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Objective—Plasma lipoprotein levels are determined by the balance between lipoprotein production and clearance. Recently, angiopoietin-like protein 4 (ANGPTL4) was uncovered as a novel endocrine factor that potently raises plasma triglyceride levels by inhibiting triglyceride clearance. However, very little is known about ANGPTL4 in human. Here we set out to identify physiological determinants of plasma ANGPTL4 levels in humans, focusing on the effect of energy restriction and plasma FFAs.

Methods and Results—We developed an ELISA for quantitative measurement of ANGPTL4 in human plasma. Using this assay we found major variations in baseline plasma ANGPTL4 levels between individuals. Within an individual, plasma ANGPTL4 levels remain stable throughout the day but increase significantly in response to long-term fasting, chronic caloric restriction, and endurance exercise. Intralipid injection as well as treatment with a β-adrenergic agonist, both of which lead to elevated plasma FFA levels, increased plasma ANGPTL4 levels compared to control treatment. Fatty acids markedly induced ANGPTL4 gene expression in rat hepatoma FAO cells, human primary myocytes, and mouse intestinal MSIE cells.

Conclusion—In conclusion, our results show that plasma ANGPTL4 levels are increased by fasting, caloric restriction, and exercise, which is likely mediated by elevated plasma FFAs. (***Arterioscler Thromb Vasc Biol.*** 2009;29:969-974.)

Key Words: ANGPTL4 ■ free fatty acids ■ caloric restriction

Changes in plasma lipoproteins are known to affect atherosclerosis and associated coronary heart disease (CHD). Indeed, it is well established that elevated plasma low-density lipoprotein (LDL) concentrations increase the risk for CHD, whereas elevated high-density lipoprotein (HDL) concentrations are considered atheroprotective. Plasma lipoprotein levels are determined by the balance between lipoprotein production and clearance. Recently, angiopoietin-like protein 4 (ANGPTL4) was uncovered as a novel endocrine factor that impacts plasma lipoprotein levels. ANGPTL4, which is also referred to as fasting-induced adipose factor Fiaf, is a secreted protein of about 50 kDa that belongs to the family of angiopoietin-like proteins.[1] ANGPTL4 was discovered by screening for target genes of the peroxisome proliferators activated receptors alpha and gamma, which serve as the molecular targets of the hypolipidemic fibrate and the insulin sensitizing thiazolidinedione drugs, respectively.[2,3] In mice, ANGPTL4 is produced by a number of tissues including adipose tissue, liver, skeletal muscle, heart, skin, and intestine.

Evidence abounds that ANGPTL4 plays a major role in the regulation of lipid metabolism.[4] ANGPTL4 inhibits the enzyme lipoprotein lipase, thereby suppressing clearance of TG-rich lipoproteins and thus raising plasma TG levels.[5] Inhibition of LPL occurs by inducing dissociation of catalytically active LPL dimers into inactive LPL monomers.[6] Experiments with transgenic mice overexpressing ANGPTL4 suggest that ANGPTL4 inhibits LPL via paracrine and endocrine signaling.[7–10] Furthermore, ANGPTL4 stimulates adipose tissue lipolysis, leading to elevation of plasma free fatty acid levels.[5,10,11] Analogous to numerous other protein hormones, ANGPTL4 is proteolytically processed to produce N-terminal and C-terminal fragments that can be detected in human serum.[12,13] Processing seems to occur in a tissue-specific manner. Indeed, the liver actively cleaves ANGPTL4, whereas human adipose tissue shows no proteolytic activity.[12]

In addition to governing clearance of plasma lipoproteins, ANGPTL4 has been suggested to play a role in angiogenesis. It has been shown that ANGPTL4 is a structural and likely regulatory component of the extracellular matrix that affects numerous aspects of endothelial function including angiogenesis, endothelial cell migration, vascular leakage, and cell adhesion.[14–19]

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Presently, very limited information is available about ANGPTL4 in human. It has been shown that carriers of a rare sequence variant of the ANGPTL4 gene have elevated plasma HDL and decreased TG levels.[20] However, because of the absence of a commercial assay to quantitatively assess ANGPTL4, very little is known about plasma ANGPTL4 levels in human. Here, we describe the development, validation, and application of a novel ELISA for quantitative determination of ANGPTL4 in human plasma. We used this newly developed ELISA to identify physiological determinants of plasma ANGPTL4, focusing on the role of fasting and plasma FFAs.

