generation of biological responses utilized by $1\alpha,25(\text{OH})_2\text{D}_3$. In the genomic pathway (left side), occupancy of the nuclear receptor for $1\alpha,25(\text{OH})_2\text{D}_3$ (VDR_{nuc}) by a ligand leads to an up- or downregulation of genes subject to hormone regulation. More than 50 proteins are known to be transcriptionally regulated by $1\alpha,25(\text{OH})_2\text{D}_3$ [see Table 15.1 and Reference 26]. In the membrane-initiated pathway (right side), occupancy of a putative membrane receptor for $1\alpha,25(\text{OH})_2\text{D}_3$ by a ligand is believed to rapidly lead to activation of a number of signal transduction pathways, including adenylate cyclase, phospholipase C, protein kinase C (PKC), mitogen-activated protein kinase (MAP kinase), and/or opening of voltage-gated L-type Ca $^{2+}$ channels, which are either individually or collectively coupled to generation the biological response(s).

sufficient evidence to suggest that the protein is vitamin D-sensitive, they cannot be used exclusively to conclude that the regulation is mediated via altered gene transcription. These data do not distinguish between transcriptional regulation vs. alterations in stability of the message nor do they guarantee that the protein level is indeed altered by $1\alpha,25(\text{OH})_2\text{D}_3$. Identification of a VDRE within the promoter region of the gene supplies additional support for vitamin D regulation but is not, in itself, indicative of altered transcription. Measurement of vitamin D-dependent nuclear transcription coupled with data supporting altered protein levels provides the best evidence of $1\alpha,25(\text{OH})_2\text{D}_3$ regulation of a specific gene product. Unfortunately,

these types of analyses have not been completed for many of the gene products presumed to be under regulation by $1\alpha,25(\text{OH})_2\text{D}_3$. Additionally, we cannot assume that lack of such evidence indicates the gene product is not vitamin D regulated. The goal of this review is to present an updated list of proteins whose mRNA levels are known to be modulated by vitamin D status. Additionally, supporting data regarding the presence of a recognized VDRE and/or nuclear transcription data are supplied where available.

Table 15.1 lists gene products whose message levels are known to be sensitive to $1\alpha,25(\text{OH})_2\text{D}_3$.²⁶ This alphabetical list contains genes associated with

TABLE 15.1

Genes Under Regulation by $1\alpha,25(\text{OH})_2\text{D}_3$

Gene	Regulation	Evidence	Tissue/Cell	Ref.
α -tubulin	Down	mRNA	Chick intestine	130
Aldolase subunit B	Up	mRNA	Chick kidney	131
Alkaline phosphatase	Up	mRNA	Rat intestine	132
			Chick intestine	131
			TE-85 cells	133
ATP synthase	Up	mRNA	Rat intestine	134
			Chick intestine	131
	Down	mRNA	Chick kidney	131
c-FMS	Up	mRNA	HL-60 cells	135
c-FOS	Up	mRNA	MG-63 cells	136
			HL-60 cells	135
c-KI-RAS	Up	mRNA	BALB-3T3 cells	137
c-MYB	Down	mRNA	HL-60 cells	135
c-MYC	Up	mRNA	MG-63 cells	136
	Down	mRNA	U937 cells	138
			HL-60 cells	139
		Transcription	HL-60 cells	140,
				141
Calbindin D _{28K}	Up	mRNA	Chick intestine	142
		Transcription	Mouse kidney	143
			Chick intestine	144
				2
Calbindin D _{9K}	Up	mRNA	Mouse kidney	144
		VDRE	Rat	145
Carbonic anhydrase	Up	mRNA	Marrow cells	146
		Transcription	Myelomonocytes	147
CD-23	Down	mRNA	PBMC	148
Collagen type I	Down	mRNA/VDRE	Rat	149
Cytochrome oxidase subunit I	Up	mRNA	Rat intestine	134
			Chick intestine	131
	Down	mRNA	Chick kidney	131
Cytochrome oxidase subunit II	Up	mRNA	Chick intestine	131
	Down	mRNA	Chick kidney	131
Cytochrome oxidase subunit III	Up	mRNA	Rat intestine	134
			Chick intestine	131
	Down	mRNA	Chick kidney	132
Cytochrome B	Down	mRNA	Chick kidney	131

TABLE 15.1 (CONTINUED)

Genes Under Regulation by $1\alpha,25(\text{OH})_2\text{D}_3$

Gene	Regulation	Evidence	Tissue/Cell	Ref.
Fatty-acid binding protein	Down	mRNA	Chick intestine	131
Ferredoxin	Down	mRNA	Chick kidney	150
Fibronectin	Up	mRNA	MG-63,TE-85, HL-60 cells	151
γ -interferon	Down	mRNA	T-lymphocytes	152
			PBMC	153
Glyceraldehyde-3-phosphate dehydrogenase	Up	mRNA	BT-20 cells	154
GM-colony stimulating factor	Down	mRNA	T-lymphocytes	155
Heat shock protein-70	Up	mRNA	PBMC	156
Histone H ₄	Down	mRNA/ Transcription	HL-60 cells	157
1α -hydroxy-vitamin D-24-hydroxylase	Up	mRNA	Rat kidney	158
		mRNA/ Transcription	Rat kidney	159
Integrin _{vβ3}	Up	mRNA/ Transcription	Avian osteoclast precursor cells	160
		VDRE	Avian gene	161
Interleukin 6	Up	mRNA	U937	162
Interleukin 1	Up	mRNA	U937 cells	162
				163
Interleukin 2	Down	mRNA	T-lymphocytes	153
Interleukin 3 receptor	Up	mRNA	MC3T3 cells	164
Matrix gla-protein	Up	mRNA	UMR106-01, ROS 25/1, ROS 25/4 cells	165
Metallothionien	Up	mRNA	Rat keratinocytes	166
			Mouse liver/kidney/skin	166
			Chick kidney	131
Monocyte derived neutrophil-activating peptide	Up	mRNA/ Transcription	HL-60 cells	167
NADH DH subunit I	Down	mRNA	Chick kidney	131
NADH DH subunit III	Up	mRNA	Chick intestine	131
NADH DH subunit IV	Up	mRNA	Chick intestine	131
Nerve growth factor	Up	mRNA	L-929 cells	168
Osteocalcin	Up	mRNA	ROS 17/2.8	169
			ROS 25/1	165
		VDRE	ROS 17/2.8	170
			Rat	171
				172
Osteopontin	Up	mRNA	ROS 17/2.8	173
		VDRE	ROS 17/2.8	174

(continued)

TABLE 15.1 (CONTINUED)

Genes Under Regulation by $1\alpha,25(\text{OH})_2\text{D}_3$

Gene	Regulation	Evidence	Tissue/Cell	Ref.
Plasma membrane calcium pump	Up	mRNA	Chick intestine	175
Pre-pro-PTH	Down	mRNA	Rat	176
		mRNA/ Transcription	Bovine parathyroid	177
Prolactin	Up	mRNA	GH ₄ C ₁ cells	178
Protein kinase inhibitor	Down	mRNA	Chick kidney	179
Protein kinase C	Up	mRNA/ Transcription	HL-60 cells	180
PTH	Down	mRNA	Rat parathyroid	181
				182
PTH-related protein	Down	mRNA/ Transcription	TT cells	183
Transferrin receptor	Down	mRNA	PBMC	153
Tumor necrosis factor- α	Up	mRNA	U937 cells	162
		Transcription	HL-60 cells	184
VDR	Up	mRNA	Rat intestine	185
			Rat pituitary	186
			MG-63 cells	181
				136

^a Regulation by $1\alpha,25(\text{OH})_2\text{D}_3$.

mineral homeostasis, autoregulation and vitamin D metabolism, cell differentiation and proliferation, bone matrix protein, extracellular matrix proteins, oncogenes, chromosomal proteins, growth factors, signal transduction proteins, peptide hormones, and energy metabolism. Of the 51 gene products listed, all have been reported to be $1\alpha,25(\text{OH})_2\text{D}_3$ sensitive in terms of altered mRNA level. However, $1\alpha,25(\text{OH})_2\text{D}_3$ regulated transcription has only been reported in 11 of the genes and the presence of a VDRE in four. Several of the genes are characterized as $1\alpha,25(\text{OH})_2\text{D}_3$ sensitive supported by data indicating altered mRNA levels, transcription, and the presence of a VDRE: integrin $_{\alpha\text{v}\beta 3}$ and 1(OH)hydroxyvitamin-D-24-hydroxylase. It should be noted that many of these genes encode proteins whose levels are indeed sensitive to vitamin D status.

15.5.2 Rapid Nongenomic Response

Studies²⁷ suggest that not all of the actions of $1\alpha,25(\text{OH})_2\text{D}_3$ can be explained by receptor–hormone interactions with the genome. Rapid actions of $1\alpha,25(\text{OH})_2\text{D}_3$ have been observed at both the cellular (e.g., calcium transport across a tissue) and subcellular level (membrane calcium transport, changes in intracellular second messengers). Table 15.2 summarizes the cell types in which rapid responses to $1\alpha,25(\text{OH})_2\text{D}_3$ have been shown. In comparison to our understanding of the interaction of $1\alpha,25(\text{OH})_2\text{D}_3$ with its nuclear VDR

TABLE 15.2
Distribution of Rapid Responses to $1\alpha,25(\text{OH})_2\text{D}_3$

Organ/Cell/System	Response Studied	Ref.
Intestine	Rapid transport of intestinal Ca^{2+} (Transcaltachia);	11, 187, 188
	CaCo-2 cells, PKC, G proteins	189
	Activation of PKC	187, 190
Colon	PKC effects subcellular	191
	distribution regulation of $25(\text{OH})\text{D}_3$ -24-hydroxylase	192, 193
Fibroblasts	Accumulation of cGMP near VDR_{nuc}	194
		37, 195
Osteoblast	ROS 17/2.8 cells	
	Ca^{2+} channel opening	12
	Cl^- channel opening	196
	UMR-106 cells	
	Ca^{2+} channel opening by $24\text{R},25(\text{OH})_2\text{D}_3$	191
Liver	Lipid metabolism;	197, 198
	Activation of PKC and MAP kinase	199
		199
Muscle	PKC & Ca^{2+} effects	200–203
Promyelocytic leukemic cells	Aspects of cell differentiation	204, 205
	PKC effects	206, 207
Keratinocytes	Alter PKC subcellular	208
	distribution	
Parathyroid cells	Phospholipid metabolism	209
	Cytosolic Ca^{2+}	210
Lipid bilayer	Activation of highly purified	211
	PKC	

Note: The reader should compare the information in this table with the concepts illustrated in Figs. 15.2 and 15.3 which summarize the vitamin D endocrine system and signal transduction pathways utilized by $1\alpha,25(\text{OH})_2\text{D}_3$ for generation of biological responses.

and the plethora of details concerning regulation of gene transcription, it is clear at the time of preparation of this review that the field of nongenomic responses is only in its infancy.

A particularly well-studied system in the author's laboratory is the duodenum of the vitamin D-replete chick where $1\alpha,25(\text{OH})_2\text{D}_3$ stimulates transcaltachia or "the rapid hormonal stimulation of Ca^{2+} transport" in the vitamin-D replete chick.¹¹ Both the chick transcaltachic response and the rat ROS 17/2.8 osteoblast cell membrane Ca^{2+} transport occur within 2 to 4 minutes after treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ in a biphasic manner.^{11,12} For both systems it has been proposed that there is a membrane receptor for $1\alpha,25(\text{OH})_2\text{D}_3$ with ligand binding properties that are different from that of the nuclear/cytosol receptor; in each system the model suggests that the ligand-receptor complex mediates the signal transduction of the hormone via opening of voltage-gated Ca^{2+} channels so as to initiate the biological response(s).^{28,29}

The process of transcalcitachia is not inhibited by genomic inhibitors such as actinomycin D or protein synthesis inhibitors like cycloheximide, but is inhibited by Ca^{2+} channel blockers like nifedipine. In addition, inhibitors of PKC, such as H7, and an inhibitor of phospholipase C, U73122, inhibit transcalcitachia; while mastoparan, which is an activator of G-proteins, stimulates transcalcitachia. Transcalcitachia, induced by $1\alpha,25(\text{OH})_2\text{D}_3$ in the intestine, appears to involve the internalization of calcium in endocytic vesicles at the brush border membrane which then fuse with lysosomes and travel along microtubules to the basal lateral membrane where exocytosis occurs.^{30,31} Thus, it is not surprising that transcalcitachia is inhibited both by colchicine, an antimicrotubule agent, and by leupeptin, an antagonist of lysosomal cathepsin B which is associated with lysosomal vesicles.

Other effects of $1\alpha,25(\text{OH})_2\text{D}_3$ that do not appear to be mediated by the nuclear receptor are phosphoinositide breakdown,³² enzymatic activity in osteoblast-derived matrix vesicles,³³ certain secretion events in osteoblasts,³⁴ rapid changes in cytosolic Ca^{2+} levels in primary cultures of osteoblasts and osteosarcoma cells,^{12,35,36} and increases in cyclic guanosine monophosphate levels in fibroblasts.³⁷

These rapid effects appear to be mediated by a membrane receptor-like protein for $1\alpha,25(\text{OH})_2\text{D}_3$.³⁸ Evidence has been presented which supports the view that the VDR_{mem} is located on the external surface of the cell.^{39,40} Also, a cell membrane-binding protein for $1\alpha,25(\text{OH})_2\text{D}_3$ which has been implicated with transcalcitachia has been isolated and purified approximately 4000 fold.⁴⁰ Other steroid hormones, estrogen,⁴¹ progesterone,^{42–45} testosterone,⁴⁶ glucocorticoids,^{47,48} corticosteroid,⁴⁹ and thyroid^{50,51} have also been shown to have similar membrane effects that result in the rapid onset of biological responses; these are reviewed by Nemere, Zhou, and Norman.³⁸

Thus, the integration of the generation of the production of biological responses by the steroid hormone $1\alpha,25(\text{OH})_2\text{D}_3$ is mediated by both genomic as well as rapid responses. Figure 15.2 provides a summary describing our current understanding of how this steroid hormone can activate the generation of second messengers in the cytosol by both a series of systems operative in the cell cytosol and plasma membrane and, in the nucleus of the cell.

15.6 Nuclear Receptor for $1\alpha,25(\text{OH})_2\text{D}_3$

15.6.1 Structural Domains

The VDR_{nuc} belongs to a superfamily of ligand-dependent nuclear receptors^{10,52,53} which includes receptors for glucocorticoids (GR), progesterone (PR), estrogen (ER), aldosterone, androgens, thyroid hormone (T_3R), hormonal forms of vitamins A (RAR, RXR) and D (VDR), and many orphan receptors (Fig. 15.7) (see References 54 and 55 for a more detailed discussion

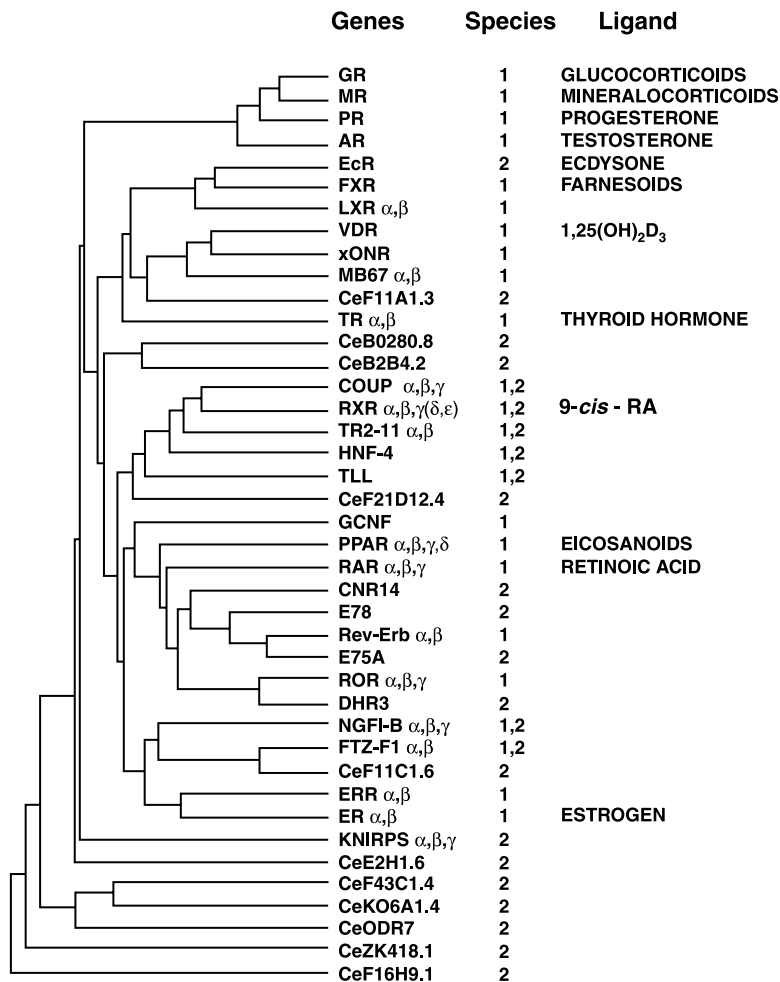
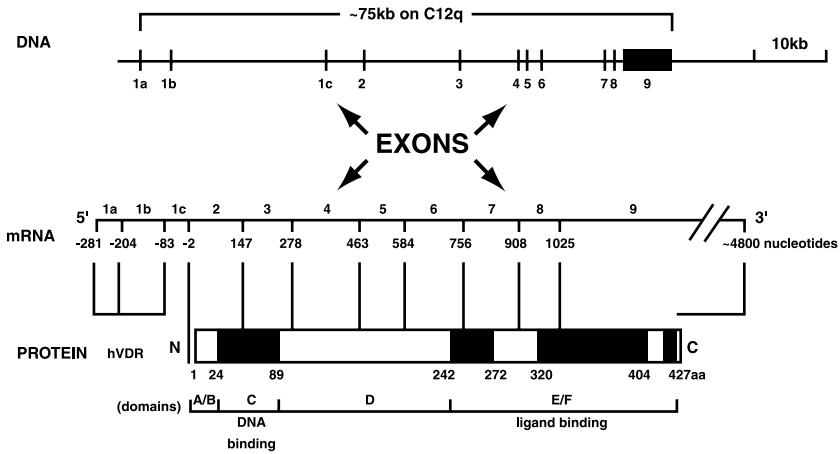


FIGURE 15.7
Nuclear receptor superfamily. This figure summarizes the hypothesized evolutionary relationships for the extended family of known nuclear receptors and related orphan receptors present in vertebrates and invertebrates (*Caenorhabditis elegans*, *Drosophila*). The relationships are based on the extent of homology for the nucleotide sequence of the cDNA of the individual protein. For further details, see References 54 and 55. (Modified from Mangelsdorf et al., *Cell*, 83, 835, 1995.)

of the evolutionary relationships). Comparative studies of these receptors reveal that they have a common structural organization consisting of five domains,⁵⁶ which are shown in Fig. 15.7. The different domains act as distinct modules that can function independently of each other.⁵⁷⁻⁵⁹

Figure 15.8 diagrams the relationships for the VDR_{nuc} among its gene, mRNA, and receptor protein; additional information is presented in recent reviews.^{60,61} The DNA-binding domain, C, is the most conserved domain throughout the family. About 70 amino acids fold into two zinc finger-like

**FIGURE 15.8**

Schematic model of the VDR_{nuc} gene, mRNA, and protein. The gene for the VDR_{nuc} is located on human chromosome 12 and spans approximately 10 kb. The gene has 11 exons which are processed to yield a full-length mRNA of 4800 nucleotides. The VDR_{nuc} protein is comprised of 427 amino acids. The numbers below the hVDR indicate the amino acid residue boundaries for the various domains. Nuclear receptors consist of 5 domains (A–E) based on regions of conserved amino acid sequence and function. The C domain, the most highly conserved domain, is the DNA-binding domain and it defines the superfamily. It contains two zinc finger motifs. The E domain is less conserved and is responsible for ligand binding, dimerization, and transcriptional activation. Subdomains within the domain include ligand1 and ligand2, τ_i or transcriptional inhibition, and dimerization which contains 9 heptad repeats as first described.⁶² Domains A/B and D have the least sequence homology.^{60,61} (Modified from Pike, *Vitamin D*, Academic Press, 1977).

motifs. Conserved cysteines coordinate a zinc ion in a tetrahedral arrangement. The first finger, which contains four cysteines and several hydrophobic amino acids, determines the DNA response element specificity. The second zinc finger, which contains five cysteines and many basic amino acids, is also necessary for DNA binding and is involved in receptor dimerization.^{52,58,62,63}

The next most conserved region is the steroid-binding domain (region E). This region contains a hydrophobic pocket for ligand binding and also contains signals for several other functions including dimerization,^{64,67} nuclear translocation, and hormone-dependent transcriptional activation.^{57,58,68}

The A/B domain is also known as the immuno- or transactivation domain. This region is poorly conserved in amino acids and in size, and its function has not been clearly defined. The VDR has the smallest A/B domain (25 amino acids) of the known receptors; the mineralocorticoid receptor has the largest A/B domain (603 amino acids). An independent transcriptional activation function is located within the A/B region^{52,58,59} which is constitutive in receptor constructs lacking the ligand-binding domain (region E). The relative importance of the transcriptional activation by this domain depends on the receptor, the context of the target gene promoter, and the target cell-type.⁶⁹

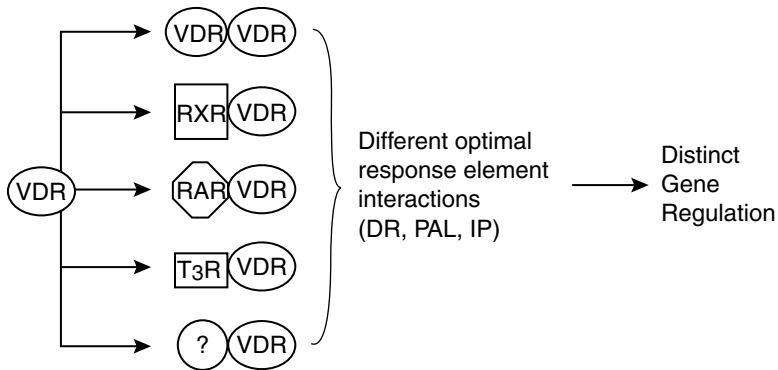
Domain D is the hinge region between the DNA-binding domain and the ligand-binding domain. The hinge domain must be conformationally flexible because it allows the ligand-binding and DNA-binding domains some flexibility for their proper interactions. The VDR hinge region contains 65 amino acids and has immunogenic properties.⁷⁰

15.6.2 Receptor Dimerization

The superfamily of nuclear receptors has been classified into subgroups based on their dimerization properties, DNA-binding site preferences, and cellular localization. Group I includes the receptors for glucocorticoids, estrogen, mineralocorticoids, progesterone, and androgens. These receptors bind as homodimers to palindromic DNA response elements. Group II includes the receptors for VDR_{nuc}, T₃R, RAR, RXR, ecdysone, and several orphan receptors. These receptors bind as homodimers or heterodimers to direct repeats, palindromic and inverted palindromic DNA response elements. Group III includes the receptors for reverb A, ROR, SF-1, and NGFI-B. No ligands have yet been identified for these receptors and they bind DNA response elements as monomers or heterodimers.

As a class, the group II receptors bind non-steroid conformationally flexible ligands (where vitamin D is classified as a seco-steroid rather than as a steroid). The group II receptors have more flexibility in the types of DNA response elements they can recognize and in the types of dimeric interactions they participate in than the group I receptors. All of the group II receptors can form heterodimers with RXR,^{71,72} and other heterodimeric interactions have also been reported.⁷³ The VDR_{nuc} can bind to DNA response elements as homodimers and as heterodimers with RAR, RXR, and T₃R.^{73,74} The ability to form heterodimers with other receptors allows for enhanced affinity for distinct DNA targets, generating a diverse range of physiological effects as shown in Fig. 15.9.

The first zinc finger determines the sequence specificity of the DNA element. The second zinc finger is aligned by the binding of the first finger to the DNA and is involved in the protein-protein contacts responsible for the cooperativity of binding. The spacing of nucleotides between the two half sites is important for the DNA-binding specificity because of the asymmetric dimer interface formed by the DNA-binding domains of a heterodimer pair. Ligand binding may function to modulate receptor dimerization. In fact, VDR_{nuc} has been shown to exist as a monomer in solution in either the presence or absence of ligand. When DNA is present, in the absence of ligand, the VDR_{nuc} binds to the DNA as monomers and homodimers. The addition of ligand stabilizes the bound monomer which favors the formation of VDR_{nuc}-RXR (or other) heterodimers. The presence of the ligand decreases the rate of monomer-to-homodimer conversion and enhances the dissociation of the dimer complex. The presence of the RXR ligand, 9-*cis*-retinoic acid, has the opposite effect on heterodimerization formation; it enhances the binding of

**FIGURE 15.9**

Schematic of possible dimeric interactions of VDR_{nuc} with other receptors. The VDR_{nuc} can bind to DNA as a homodimer or as a heterodimer with a variety of other group 2 receptors, i.e., RXR (retinoid X receptor), RAR (retinoic acid receptor), T_3R (thyroid receptor), and perhaps other receptors or factors not yet identified. Each dimer pair has an enhanced affinity for distinct DNA targets allowing a small family of receptors to generate a diverse range of physiological effects.

RXR homodimers to DR+1 elements.⁷⁵ Ligand bound to VDR_{nuc} enhances the binding of RXR- VDR_{nuc} heterodimers to DR+3 elements. There are also other possible protein–protein interactions that can involve VDR_{nuc} including association with AP-1, EE1A/TFIID, TFIIB. These protein–protein interactions can be determined by the concentration of the protein partner and/or by the concentration of ligand or both, as well as by the nature of the DNA target site itself.

15.6.3 Hormone Response Elements

Each zinc finger appears to be encoded by separate exons as shown by the genomic structure of the ER,⁷⁶ PR,⁷⁷ and the VDR_{nuc} .⁷⁸ Most of the knowledge of how zinc fingers interact with DNA response elements has been gained by studies of GR and ER. The palindromic nature of GR and ER response elements suggested that these hormone receptors would bind to DNA as symmetrical dimers. Subsequent studies have confirmed that GR and ER bind as homodimers to their response elements.^{79,80} The principal ER-dimerization domain is in its ligand-binding domain.⁸¹ Both the ER and GR contain additional residues in the DNA-binding domain that are also important for dimerization. When the GR and ER DNA-binding domains are translated, they cannot dimerize alone but in the presence of the correct palindromic response element, they bind to DNA as a dimer in a cooperative manner.⁸² The five amino-acid-stretch between the first two coordinating cysteines of the second zinc finger is designated the “D” box⁸³ and mediates spacing requirements critical for cooperative dimer binding to palindromic HREs, probably through a dimer interface involving these residues in each monomer.^{84,86}

Using the GR and ER as models of receptor–DNA interactions, the binding of VDR_{nuc} to DNA has also been examined. Since VDR_{nuc} can bind to DNA as a heterodimer, often with RXR, VDR_{nuc} and other group II receptors seem to display more variety in how they bind to their response elements.^{66,78,87} The primary response element for the group II receptors is a direct repeat instead of an inverted palindrome; the protein–protein contacts are non-equivalent. There is an asymmetrical dimerization interface. Amino acid residues, designated the T/A box in the hinge region (domain D) just adjacent to the DNA-binding domain, are involved. The T/A-box residues form an α -helix making backbone and minor groove interactions which are involved in intramolecular packing against residues in the tip of the first zinc finger and determine the spacing requirements for the heterodimer pair. The P-box is the DNA-recognition helix at the C-terminal base of the first zinc finger where specific base contacts with the DNA are made. The D-box is at the N-terminal base of the second zinc finger and together with additional residues from the second zinc finger form part of the dimerization interface.⁸⁸

Table 15.3 summarizes examples of hormone response elements for VDR_{nuc}. The natural response elements for the group II receptors appear to consist of a direct repeat of the hexamer AGGTCA. The spacing of the direct repeat determines the receptor preference: VDR_{nuc} prefers a three base pair space, T₃R prefers four base pairs, and RAR prefers five base pairs.⁸⁹ RXR, RAR, T₃R, VDR_{nuc} spacing optimum on a palindrome is no nucleotides between half-sites. Spacing on inverted palindromes depends on the overhang of the dimeric partners: 11 for VDR_{nuc}–RAR; VDR_{nuc}–RXR is predicted to be 7 to 8, but actually is 9; RXR appears to use a slightly different contact interface when it heterodimerizes with VDR_{nuc} than with other receptors.⁹⁰ Free rotation around the hinge (domain D) enables the same interaction of the ligand-binding domains of both receptors on each response element. The steric requirements of the T/A boxes give the receptor its asymmetry when binding to direct repeats and inverted palindromes and determines the optimal spacing, illustrated in Fig. 15.10.

TABLE 15.3
Hormone Response Elements for the Nuclear Vitamin D Receptor (VDR_{nuc})

Gene	Hormone Response Element	Ref.
hOsteocalcin	GGGTGA acg GGGGCA	212
rOsteocalcin	GGGTGA atg AGGACA	172
mOsteopontin	GGTCA cga GGGTCA	174
rCalbindin D _{9k}	GGGTGA cgg AAGCCC	21
mCalbindin D _{28k}	GGGGGA tgt GAGGAG	213
24R-Hydroxylase	AGGTGA gtg AGGGCG	214
DR+3	AGGTCA agg AGGTCA	89
CONSENSUS	GGGTGA nnn GGGNCNAA	

Note: A comparison of reported VDREs. The two half-sites are listed as uppercase letters. The sequences are –500 to –486 of human osteocalcin, –456 to –438 of rat osteocalcin, –758 to –740 of mouse osteopontin, –488 to –474 of rat calbindin D_{9k}, and –199 to –184 of mouse calbindin D_{28k}.

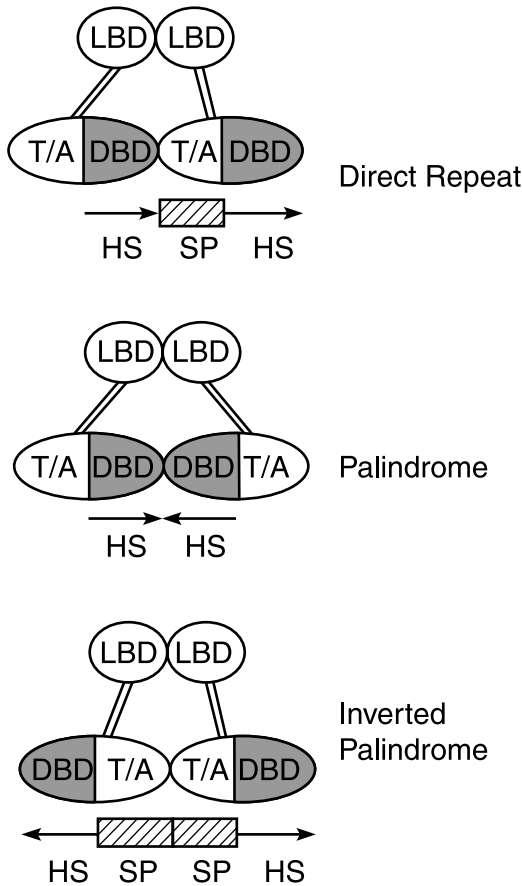


FIGURE 15.10

Mechanism of receptor dimers binding to DNA response elements. Group II receptors can bind to three types of response elements, which are direct repeats, palindromes, and inverted palindromes. The spacing (SP), number of base pairs between half-sites (HS), is determined by steric constraints of the T/A box. The orientation of the DNA half-sites is shown with arrows. The flexible hinge domain allows the formation of the same dimerization interface between ligand-binding domains (LBD) regardless of the orientation of the DNA half-sites.

15.6.4 Ligand Binding

The ligand-binding domain of group II receptors has been further dissected (see Fig. 15.8). Subdomains ligand 1 and ligand 2 are nearly identical among receptors of the same binding specificity but are different among receptors of different binding specificity.^{91,93} Surprisingly, there is greater homology between the ligand binding subdomains of RAR (α , β , γ) and T₃R (α , β) than there is between RAR and RXR. The τ_i subdomain is highly conserved among all nuclear hormone receptors and is a putative transcriptional inactivating domain. Inactivation of this domain is relieved by ligand binding.

The dimerization domain consists of 8 to 9 heptad repeats of hydrophobic amino acids. The heptads contain leucine or other hydrophobic residues such as Ile, Val, Met, or Phe at positions one and eight or charged amino acids with hydrophobic side chains such as Arg or Gln in the fifth position. In an ideal coiled-coil α -helix, these amino acids would form a hydrophobic surface along one face of the helix that would act as a dimerization interface.⁶⁵ Deletion/mutation analysis of the VDR_{nuc} ligand-binding domain has shown that Asp-258 and Ile-248 are involved in heterodimerization with RXR. Leu-254 and -262 are critical for heterodimerization. A mutant that is truncated at amino acid 190 becomes constitutively transcriptionally active. Other amino acids identified as being important for heterodimerization are 325–332, 383–390, and 244–263. Residues 403–427 are important for ligand binding [$1\alpha,25(\text{OH})_2\text{D}_3$].⁹⁴

The location of the dimerization subdomain between the two ligand-binding subdomains links dimerization with ligand binding and has regulatory implications. The dimerization subdomain appears important for heterodimerization of the group II receptors.^{66,67} This subdomain is similar to two known dimerization domains present in other transcription factors, the leucine zipper and helix-loop-helix motifs^{87,95,96} and is sometimes called a “regulatory zipper”. The group II receptors are the only members of the superfamily that have these conserved heptad repeats. The dimerization interfaces are precisely designed to accommodate the formation of homo- or heterodimers on symmetrical or asymmetrical response elements. There is considerable flexibility in response element specificity but this follows precise rules. By promiscuous dimerization, small families of structurally related proteins can result in large numbers of transcription factors with distinct functional properties such as binding affinities for specific response elements and inducibility by specific ligands.⁹⁷

Recently, the ligand-binding domain of three steroid receptors has been determined via X-ray crystallographic techniques which have allowed for the first time a definitive view of how ligands interact with the ligand-binding domain of their cognate receptors. The new structures include the human RXR- α ,⁶⁴ the rat thyroid receptor,⁹⁸ and the human estrogen receptor.⁹⁹ A striking finding is that all three structures were essentially identical. For each receptor, approximately 65% of the amino acid residues in the ligand-binding domain are arranged in 11 or 12 α -helices, forming a three-layered structure surrounding a large hydrophobic pocket which can accommodate the appropriate ligand. Thus, it seems likely that the overall topology of the VDR_{nuc} ligand-binding domain will be similar to that of these three structures.

Figure 15.11 presents a schematic model of the VDR_{nuc} interaction with its heterodimer partner and the subsequent interaction with the promoter of genes selected for modulation, as well with other proteins (coactivators, TATA-binding protein, etc.) to generate a competent transcriptional complex. During the past decade there has been a continuing evolution of understanding and complexity concerning the details of what constitutes a competent

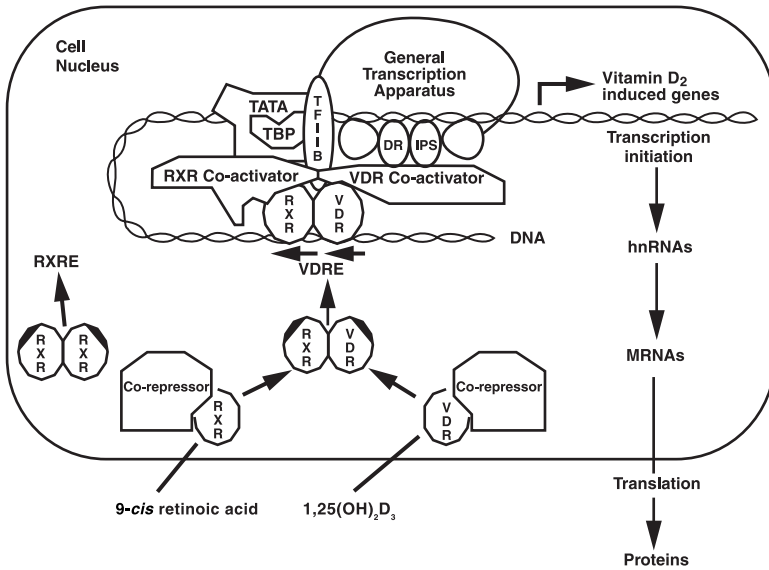


FIGURE 15.11

Model of $1\alpha,25(\text{OH})_2\text{D}_3$ and VDR_{nuc} activation of transcription. The VDR after binding its cognate ligand $1\alpha,25(\text{OH})_2\text{D}_3$ forms a heterodimer with RXR. This heterodimer complex then interacts with the appropriate VDRE on the promoter of genes (in specific target cells) which are destined to be up- or downregulated. The heterodimer–DNA complex then recruits necessary coactivator proteins, TATA, TBP, TFIIB, and other proteins to generate a competent transcriptional complex capable of modulating mRNA production. (Modified from Haussler et al., *Vitamin D*, Academic Press, 1977.)

transcriptional complex. Additional viewpoints and information can be found in recent review articles.^{100–104}

15.7 Genetics and the Vitamin D Endocrine System

15.7.1 Mutations in the VDR_{nuc}

Vitamin D-dependent rickets-type II is a rare genetic disease. Genetic analysis has shown that it is autosomal recessive. Less than 30 kindreds have been reported. The combination of symptoms, i.e., defective bone mineralization, decreased intestinal calcium absorption, hypocalcemia, and increased serum levels of $1\alpha,25(\text{OH})_2\text{D}_3$, suggest end-organ resistance to the action of $1\alpha,25(\text{OH})_2\text{D}_3$. Patients do not respond to doses of vitamin D, $25(\text{OH})\text{D}_3$ or $1\alpha,25(\text{OH})_2\text{D}_3$.

The unresponsiveness to $1\alpha,25(\text{OH})_2\text{D}_3$ has been demonstrated to arise from defects in the gene coding for the VDR_{nuc} . Table 15.4 summarizes the

TABLE 15.4
Genetic Analysis of the Nuclear Receptor for 1 α ,25(OH) $_2$ D $_3$: Site of Mutation in the Nuclear Receptor for 1 α ,25(OH) $_2$ D $_3$

VDR Domain	Mutation	Functional Consequence	Ref.
DNA-binding domain	R30Stop	Premature termination- no DNA	215
	R73Stop	binding, no ligand binding	216
	R88Stop		215
	Point mutation intron 4 results in premature stop codon		217
	R30G	Mutations occurring at highly	108
	G33D	conserved amino acid residue within	218
	H35Q	the first and second zinc fingers.	219
	K42I	Mutation interferes with the ability of	107
	K43E	the receptor to interact normally with	107
	F44I	DNA	107
	G46D		220
	R50Q		221
	R70D		108
	R73Q		218
	R80Q		222
	K91N/E92Q		223
Hinge region	148Stop	Premature termination — no ligand	216
	Q152Stop	binding	105
Ligand binding domain	Y295Stop	Premature termination — no ligand	106
		binding	
	C190Y	Familial VDDRII	224
	S208G	Phosphorylation that modulates	225
		transcription	
	S208A	No enhancement of transcription	
	F244G	Impaired transactivation; no RXR	226
		dimers	
	K246G	Impaired transactivation	226
	L254G	Impaired transactivation; no RXR	226
	Q259G	dimers	
	L262G		
	C288G	Impaired ligand binding	227
	H305Q	Decreased binding (slight); decreased	228
		transactivation	
	I314S	Impaired transactivation and RXR	229
		dimerization	
	C337G	Impaired ligand binding	227
	C369G	Impaired transactivation and RXR	226
	R391C	dimerization	229

locations and nature of known mutations in the VDR_{nuc}. Two types of abnormalities have been defined by binding studies: receptor-negative and receptor-positive phenotypes. The mutations identified in the receptor-negative phenotype involve a mutation that introduces a premature stop codon in the message. The resulting truncated protein is not able to bind ligand. The receptor-positive phenotype arises from one of several missense mutations localized within the zinc finger domains of the DNA-binding domain. Several of these mutant receptors have been demonstrated to be defective

in their ability to bind to DNA-cellulose and unable to mediate $1\alpha,25(\text{OH})_2\text{D}_3$ -stimulated gene transcription *in vitro*.^{105–108}

15.7.2 Knockout of the VDR_{nuc}

An animal model of vitamin D-dependent rickets-type II (VDDRII) was engineered most recently by targeted disruption of DNA encoding the first and the second zinc finger of the DNA-binding domain of the VDR, respectively, by two different groups independently.^{109,110} The resultant animals were phenotypically normal at birth. No defects in development and growth were observed before weaning, irrespective of reduced expression of vitamin D target genes. After weaning (3 weeks after birth), however, the VDR null mutant mice showed marked growth retardation. Such growth retardation became apparent only after weaning, even when it was either hastened or delayed. Most of the null mutant mice died by 15 weeks. No overt abnormalities, however, were found in the heterozygotes even at 6 months. All of the VDR null mutant mice developed alopecia and had few whiskers by 7 weeks. The serum levels of calcium and phosphate were reduced at 4 weeks, with markedly elevated serum alkaline phosphatase activity present in the null mutant mice while in older VDR-deficient mice, these abnormalities became more prominent. These observations in the VDR null mutant mice are similar to those in a human vitamin D-dependent rickets-type II disease, in which mutations in VDR gene have been identified in several families, although this disease is not lethal.