Materials and Methods

For detailed information on the human interventions, Western blot, and quantitative RT-PCR, please see the supplemental materials (available online at http://atvb.ahajournals.org).

ELISA

96-well microtiter plates were coated with the antihANGPTL4 polyclonal goat IgG antibody (AF3485, R&D Systems) at 0.16 μg/well and incubated overnight at 4°C. This antibody was obtained by immunization of goats with NS0-derived recombinant human angiopoietin-like 4 (AA 26 to 406). After 4 washes with 300 μL PBS-Tween20 0.1% (=washing buffer), 300 μL of blocking solution (PBS containing 1% BSA) was added per well and left for 1 hour at room temperature under gentle agitation. 100 μL of 20-fold diluted human plasma was applied to each well, followed by 2-hour incubation at room temperature under gentle agitation. A standard curve of increasing amounts of recombinant human ANGPTL4 (3485-AN, R&D Systems) was prepared (0.3 to 2.1 ng/well) in TBS-Tween20 0.5%, containing 0.1% BSA, and incubated under similar conditions. After rinsing 4 times with washing buffer, 100 μL of diluted biotinylated anti-hANGPTL4 polyclonal goat IgG antibody (BAF3485, R&D Systems) was added at 0.02 μg per well, followed by another 2-hour incubation. After rinsing 4 times with washing buffer, streptavidine-conjugated horseradish peroxidase was added for 1 hour. After, rinsing 4 times with washing buffer, subsequent reaction with tetramethyla-benzidine substrate reagent was allowed to proceed for 6 minutes. The reaction was stopped by addition of 50 μL of 10% H_2SO_4, and the absorbance was measured at 450 nm on a MultiSkan Ascent spectrophotometer (Thermo Scientific, Breda, the Netherlands).

The ELISA assay used is virtually identical to the DuoSet Elisa hANGPTL4 offered commercially by R&D systems with several minor modifications. Importantly, absorbance values remain within the linear range of the standard curve, which does not exceed 2.1 ng/mL.

Cell Culture

Partially confluent HepG2 cells were transfected with a GFP control vector (pEGFP-N2) or the expression vector pcDNA3.1/V5-HisA encoding mAngptl4 or hANGPTL4. After transfection, cells were incubated in serum-free DMEM. 24 hours posttransfection, medium and cell lysates were collected and used for ANGPTL4 Elisa assay.

Human primary myocytes were prepared from 3 individuals as previously described.[28] Rat hepatoma FAO cells, human primary myocytes, and mouse intestinal MSIE cells were grown in DMEM containing 10% (vol/vol) fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were incubated with albumin only (control), albumin-bound oleic or linoleic acid (100 μmol/L), or synthetic PPAR agonists for 24 hours, followed by RNA isolation and qRT-PCR. Wy14643 was used at a concentration of 5 μmol/L (FAO, MSIE). GW501516 was used at 1 μmol/L (FAO, MSIE) or 10 μmol/L (primary myocytes), and TTA (Tetradecylthioacetic Acid) was used at 100 μmol/L.
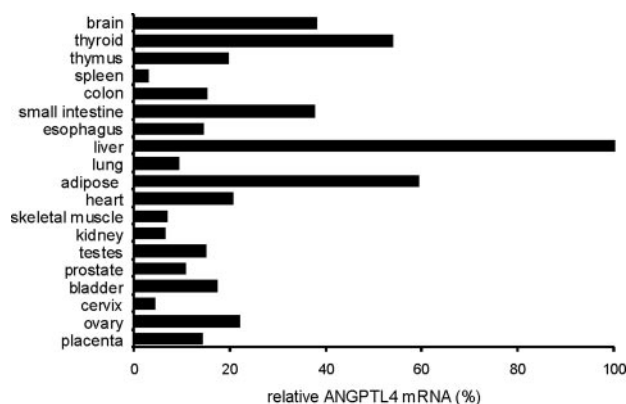

Figure 1. Expression profile of ANGPTL4 mRNA in human tissues. The FirstChoice Human Total RNA Survey Panel (Ambion) was reverse transcribed and used for qPCR using primers specific for human ANGPTL4. Expression levels are expressed relative to liver, which showed the highest expression level (100%).