In the VDR null mutant mice at 3 weeks, the serum levels of $1\alpha,25(\text{OH})_2\text{D}_3$, $24\text{R},25(\text{OH})_2\text{D}_3$, and $25(\text{OH})\text{D}_3$ were the same as those in the heterozygous and wild-type mice. However, a marked increase (10 times) in serum $1\alpha,25(\text{OH})_2\text{D}_3$ and a clear reduction (to almost undetectable levels) in serum $24\text{R},25(\text{OH})_2\text{D}_3$ developed in the VDR null mutant mice at 4 weeks and persisted at 7 weeks. Immunoreactive PTH levels were also raised sharply after weaning and the size of the parathyroid glands in the 70-day-old VDR-ablated mice was increased more than 10-fold. These observations establish that VDR is essential for regulations of these enzymes by $1\alpha,25(\text{OH})_2\text{D}_3$ after weaning, again supporting the idea that $1,25(\text{OH})_2\text{D}_3$ (VDR) plays a critical role only after weaning. The authors suggest that a functional substitute for $1\alpha,25(\text{OH})_2\text{D}_3$ is present in milk.

Severe bone malformation was induced by the inactivation of VDR after weaning. Radiographic analysis of VDR null mutant mice at 7 weeks revealed growth retardation with loss of bone density. A 40% reduction in bone mineral density was observed in the homozygote mutant mice. In gross appearance and on X-ray analysis of tibia and fibula, typical features of advanced rickets were observed, including widening of epiphyseal growth plates, thinning of the cortex, fraying, cupping, and widening of the metaphysis. In addition, orderly columns of hypertrophic chondrocytes were lost, and the layers of cartilage were widened with inadequate mineralization. In cancellous bone adjacent to the growth plates, marked increases in the extent

and width of osteoid seams were noted, and bone surfaces were surrounded by numerous osteoblastic cells. The number of osteoclasts appeared not to be reduced in bone from VDR null mutant mice when compared to that of normal mice. These findings indicated that $1,25(\text{OH})_2\text{D}_3$ can stimulate, but is not essential for, osteoclast formation *in vivo*, and that other factors can induce the osteoclast formation when VDR-mediated actions are absent.

The male and female VDR null mutant mice were infertile. The uterus had not matured in the female VDR knockout mice at 7 weeks. This uterine hypoplasia is not due to inability of the uterus to respond to estrogen. Only primary and secondary, but no mature, graafian follicles were observed in the null mutant mice, which indicates a lack of estrogen synthesis in the mutant ovaries. In contrast, male reproductive organs appeared normal in VDR null mutant mice. In addition, no obvious difference between the null mutant and wild-type mice at 3 or 7 weeks was detected in the proportional change of immunological cell population when cells from the spleen, thymus, mesenteric lymph node, and bone marrow were analyzed.

15.7.3 Knockout of the $25(\text{OH})\text{D}-24\text{-Hydroxylase}$

$24\text{R},25(\text{OH})_2\text{D}_3$ is the second major dihydroxylated metabolite of vitamin D_3 , which is found in significant concentrations in the serum of humans,^{111–113} rats,¹¹⁴ and chicks.¹¹⁵ Although the production of $24\text{R},25(\text{OH})_2\text{D}_3$ by the kidney is tightly regulated,¹¹⁶ the biological importance of this compound is still the subject of uncertainty and question.^{4,117} While several possible biological roles and sites of action have been suggested for $24\text{R},25(\text{OH})_2\text{D}_3$, including regulation of parathyroid hormone release from the parathyroid gland,^{117,118} most studies concerning this vitamin D metabolite have focused on its possible actions on bone biology.^{119–121} The possible existence of a nuclear or cytosolic-binding protein for $24\text{R},25(\text{OH})_2\text{D}_3$ was reported in the chick parathyroid gland,¹²² the long bone of rat epiphysis,¹²³ and chick tibial fracture-healing callus;¹²⁴ however, there has been no general confirmation of these early findings. Also, several more recent reports have described specific actions or accumulation of $24\text{R},25(\text{OH})_2\text{D}_3$ in cartilage,^{123,125} and the bone fracture-healing callus tissue.^{121,126,127}

In order to address the physiological functions of $24\text{R},25(\text{OH})_2\text{D}_3$, a strain of mice deficient for the $25(\text{OH})\text{D}-24\text{-hydroxylase}$ enzyme has been generated recently¹²⁸ through homologous recombination in embryonic stem cells. The targeted mutation effectively deleted the heme-binding domain of the cytochrome P-450 enzyme, ensuring that the mutated allele could not produce a functional protein. The analysis of the phenotype of the knockout animals revealed fascinating and previously unrecognized roles for $24\text{R},25(\text{OH})_2\text{D}_3$. About half of the mutant homozygote mice born from heterozygote females died before weaning. Bone development of those survivors was abnormal in homozygous mutants born of homozygous females. Histological analyses of the bones from these mice revealed an accumulation

of unmineralized matrices at sites of intramembranous ossification, particularly the calvaria and exocortical surface of long bones. However, the growth plates from these mutant animals appeared normal, suggesting that $24R,25(OH)_2D_3$ is not a major regulator of chondrocyte maturation *in vivo*.

This study confirms the earlier reports that the presence of $24R,25(OH)_2D_3$ in collaboration with $1\alpha,25(OH)_2D_3$ is essential to the normal operation of the vitamin D endocrine system.

15.8 Summary

$1\alpha,25(OH)_2D_3$ is responsible for the generation of a wide array of biological responses throughout the far reaching network of its vitamin D endocrine system. In each instance, the initiation of a biological response is dependent upon the specific interaction of $1\alpha,25(OH)_2D_3$ as a ligand with the VDR_{nuc} , the VDR_{mem} , or the plasma transport-binding protein, DBP (see Fig. 15.12). While this chapter has focused upon the biological properties of only the VDR_{nuc} and the VDR_{mem} , based on evidence accumulated to date, we have concluded that DBP, VDR_{nuc} , and VDR_{mem} proteins are conformationally specific. That is, each will optimally bind a different shape of $1\alpha,25(OH)_2D_3$. Thus, one possible way in which the vitamin D endocrine system is able to achieve such

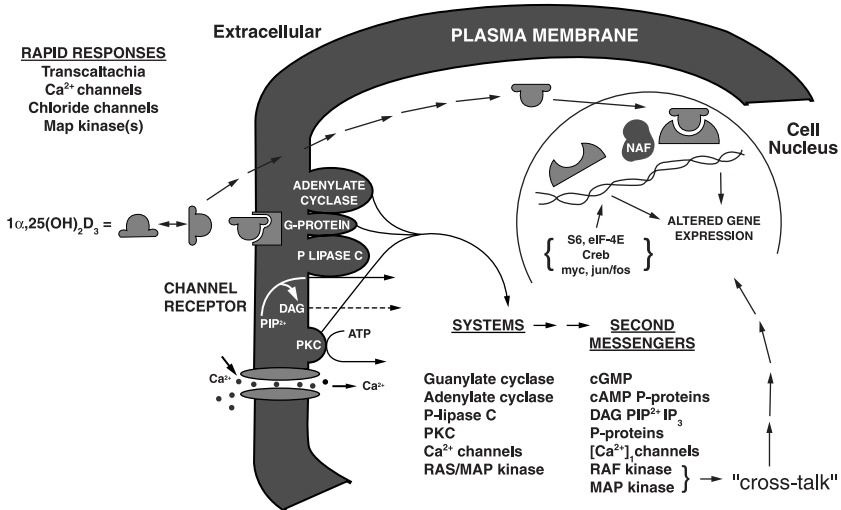


FIGURE 15.12

$1\alpha,25(OH)_2D_3$ and signal transduction; a working model. The upper left inset lists the rapid responses generated by $1\alpha,25(OH)_2D_3$ whose actions are believed to be described by this schematic model. $1\alpha,25(OH)_2D_3$ can initiate biological responses via its nuclear receptor and a putative cell membrane receptor⁴⁰ which rapidly generates the appearance of second messengers, some of which modulate via cross-talk selective events in the nucleus.

diversity in its biological responses is through utilization of receptors and transport proteins (DBP) which have evolved to be selectively responsive to different subsets within the myriad shapes available for the conformationally flexible $1\alpha,25(\text{OH})_2\text{D}_3$. Further studies are in progress to test experimentally various ramifications of this hypothesis.

Abbreviations

$1\alpha,25(\text{OH})_2\text{D}_3$	$1\alpha,25$ -dihydroxyvitamin D_3
VDRE	$1\alpha,25(\text{OH})_2\text{D}_3$ nuclear response element
TPA	12-0-tetradecanoylphorbol-13 acetate
25OHD_3	25 -dihydroxyvitamin D_3
$24\text{R},25\text{OHD}_3$	$24\text{R},25(\text{OH})_2$ -vitamin D_3
ER	estrogen nuclear receptor
GR	glucocorticoid nuclear receptor
Gc	group-specific component
IU	International Unit
HRE	nuclear receptor hormone response element
VDR_{nuc}	nuclear receptor for $1\alpha,25(\text{OH})_2\text{D}_3$
PTH	parathyroid hormone
PR	progesterone nuclear receptor
VDR_{mem}	putative membrane receptor for $1\alpha,25(\text{OH})_2\text{D}_3$
transcaltachia	rapid hormonal stimulation of Ca^{2+} transport
RDA	Recommended Dietary Allowance
RAR	retinoid acid nuclear receptor
RXR	retinoid x-nuclear receptor
T_3R	thyroid hormone nuclear receptor
DBP	vitamin D binding protein
VDDR II	vitamin D-dependent rickets type II
VDR	vitamin D receptor

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16

Vitamin E and Gene Expression

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16.1 Introduction

Free radicals and reactive oxygen species (ROS) are generated continuously in living cells, damaging cellular constituents if remaining uncontrolled. ROS cause DNA base modifications, DNA and protein cross-links, and strand breaks.¹ Proteins are also targets of ROS attack, resulting in oxidation of amino acids that can generate inactive enzymes or proteins with increased susceptibility to proteolytic attack.^{2,3} In addition, oxidation of cellular membranes gives rise to peroxyl radicals and lipid epoxides.⁴ Accordingly, tissues are equipped with enzymatic (catalase, superoxide dismutase, and glutathione peroxidase) and non-enzymatic (vitamins A, C, and E as well as sulfur-containing amino acids) antioxidant defense systems.

Among the dietary antioxidants, vitamin E is the most abundant and efficient scavenger of hydroperoxyl radicals in biological membranes. In addition to its effects on lipid radicals, vitamin E protects cells by regulating cellular oxido-reductive status. Recent molecular and cellular investigations have demonstrated that antioxidants such as vitamin E can directly affect redox-sensitive signal transduction cascades and, consequently, the control of gene expression. Furthermore, the beneficial effects of vitamin E in several diseases for which oxidative stress has been implicated as a contributing factor have been demonstrated. This review will discuss the interactions between vitamin E and gene expression and their implications for chronic and inflammatory diseases.

16.1.1 Tocopherol Structures, Homologs, and Bioactivity

Naturally occurring vitamin E or tocopherols have four major isoforms, each of which exerts differential biopotencies (Fig. 16.1).

These differences in biopotency are due mainly to differences in the stereospecificity of carbon atoms 2, 4' and 8', the presence of ring methyl groups,

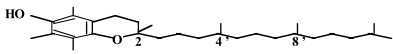
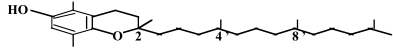
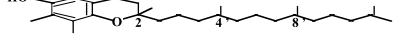
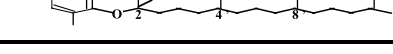
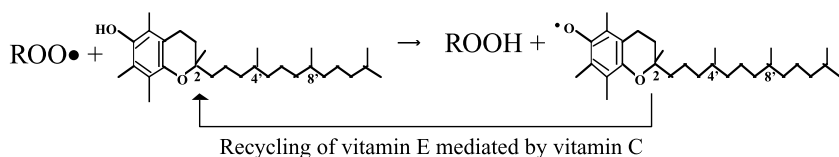
Vitamin E homologs	Structures	Biological activity (%)*
α-Tocopherol		100
β-Tocopherol		60
γ-Tocopherol		25
δ-Tocopherol		27

FIGURE 16.1
The structures and biological activities of tocopherols.
* As determined by relative activities with lipid peroxyl radicals.⁵

**FIGURE 16.2**

Peroxyl radical quenching reaction by tocopherol.

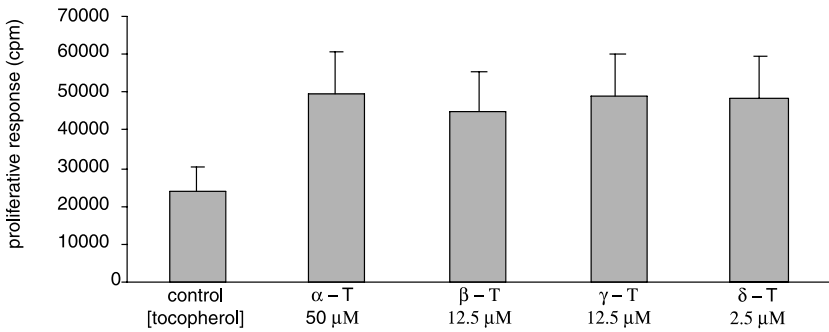
the number of carbon atoms, and the point of attachment in the side chain. Furthermore, the recycling rate, as well as differential affinity of tocopherol-binding proteins (which affect incorporation of tocopherols into lipoproteins), affects tocopherol bioavailability.⁶ The antioxidant property of vitamin E is exerted through the phenolic hydroxyl group, which reacts with peroxyl radicals to form tocophydroxyl radicals (Fig. 16.2). This effectively interrupts the chain of peroxidation reaction, and tocopherol radicals are subsequently reduced and recycled.

16.1.2 Tocopherol Stereoisomers: Differential Effects on Gene Expression

Currently, only limited data are available for determining the relationship between tocopherol isomer bioactivity and gene expression. Even tocopherol isomers with substantially different antioxidant activity, such as α - and δ -tocopherol (Fig. 16.1, 100 and 27%, respectively), may exhibit similar influences on gene expression. For example, mRNA expression of α -tocopherol transfer protein (α -TTP), a 32 kDa protein which transports α -tocopherol to the nascent very-low-density lipoprotein (VLDL), can be induced to a similar degree by both isomers.⁷

Differential transcriptional regulation by tocopherol isomers has been demonstrated for the low-density lipoproteins (LDL)-scavenger receptor (SR) gene.⁸ LDL-SR mediates the uptake of modified LDL by macrophages (M ϕ). Both α -tocopherol and γ -tocopherol downregulate LDL-SR activity as well as LDL-SR mRNA expression and binding activity of transcription factor AP-1 in a dose-dependent manner. α -Tocopherol has been shown to reduce LDL-SR mRNA expression and AP-1 binding activity more significantly than γ -tocopherol. Differential regulation of gene expression by the different tocopherol isomers has also been observed in vascular smooth muscle cells, where α -tocopherol, but not β -tocopherol, downregulates proliferation of vascular smooth muscle cells. These differential effects on proliferation have been attributed to their varied effects on protein kinase C activity (PKC); α -tocopherol inhibits PKC activity whereas β -tocopherol has no effect. The inhibition of PKC activity in smooth muscle cells by α -tocopherol has been shown to occur via a decreased activation of transcription factor AP-1.⁹

Recently, we demonstrated that the concentration needed to induce maximal increases in mitogen-induced T-cell proliferation differs among tocopherol

**FIGURE 16.3**

Effect of tocopherol homologs on concanavalin A (1.5 mg/L)-induced proliferation of mouse spleen T-cells. (From Wu et al., *Free Radic. Biol.*, 28(4), 647, 2000. With permission.)

isomers.¹⁰ In this study, δ -tocopherol, with the lowest antioxidant capacity, required 20-fold less concentration (2.5 μ M) than α -tocopherol, which has the highest antioxidant capacity (50 μ M), to induce the same degree of T-cell proliferation (2-fold induction compared to control) (Fig. 16.3). Based on these data, the differential effects of tocopherol isomers on T-cell proliferation appear to be due to their regulation of gene expression through a nonantioxidant activity. At the level of gene expression, δ -tocopherol might induce a higher transient transcriptional activation than the other isomers, possibly through one or more upstream signal transduction pathways.

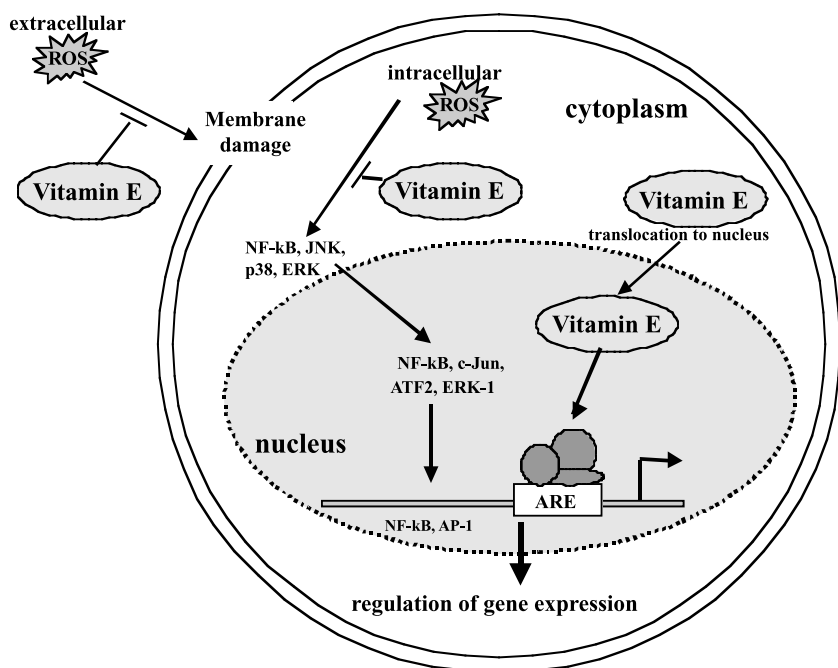
In the same study,¹⁰ δ -tocopherol was also more effective in inducing cell death than was α -tocopherol. Although the mechanism of tocopherol-induced cell death is currently not known, tocopherol isomers may regulate expression of genes involved in necrosis or apoptosis differentially.

In the diet, non- α -tocopherol stereoisomers predominate; the intake of γ -tocopherol in humans is about 2 to 4 times that of α -tocopherol. Although the intestinal absorption of individual tocopherol isomers is similar, α -tocopherol is found in its highest concentration in blood and tissues, mainly because α -TTP selectively supplements nascent VLDL with α -tocopherol isomers.

α -Tocopherol, the isomer with the highest antioxidant capacity and the predominant form of tocopherol in plasma, is also preferentially incorporated into plasma lipoproteins. Consequently, most reported data demonstrating the effects of vitamin E on gene expression focus on the effects of α -tocopherol.

16.2 Modes of Vitamin E Action

Two modes of action for vitamin E on molecular physiology of cells have been proposed: antioxidant and non-antioxidant. While some studies^{9,11,12} have shown that the effect of tocopherol on gene expression is mediated

**FIGURE 16.4**

Proposed modes of antioxidant action of vitamin E on signal transduction. Vitamin E scavenges extracellular ROS and protects hydroperoxyl radical-induced membrane damage.¹³ Intracellular ROS-induced activation of transcription factors such as NF-kB and AP-1 is also inhibited by vitamin E.¹⁴ Vitamin E can translocate to the nucleus and affect transcriptional activation by altering the RNA polymerase complex.¹⁵ Vitamin E is proposed to regulate gene expression via the antioxidant responsive element (ARE) by interacting with ARE-associated transcription factors.

through its non-antioxidant function, most studies indicate that vitamin E alters gene expression by direct modulation of ROS, which in turn have been shown to influence signalling pathways and hence transcription factor activity (Fig. 16.4).

ROS (H_2O_2 , $\text{O}_2^{\bullet-}$, and $\text{HO}^{\bullet}$) are byproducts of general metabolism in most cell types. Free radicals can originate exogenously, as seen in certain components of tobacco smoke, air pollutants, exposure to UV radiation, and through the metabolism of certain solvents, drugs, and pesticides, as well as via the oxidative respiratory chain reactions occurring in mitochondria.