Results

To better characterize ANGPTL4 in human, we first determined ANGPTL4 gene expression levels in a large number of human tissues. In general, ANGPTL4 expression was relatively ubiquitous. Highest expression levels were found in liver, followed by adipose tissue, thyroid, brain, and small intestine (Figure 1).

To better understand the role of ANGPTL4 in human, we developed an ELISA assay to quantitatively assess serum or plasma ANGPTL4 levels in human subjects. A standard curve was generated using recombinant human ANGPTL4, showing a clear linear relationship between ANGPTL4 concentration and absorbance at 450 nm (Figure 2A). As shown by Western blot, the antibody used in the ELISA detected a single band at 50 kDa, corresponding to the molecular weight of full-length ANGPTL4 (Figure 2B). The same band and an additional band corresponding to the N-terminal truncated portion of ANGPTL4 were detected using an antibody directed against a N-terminal peptide-epitope (Figure 2C). To test the specificity of the assay, HepG2 cells were transiently transfected with expression vectors encoding mouse Angptl4, human ANGPTL4, or EFGP, and cells and medium were harvested 24 hours thereafter. ANGPTL4 was specifically detected in medium and cell lysate of HepG2 cells transfected with hANGPTL4, indicating the absence of any cross-reactivity with mAngptl4 (Figure 2D). As a final verification, we confirmed the increase in plasma ANGPTL4 levels in patients after treatment with the PPARα agonist fenofibrate (Figure 2E), as previously demonstrated by semiquantitative Western blot.[12]

We first determined a number of basic characteristics of the hANGPTL4 ELISA assay. The intraassay coefficient of variation (CV) for the assay was determined at 6.8%. The within-subject intraday CV, which was determined by measuring plasma ANGPTL4 levels at numerous time points throughout the day, was slightly higher at 10%, whereas the within-subject interday CV was similarly low at 14%. In contrast to the low variability within subjects, we found a large interindividual variation in plasma ANGPTL4 levels, as shown by a between-subject CV of 78%. These data indicate
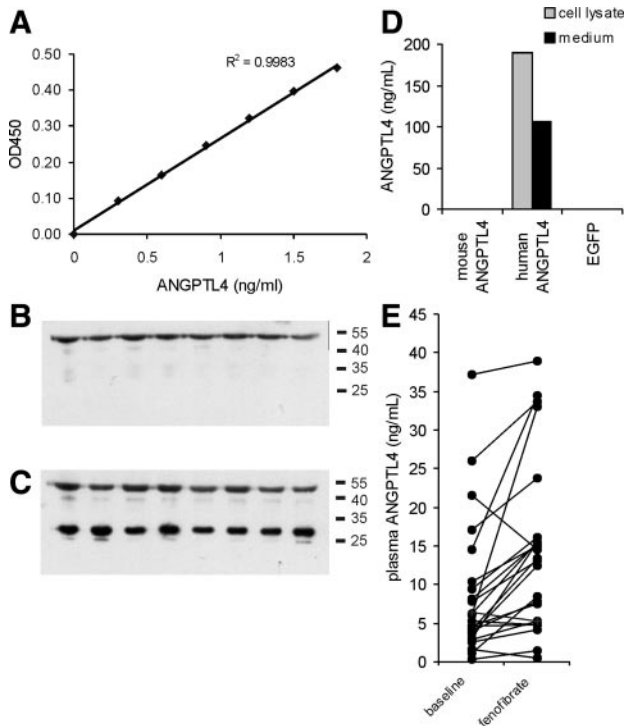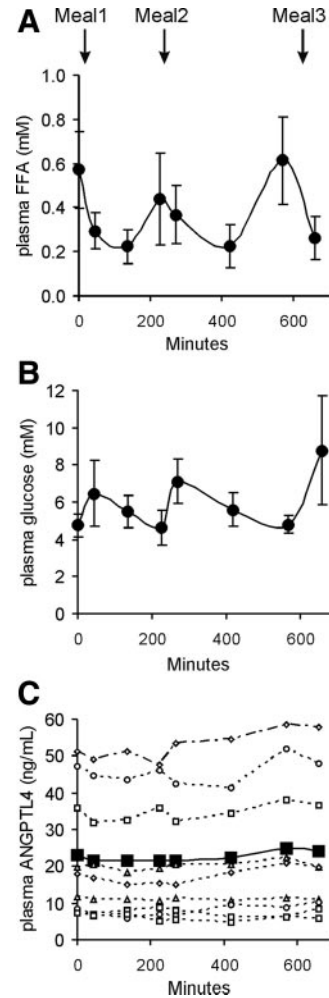
Figure 2. Characteristics of the ANGPTL4 Elisa assay. A, Standard curve showing linear relationship between concentration of ANGPTL4 protein and absorbance. Plasma samples were diluted 20- to 50-fold to fall within the linear range. B, Western blot of human blood plasma using the same antibody as used in the Elisa assay. C, Western blot of human blood plasma using an antibody directed against a N-terminal epitope of ANGPTL4.[12] Same individuals are shown as in panel B. D, Concentration of ANGPTL4 in medium and cell lysate of HepG2 cells 24 hours after transfection with GFP control vector or expression vector encoding ANGPTL4. E, Plasma ANGPTL4 levels in 25 individuals before and after 4 weeks of fenofibrate treatment (study 1).[12]