ROS have been shown to induce lipid peroxidation and subsequent transactivation or repression of effector gene expression resulting in oxidative stress-induced cell damage. For example, exposure of liver cells to ROS results in the induction of lipid peroxidation accompanied by a significant increase in the constitutive expression of procollagen type I mRNA, which leads to the development of liver fibrosis.¹⁶ This fibrogenic effect induced by ROS has been shown to be eradicated almost completely by treatment of cells with α -tocopherol.¹⁶

ROS are also involved in the induction of apoptosis, which can be inhibited by various antioxidants. For example, increased ROS have been shown to activate prostate apoptosis response-4 (Par-4, a product of a gene upregulated in prostate cancer cells undergoing apoptosis), and pretreatment of cultures with vitamin E can prevent Par-4 induction.¹⁷

Vitamin E can protect against ROS-induced vascular cell dysfunction mediated through increased adhesion molecule expression and monocyte recruitment. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are expressed in endothelial cells in inflammatory disease. ROS are produced in inflammatory responses and ROS-induced ICAM-1^{18, 19} and VCAM-1 expression¹⁹ are inhibited by α -tocopherol treatment.

Treatment of cells with H_2O_2 ²⁰ and ultraviolet radiation (UVA), which damages membrane lipids and reduces intracellular tocopherol²¹ and glutathione²² levels, is accompanied by a significant increase in heme oxygenase-1 (HO-1) gene expression. Free heme has been established as a source of oxidative stress capable of generating free radicals and causing cell damage. HO-1 is a rate-limiting enzyme that catalyzes the conversion of heme into carbon monoxide and biliverdin, free iron, and carbon monoxide. Thus, through catalyzing free heme, HO-1 acts as a potent antioxidant protein. Addition of α -tocopherol onto human skin fibroblasts in culture has been shown to enhance HO-1 mRNA accumulation.²³

Vitamin E can protect proteins that are sensitive to oxidative stress. For example, vitamin E protects protein thiol-groups against oxidative damage in the maintenance of enzyme activity.²⁴ Thiol-groups containing amino acids (such as cysteine residues) that are located especially in DNA-binding domains of transcriptional factors²⁵ can be oxidized, resulting in a loss of transcriptional regulatory activity. Conserved cysteine residues and their critical role in DNA-binding activity have also been demonstrated in redox state-sensitive transcription activators such as NF κ B. Mutations in the conserved residue (Cys61) within the N-terminal basic region of the p50 subunit of NF κ B impair the stimulation of p50 DNA-binding activity.²⁶ Other transcription factors such as the *myb* protooncogene^{27,28} and Jun proteins, which have been implicated in redox state-related regulation of gene expression,²⁹ also contain conserved cysteine sequences in the DNA-binding domain. Thus, it appears that the protective effect of vitamin E on gene expression is mediated through prevention of oxidative damage to the thiol-groups in NF κ B, *myb*, and Jun proteins.

Vitamin E can exert its non-antioxidant effects by modulating the activity of enzymes that are involved in ROS production. For instance, in human monocytes, several subunits of NADPH-oxidase (which generate superoxide anion $O_2^{\bullet-}$) exist in two major fractions: membrane-bound (b_{558}) and cytosolic subunits (p47^{phox}, p67^{phox}, Rac1/1, and p40^{phox}).³⁰ PMA-induced PKC phosphorylates the p47^{phox} subunit and results in a subsequent translocation and assembly of active form of NADPH-oxidase. α -Tocopherol has been shown to inhibit phosphorylation and translocation of p47^{phox} as well as PKC activity, resulting in a reduced production of $O_2^{\bullet-}$.³⁰ In the same study, addition of

2-carboxy-2,5,7,8-tetramethyl-6-chromanol (Trolox or CTMC), which is a water-soluble vitamin E derivative with free-radical scavenging activity, did not show any effect. Collectively, these data suggest that the inhibitory effect of vitamin E on PMA-induced $O_2^{\bullet-}$ production in human monocytes might be due to the non-antioxidant action of vitamin E. In addition, vitamin E has been shown to be localized to the nucleus³¹ and to exert a non-antioxidant inhibitory effect on PMA-induced RNA-polymerase activity in isolated rat liver nuclei.¹⁵

The transcriptional regulation of antioxidant genes (NADPH quinone oxidoreductase, glutathione S-transferase, glutathione-S-transferase (GST) Ya subunit), are mediated by *cis*-acting elements called the antioxidant responsive element (ARE, 5'-GTGACTCAGCG-3'). The major function of these enzymes is to protect cells against oxidative radicals and against the toxic effects of xenobiotics by metabolizing them.^{23,24}

Other oxidative stress-sensitive proteins such as collagenase α -1 gene³⁵ and HO-1³⁶ also contain ARE. Treatment of mice hepatocytes with α -tocopherol has been shown to inhibit basal levels of collagenase α -1³⁵ and phorbol ester-induced HO-1 gene expression.³⁶ Several transcription factors, such as Cap'n'Collar transcription factors Nrf1³⁷ and Nrf2^{37,38} as well as Jun and Fos family proteins, recently have been shown to bind ARE.³⁹ Whether the inhibitory effects of α -tocopherol on collagenase α -1 and HO-1 gene expression are mediated via Jun, Fos, Nrfs, or by other transcription factors remains to be determined.

16.3 Vitamin E and Gene Expression in Human Disease

Growing evidence suggests that free radical damage contributes to the etiology of many chronic diseases, such as diabetes, emphysema, cardiovascular and inflammatory diseases, cataracts, male infertility, severe collagen diseases, lung fibrosis, neurodegenerative conditions, kidney and liver damage, and cancer. Epidemiological studies have reported an inverse correlation between increased intake of dietary antioxidants such as vitamin E and a lower risk for a number of these diseases. Below we review current findings on the effect of vitamin E on gene regulation in the progression of several human diseases.

16.3.1 Vitamin E, Immune Function, and Immunity-Related Diseases

Cells of the immune system secrete a wide variety of potent substances that, while necessary for normal host defense, can promote inflammation and tissue damage if produced in excess. Among these substances are numerous cytokines, reactive oxidant species, free radicals, and pro-inflammatory

factors, including various eicosanoids, such as prostaglandins, the end products of the arachidonic acid cascade.

Many reactive oxygen intermediates (ROI) are produced by immune cells as part of their normal function of defense against the wide array of foreign antigens to which they are exposed. The oxidant/antioxidant balance is particularly important for immune function, not only for maintaining cellular integrity, but also for controlling signal transduction and gene expression. Indeed, secreted ROI contribute widely to redox changes in the milieu to which leukocytes must adjust, and have thus developed sophisticated antioxidant defense mechanisms to protect themselves from oxidative damage.

It is noteworthy that cells of the immune system are particularly sensitive to redox changes because their plasma membrane contains a higher percentage of polyunsaturated fatty acids (PUFA) relative to other cell types. Indeed, it is well established that immune cells, particularly of the myeloid lineage, contain higher levels of vitamin E than other cells.^{40,41}

It is known that in T-lymphocytes, oxidative stress results in decreased IL-2 production, reduced protein tyrosine phosphorylation, and reduced intracellular calcium mobilization.⁴² Moreover, it has been hypothesized that vitamin E may exert some of its immunostimulatory effects by directly or indirectly increasing IL-2 production.⁴³

Numerous studies have shown vitamin E deficiency to be associated with impaired immune function. In fact, vitamin E deficiency has been shown to diminish the ability of immune cells to mount a defense against infectious microorganisms, produce adequate levels of antibodies against specific antigens, and generate delayed-type hypersensitivity reactions.⁴⁴ Interestingly, vitamin E deficiency has been linked to increased production of IL-6 in response to bacterial endotoxin.⁴⁵ Furthermore, in a study conducted on an elderly population, vitamin E supplementation was shown to inhibit IL-6 production from peripheral blood mononuclear cells.⁴⁶

Aging is also associated with impaired T-cell-mediated function.^{42,48} The age-associated changes in T-lymphocyte function are due in part to increased production of PGE₂, a macrophage (M ϕ) product known to inhibit IL-2 secretion, T-cell proliferation, and T-cell cytotoxicity.⁴⁸ We have shown that the age-related increase in PGE₂ is due to increased mRNA and protein expression of the inducible form of cyclooxygenase, COX-2 (Fig. 16.5).^{49,50}

In addition, we have shown that these changes contribute to the decline in T-cell-mediated function and IL-2 production by T-cells from aged animals.^{47,48} Furthermore, the immunostimulatory effect of vitamin E on T-cells from aged mice is due in part to a reduction in the PGE₂ levels from M ϕ of old mice (Fig. 16.6).^{48,50}

The vitamin E-induced decrease in PGE₂ production is mainly mediated through inhibition of M ϕ COX-2 activity.⁵⁰ The effects of vitamin E on COX-2 activity were thought to occur at a post-transcriptional level since no effect of vitamin E on COX-2 mRNA and protein levels was observed.⁵⁰ We recently showed that the post-transcriptional effect of vitamin E on COX-2 occurs through a reduction of nitric oxide (NO $\cdot$) resulting in a decreased

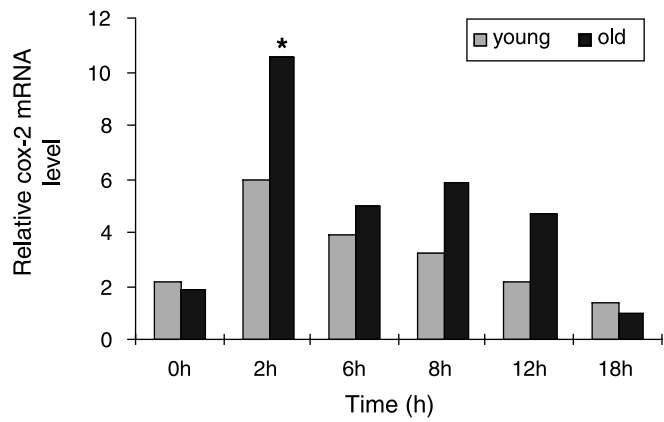


FIGURE 16.5
Relative COX-2 mRNA levels in macrophages (M ϕ) from young and old C57BL/6NIA mice. M ϕ from young (6 months) and old (24 months) were stimulated with 5 μ g/ml LPS at the times indicated and mRNA levels were detected by RNase protection assay. The relative values were calculated based on the expression of control β -actin mRNA using densitometry. Values are the mean of three independent experiments for 0, 2, 6, and 8 h. The other time points represent values from a single experiment. The standard deviations for 0, 2, 4, 6, and 8 h are 0.7, 0.6, 4.9, and 0.6 in young, and 0.2, 1.7, 4.8, and 2.9 in old, respectively. (From Hayek et al., *J. Immunol.*, 159(5), 2445, 1997. With permission.)

* Significantly higher than young mice at $p < 0.03$.⁴⁹

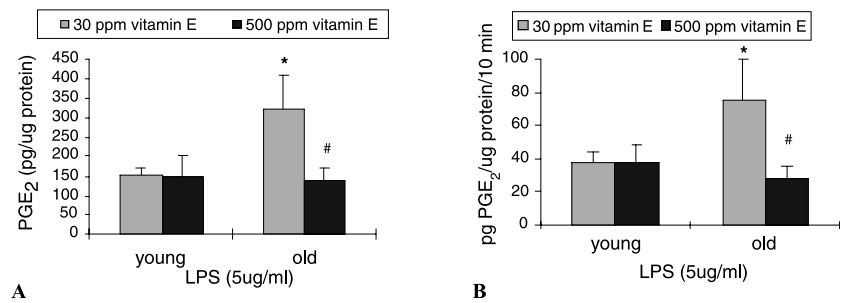


FIGURE 16.6
Effect of vitamin E on the production of accumulated PGE₂ (A) and on the activity of COX-2 (B) from peritoneal M ϕ isolated from young and old mice. Young (6 months) and old (24 months) mice were fed semisynthetic diets containing adequate (30 ppm) or high (500 ppm) dl- α -tocopheryl acetate for 30 days. Peritoneal M ϕ were isolated and cultured in the presence of 5 μ g/ml LPS for 24 hours at 37°C. Supernatants were collected for measurement of accumulated PGE₂ production and COX activity in the presence of substrate arachidonic acid (AA). (From Wu et al., *Am. J. Physiol.*, 275, 2449, 1998. With permission.)

* Significantly higher than young mice fed 30 ppm vitamin E at $p < 0.05$. # Significantly lower than old mice fed 30 ppm vitamin E at $p < 0.05$.⁵⁰

formation of peroxynitrite.⁵¹ Moreover, we, as well as others, have shown that M ϕ from old mice have a significantly higher production of NO $\cdot$.⁵¹ Other researchers have also found that the inducible nitric oxide synthase (iNOS) expression from murine M ϕ is markedly decreased upon addition of vitamin E *in vitro*.^{52,53}

The effects of oxidative stress on the transcriptional events occurring during various immune responses, although extensive, still remain controversial. For instance, one study has demonstrated that oxidation promotes a lower DNA-binding activity of the nuclear transcription factors NFAT and NF κ B while increasing transactivation of the transcription factor AP-1 in T-lymphocytes.⁴² In contrast, various oxidants have been shown to induce the production and secretion of prooxidative cytokines such as IL-1, IL-6, and TNF α from phagocytes in response to inflammatory stimuli mainly via activation of NF κ B.⁵⁴ Another study has shown that vitamin E derivatives can decrease elevated levels of NF κ B in TNF- α -stimulated Jurkat T-lymphocytes in a concentration-dependent manner.⁵⁵ Transfection studies using macrophagic TR-1 cells (derived from the THP-1 cell line) have shown that LPS-stimulated transfectants carrying the TNF- α gene promoter demonstrate decreased transcription of this gene upon culture with vitamin E succinate (50 μ M concentration).⁵⁶ Gel shift assays in this study showed that NF κ B DNA-binding activity of the stimulated transfectants decreased in the presence of the antioxidant. Discrepancies in NF κ B binding across these studies might be explained by variations in culture conditions as well as by the biological nature and/or combination of oxidants or stimuli used.

As indicated in 16.1.2, another gene whose transcription is altered by oxidation and which is downmodulated by vitamin E is the scavenger receptor type A in M ϕ . Teupser et al.⁸ demonstrated that α -tocopherol (100 μ M) supplementation decreased the mRNA expression of the scavenger receptor SR-A as well as its activity by 60% in a dose-dependent manner. This effect was accompanied by reduced AP-1 DNA-binding activity in these cells. γ -Tocopherol in comparison demonstrated only a weak suppression of SR-A transcription.⁸

A study involving the mRNA expression of tissue factor (blood clotting factor) in U937 and THP-1 cells has also shown that vitamin E treatment (50 μ M α -tocopherol) of these cells substantially diminishes the elevated copper-induced tissue factor levels found in them.⁵⁷ These findings are interesting in that they corroborate the notion that monocytes are constantly exposed to oxidizing conditions, and that *in vitro* at least, antioxidants such as vitamin E can downmodulate at a transcriptional level the expression of various genes needed to embark on a rapid inflammatory response.

Increased cell–cell and cell–matrix interactions are hallmarks of transmigration events and leukocyte homing to vascular endothelium during inflammatory reactions. Interestingly, vitamin E (50 to 100 μ M) has been shown to block the adhesion of monocytes,^{58,59} Jurkat T-cells,⁶⁰ and other leukocytes to endothelial cells *in vitro*, and is correlated with the downmodulation of CD11b and VLA-4 adhesion molecule expression on leukocytes.⁵⁸

Similarly, in three independent studies, IL-1 or TNF- α -primed U937 monocytes showed decreased adhesion to human umbilical vein endothelial cells *in vitro* upon treatment with vitamin E succinate (20 to 60 μ M, 20 to 72 h) or α -tocopherol; this decrease was accompanied by a marked reduction in the monocyte expression of the VCAM-1, ICAM-1, and E-selectin adhesion molecules in a time- and dose-dependent manner.^{59,61,62} However, although it is tempting to speculate that these effects are due to decreases in NF κ B activation, experimental data showing the activation of NF κ B in this regard have not fully supported this hypothesis.⁶²

Impaired antioxidant defenses are associated with disease progression in HIV-infected individuals. Acquired immunodeficiency syndrome (AIDS), which is characterized by lymphadenopathy, reduced cytokine levels, and overall impaired immune function, is also associated with lower tissue vitamin E levels in mice and humans compared to noninfected hosts.^{63–66} Hence, the possibility of ameliorating the disease status of AIDS patients with vitamin E has been studied *in vitro* and *in vivo*.^{55,67,68} Indeed, vitamin E supplementation in a murine model of AIDS (via LP-BM5 murine leukemia virus infection) has shown to decrease the retrovirus-induced increase in mononuclear phagocyte IL-6 and TNF- α production.⁶⁴ Interestingly, a study conducted by Hirano et al.⁶⁹ has shown that a particular phosphodiester compound of vitamin C and vitamin E can inhibit NF κ B-dependent transcription of the HIV-1 promoter in TNF- α -treated human-cultured astrocytoma cells.

Vitamin E supplementation has become an area of therapeutic interest with regard to rheumatic diseases, which are characterized by changes in immune function. In the synovium of rheumatoid arthritis (RA) patients, transcriptional changes occur that reflect the genetic responses to a hypoxic/anoxic environment.⁷⁰ Changes in hypoxia-induced factor (HIF)-1 activation most directly involve the participation of various transcription factors and hypoxia responsive genes which ultimately play a significant role in the pathology of synovitis. The vascular endothelial growth factor (VEGF), the key to angiogenic and fibrinogenic events, is upregulated in the synovium of arthritic patients. Under hypoxic conditions, the hematopoietic factor erythropoietin is upregulated in the inflamed synovium as well. TGF- β , TNF- α , IL-1, IL-8, IL-6, NF κ B, AP-1, Sp-1, collagen, ELAM-1/ICAM-1, LFA-1, VCAM-1, VLA-4 CD11a/CD18, as well as COX-2 have demonstrated increased gene expression in the arthritic synovium.⁷¹

Furthermore, patients with RA exhibit local vitamin E deficiencies compared to control subjects (lower vitamin E levels in the joint), and although significant anti-inflammatory effects have not been demonstrated with vitamin E administration thus far, vitamin E does have an analgesic effect in inflamed joints that appears to be independent of its antioxidant properties.^{71,72}

Vitamin E might subvert inflammation by suppressing COX-2 activity. Indeed, COX-2 inhibitors are currently being tested in various animal models of experimental arthritis.⁷³ Another potential strategy for suppressing

chronic synovitis via vitamin E includes attempts to downregulate the transcription of genes such as TNF- α .

16.3.2 Vitamin E and Control of Cellular Proliferation and Cancer

Extensive *in vitro* and *in vivo* studies have demonstrated the efficacy of vitamin E (particularly α -tocopheryl succinate or α -tocopheryl acetate) in promoting the inhibition of cell growth and proliferation in various rodent and human cells.^{74,75}

Several mechanisms of action have been postulated to explain vitamin E's capability of reducing malignant cell growth. Overall, the effect of vitamin E results in a reduced expression of some transcription factors as well as reduced responses to specific growth factors, thus modulating the progression of cell-cycle events. While the exact biochemical reactions by which vitamin E modulates these pathways have not been completely delineated, it has been suggested that vitamin E's role in scavenging peroxy radicals contributes to phosphorylation events leading to distinct changes in kinase activities that affect gene transcription. For instance, increased activity of the cyclin-dependent kinase cdc2 (cdk1) resulting in increased expression of the p53 tumor suppressor gene have been observed.⁷⁶ As described below, vitamin E has also been implicated in the regulation of TGF- β and p21 genes. Inhibition of gene expression and activity of NFAT, NF κ B, or other Rel family members by vitamin E has also been noted in various human and rodent tumor cells.⁷⁴

One of the proposed mechanisms by which vitamin E subverts cell growth is via inhibition of PKC activity. Azzi and colleagues^{9,77} have shown that α -tocopherol and β -tocopherol inhibit cellular proliferation and PKC activity of Chinese hamster ovary cells (CHO). These effects, however, are not seen for HeLa cells, suggesting that there are different cell-specific pathways of cellular proliferation in which vitamin E can act. Such pathways are also likely to be differentially affected by the specific actions of the various tocopherol isoforms tested. Other important pathways where vitamin E has been shown to intervene with cell growth and proliferation involve decreases in upregulated adenylate cyclase activity, and decreased expression of the transcription factors c-myc, H-ras, and E2F.^{74,78} For example, vitamin E succinate has been shown to decrease c-myc and H-ras mRNA levels in B16 melanoma cells *in vitro*.⁷⁸ In addition, d- α -tocopheryl succinate (11.3 μ M) has been shown to inhibit growth and to downregulate N-myc and H-ras mRNAs in murine neuroblastoma cells (NBP2).⁷⁹

It has been established that prostaglandins are factors that can enhance cancer development and progression, albeit in a tissue-specific as well as prostaglandin-specific manner.⁸⁰ Once again, overexpression of COX-2 mRNA and protein has been observed in numerous cancer types. Vitamin E, by downregulating the production of accumulated prostaglandins, could potentially subvert the invasive growth of cancer cells. It is noteworthy that vitamin E seems to strengthen the cytotoxic effects of various

chemotherapeutic agents and NSAID on tumor cells when administered in synergistic combination.⁸⁰ Described below are several examples of the experimental evidence found for the effects of vitamin E on several types of cancer *in vitro*.