Figure 3. Plasma ANGPTL4 shows little diurnal variation. Blood plasma was sampled from 9 subjects before, after, and in-between meals for measurement of FFA (A), glucose (B), and ANGPTL4 (C; study 3). Dotted lines represent plasma ANGPTL4 levels of individual subjects. Straight lines represent means of 9 subjects. Error bars represent SD.

that plasma ANGPTL4 levels are relatively constant within an individual, yet differ markedly between subjects.

As plasma ANGPTL4 levels were highly variable between individuals, we were interested to study the potential relationship with plasma TG, which have been shown to be impacted by ANGPTL4 overexpression or deletion in mice. Interestingly, in a samples of 36 middle-aged women (study 2), we did not find any association between plasma ANGPTL4 and plasma TG levels (data not shown). Similarly, no association was found with BMI or other plasma metabolic parameters such as insulin, glucose, and adiponectin.

In an effort to ascertain the physiological determinants of plasma ANGPTL4 levels, we first determined the circadian rhythm of plasma ANGPTL4 in 9 subjects who took their meals at specific times. While normal postprandial responses in plasma free fatty acids and glucose were observed (Figure 3A and 3B), plasma ANGPTL4 levels remained remarkably stable (Figure 3C). As mentioned above, however, major variation in plasma ANGPTL4 levels was observed between subjects.

To metabolically challenge subjects, 4 young males fasted for 48 hours.[23] Plasma ANGPTL4 levels went up in all individuals (Figure 4A, mean relative increase: 80%, $P<0.01$). Plasma ANGPTL4 also significantly increased in

obese patients subjected to 25 days of severe food deprivation (Figure 4B, mean relative increase: 79%, $P<0.001$).[24] These data indicate that plasma ANGPTL4 levels increase in response to both short-term and long-term energy restriction, which are associated with elevated plasma FFA levels (supplemental Figure I).

Expression of ANGPTL4 in numerous tissues was highly sensitive to free fatty acids, as shown by dramatic induction of ANGPTL4 mRNA by the fatty acids oleic acid and linoleic acid in rat FAO hepatoma cells, primary human myocytes, and mouse intestinal MSIE cells (Figure 5). ANGPTL4 induction was mimicked by synthetic agonists of PPARs, which serve as receptors for fatty acids, indicating a role for PPARs. These data suggest that the effect of severe energy restriction on plasma ANGPTL4 may be mediated by elevated plasma FFAs.

Another physiological stressor that increases plasma FFA is endurance exercise. Remarkably, we found that fasting plasma ANGPTL4 levels further increased during endurance exercise and especially during subsequent recovery, concur-
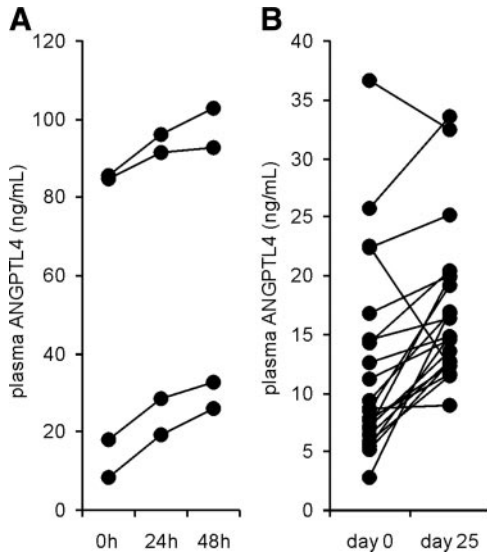
Figure 4. Plasma ANGPTL4 levels are increased by fasting and caloric restriction. A, Plasma ANGPTL4 was measured in 4 subjects after a meal, and after 24 hours or 48 hours of fasting (study 4). B, ANGPTL4 levels were measured in overnight fasting plasma from 22 overweight males before and after 25 days of caloric restriction (study 5).

Figure 6. Plasma ANGPTL4 levels increase during exercise in fasted but not fed state. A, After overnight fasting, 7 subjects performed 2 hours of endurance exercise followed by 4 hours of postexercise recovery while fasted (gray bar) or with glucose ingestion (white bar; study 6). To normalize for large differences in baseline ANGPTL4 values, levels are shown as % change over baseline (=fasted state, preexercise). B, Plasma ANGPTL4 levels were determined in 9 subjects before (baseline) and after a 6-hour hyperinsulinaemic-euglycaemic clamp with simultaneous infusion of either glycerol or lipid emulsion (study 7). The percentage change in plasma ANGPTL4 from 0 to 6 hours is shown. C, Plasma ANGPTL4 levels in 9 males before (baseline) and after receiving a 3-hour infusion of salbutamol with or without simultaneous administration of acipimox (study 8). The percentage change in plasma ANGPTL4 from 0 to 3 hours is shown. Error bars represent SEM. *Significantly different according to Student t test ($P<0.05$).

rent with an increase in plasma FFAs (Figure 6A and supplemental Figure II).[25] Importantly, the increase in plasma ANGPTL4 was entirely abolished when subjects were given oral glucose, which elicits insulin release and thereby suppresses plasma FFA levels. To further explore the impact of plasma FFA, plasma ANGPTL4 levels were measured in subjects who underwent a hyperinsulinemic clamp while receiving an infusion of either glycerol (control) or lipids together with heparin, which causes a massive increase in plasma FFAs (Supplemental Figure II).[26] Whereas plasma ANGPTL4 declined significantly on control treatment, which

may reflect a direct effect of insulin or may be attributable to an insulin-induced suppression of plasma FFAs, the decrease in plasma ANGPTL4 was significantly diminished after lipid infusion (Figure 6B). Similarly, treatment with the
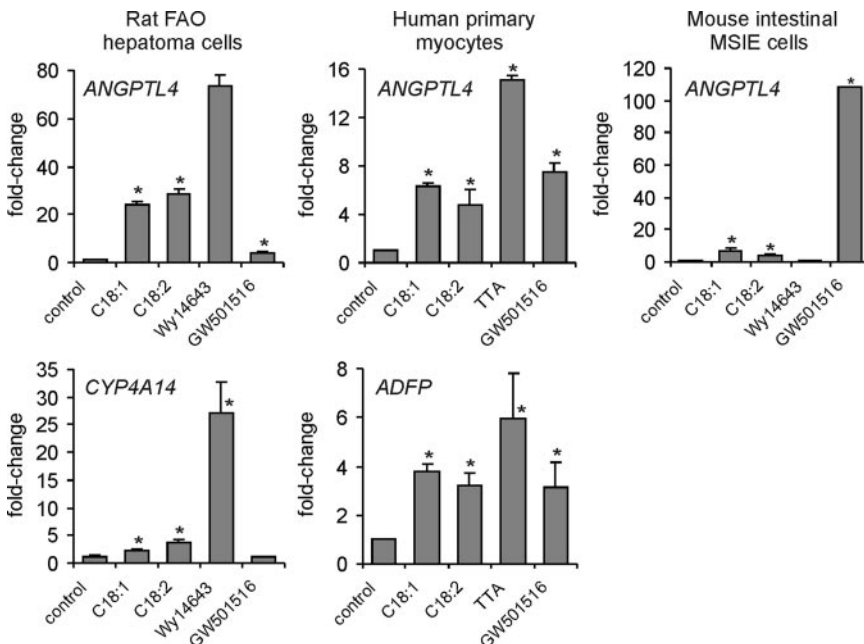
Figure 5. Fatty acids markedly induce ANGPTL4 mRNA. Cultured cells were incubated with oleic or linoleic acid, Wy14643, GW501516, or tetradecylthioacetic acid (TTA) for 24 hours, followed by RNA isolation and qRT-PCR. CYP4A14 or ADFP served as positive PPAR control genes. Error bars represent SEM. *Significantly different according to Student t test ($P<0.05$).