16.3.2.1 Breast cancer

Studies using the estrogen receptor negative (ER-) BT20 human breast cancer cell line showed that vitamin E decreased both the phosphorylation and the transactivation of the transcription factor E2F1 which is involved in cell cycle regulation.^{81,82} Moreover, changes in increased cyclin A binding to E2F1 (which negatively regulates E2F1 function) as well as increased binding of p21 to cyclin A and to the cyclin-dependent kinase 2 (cdk2) were found, demonstrating that vitamin E is capable of activating signal transduction pathways that can prevent cell cycle arrest. In addition, these investigators showed that vitamin E could inhibit the transcriptional activation of the E2F1-responsive gene, *c-myc*.^{81,82}

Yu et al.⁸³ reported that increases in AP-1 transactivation, prolonged c-jun N-terminal kinase (JNK) and c-jun activation (as seen by mRNA and protein levels) in MDA-MB-435 breast cancer cells can occur upon treatment with α -tocopheryl succinate. These changes paralleled the increased apoptotic events observed in the cancerous cells with vitamin E treatment.^{84,85} Studies with this cell line using α -tocopheryl succinate have also revealed increased mRNA and protein expression of the cytokines TGF- β 1, TGF- β 2, TGF- β 3 as well as of the TGF- β type II receptor.⁸⁶ In addition, vitamin E succinate has been shown to inhibit cellular proliferation in a dose-dependent manner and to induce apoptosis of reticuloendotheliosis virus-transformed avian lymphoid cells, RECC-UTCA-1, *in vitro*. These changes are paralleled by decreased c-myc mRNA levels.^{87,88} Using the ER+ human breast cancer cell line MCF-7, vitamin E succinate (19 μ M) has been shown to arrest DNA synthesis, inhibit cell proliferation, and induce apoptosis. The antioxidant facilitated a persistent expression of c-jun mRNA and protein levels, as well as elevated AP-1-binding activity, suggesting that c-jun is involved in the apoptotic process induced in these cells upon vitamin E treatment.⁸⁹ Interestingly, a recent report showed that vitamin E exerts its inhibitory effect on breast cancer cell growth irrespective of ER status.⁹⁰

Moreover, Yu et al.^{84,85} have also demonstrated that treatment of murine EL4 T lymphoma cells with α -tocopheryl-succinate (20 μ g/ml) can induce cells to undergo apoptotic cell death *in vitro* (95% cell death) as seen by DNA fragmentation analysis. Concomitant with these changes, reduced c-myc mRNA expression and increased bcl-2, c-fos, and c-jun mRNA levels have been observed 3 to 6 hours post-vitamin E treatment.^{84,85} Additionally, gel mobility assays have shown increased AP-1 DNA-binding activity and decreased c-myc DNA-binding activity 12 to 24 hours post-vitamin E treatment.^{84,85}

16.3.2.2 Colorectal Cancer

In colorectal cancer biopsies, increased ratios of arachidonic acid to eicosapentaenoic acid in mucosal tissues have been observed in diseased patients relative to controls.⁹¹ These increased ratios are associated with the progression of adenomas to carcinomas and reflect increased substrate availability for PGE₂ synthesis. As mentioned previously, increased PGE₂ has been reported in colorectal adenomas and carcinomas. Similarly, COX-2 transcription has been shown to be elevated in most human colorectal cancers.^{92,93} It is interesting that such changes in tissue fatty-acid expression reflect changes that could be caused by differences in fatty acid utilization to generate eicosanoids and could contribute to the development of tumor malignancy. It has been postulated that changes in arachidonic acid metabolism may stimulate cell proliferation via activation of PKC and other kinases.^{94,95}

These observations strongly implicate COX-2 in the induction or progression of the malignant state of colorectal tumors, and suggest, furthermore, that PKC might be one of the important signaling pathways through which certain tumors are initiated or maintained. Since, as mentioned earlier, vitamin E exerts an inhibitory effect on COX-2 activity, vitamin E might be beneficial in circumventing colon cancer pathogenesis and/or progression via inhibition of COX-2 activity.

Vitamin E is known to induce apoptosis of colorectal cancer cells. One suggested mechanism of action for this induction involves the activation of p21^{WAF1/CIP1} (cell-cycle inhibitor factor) and c/EBP β , in a manner that is independent of p53 activation.⁹⁶

16.3.2.3 Myelodysplastic Syndromes

Myelodysplastic syndrome (MDS) patients exhibit impaired granulopoiesis and thrombopoiesis, decreased blast-forming units–erythroid progenitor cells (BFU–Es) formation and decreased responses to erythropoietin and cytokines leading to anemia. Furthermore, functional defects in mature myeloid cells have been observed in 50% of patients. α -Tocopherol has been shown to have a protective effect on erythroid progenitor cells and BFU-E growth *in vitro* (increasing number and function of mature cells through α -tocopherol treatment) and might be coupled with retinoic acid therapy to improve benefits of the treatment.^{67,97} Retinoids have been shown to improve hematopoiesis and immune response against infection. Some of the effects of retinoids might be mediated through decreased gene expression and secretion of TNF- α in monocytes. Hence, it is plausible that α -tocopherol might also act in subverting myeloid defects by preventing hematopoietic failure in MDS patients via a similar mechanism of TNF- α downregulation. One study has reported a strong synergism of all trans-retinoic acid (25 to 45 mg/m²/d), G-CSF (granulocyte-colony stimulating factor) (1 mg/kg/d), erythropoietin (500 U/d), and α -tocopherol (400 mg/d) in ameliorating the number and function of hematopoietic cells of MDS patients. This study demonstrated that the combination therapy improved white blood cell counts significantly

in an 8-week period, increasing hemoglobin levels in all patients, and reducing levels of TNF- α expression in a subgroup of patients.⁹⁷

16.3.3 Vitamin E and Cardiovascular Diseases

Epidemiological studies have shown an inverse relationship between coronary artery diseases and plasma vitamin E levels,^{98–100} but the precise mechanism underlying vitamin E's beneficial effects has not been elucidated fully. However, a broad range of benefits, such as promoting and modulating vascular function, has been proposed for vitamin E.

The most widely studied model of vitamin E's protective effects in atherosclerosis involves protection against low-density lipoprotein (LDL) oxidation. Vitamin E, present in the LDL as well as in the extracellular fluid of the arterial wall, protects LDL against oxidative modification. Vitamin E incorporated into subendothelial spaces also protects LDL from oxidative modification by resident vascular cells such as smooth muscle cells, endothelial cells, and M ϕ . Under conditions of increased oxidative stress and in the absence of adequate antioxidant protection, accumulation of oxidized LDL (ox-LDL) occurs, resulting in monocyte chemotaxis, inhibition of M ϕ egress, foam cell formation, and endothelial dysfunction.¹⁰¹ In addition, ox-LDL itself induces endothelial expression and secretion of cytokines, growth factors, and several cell surface adhesion molecules, which result in the recruitment of circulating monocytes into the intima where they differentiate into M ϕ and foam cells. In response to growth factors, smooth muscle cells proliferate in the intima, resulting in the narrowing of the lumen. Moreover, oxidized LDL can inhibit endothelial production of nitric oxide, a potent vasodilator.¹⁰² Thus, vitamin E can protect against the development of atherosclerosis by retarding LDL oxidation, and inhibiting the proliferation of smooth muscle cells and monocyte adhesion to endothelial cells.

Moreover, vitamin E has been shown to prevent ox-LDL-induced endothelial cell dysfunction by inhibiting PKC activation.¹⁰³ Phorbol 12-myristate 13-acetate (PMA)-induced activation of platelet aggregation can be blocked by vitamin E supplementation via inhibition of PKC-dependent protein phosphorylation.¹⁰⁴ In addition, calphostin C, a potent inhibitor of PKC, has been shown to attenuate ox-LDL-induced inhibition of endothelium-dependent relaxation.¹⁰⁵ Furthermore, PKC can also be activated via increased DNA-binding activity of NF κ B in human endothelial cells.¹⁰⁶

Thus, vitamin E-induced modulation of PKC appears to play an important role in gene expression involved in endothelial dysfunction. However, the inhibitory effects of vitamin E on PKC have not been observed in some studies. For example, in human endothelial cells, vitamin E was shown to inhibit agonist-induced monocyte adhesion in a time- and concentration-dependent manner.⁶² This inhibition was correlated with a decrease in steady-state levels of E-selectin mRNA and cell surface expression of E-selectin. Inhibition of E-selectin was not due to PKC activation, as vitamin E treatment did not

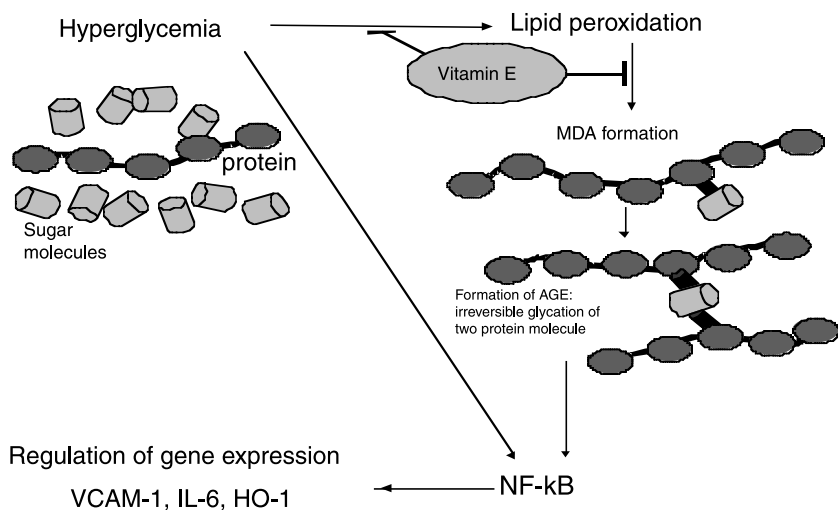
suppress phosphorylation of PKC substrates. In addition, activation of the transcription factor NFκB was reported to be unnecessary for E-selectin expression since electrophoretic mobility shift assays failed to show vitamin E-induced decreases in activation of this transcription factor.⁶²

16.3.4 Vitamin E and Diabetes

The classic markers of oxidative stress, e.g., lipid peroxides, oxidized LDL, conjugated dienes, superoxide anion, and isoprostane, are elevated in diabetes. Although the exact mechanism of oxygen-derived reactive oxygen species (ROS) generation in diabetes has not been clearly defined, elevated glucose levels appear to contribute greatly. Hyperglycemia, a classic symptom of diabetes, has been shown to increase autooxidation of glucose, to increase advanced glycation end products (AGE), and to induce NADPH-oxidase activity. AGE are formed during non-enzymatic glycation and oxidation (glycoxidation) reactions by binding of sugar molecules (aldoses) onto free amino groups of proteins or lipoproteins. This process is accelerated in diabetics due to their elevated levels of glucose (hyperglycemia) and has been implicated in the pathogenesis of diabetic vascular complications. For example, treatment with AGE has been shown to increase vascular endothelial growth factor (VEGF) mRNA with a concomitant development of retinal vascularization resulting in diabetic retinopathy.¹⁰⁷ Receptors for AGE (RAGE) are present on the cell membrane of endothelial cells, and blockade of RAGE by specific antibodies can correct the vascular complications observed in diabetics. AGE treatment can induce endothelial cell (EC) oxidant stress, including the generation of thiobarbituric acid reactive substances (TBARS) and activation of NFκB. NFκB has been shown to mediate AGE-induced expression of IL-6 mRNA in human bone-derived cells.¹⁰⁸ Moreover, AGE have been suggested to increase endothelial expression of adhesion molecules such as VCAM-1 by inducing oxidative stress with subsequent activation of nuclear transcription factor NFκB.¹⁰⁹

Glycation of hemoglobin (G-Hb) in red blood cells (RBC) has been shown to increase significantly in the presence of H₂O₂ as well as with higher glucose concentrations. This increase in G-Hb can be blocked when RBC are pre-treated with vitamin E. Vitamin E can also inhibit the formation of malondialdehyde (MDA), an end product of lipid peroxidation. Furthermore, treatment of RBC with MDA, or with a combination of glucose and MDA, has shown higher G-Hb formation with glucose-MDA in contrast to glucose alone.¹¹⁰ In addition, hyperglycemia enhances lipid peroxidation and increases MDA accumulation.¹¹¹

Collectively, these findings suggest that both hyperglycemia as well as hyperglycemia-induced lipid peroxidation leading to an increased formation of MDA can stimulate glycation of proteins in diabetes. Vitamin E is capable of blocking glycation of proteins by inhibiting MDA formation¹¹⁰ (Fig. 16.7).

**FIGURE 16.7**

Hyperglycemia-induced protein glycation. Increased levels of glucose induce lipid peroxidation, and lipid peroxidation produces MDA.¹¹⁰ MDA stimulates glycation of proteins leading to altered gene expression, which is mediated primarily through NFκB.¹¹² Hyperglycemia also directly results in activation of NFκB.¹¹³ Vitamin E blocks the glycation of proteins by inhibiting the formation of MDA and consequently blocking hyperglycemia-associated complications.

16.3.5 Role of Vitamin E in the Reversal of Renal and Hepatic Tissue Damage

16.3.5.1 Renal Injury and Immunoglobulin A (IgA) Nephropathies

It has been established that diets deficient in vitamin E promote renal enlargement, proteinuria, mild tubulointerstitial disease, and a reduced glomerular filtration rate.¹¹⁴ The mechanism of renal tissue damage is correlated to oxidative stress and increased net DNA content and synthesis in the kidney, mainly in the renal tubular epithelium. Gene transcription in damaged renal tissue is upregulated for various transcription factors, cytokines, and extracellular matrix factors. For instance, a study in which rats were fed a vitamin E-deficient diet revealed that mRNA levels were increased for factors such as c-myc, the histone H2b, TGF-β1, and collagens I, III, and IV.¹¹⁴ Moreover, glutathione peroxidase mRNA expression in the kidney was also decreased with the vitamin E-deficient diet. The induction of such factors upon vitamin E deficiency in the kidney corresponds to those typically observed with oxidative stress.¹¹⁴

In vitro studies have also shown that the genes for the previously listed factors are upregulated in rodent kidney mesangial cells exposed to non-cytolytic doses of hydrogen peroxide.¹¹⁴ Interestingly, in the rats fed the vitamin E-deficient diet, whole kidney catalase mRNA levels were reduced, suggesting the involvement of an additional mechanism by

which clearance of hydrogen peroxide with vitamin E deficiency can be compromised in the kidney.¹¹⁴

These findings suggest that reducing the prooxidant status of renal tissue with vitamin E supplementation might lead to downregulated transcription of various oxidative stress-induced genes, thus potentially lowering tissue damage in the kidney.

Reactive oxygen species (ROS) suppress *in vitro* synthesis of erythropoietin. Vitamin A supplementation has been shown to increase erythropoietin synthesis in renal and hepatic tissue.¹¹⁵ One study demonstrated that erythropoietin expression could be increased significantly relative to control tissue in a rat model of reperfused hypoxic kidney when vitamin A (retinol acetate, 0.5 µg/ml), vitamin E (α-tocopherol, 0.5 µg/ml), and vitamin C (10 µg/ml) were added in combination for 24 h.¹¹⁵ In hepatoma cell cultures, while vitamin A specifically increased erythropoietin production, neither C nor E (0.05 to 500 µg/ml), when added separately, increased erythropoietin gene expression. This study suggested a strong potential for vitamins E and C to protect cells from oxidative damage but demonstrated that vitamin A was specific in stimulating erythropoietin production.¹¹⁵

In various animal species, including rodents, TNF-α levels and IL-1 renal dysfunction due to sepsis or ischemia-reperfusion injury are dramatically increased from Mφ and renal mesangial cells.^{116,117} Such cytokines promote kidney parenchymal damage either directly via apoptosis, or by recruitment of neutrophils, which release ROS and proteases.¹¹⁶ In such models of ischemia-reperfusion, mesangial cells have been shown to exhibit an increased activation and transcription of TNF-α, hypoxia-induced factor (HIF)-1, p38 MAPK, and JNK.^{115,116,118} It has been speculated that inhibition of TNF-α production by vitamin E supplementation could be considered a therapeutic strategy to ameliorate oxidative injury in the kidney.¹¹⁶

In IgA nephropathy, it has been shown that IgA-containing immune complexes stimulate the production of oxygen free radicals in mesangial cells *in vitro*.¹¹⁹ Studies conducted by Kuemmerle et al.^{120,121} and by Trachtman et al.¹¹⁹ have shown that α-tocopherol (100 IU/kg diet) can reduce proteinuria in experimentally induced IgA nephropathy (from bovine γ-globulin injection) in rats as early as 4 weeks of treatment and by as much as 50%. Such studies revealed that reduced renal plasma flow also was restored to normal levels upon vitamin E treatment. In addition, TGF-β mRNA expression, which was elevated with proteinuria and IgA deposition in the renal mesangium, was significantly decreased between 4 and 8 weeks of vitamin E administration. Furthermore, lipid peroxidation of LDL has also been associated with tissue damage in glomerulosclerosis. It has been shown that an important mechanism by which this might occur involves upregulation of collagen gene expression by LDL (including collagen IV) in mesangial cells. Vitamin E (at a 50 µM dose) markedly downregulated collagen mRNA expression from cultured human mesangial cells exposed to LDL.¹²² Additionally, in a rat model of reflux pyelonephritis, vitamin E (24 IU/kg/day) has been proven effective in decreasing the degree of inflammation of tissue as assessed histologically.¹²³

16.3.5.2 Liver Injury

In a study by Pietrangelo et al.¹²⁴ the anti-fibrinogenic properties of vitamin E were tested on gerbils to assess the molecular pathways involved in iron-induced hepatic fibrosis. With administration of an α -tocopherol-enriched diet (250 mg/kg) over 4 months, animals treated with high doses of iron (1 mg/g) showed significant decreases in collagen mRNA levels, despite exhibiting persistent liver damage. Although small, reduced levels of accumulated TGF- β mRNA produced by M ϕ and Kupffer cells were also observed.

Nonparenchymal cell proliferation (macrophagic population and fat storing cells) was arrested in contrast to control animals. Iron-dosed animals not supplemented with vitamin E developed severe hepatic cirrhosis. This study demonstrated that vitamin E supplementation could prevent liver cirrhosis by arresting fibrinogenesis during experimentally induced hepatic fibrosis by arresting collagen-mediated nonparenchymal cell proliferation.¹²⁴

Acute liver damage in the rat can also be induced by intragastric administration of carbon tetrachloride (CCl₄).¹²⁵ In this experimental model, increased MCP-1 gene expression could be detected as early as 12 h, peaking at 48 h after lipid peroxidation events had initiated. Increased expression of MCP-1 could be detected prior to monocyte/M ϕ infiltration. Vitamin E supplementation was shown to decrease MCP-1 mRNA as well as protein levels. These changes paralleled decreased numbers of infiltrating M ϕ in the liver. The results suggested a significant role for MCP-1 in the recruitment of inflammatory cells during liver injury, and that these events could be subverted by subsequent administration of vitamin E.

Another report has demonstrated that increased DNA-binding activation of AP-1 from lipid peroxidation induced by CCl₄ can be completely blocked in the liver by treatment with α -tocopherol in rats.¹²⁶ Interestingly, in the inflammatory events taking place in hepatic cholestatic injury, NF κ B and TNF- α gene expression have been shown to be upregulated in M ϕ .¹²⁷ Current investigations are focusing on the ability of vitamin E to downmodulate these genes during hepatic injury.