β-adrenergic agonist salbutamol, which causes a marked increase in plasma FFA (Supplemental Figure II),[27] significantly raised plasma ANGPTL4 levels. This response was entirely blunted when subjects were simultaneously given acipimox, a lipolysis inhibitor that reduces plasma free fatty acids (Figure 6C). These data suggest that plasma FFAs increase plasma ANGPTL4 levels.

Discussion

Very little is known about determinants of plasma ANGPTL4 levels in humans. Our data point to a large interindividual variation in plasma ANGPTL4 levels. In contrast, plasma ANGPTL4 levels are very constant within an individual, which is true during normal circadian rhythm as well as between days. From our data it is evident that major physiological stressors such as fasting and exercise are required to elicit significant changes in plasma ANGPTL4. Specifically, we observed that 48 hours of fasting and 25 days of severe caloric restriction both raised plasma ANGPTL4 by an average of 80%. Fasting plasma ANGPTL4 levels were further stimulated by endurance exercise and postexercise recovery. Additional experiments indicated that the increase in plasma ANGPTL4 levels is likely mediated by elevated FFAs via activation of ANGPTL4 gene transcription via PPARs. Alternatively, it may be argued that instead of or in addition to FFAs raising plasma ANGPTL4, ANGPTL4 may raise plasma FFAs via stimulation of adipose tissue lipolysis. Studies using Angptl4 knock-out or transgenic mice,[10] as well as i.v. injection of recombinant Angptl4[5] support a potent prolipolytic activity of ANGPTL4 in adipose tissue. It is possible that both mechanism operate in concert as part of a positive feedback loop.

The increase in plasma ANGPTL4 levels during fasting is in line with a wealth of mouse data showing induction of Angptl4 gene expression by fasting in numerous tissues.[2,10,11,29,30] By inhibiting lipolysis of TG-rich lipoproteins, which results in decreased tissue uptake of plasma TG-derived fatty acids, while at the same time increasing plasma FFAs by stimulating adipose tissue lipolysis, we hypothesize that ANGPTL4 may cause a shift in overall fuel use from plasma TG toward TG stored in the adipose tissue. Depending on whether the tissue primarily uses plasma TG-derived fatty acids or FFA,[31] ANGPTL4 upregulation is expected to either cause a decrease or increase in net fatty acid uptake. Upregulation of ANGPTL4 by fasting may thus be aimed at promoting use of fat in favor of fat storage. This mechanism may be supported by the above-mentioned positive feedback loop in which ANGPTL4 stimulates release of FFAs, which in turn stimulates ANGPTL4 production.

Depending on the type of tissue involved, the effect of fatty acids on ANGPTL4 mRNA can be mediated by different PPAR isotypes. Our results suggest that fatty acids induce ANGPTL4 mRNA via PPARα in rat hepatoma cells and via PPARβ/δ in mouse intestinal cells MSIE cells. In human myocytes, both PPARα and PPARβ/δ likely play a role in ANGPTL4 gene induction by fatty acids.

Using semiquantitative Western Blot, we previously showed that 4 weeks of treatment with the PPARα agonist fenofibrate

significantly raised plasma levels of the N-terminal ANGPTL4 fragment.[12] Our present ELISA data similarly reveal a marked increase in plasma levels of full-length ANGPTL4 on fenofibrate treatment. As fibrates primarily target liver, these data suggest that liver is the most important source of plasma ANGPTL4, which is supported by the dominant expression of ANGPTL4 in human liver.

Previously, Xu and colleagues found that plasma ANGPTL4 levels were decreased in obese diabetics compared to obese nondiabetics or normal weight individuals.[7] It should be emphasized that, in contrast to the antibody used by Xu and colleagues, the antibody used in our ELISA specifically detects the full-length ANGPTL4 protein and does not recognize any of the truncated variants of ANGPTL4. It is known that ANGPTL4 is cleaved into N- and C-terminal fragments of about equal size, which may each carry an entirely different function.[12] N-terminal ANGPTL4 has been shown to potently inhibit lipoprotein lipase, whereas C-terminal ANGPTL4 influences endothelial cell migration and function, possibly via suppressing the Raf/MEK/ERK signaling cascade.[32] Future research will have to better address possible changes in full-length ANGPTL4 in diabetic patients.

Because Angptl4 overexpression markedly elevates plasma TG levels in mice,[7–10] it is of interest to study whether in humans a positive correlation may exist between plasma ANGPTL4 and plasma TG levels. In a limited sample of 36 women, we could not find such a correlation, nor between plasma ANGPTL4 and other metabolic parameters. Although these data might argue against a major impact of ANGPTL4 on plasma TG levels in humans, recent studies indicate that a rare sequence variant of ANGPTL4 that gives rise to a dysfunctional protein is associated with decreased plasma TG levels.[20,33–35] More extensive investigations using large cohorts will need to be carried out to further explore the relation between plasma ANGPTL4 and various plasma lipid parameters.

In conclusion, our data show major variation in plasma ANGPTL4 levels between individuals. Within an individual, plasma ANGPTL4 levels remain stable throughout the day but increase in response to fasting, chronic caloric restriction, and endurance exercise. Long-term changes in plasma ANGPTL4 levels are likely mediated by changes in plasma FFA, which potently raise ANGPTL4 gene expression.

Disclosures

None.

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Supplemental methods
Human interventions

Study 1: Blood was taken after an overnight fast from 25 male mildly hyperlipidemic subjects before and after a 4-week treatment with 250 mg of micronized fenofibrate daily. All subjects had significant coronary artery disease as documented by angiography [21].

Study 2: A fasting blood sample was taken in 36 apparently healthy post-menopausal women varying in body mass index from 20.7 to 35 kg/m^2 (age: 56.5±4.2 years, BMI: 25.4±3.3 kg/m^2, fasting glucose: 5.5 ±0.4 mM). The women participated in a randomised, open label, placebo-controlled trial that investigated the effects of daily wine or grape juice consumption on markers of insulin sensitivity. Blood samples were obtained from the antecubital vein of the forearm and collected in EDTA-coated tubes [22].

Study 3: Two male and seven female subjects between the age of 24 and 32 years came to the laboratory in fasted state (BMI: 19.1–26.6, mean 22.4 kg/m^2; fasting glucose: 4.8 ±0.6 mM). Blood samples were taken via finger prick at selected time before, after and in between meals. Subjects maintained their normal pattern of activities including meal choice.

Study 4: Four healthy male volunteers, between 19 and 22 years of age (mean 20.6), were recruited from the Wageningen student population (age:19-22, mean 20.6; BMI: 20.4–22.6, mean 21.3 kg/m^2; fasting glucose: 4.9–5.7, mean 5.2 mM). Volunteers received an identical meal at 17.00, before the start of a 48-h fasting period. At baseline and after 24h and 48 h of fasting, blood was drawn from the antecubital vein of the forearm into EDTA-coated tubes [23].

Study 5: Participants were overweight men with a mean (± SEM) age of 34.8±1.3 y and body mass index (in kg/m2) of 28.8±0.5. All 22 subjects reduced their energy intake to 2.1 MJ/d by means of a very-low-energy diet for the duration of 25 days. Fasting blood samples were obtained from the antecubital vein of the forearm and collected in EDTA-coated tubes [24].

Study 6: Seven healthy, untrained male volunteers participated in the study (age: 22.7±0.6 years, BMI: 23.8±1.0 kg/m^2, height: 1.79±0.03 m, VO$_2$max: 50.5±2.4 mL/min/kg). After an overnight fast, subjects came to the laboratory at 8:00 AM. After local anesthesia, a Teflon cannula was inserted in an antecubital vein for sampling of blood. Subjects rested on a bed, and a baseline blood sample was taken. Immediately thereafter, subjects ingested 1.4 g/kg bodyweight glucose or water. Subject exercised at 50% VO2 max for 2 h and then rested for 4 h. Blood was samples at regular intervals throughout the study. Also, at regular intervals subjects ingested 0.35 g/kg bodyweight glucose or water. All subjects underwent the experimental protocol two times, once with glucose ingestion and once while fasting [25].