TGF- β 1 and pro-collagen type I gene expression are also upregulated by a prooxidant status in the liver, and are considered hallmarks of liver fibrosis. Expression of these genes has been downregulated with long-term treatment of vitamin E *in vivo* in the rat.^{128,129} In addition, vitamin E treatment of hepatic fat-storing cells has been shown to decrease mRNA expression of both genes *in rats*.^{128,129}

In another report, long- and short-term supplementation with d- α -tocopherol (40 IU/day for 8 weeks or 450 IU for 48 h, respectively) of mice with hepatic injury has revealed a marked decrease in collagen- α 1 type I mRNA (70 and 60%, respectively).³⁵ Moreover, reporter assays using hepatic stellate cells to look at collagen gene expression have allowed the characterization of an α -tocopherol responsive element in the collagen gene, suggesting that vitamin E can mitigate oxidative stress in the liver by modulating collagen gene expression *in vivo*.³⁵

α -Tocopherol is metabolized in the liver with lipoproteins and is secreted into the plasma in conjunction with very low-density lipoprotein (VLDL) via the α -tocopherol transfer protein (α TTP). α TTP and its mRNA are expressed in lower levels in the plasma and liver of vitamin E-deficient rats relative to control rats, or rats given a vitamin E-enriched diet (5g/kg diet α -tocopheryl acetate).¹³⁰ In addition, it has been shown that α -tocopherol and δ -tocopherol are capable of inducing α -TTP mRNA in the liver of rats fed a vitamin E-rich diet (with either stereoisomer or with both in combination).^{7,131} Interestingly, decreases in α TTP mRNA have been detected as well in tumor modules of patients with early stages of hepatic carcinogenesis,¹³² suggesting that aberrant α TTP expression leading to vitamin E deficiency might lead to tumor development in the liver.

16.3.6 Vitamin E and Neurodegenerative Conditions

16.3.6.1 Ataxias

Among the inherited idiopathic ataxias, ataxia with isolated vitamin E deficiency (AVED) is an autosomal recessive disorder associated with varied neurological manifestations, including progressive ataxia, araflexia, impaired position sense, sensory loss, and pyramidal signs, and is often linked to cardiomyopathy.¹³³ The age of onset of this condition is 4 to 18 years of age. AVED has been linked to chromosome 8q13 in which mutations in the α TTP gene result in an impaired ability to incorporate α -tocopherol into lipoproteins that are secreted by the liver. Tamaru and colleagues have suggested that exon skipping of all transcripts for the α TTP gene results in impaired splicing mechanisms (complete inactivation of the splicing site) that lead to the inability of the α TTP protein to function correctly in the absorption of vitamin E, consequently contributing to the development of AVED ataxia.¹³⁴ α -Tocopheryl-acetate has been used therapeutically to arrest neurological loss in this as well as other ataxias.¹³³

Friedreich's ataxia is also an autosomal recessive disorder with an incidence of 1 in 50,000 individuals and is associated with DNA-triplet repeat expansions (GAA) localized to chromosome 9, where the frataxin gene has been mapped. It has been suggested that this type of ataxia might be a result of defects in vitamin E metabolism. In Friedreich's ataxia, genetic abnormalities in the frataxin gene often result in a loss of function of the frataxin protein, resulting in undetectable or low levels of frataxin mRNA.¹³⁵ The function of the frataxin protein is not completely understood but it is thought that reduced frataxin in the heart and spinal cord is the primary cause of neural degeneration in these patients.¹³³ Interestingly, in a study conducted by Yokota et al.,¹³⁶ four individuals with Friedreich's-like ataxia accompanied by retinitis pigmentosa and with isolated vitamin E deficiency were tested. Although absorption of vitamin E was found to be normal in all four cases, decreases in serum levels of vitamin E were markedly worsened, and were associated with a specific point mutation observed in

the α TTP gene, suggesting that the degree of adequate or inadequate vitamin E absorption in these individuals was dependent on the severity of genetic defects affecting the α TTP gene.¹³⁶

16.3.6.2 Amyotrophic Lateral Sclerosis and Other Neuronal Abnormalities

Amyotrophic lateral sclerosis is a high-incidence motor neuron disease characterized by progressive neurodegeneration and cell death of lower motor neuron groups in the spinal cord and brain stem, and also of upper motor neurons in the motor cortex. The result of such neurodegenerative effects includes progressive muscle weakness and wasting. Vitamin E has been shown to retard the onset of motor neuron disease where oxidative stress contributes to the early stages of motor neuron injury.¹³⁷

In a study conducted by Ghadge et al.,¹³⁸ mutant superoxide dismutase genes (SOD) were expressed in neuronal cells via adenoviral gene transfer and were shown to induce cell death in differentiated PC12 cells, superior cervical ganglion neurons, and hippocampal pyramidal neurons,¹³⁸ cells that displayed high rates of superoxide radicals. In contrast, cell death was prevented by administration of vitamin E (100 μ M dose), copper chelators, glutathione, Z-Val-Ala-Asp-(o-methyl)-fluoromethyl Ketone (ZVAD) and Tyr-Val-Ala-Asp-aldehyde (YVAD) caspase inhibitors, as well as by the constitutive expression of bcl-2.¹³⁸

Another study looking at mice with mutant Cu/Zn superoxide dismutase showed that neurodegeneration was slowed down markedly when these mice were cross-bred into transgenic strains overexpressing bcl-2 and thus inhibiting apoptotic pathways leading to neuronal cell death.^{137,139} These findings are relevant because downregulation of Cu/Zn SOD induces apoptosis in PC12 neuronal cells,¹⁴⁰ and furthermore, missense mutations in the SOD1 gene (leading to impaired enzymatic activity) have been shown to contribute to the neurodegenerative effects seen in human familial amyotrophic lateral sclerosis (20% of cases). Blocking SOD1 with antisense oligonucleotides has been shown to improve neuronal cell survival. In addition, treatment of dying cells with vitamin E clearly rescues the cells from apoptosis.¹⁴¹ Noteworthy is the observation that a combination of vitamin E and neural growth factor supplementation has proven to be most effective in blocking apoptosis of such cells.¹³⁸

The effects of vitamin E on gene expression of neuronal cells exposed to oxidative stress *in vitro* were examined in a study by Post et al.¹⁴² Clonal hippocampal HT22 cells were subjected to haloperidol (HP), a dopamine receptor antagonist that induces cell death by oxidative stress. As a result, intracellular glutathione levels were found to be reduced in parallel to increased peroxide levels, while the DNA-binding activity and transcription levels of NF κ B were also increased. Treatment with α -tocopherol (200 μ M) blocked NF κ B activation in reporter assays, and in addition, suppressed NF κ B DNA-binding activity.¹⁴² Moreover, overexpression of I κ B α

suppressed NF κ B transcription protecting the HT22 cells from neurotoxicity. Vitamin E thus partially protected the cells against HP oxidation, most likely by blocking lipid peroxidation events, and subsequently by suppressing HP-induced activation of NF κ B.¹⁴²

Finally, in an *in vitro* study by Noh et al.,¹⁴³ murine neuronal cortical cells subjected to zinc-mediated oxidative injury were rescued from neurotoxicity by administration of the vitamin E analog, Trolox (100 μ M).¹⁴³ Although not proven directly, a mechanism by which vitamin E suppresses oxidative damage of neuronal cells indicates an inactivation of PKC. PKC can become activated upon zinc influx, leading to intracellular calcium entry and neuronal death.¹⁴³ Vitamin E is known to inhibit PKC activation.¹⁴⁴ Vitamin E could function in a manner very similar to that of Trolox in such cells, downregulating PKC activity and/or other kinase pathways, and leading to a consistent block of cytotoxic events resulting in prevention of neural degeneration.

Noteworthy is that COX-2 gene expression has been shown to be upregulated in various models of brain peripheral inflammation and is associated with neurodegeneration.^{145,146} We have shown that vitamin E downregulates COX-2 activity in M ϕ .⁵⁰ Current investigations are under way to test the ability of COX-2 inhibitors and vitamin E to downregulate the expression and activity of cyclooxygenases potentially involved in promoting apoptosis and neurodegeneration of brain cells.

16.4 Conclusion

Vitamin E is the most effective lipid-soluble antioxidant known to protect plasma and intracellular membranes against the harmful effects of free radicals. Mounting evidence points to vitamin E as an important regulator of intracellular signal transduction, and thus, an important regulator of the expression of genes coding for key metabolic proteins. Most of vitamin E's effect in this regard is mediated through its control of oxidant-sensitive transcription factors. There is, however, some evidence that vitamin E might mediate its effect through a non-antioxidant, and as yet unknown, mechanism. Vitamin E regulation of gene regulation has implications for many biologic functions including those of vascular, neuronal, and immune systems as well as the diseases related to them. There is growing interest not only in free radicals, but also in predisposing genetic factors, and the roles these might play in increasing the susceptibility of individuals to acute and chronic diseases. The ability of vitamin E to regulate gene expression directly or indirectly under physiological and pathological conditions has great implications for determining the dietary requirement of this vitamin and strongly supports the need for further research in this area.

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Differential Regulation and Function of Glutathione Peroxidases and Other Selenoproteins

Xin Gen Lei

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17.1 Introduction

A dozen of selenium (Se)-dependent proteins identified in mammals (Table 17.1) share at least two distinct features. First, Se in the polypeptide is covalently bound in a moiety of selenocysteine encoded by thymine-guanine-adenine (TGA), normally a stop codon. Second, Se availability regulates expression of not only their protein and activity, but also their mRNA levels in cells or tissues. In prokaryotes, Se is incorporated into selenocysteine co-translationally. The process is directed by a special stem-loop structure of mRNA, requiring four unique gene products. Much less is certain about the eukaryotic Se-incorporation. The dramatic impact of Se status on selenoprotein gene expression and the differential responses of various selenoproteins to Se depletion or repletion are fascinating, although the mechanism of Se regulation and the physiological implication remain largely unclear. A comprehensive understanding of selenoprotein biosynthesis in eukaryotes would help us in tackling these problems, and gene-knockout mouse models provide us with unprecedented tools. Because cellular glutathione peroxidase (EC 1.11.1.9; GPX1) is the first identified,^{1,2} the most abundant,³ and the best-studied biochemical functional form of body Se, this chapter focuses on the expression and Se regulation of GPX1 and its three family members in comparison with those of other selenoproteins.

17.2 Characterization of Glutathione Peroxidases and Other Selenoproteins

The Se-dependent GPX family consists of GPX1, GPX2, GPX3, and GPX4.^{4–7} Regardless of the physiological relevance, all of these selenoperoxidases reduce H_2O_2 and hydroperoxides using reduced glutathione (GSH) *in vitro*. Only GPX4 efficiently reduces phospholipid hydroperoxides, and thus its activity is distinguishable from that of the others. In the routine assay, it is not possible to specify the precise contribution of the individual GPX enzymes to the total GPX activity using H_2O_2 as a substrate. However, total GPX activity is often interchangeably described as GPX1 activity because it numerically accounts for more than 90% of the total GPX activity in most tissues.³ The same analogy is also used in this chapter for simplicity. Structurally, GPX4 is a monomer of 19 kDa, the other three are tetramers with respective subunits of approximately 22 to 23 kDa. In addition, GPX3 is an extracellular glycoprotein, whereas the others are intracellular enzymes.

TABLE 17.1
Characterized Mammalian Selenoproteins

Name	Size (kDa)	Role	Distribution
<i>1. Glutathione peroxidase (GPX) family</i>			
GPX1 or cGPX (cytosolic, cellular, or classical GPX)	88	Antioxidative, GSH-dependent reduction of hydroperoxides	Ubiquitous
GPX2 or GPX-GI (gastrointestinal GPX)	88	GSH-dependent reduction of hydroperoxides	Mainly gastrointestinal tract
GPX3 or plasma GPX (extracellular GPX)	92	GSH-dependent reduction of hydroperoxides	Plasma and interstitial and extracellular space in lung, intestine, and kidney
GPX4 or PHGPX (phospholipid hydroperoxide GPX)	19	Reduction of phospholipid hydroperoxides	Ubiquitous, abundant in testis
<i>2. Iodothyronine 5'-deiodinases (ID)</i>			
ID1 (type 1)	28	Conversion of T ₄ to T ₃ , inactivation of T ₄ and T ₃	Brain, kidney, liver, and thyroid
ID2 (type 2)	30	Conversion of T ₄ to T ₃	Adipose tissue heart, muscle, pituitary, and thyroid
ID3 (type 3)	32	Inactivation of T ₄ and T ₃	Brain, placenta, and skin
<i>3. Thioredoxin reductases (TR)</i>			
TR1	110	NADPH-dependent reduction of thioredoxin	Ubiquitous
TR2	130	NADPH-dependent reduction of thioredoxin	Adrenal, heart, kidney, testis
TR3	114	NADPH-dependent reduction of thioredoxin	Ubiquitous
<i>4. Selenophosphate synthetase</i>			
	48	ATP-dependent activation of selenium for biosynthesis of selenocysteine	Ubiquitous
<i>5. Selenoproteins (Sel)</i>			
Sel-P	57	Unknown	Plasma
Sel-W	10	Unknown	Ubiquitous
15-kD Sel	15	Unknown	Ubiquitous

17.2.1 Cellular Glutathione Peroxidase (GPX1)

As mentioned above, GPX1 was the first mammalian selenoprotein, identified in 1972 by Rotruck et al.¹ who found that H₂O₂-dependent hemolysis is no longer prevented by glucose in erythrocytes from Se-deficient rats that have low GPX1 activity. Retrospectively, two important findings in 1957 by Mills⁸ on the protection of GPX against hemoglobin oxidation and by Schwarz and Foltz⁹ on the essentiality of Se against liver necrosis in rats conceived this milestone discovery in Se biology. It seems that GPX1 activity is detectable in various tissues of mammals and birds studied so far. The GPX1 gene cloned from mice^{10,11} is 5.2 kb and contains two exons and only one intron. There is more than 80% sequence identity in the coding region among various mammalian GPX1 genes.⁵ The human GPX1 gene localizes to a single site on chromosome 3.¹² In general, GPX1 proteins consist of 201 amino acids and the selenocysteine residual locates at the 47th residual from the N-terminal of the peptides.⁵ Approximately 75% of the GPX1 enzyme is found in cytosol, and the remaining 25% in mitochondria.¹³ Knockout of the GPX1 gene in mice results in the disappearance of this enzyme in both of the locations.¹⁴

The GPX1 enzyme does not form any ternary complexes with GSH and H₂O₂ and has no fixed K_m for either substrate.^{13,15,16} Because the liver GPX1 protein contains 60% of total Se in the tissue³ and its expression fluctuates so readily and widely with alterations of Se status, GPX1 has been suggested as a Se buffer to serve a homeostatic function in Se metabolism,^{5,17} instead of an antioxidant enzyme. However, we and others have generated solid evidence from the GPX1 knockout mice¹¹ that GPX1 is the mediator of body Se in protecting mice against acute, lethal oxidative stress.^{18–21}

17.2.2 Gastrointestinal Glutathione Peroxidase (GPX-GI or GPX2)

The GPX2 cDNA isolated by Chu et al.²² shares 61% sequence identity with GPX1. Initially, GPX2 was considered the main form of GPX activity in gastrointestinal tissues and played an important role in detoxifying hydroperoxides from digesta.²² A recent study with the GPX1 knockout mice indicates that GPX2 is expressed in the mucosal epithelium of intestines and contributes nearly the same portion to the total GPX activity as GPX1.²³ There is relatively little information on the regulation or physiological function of GPX2 expression *in vivo*.

17.2.3 Extracellular Glutathione Peroxidase (Plasma GPX or GPX3)

Although plasma GPX3 was initially considered the same enzyme as GPX1, these two enzymes are distinctly different in their activity response to Se supply, immunological property, specific activity, heat stability, and mobility in gel electrophoresis.^{24–26} The human GPX3 cDNA²⁷ encodes 226 amino acids and has selenocysteine at residue 73. The deduced peptide shows 44% homology with

human GPX1. The human GPX3 gene consists of five exons spanning approximately 10 kb and is localized in chromosome 5, q32.²⁸

The purified GPX3 has an apparent molecular mass of 92 kDa.²⁵ The hydrophobic core of signal peptide in rat GPX3 seems to be the first 19 deduced amino acids in the N-terminal.²⁹ Kidney is the primary site to produce GPX3 in humans and rodents.²⁹ Intestinal epithelia of both species³⁰ and a number of cell lines³¹ also express GPX3. Although *in vitro* GPX3 reduces peroxides and even phospholipid hydroperoxides,³² the low concentration of GSH in plasma seems to preclude its physiological action.²⁵ Probably, GPX3 protects against peroxides in the renal extracellular space,³³ lung epithelial lining fluid and interstitial space,³⁴ and intestinal intercellular space.³⁰ A wide antioxidant role of GPX3, including a possible role in embryogenesis, has also been speculated.³⁵

17.2.4 Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPX or GPX4)

The identification of GPX4 as the second intracellular GPX by Ursini et al.³⁶ was not fully recognized until the cloning of pig GPX4 cDNA.³⁷ There is 95% homology among amino acid sequences deduced from the GPX4 cDNA of rat,^{5,38} mouse,³⁹ and human.⁴⁰ In contrast, the homology between GPX1 and GPX4 is less than 40%. Both the pig (2.8kb) and mouse (4.0 kb) GPX4 genes contain seven exons and six introns, with putative regulatory elements or binding sites for transcriptional factors.^{41,42}

There are two forms of GPX4: the long form (23 kDa) with a leader sequence for transportation to mitochondria, and the short form (20 kDa) or the non-mitochondria form.⁴³ Although GPX4 reduces phospholipid hydroperoxides,⁴⁴ it has a relatively low rate of constant for H₂O₂. There are abundant GPX4 activity and mRNA in testis^{45,46} and rat epididymal spermatozoa,⁴⁷ indicating a possible involvement in sperm maturation.⁴⁸ Recently, Flohé, Ursini, and colleagues⁴⁹ discovered that GPX4 exists as a soluble peroxidase in spermatids, but loses its activity in mature spermatozoa and persists as an oxidatively cross-linked insoluble protein. In the midpiece of mature spermatozoa, GPX4 accounts for 50% of the capsule material and is the protein previously called sperm mitochondria capsule selenoprotein.

17.2.5 Other Selenoproteins

Three types of iodothyronine deiodinases are identified as selenoenzyme.⁵⁰⁻⁵² These enzymes regulate the activity level and distribution of thyroid hormones by catalyzing step-wise deiodination. Three types of thioredoxin reductases (TR)⁵³⁻⁵⁵ contain selenocysteine as an additional redox center. These pyridine nucleotide-disulfide oxidoreductases are homodimers of 55 to 57 kDa subunits containing (flavin adenine dinucleotide) FAD.⁵⁴ A human selenophosphate synthetase, equivalent to selenophosphate synthetase

(SELD) of *E. coli*, also contains selenocysteine encoded by TGA.⁵⁶ Selenoprotein P (Sel-P) contains ten TGAs in the open reading frame of the cloned cDNA.⁵⁷ Multiple forms of Sel-P are found in rat plasma^{58,59} and account for 65% of the plasma Se.⁶⁰ It has been localized adjacent to endothelial cells⁶¹ and found to be able to reduce 1-palmitoyl-2-(13-hydroperoxy-cis-9,trans-11-octadecadienoyl)-3-phosphatidylcholine hydroperoxide,⁶² implying a GPX4-like antioxidant function in extracellular fluids. Selenoprotein W (Sel-W) has a molecular mass of 9.5 to 10 kDa.⁶³ The cDNA isolated from rat skeletal muscle contains 672 bases and the selenocysteine codon, TGA, is in the position corresponding to amino acid residual 13.⁶⁴ It exists in all the tissues tested and may relate to white muscle disease in Se-deficient animals. A prominent ⁷⁵Se-labeled, 15 kDa protein detected in human T-cells is also considered a new selenoprotein.⁶⁵

17.3 Differential Regulation of GPX1 and Other Selenoproteins by Selenium

Numerous studies have demonstrated that expression of GPX1 and other selenoproteins is affected by Se status in animal tissues or cultured cells.^{4–6} Animals fed Se-deficient diets for several weeks exhibit undetectable liver GPX1 activity^{66–68} and a rapid exponential decline of liver GPX1 protein.⁶⁹ More strikingly, GPX1 mRNA falls dramatically in Se deficiency, as reported by all groups^{70–73} except for one.⁷⁴ Apparently, Se is not used for the transcription of GPX1 mRNA. Instead, Se deficiency must exert its impact on the steady-state levels of GPX1 mRNA through regulatory pathways. In nearly all cases, GPX1 expression is modulated by Se supply more than that of any other selenoprotein and thereby is considered to be differentially regulated by Se.

17.3.1 Comparisons of GPX1 with Other GPX Enzymes

A striking difference in Se regulation of GPX1 and GPX4 expression has been observed by Weitzel et al.⁷⁵ in mice and by Lei et al.⁴⁶ in rats. While mouse liver GPX1 activity falls to near zero within 130 days of Se depletion, liver GPX4 retains 30% residual activity. In Se-deficient rats, GPX1 activity and mRNA are reduced to 1 and 6% of the Se-adequate level in liver, respectively, whereas GPX4 activity is reduced only to 25 to 50% in various tissues. Liver GPX4 mRNA is not significantly affected. Testis has 15-fold higher GPX4 activity and 45-fold higher GPX4 mRNA than liver, and testis GPX4 mRNA is almost completely resistant to Se deficiency. Similar effects of Se on GPX1 and GPX4 expression have also been shown in other studies.^{76–78} In general, GPX3 activity in plasma and GPX3 mRNA

in tissues respond to Se repletion quicker than those of GPX1. In patients with low Se and GPX activity in plasma and erythrocytes, supplementing Se for up to 2 weeks does not enhance the low GPX1 activity in erythrocytes,²⁴ but produces detectable increases in plasma GPX3 activity within 6 hours.⁷⁹ In three human cell lines, GPX3 mRNA is also more resistant to Se deprivation than that of GPX1.³¹ Likewise, GPX2 seems to have a higher mRNA stability in Se deficiency and a faster protein synthesis rate upon Se repletion than that of GPX1 in cultured cells.⁸⁰ Although more research is needed to rank the Se-dependence of GPX2 expression *in vivo*, it seems clear that Se availability affects the expression of GPX1 most, GPX4 least, and GPX3 in between. The overall relative reduction of different GPX mRNA or activity levels by dietary Se deficiency in mice, summarized from six major studies conducted by us during last few years, follows the exact same pattern (Fig. 17.1).

17.3.2 Comparisons of GPX Enzymes with Deiodinases

Distinct patterns of GPX1, GPX4, and type-1 iodothyronine 5' deiodinase (ID1) expression exhibit in three tissues of rats fed diets containing Se from 0.003 to 0.405 mg/kg.⁷⁷ In liver, severe Se deficiency produces losses of GPX1 activity and mRNA by nearly 100%, GPX4 activity by 75%, ID1 activity by 95%, and ID1 mRNA by 50%. In heart, GPX1 mRNA and activity are reduced by Se deficiency to the same extent as in liver, while GPX4 activity

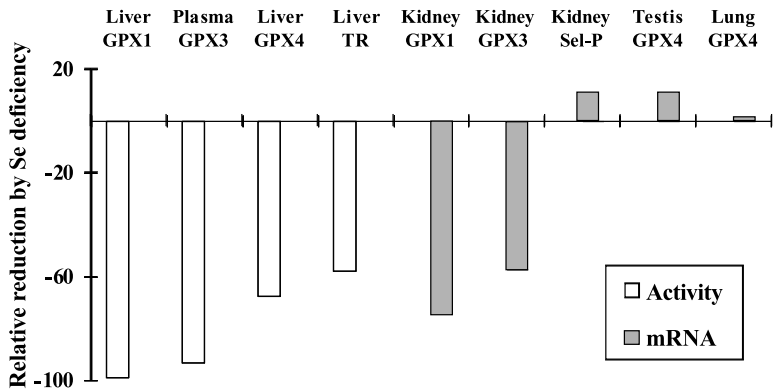


FIGURE 17.1 Relative reduction of mRNA and activity levels of GPX enzymes and other selenoproteins by dietary selenium deficiency in various mouse tissues. Data were collected from six experiments conducted in the author’s laboratory during the past 4 years.^{3,18–20,127,128} A total of 223 young or adult mice (3 weeks to 2 months of age) were fed a selenium-deficient (0.02 mg/kg) or a selenium-adequate (0.3 to 0.5 mg/kg) torula yeast diet for 5 to 13 weeks. Tissues were collected for different assays after these animals were anesthetized with carbon dioxide and killed by exsanguination. The reduction in mRNA or activity levels in the selenium-deficient animals is expressed as the percentage of the pertinent selenium-adequate controls within each individual experiment and then pooled among the six experiments.

is reduced only 60%. In thyroid, Se deficiency causes increases of ID1 activity by 15% and ID1 mRNA by 95%, no change in GPX4 activity, and significant decrease in GPX1 activity. Levels of GPX4 mRNA are not affected by Se deficiency in liver or heart, but increased 52% in thyroid. These data clearly indicate a tissue specificity of differential regulation of GPX enzymes from that of ID1.

In second generation Se- or iodine-deficient rats, Se deficiency results in changes of GPX1, GPX4, and ID1 expression in liver and thyroid similar to or slightly different from those seen in the first generation rats.⁸¹ When iodine deficiency induces mRNA of the three enzymes from 2- to 5-fold in thyroid, only GPX1 and ID1 activities are increased. In contrast, GPX4 activity is decreased. In the combined deficiency of Se and iodine, ID1 activity is increased, GPX1 activity is unaltered, and GPX4 is decreased while mRNA levels of all three enzymes are increased. Thus, iodine deficiency may act as an oxidative stress in thyroid to induce mRNA expression of selenoenzymes at limited Se supply, and Se is preferentially used by ID1 and GPX1 to maintain thyroid function. Iodine deficiency also increases brain type-2 ID or ID2 activity by up to 3-fold in 4 to 11 day old rats, regardless of Se status.⁸² However, Se or iodine deficiency has little effect on the expression of these three enzymes in most brain regions studied.⁸³ At excessive Se supply (2 mg/kg), hepatic ID1 and GPX1 activities are not elevated in rats.⁸⁴ A lower dietary Se level is required to maximize tissue ID1 activity than that of GPX1.⁸⁵

Differential regulation of ID1 and GPX1 expression has also been illustrated in cultured porcine kidney epithelial cell line LLC-PK1.⁸⁶ Thyroid-stimulating hormone induces a larger increase in ID1 expression in Se-deficient FRTL-5 cells than that in the Se-repleted cells, indicating an interaction of Se and hormone in the regulation of selenoenzyme expression.⁸⁷

17.3.3 Comparisons of GPX Enzymes with Selenoprotein P

Plasma concentration of Sel-P falls at approximately the same rate as GPX3 activity in rats fed a Se-deficient diet.⁸⁸ When rats are fed graded levels of dietary Se from 0.01 to 2.0 mg/kg, the elevations of plasma Sel-P concentration precede those of plasma GPX3 and liver GPX1 activities and reach a plateau at a lower level of Se. The GPX enzymes do not seem to respond to an injection of Se as strongly as Sel-P.⁸⁹ When an intraperitoneal injection of Se at 50 µg/kg body weight in Se-deficient rats enhances Sel-P from 7 to 43% of the Se-adequate control level by 12 hours, there is only a 2 to 3% increase in liver GPX1 and plasma GPX3 activities. In Se-deficient rats, Sel-P mRNA is 19% of the control while GPX1 mRNA is only 3% of the control.⁷¹ There is also a tissue dependence of differential regulation of Sel-P from GPX1 or ID1.⁶⁶ When Se deficiency reduces rat liver GPX1 mRNA by 89%, abolishes GPX1 activity, and reduces ID1 mRNA and activity by 69 and 70%, respectively, Sel-P mRNA is lowered by only 14%. Thus, Sel-P is

more resistant to Se deprivation than GPX1 or ID1 in rat liver. However, Sel-P mRNA is decreased more by Se deficiency than that of ID1 mRNA in rat kidney. Similar resistance of Sel-P expression to dietary Se deficiency has also been illustrated in our recent mouse studies (Fig. 17.1). Compared with the Se-adequate controls, the Se-deficient mice have slightly increased levels of Sel-P mRNA in kidney, just as those of GPX4 mRNA in lung and testis, while GPX1 and GPX3 mRNA levels in kidney are reduced by 74 and 57%, respectively.

17.3.4 Comparisons of GPX Enzymes with Selenoprotein W

Differences in Se regulation of the Sel-W protein from that of the GPX activities in several tissues have been illustrated in rats fed diets containing Se ranged from 0.004 to 4.0 mg/kg.⁹⁰ The GPX1 activity is saturated at 0.1 mg Se/kg in the brain, and at 0.06 mg Se/kg in both testes and spleen. In muscle, GPX1 activity increases further when 1.0 and 2.0 mg Se/kg are fed. In contrast, the amounts of Sel-W protein in muscle do not change until 0.06 mg Se/kg is fed and increase rapidly up to the plateau level at 1.0 mg Se/kg. Both brain and spleen Sel-W levels increase linearly with the Se concentration up to 0.1 mg Se/kg. Marked increase of Sel-W in testis occurs at 0.01 mg Se/kg with no further elevation beyond that level. In Se-deficient sheep, brain Se content and GPX1 activity are reduced by 50 and 30%, respectively, whereas Sel-W level remains unaltered.⁹¹ Thus, Sel-P may be preferentially protected than GPX1 in brain in Se deficiency.

17.3.5 Comparisons of GPX Enzymes with Thioredoxin Reductase

When rats are fed Se-deficient diets for 14 weeks following weaning, thioredoxin reductase (TR) activity in liver and kidney are decreased to 4.5 and 11% of the controls, respectively, without change in brain.⁹² Meanwhile, plasma GPX3 and Sel-P are reduced to 0.9 and 7.1%, respectively, and GPX1 reduced to 1.2, 4.3, and 70% of the controls in liver, kidney, and brain, respectively. At 12 or 48 hours after an intraperitoneal injection of Se at 50 µg/kg body weight, TR activity in liver is replete to a relatively higher level than those of liver GPX1 and plasma GPX3, but to a lower level than that of the Sel-P concentration.⁹² In mice, hepatic TR activity is also more resistant to dietary Se deficiency than that of GPX enzymes (Fig. 17.1). In contrast, TR in four human cell lines have consistently lower responses to Se than GPX1,⁹³ indicating the possible existence of a Se-unresponsive isoenzyme or a residual disulfide reductase activity in the Se-free truncated protein produced in Se deficiency. In another study, the increased TR activity in cells⁹⁴ is not directly related to the increase in protein amount but rather the increase in the specific activity of the enzyme. Primary human fibroblasts express greater TR and GPX4 proteins than those of melanocytes, and keratinocytes express little of these two enzymes.⁹⁵

17.4 Regulation of GPX Enzymes and Other Selenoproteins by Non-Selenium Factors

Many factors other than Se can influence expression of selenoproteins. As discussed above, iodine modulates expression of three selenoenzymes in thyroid at least as much as Se. In cultured cardiomyocytes, α -tocopherol (200 μ M) increases GPX1 activity and mRNA levels up to 2-fold, and this action is independent of oxygen tension, Se, and *de novo* synthesis of GPX1 transcripts.⁹⁶ A marginal effect of high levels of α -tocopherol on tissue GPX4 or GPX1 activity has also been seen in mice under acute oxidative stress.⁹⁷ Expression of GPX1 mRNA, protein, and activity are reduced by as much as 60% by iron deficiency in various tissues⁹⁸ or copper deficiency in liver of rats.⁹⁹

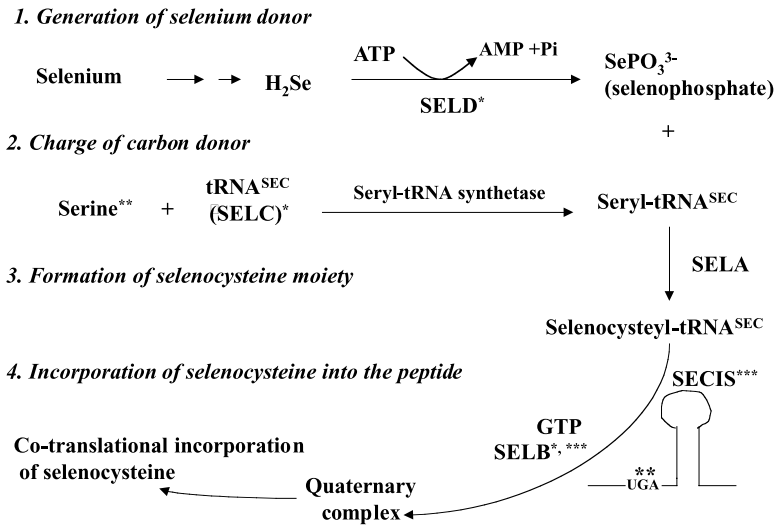
Female rats have 1- to 2-fold higher levels of GPX1 activity and mRNA than the males,¹⁰⁰ but no such difference in mice. There are two potential estrogen-regulatory elements in the upstream sequences of GPX1 gene.¹⁰¹ Testis GPX4 expression depends on gonadotropin as hypophysectomy causes a rapid decline of the activity and gonadotropin treatment partially restores the activity.⁴⁵ However, the age or gonadotropin-dependent expression of GPX4 in testis is due to differentiation stage-specific expression in late spermatids, rather than from a direct gene transcriptional activation by testosterone.⁴⁸ There are oxygen responsive elements in the 5' flanking region of human GPX1 gene,¹⁰² and oxygen tension induces GPX1 expression in cultured cells.¹⁰³ Liver GPX1 mRNA, protein, or activity in rodents is affected by nafenopin,¹⁰⁴ paraquat,^{19,21} and many factors related to redox status.⁶ In addition, GPX1 and other selenoproteins are affected by development.¹⁰⁵

17.5 Mechanisms of Selenium Incorporation and Regulation

There are two apparent ways in which Se can influence selenoprotein expression. As a cofactor, Se is incorporated into these proteins through the co-translational synthesis of selenocysteine. As a regulator, Se affects the expression of selenoprotein genes at different levels. However, it is difficult to distinguish these two types of Se action in many studies reported. To understand the hierarchy of Se used for the synthesis of various mammalian selenoproteins, we need to know the eukaryotic Se-incorporation system. That system in prokaryotes has been well characterized and may serve as a good reference.

17.5.1 Co-Translation of Selenocysteine in Prokaryotes

Using a series of *E. coli* mutants unable to synthesize Se-dependent formate dehydrogenases, Böck and his colleagues discovered four genes and their

**FIGURE 17.2**

Schematic pathways of selenoprotein biosynthesis by prokaryotes. The process requires four unique gene products (*SELA*, *SELB*, *SELC*, and *SELD*) and two special mRNA sequence elements: the UGA codon for selenocysteine and an immediate downstream stem-loop selenocysteine insertion sequence (SECIS) for directing the co-translation of selenocysteine at UGA. Single asterisk (*) indicates that the eukaryotic equivalents of *SELB*, *SELC*, and *SELD* have been identified: the SECIS-binding protein to serve as a translational regulator, the selenocysteine tRNA, and the selenophosphate synthetase to generate activated selenium donor. Double asterisks (**) indicate that both prokaryotic and eukaryotic selenocysteines are encoded by UGA and their carbon sources are from serine. Triple asterisks (***) indicate that (1) the stem-loop SECIS required for selenocysteine translation is located in the 3' untranslated region of eukaryotic selenoprotein mRNA instead of the open read frame of prokaryotic selenoprotein mRNA; and (2) there is more than one SECIS-binding protein in eukaryotes and the elongation mechanism is probably much more sophisticated than the action of *SELB* in prokaryotes. Eukaryotic selenoprotein genes cannot be directly expressed in prokaryotic hosts.

products required for the synthesis and insertion of Se into selenoproteins (Fig. 17.2).^{7,106} The four genes are named *selA*, *selB*, *selC*, and *selD* and encode selenocysteyl-tRNA synthetase, an elongation factor, selenocysteyl-tRNA (tRNA^{SEC}), and selenophosphate synthetase, respectively. Biosynthesis of selenocysteine is initiated by the generation of an active Se donor (selenophosphate) and carbon source (serine). The active form of Se is formed from selenide and ATP under the catalysis of selenophosphate synthetase (*SELD*), and serine is esterified to the 3' terminal of the novel selenocysteyl-tRNA (*SELC*) that contains the anticodon for uracil-guanine-adenine (UGA), by the normal seryl-tRNA synthetase. Selenocysteyl-tRNA synthetase (*SELA*) catalyzes the dehydration of L-serine in the seryl-tRNA to form aminoacrylyl-tRNA^{SEC}, and selenophosphate replaces the side-chain oxygen in serine and produces selenocysteine moiety (still esterified to the tRNA^{SEC}). The novel elongation factor (*SELB*) specifically recognizes the selenocysteyl-tRNA, presumably by binding to the stem-loop structure of 3' untranslated region (UTR) of mRNA. These three components,

plus guanosin-triphosphate (GTP), form a functional quaternary complex on the ribosome, and selenocysteine is incorporated into the growing peptide at the position corresponding to that of UGA in the mRNA.

17.5.2 Comparison of Prokaryotic and Eukaryotic Selenoprotein Biosynthesis

Because selenoprotein biosynthesis requires highly species-specific translation machinery, mammalian selenoprotein genes cannot be directly expressed in *E. coli* and vice versa. Nevertheless, these two types of organisms seem to share common features in the overall biosynthesis of selenoproteins. First, selenocysteine in both organisms is encoded by UGA. Second, both require a stem-loop structure of the mRNA for distinguishing UGA as the selenocysteine codon from that of termination codon, although the stem-loop is located immediately downstream the UGA codon in *E. coli* and in the 3'UTR of mRNA in mammals. Deletion of this conserved stem-loop mRNA, called selenocysteine insertion sequence motif (SECIS) in human GPX1, completely abolishes selenocysteine insertion.¹⁰⁷ This motif is functionally interchangeable among various selenoprotein genes¹⁰⁸ and is able to direct selenocysteine integration at predetermined positions of heterologous gene.¹⁰⁹ Third, the carbon source of eukaryotic selenocysteine is also from serine.¹¹⁰ Last, the identified components of the eukaryotic selenocysteine incorporation machinery may be similar to three respective gene products in *E. coli*.^{111–113}

A special tRNA, the SELC equivalent, is essential for early embryonic development¹¹⁴ (see below). Two selenophosphate synthetases,^{56,112,115} the SELD equivalent, have been identified from mice and human. Thus, Se may be activated in the same way in eukaryotes as in prokaryotes. One of the enzymes is Se-dependent, indicating a possible autoregulation mechanism.⁵⁶ Two SECIS-binding proteins, the SELB equivalent, have also been identified,^{113,116} although the role and complexity of these binding proteins in eukaryotic selenocysteine insertion may exceed those of SELB in prokaryotes.¹¹⁶ Moreover, high levels of rat TR can be expressed in *E. coli* when the open reading frame of the gene is fused with the SECIS element of the bacterial selenoprotein formate dehydrogenase H.¹¹⁷ Co-expression with *selA*, *selB*, and *selC* genes further enhances the production of and the specific activity of the recombinant protein. Thereby, the species-specific translation of selenoproteins is probably dictated by the characteristics of the pertinent SECIS motif. In spite of this, there is a good similarity in selenoprotein expression between eukaryotes and prokaryotes, and *E. coli* can be used as an efficient host to produce recombinant selenoproteins of mammalian sources.

17.5.3 Sites of Selenium Regulation

Although the tissue specificity of GPX1 and other selenoprotein expression is determined mainly at the transcriptional level,¹¹⁸ transcription rate of GPX1

mRNA, as well as other selenoprotein mRNA, is not affected by Se deficiency.^{66,73,77} Thus, the loss of GPX1 mRNA in Se deficiency is post-transcriptional, probably due to accelerated degradation.^{5,76}

It appears that GPX1 mRNA has the lowest stability in Se deficiency because it is reduced more dramatically than that of any other selenoprotein at this circumstance. A recent study by Moriarty et al.¹¹⁹ indicates that Se deprivation does not alter levels of either nuclear pre-mRNA or nuclear mRNA, but reduces the level of cytoplasmic mRNA in a nonsense codon-mediated and translational-dependent way.

Overall, Se-dependent differential mRNA stability does not account for the complete changes of selenoprotein expression with the alteration of Se status. For example, reductions of enzyme activity or protein often exceed that of mRNA.^{4,119} Activities of GPX1 and GPX4 are reduced by Se deficiency in rat testes without comparable changes in their mRNA levels.⁴⁶ In addition, the chemical form of supplemental Se affects the nature of selenoprotein synthesis.¹²⁰ Based on these observations and the assumption that eukaryotic selenoprotein biosynthesis follows pathways similar to those of prokaryotes, the rate of synthesis would depend on the availability of selenocysteine-loaded tRNA^{sec}. For each individual selenoprotein, this availability could be decided by its characteristic mRNA stability and SECIS efficiency at different status of Se.⁶ As there are possibly multiple forms of SECIS-binding proteins in eukaryotes,^{113,116} the same availability may also be regulated by the structural specificity and distribution of these proteins and their ability to form functional complexes with different SECIS.

Because the UGA-mediated translation of selenocysteine is a rather inefficient process,¹²¹ there would be a strong competition between termination (production of truncated proteins) and elongation (co-translation of selenocysteine) at the UGA codon under limited Se supply. Although different SECIS elements are functionally interchangeable, their efficiencies in directing Se incorporation are not equal. The SECIS element from ID1 is more efficient than that of GPX1, but less efficient than that of Sel-P in directing the expression of ID1.¹⁰⁸ The GPX4 SECIS element is more efficient than that of ID1 or Sel-P in suppression of UGA by reporter-gene assays.¹²² Among three GPX enzymes, SECIS efficiency ranks in the order of GPX4 > GPX1 > GPX2 and the augmentation of SECIS efficiency by Se is highest for GPX1, marginal for GPX2, and intermediate for GPX4.⁸⁰ Consistently, the tentative efficiency order of different SECIS elements matches well the relative susceptibility of the pertinent protein and mRNA to Se deficiency.⁶

Based on the above described Se-dependent differential mRNA stabilities and different SECIS efficiencies of selenoproteins, Flohé et al.⁶ have proposed that the differential response of any given selenoprotein to Se availability can be achieved by the formation of complex among the translational factor SELB (binding protein), selenocysteine-loaded tRNA^{sec}, and the specific mRNA. If the binding of tRNA^{sec} to SELB is determined by Se availability, this binding would specifically affect the affinity of a particular SECIS motif to SELB or the binary complex SELB-tRNA^{sec}. Conceivably, the alteration of affinity may determine

the feasibility in forming the three components complex. Those mRNAs that form productive ternary complex first because of the largest affinity increases would be translated efficiently and resistant to degradation. Therefore, the Se-dependent mRNA stability and SECIS efficiency of the individual selenoprotein dictate its own fate in the preferential biosynthesis at limited Se supply. Experimentally, Se regulation of GPX1 mRNA in cultured cells requires the presence of intact SECIS,^{123,124} although its presence, along with the UGA codon, does not necessarily confer an efficient selenocysteine biosynthesis.¹²⁵ In Se deficiency, more GPX1 mRNA may be released from polysomes and degraded than that of GPX4 mRNA because of its greater decrease in translation.⁷⁶

Two important recent findings¹¹⁶ should be added to the above-discussed model. First, there is more than one SECIS-binding protein in eukaryotes and specific association of homologous or heterologous factors may be presented in the RNA-protein binding functional complex. Second, a minimal binding site in the 3'UTR of selenoprotein mRNA is necessary. However, only binding itself seems to be insufficient for selenocysteine incorporation.

17.6 Use of Gene-Knockout Approach to Study Regulation of Selenoprotein Expression

Two gene knockout mouse models have been used in Se research. One is the targeted disruption of the mouse selenocysteine tRNA gene (*Trsp*) in chromosome 7.¹¹⁴ The heterozygous mutants have 50 to 80% of the wild-type levels of tRNA^{sec} in most organs, but exhibit normal viability, fertility, and expression of GPX1. However, the homozygous mutants die shortly after implantation, and the embryos are resorbed before 6.5 days post coitum. Thus, the haploid amount of tRNA^{sec} is not limiting in selenoprotein expression, but *Trsp* expression is essential for early embryonic development. To study the role of *Trsp* expression at other stages of development in selected tissues, conditional knockout of the gene is in progress.¹²⁶

The other knockout model is the disruption of the GPX1 gene in mice. Using this model, we have demonstrated that:

1. GPX1 represents 60% of liver Se and the most abundant selenoprotein in the organ.^{3,127}
2. The expression of GPX3, GPX4, Sel-P, or TR in Se-deficient or adequate mice is independent of GPX1, and there is no change in Se channeled to the expression of these proteins by altering GPX1 expression.^{3,20,97,127–129}
3. GPX1 is the major metabolic form of body Se in protecting mice against acute,^{18–20,129} lethal oxidative stress induced by prooxidants and high levels of vitamin E do not replace that role of GPX1.⁹⁷

4. GPX1 knockout has no significant impact on mouse fertility or susceptibility to low levels of oxidative stress or other antioxidant enzymes.^{11,18,129}

These unequivocal data cannot be obtained using conventional approaches. With more selenoprotein gene knockout models being developed, our understanding of the regulation and function of selenoprotein expression will be significantly enhanced.

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Abbreviations

FAD	flavin adenine dinucleotide
GPX1	cellular glutathione peroxidase
GPX2 (GPX-GI)	gastrointestinal glutathione peroxidase
GPX3	plasma or extracellular glutathione peroxidase
GPX4 (PHGPX)	phospholipid hydroperoxide glutathione peroxidase
GSH	reduced glutathione
ID1	type-1 iodothyronine 5' deiodinase
Se	selenium
SECIS	selenocysteine insertion sequence motif
Sel	selenoprotein
Sel-P	selenoprotein P
Sel-W	selenoprotein W
TR	thioredoxin reductase
Trsp	selenocysteine tRNA gene
UTR	untranslated region

Glossary

Glutathione peroxidases: Intra- or extra-cellular selenoenzymes, homotetramer (~88 kDa) or monomer (19 kDa), catalyzing the reduction or removal of H₂O₂, lipid peroxides, or phospholipid hydroperoxides using reduced glutathione

Iodothyronine 5' deiodinases: Selenoenzymes (~28 to 30 kDa), catalyzing the conversion of T_4 to T_3 and the inactivation of both hormones, three different types based on distribution and function

Selenium: An essential trace element for animals and humans

Selenoproteins: Proteins containing selenium in the form of selenocysteine

Selenoenzymes: Enzymes containing selenium in the form of selenocysteine

Selenoperoxidases: Refer to four different selenium-dependent glutathione peroxidases that use reduced glutathione to reduce hydroperoxides

Selenocysteine insertion sequence (SECIS): A well-conserved stem-loop mRNA sequence in the 3' untranslated region of selenoprotein genes that directs selenium incorporation into the peptide

selA: Prokaryotic gene of selenocysteine synthetase (SELA) that catalyzes the formation of selenocysteine moiety from seryl-tRNA and selenophosphate

selB: Prokaryotic gene of an elongation factor (SELB) needed for co-translation of selenocysteine

selC: Prokaryotic gene of selenocysteine tRNA (SELC) that contains the anticodon for UGA that encodes selenocysteine

selD: Prokaryotic gene of selenophosphate synthetase (SELD) that activates selenium for co-translation of selenocysteine

Selenoprotein P: A selenium-dependent glycoprotein of 57 kDa, accounting for 65% of plasma selenium in rodents

Selenoprotein W: A selenium-dependent protein of 10 kDa, relatively high in muscle, low expression in selenium-deficient animals with white muscle disease

Thioredoxin reductases: Pyridine nucleotide-disulfide oxidoreductases, homodimers of 55 to 65 kDa subunits, catalyzing NADPH-dependent reduction of thioredoxin

tRNA^{SEC}: A novel tRNA that contains the anticodon for UGA and carries serine for the biosynthesis of selenocysteine

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18

Ferritin: A Novel Human Ferritin Heavy-Chain mRNA is Predominantly Expressed in the Adult Brain

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18.1 Introduction

Ferritin, a 480-kDa protein, is composed of 24 subunits of a 21-kDa heavy chain (FTH) and a 19-kDa light chain. It is a multifunctional molecule involved in detoxification, storage, and transport of iron. Transferrin is a serum glycoprotein involved in iron transport. Free intracellular iron regulates the rates of synthesis of ferritin and transferrin receptors. The accumulation of iron and synthesis of ferritin are developmentally regulated. Both ferritin and transferrin also store and transport aluminum. Studies aimed toward the characterization of the effects of aging and of certain neurodegenerative diseases, for example, Alzheimer's disease (AD), which is associated with the accumulation of iron, aluminum, ferritin, and transferrin, give information about the regulation and contribution of genes involved in iron homeostasis.

The present chapter describes the work involving molecular identification and characterization of a novel ferritin H chain cDNA from the adult and fetal human brains. New findings and a possible role of this human FTH cDNA in normal and AD brains are discussed.

18.2 Role of Iron in Brain Disorders

The brain is a highly compartmentalized organ. An adult human brain weighs about 1.5 kg, and constitutes only about 2% of the body weight. It is aerobic and gets its energy primarily by the oxidation of glucose. Several enzyme reactions involved in the oxidation of glucose require oxidation and/or reduction of Fe^{+2} and Fe^{+3} . About 20% of the oxygen consumed by the body is used by the brain. Brain cells undergo limited or regeneration, and thus, the brain has a perfect environment for the accumulation of various toxins such as aluminum. The brain is also the primary target for diseases arising due to overloading of iron as well as of iron metabolism. For example, Alzheimer's disease (AD) is a neurological disorder that affects primarily older people. AD is characterized by altered memory, cognition, and behavior. In AD, nerve cells in specific areas of the brain degenerate. Besides AD, Parkinson's disease (PD), Huntington's disease (HD), and Hallervorden-Spatz disease are some of the other neurodegenerative diseases associated with the accumulation of iron in the brain.^{1–4} The basal ganglia contain the highest levels of iron in the brain. Iron metabolism is disrupted in patients with AD and HD. Overloading of iron in the brain also leads to oxidative damage in the brain cells. However, Bartzokis and Tishler, using Magnetic Resonance Imaging (MRI) to suggest that the increase in the levels of iron in the basal ganglia in AD and HD may be regulated by independent pathways and thus the presence of these high levels of iron at the onset of the disease, propose iron to be a risk factor rather than the result of the disease.⁵ In addition to aberrations in the metabolism of iron in the AD brain, defects are also observed in the central nervous system and plasma.^{6,7}

18.3 Role of Ferritin, Transferrin, and Iron Regulatory Proteins in Iron Homeostasis

Ferritin is a ubiquitous iron-storage protein with a molecular weight of about 480,000. It is composed of two types of subunits: heavy (H, Mr 21,000) and light (L, Mr 19,000) chains.^{8,9} Ferritin is ubiquitously expressed in the tissues like heart, brain, liver, lung, etc. Isoferritins varying in subunit composition

exist in different tissues. The H and L subunits of ferritin are genetically and functionally distinct. The H chain subunit is predominantly found in cells and tissues (the heart and the brain) that have high levels of oxidative phosphorylation, while the L subunit is present in the tissues (the liver and the spleen) that store iron.¹⁰ Furthermore, ferritin H and L subunits are independently regulated and are differentially regulated in response to the intracellular iron. Han et al. have recently shown that severe iron deficiency reduced brain ferritin H protein levels significantly in all the regions of the brain, whereas ferritin L levels only in the striatum, substantia nigra, and pons were affected. Exogenously added iron in the diet increased both the H and L subunits. These data suggest post-transcriptional regulation of the two subunits in response to iron.¹¹

Ferritin binds several metal ions *in vitro* and *in vivo*.¹² An expanded role for ferritin in metal toxicity is suggested.^{13–15} Ferritin–aluminum complexes have been isolated from the brains of two AD patients. Furthermore, ferritin isolated from two AD and one normal brain had from 2 to 4 moles of aluminum bound per mole of protein (Fleming and Joshi, unpublished). It has been shown that in AD brains, the total concentration of iron and ferritin is significantly higher than their age-matched controls. Ferritin is also found to be a component of the senile plaques in the dementia of AD.¹⁶ Taken together, data show that there are elevated levels of ferritin in AD brains, aluminum–ferritin complexes could be isolated *in vitro*, and the rate of iron uptake by ferritin is reduced by aluminum.^{17,18}

Transferrin(s) is a family of glycoproteins of MW 80,000. It is a single polypeptide chain and has two metal binding sites, one each at the N- and C-terminal ends. It is the major serum protein involved in iron transport. In the brain, besides being present in the iron-containing cells, transferrin is also found in oligodendrocytes, which are important in myelin synthesis. Transferrin receptor is highly expressed on blood vessels, large neurons in the cortex, striatum, and the hippocampus.^{19,20}

In higher organisms, iron bound to transferrin and ferritin constitutes more than 90% of non-heme iron.²¹ Both the proteins also sequester other metal ions including aluminum.²²

Intracellular concentration of iron regulates the synthesis of ferritin and the stability of the transferrin receptors. This translational regulation is due to a conserved sequence (termed as the Iron Responsive Element, IRE) of 28 nucleotides in the 5' untranslated region of ferritin mRNA and the 3' untranslated region of the transferrin receptor RNA.^{19,23}

Two iron regulatory proteins (IRPs), IRP1 and IRP2, have been isolated from both rodent and human tissues. These interact with the IRE and modulate the expression of ferritin and transferrin at the mRNA level. Pinero et al. hypothesized that the alterations in the IRP/IRE binding are the event which is altered in the AD brains. They showed a change in the stability of the IRP1/IRE complex in normal vs. AD brain extracts. They propose that relatively high endogenous ribonuclease activity in the AD brain may be one of the mechanisms by which iron homeostasis is disrupted.²⁴

18.4 A Novel Ferritin H Chain mRNA in the Human Brain

Ferritin is an important protein involved in the regulation of iron and it is evident that the H subunit (FTH) is of major significance in the central nervous system. Thus, an in-depth characterization of FTH from the human brain was undertaken. A major portion of this project was carried out in collaboration between the laboratories of Dr. J.G. Joshi (Department of Biochemistry, University of Tennessee, Knoxville, TN, USA) and Dr. Marie Percy (Department of Physiology, University of Toronto and Surrey Place Center, Toronto, Ontario, Canada).

High performance liquid chromatography (HPLC) of human brain ferritin revealed five distinct molecular species of brain FTH but, only one major species of ferritin L chain was identified. There is more of the ferritin H subunit than the L in the human brain. Protein chemistry of human brain ferritin from normal and AD brains revealed no differences.^{8,21} Thus, molecular studies were initiated to address these observations. A human liver ferritin heavy chain cDNA (a gift from Dr. R. Klausner, NIH, Bethesda, MD) was used as a probe to screen a normal adult human brain cDNA library (Clontech, Palo Alto, CA, USA). Northern blot analysis of the poly(A)⁺ RNAs from human brain and liver showed two transcripts of 1.4 and 1.1 kb, respectively.²⁵ The 1.4 kb transcript was ubiquitously expressed in the human heart, brain, liver, kidney, lung, skeletal muscle, placenta, and pancreas. The level of expression, however, in each tissue was different. The 1.4 kb RNA is predominantly expressed in the brain, while its level of expression in the liver is 10 times lower.²⁶ On the other hand, the 1.1 kb RNA is predominantly expressed in the liver. Sequence analysis showed that the 1.1kb transcript is identical to the previously characterized RNA from liver and lymphocytes.²⁷ The larger transcript contained an additional 279 bp sequence at the 3' end. A genomic clone containing the 279 bp sequence was obtained.²⁸ Sequence comparison of the cDNA and the genomic clones showed that in the larger transcript, the 279 bp sequence is a part of the transcribed sequence and hence of the mature mRNA, whereas in the smaller one, it is a part of the non-transcribed sequence present only in the genomic clone.

These data suggested that differential processing of the primary transcript of the FTH mRNA in human tissues generates two mature mRNAs of 1.4 and 1.1 kb. This is due to the utilization of an alternative polyadenylation site in the precursor mRNA. Sequencing of the cDNAs confirmed this observation, wherein, one polyadenylation site is identical to the one reported to be in the liver, and the other is a part of the 279 bp fragment and is found to be 16 bp upstream to the poly(A) tract.²⁹ Primer extension and reverse-transcriptase polymerase chain reaction (RT-PCR) were used to obtain full-length cDNAs from both the normal as well as AD brains. Sequence analysis showed that the IRE and the 279 bp sequence coexist in

at least one of the FTH transcripts in the brain.⁸ Search for an L chain mRNA consisting of the 279 bp sequence was unsuccessful (Dhar and Joshi, unpublished). Thus, it is possible that the novel H chain mRNA identified in the human brain has a special role in brain homeostasis.

Percy et al. (1998) showed tissue- as well as region-specific expression of the novel FTH message within the brain — the level of expression was the highest in the amygdala, caudate nucleus, putamen, substantia nigra, and spinal cord, and lowest in the cerebellum.³⁰

In AD, hippocampus is often the most seriously affected structure. To determine whether the observed elevated levels of ferritin in the AD brain are area specific and disease dependent, and if the concentration of H chain parallels the changes in the 279 bp containing message, ribonuclease protection assays (RPA) were carried out using total RNA from human liver and brain as well as from the hippocampal regions of normal and the AD-affected brains. Furthermore, tissue *in situ* RT-PCR analysis was also carried out to visualize relative levels of only FTH mRNA containing the 279 bp sequence in its 3'UTR in different cell types. Preliminary data showed lower concentrations of the mRNA in the liver compared to the brain. However, the concentrations were identical in both the normal and the AD brains.^{8,30,31} In the normal adult hippocampus, this new message localizes strongly to non-neuronal cells, capillary endothelial cells, and to the granule cells of the dentate gyrus.³³ Future experiments involving Northern blotting, RPA analysis, and primer extension analysis are required to study the expression of the novel H chain mRNA in AD tissues.

18.5 Ferritin H Chain mRNA in Aging

The accumulation of iron and the synthesis of ferritin in the human brain is developmentally regulated. A human fetal brain contains low levels of iron and ferritin.³² By contrast, an adult human brain contains large quantities of iron and about one third of its total nonheme iron is stored in ferritin.³³

An 11-week-old human fetal brain (FB) cDNA library (a gift from Dr. Swaroop, University of Michigan, Ann Arbor, MI) was screened using the human liver ferritin H-chain cDNA as the probe. Northern blot analysis together with sequencing showed that one type of cDNA corresponded to the FTH transcript reported in liver while the other was identical to the novel FTH transcript identified in the adult human brain.^{29,34} The relatively low level of the 279 bp carrying transcript in the fetal brain observed in quantitative *in situ* hybridization experiments is indicative of developmental regulation of this message. The iron responsive element could not be detected, instead a new 54 bp sequence is observed. The role of this 54 bp sequence is not yet known.^{30,34}

18.6 Conclusions

Our understanding of the mechanisms of iron transport and homeostasis in the brain is still limited. A large number of experiments to delineate the role of these new biological tools have to be carried out. Percy et al. (1998) reported some interesting features from the predicted secondary structures of FTH RNAs containing the IRE in the 5'UTR and the novel 279 bp sequence in the 3'UTR. The residues 999–1120 (located in the novel 279 bp 3'UTR) formed three stem-loop structures. Blast sequence similarity search showed about 88% homology between the positions 1013 and 1095 of this stem-loop region and a region in the 3'UTR of human endoperoxide synthase type II cyclooxygenase-2 (COX 2).³⁰ These enzymes are rate-limiting in the synthesis of prostaglandins, which are mediators of inflammation. A role of COX-2 is also suggested in arthritis rheumatoid.^{35,36} Even though there is some homology between the 279 bp sequence and another gene not related to ferritin, the 279 bp sequence seems to be associated with the functional FTH gene and mRNA. This novel FTH cDNA maps exclusively to human chromosome 11. It maps to the same locus as the functional liver FTH cDNA.³¹ These observations suggest that it is unlikely that this entire sequence exists in any other human gene including the FTH pseudogenes, the gene for human liver ferritin L, or its pseudogenes that map to other human chromosomes than 11.

The interactions between iron, the iron-responsive element (IRE) in the 5'UTR of H and L-ferritin mRNA, and in the 3'UTR of the transferrin receptor mRNA as well as the IRE-binding protein (IRE-BP) are now well documented.¹⁹ The role of the novel FTH cDNAs from human adult and fetal brains is still unknown. Henderson et al. reported a second IRE-binding protein, IRF, an Iron Regulatory Factor, in mammalian tissues, with the highest levels in the brain.³⁷ Though the affinity of IRE to IRF is similar to that of IRE-BP, the role of IRF is still under investigation. The identification of a novel cDNA for ferritin H subunit along with a new iron-binding protein, both highly expressed in human brains, may be functionally relevant rather than a coincidence. Their roles in ferritin synthesis are unknown; however, a few interesting questions can open new avenues for exciting research — do the IRE-BP and the IRF bind to the 279 bp sequence? If yes, how does this affect the translation of ferritin H chain? Why is a part of the 279 bp sequence conserved/present in human COX-2? What is the biological role of this conserved sequence? Is there any differential transcription of the liver-like ferritin H chain vs. the longer brain-like message? Is the elongated form of FTH expressed in other animal species? Are there any factors or conditions like stress or disease that favor one form over the other? What, if any, is the role of the elongated message in AD brain? Does it influence the expression of the FTH gene differentially in the AD brain vs. the normal human brain? If so, what triggers this differential expression? Future experiments to study the biological function and role of this novel elongated FTH cDNA have to be carried out.

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