Study 7: Nine healthy lean male volunteers participated in the present study (age: 20.1±0.5 years, BMI: 21.7±0.6 kg/m2, bodyfat: 16.0±1.3%). All subjects underwent two euglycaemic–hyperinsulinaemic clamps with 40 mU/m2/min insulin and variable co-infusion of a 20% glucose solution to reach a blood glucose level of ~5 mmol/L with simultaneous infusion of glycerol (73 mL/h) or intralipid (81 mL/h) in randomised order. Test days were separated by at least 1 week. On both experimental days, subjects arrived at the laboratory at 8.00 h after an overnight fast. Blood was sampled at regular time points throughout the clamp [26].

Study 8: Nine healthy lean male volunteers participated in this study (age: 24.4±1.3 years, BMI: 22.2±0.8 kg/m2, height: 1.79±0.03 m). Upon arrival at the laboratory after an overnight fast, two Teflon cannulas were inserted into an antecubital vein of each arm. One cannula was used for the infusion of β2-adrenergic agonist and one cannula for sampling of blood. A first blood sample was taken, followed by a continuous infusion of 77 ng/min/kgFFM salbutamol maintained for 3 h. In addition, two doses of 250 mg acipimox or placebo were given orally at –120 min and time 0. Blood samples were taken at regular intervals throughout the study [27].
All human interventions were approved by the Medical Ethics Committee of the institute involved.

Electrophoresis and Western Blot

0.25 µL of human plasma was separated by SDS-PAGE and transferred to immobilon-P membrane. Western blotting was carried out using an ECL system (GE Healthcare, Diegem, Belgium) according to the manufacturer's instructions. The primary antibody was used at a dilution of 1:2500, and the secondary antibody (anti-rabbit IgG, Sigma) was used at a dilution of 1:8000. All incubations were performed in 1x Tris-buffered saline, pH 7.5, with 0.1% Tween 20 and 5% dry milk, except for the final washings, when milk was omitted. Primary antibody was the same as used for Elisa or was a human polyclonal antibody directed against the epitope CQGTEGSTDLPLAPE [12].

Quantitative PCR

cDNA was prepared from FirstChoice® Human Total RNA Survey Panel (Ambion) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). Real-Time PCR was carried out using platinum Taq polymerase (Invitrogen, Breda, the Netherlands) and SYBR green using an iCycler PCR machine (Bio-Rad Laboratories BV). Melt curve analysis was performed to assure a single PCR product was formed. hANGPTL4 cDNA was amplified using primers: 5'-CACAGCCTGCAGACACAACTC-3' and 5'-GGAGGCCAAACTGGCTTTGC-3'; m/rAngptl4 cDNA was amplified using primers: 5'-GTTTGCAGACTCAGCTCAAGG-3' and 5'-CCAAGAGGTCTATCTGGCTCTG-3'. ANGPTL4 expression data were normalized against the housekeeping genes 36B4 (FAO, MSIE) or actin (primary myocytes). Primer sequences are available upon request.

Supplemental Figures
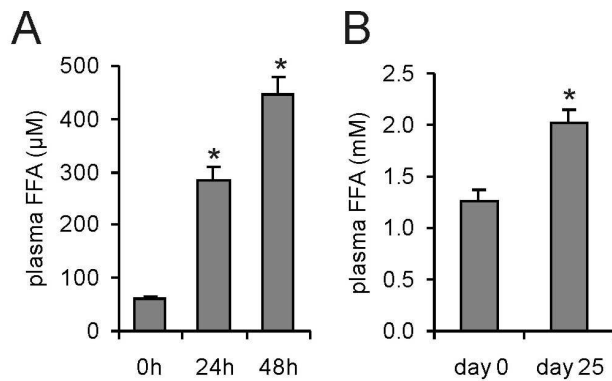
Supplemental Figure I: Plasma FFA levels are increased by fasting and caloric restriction. A) FFA levels were measured in plasma sampled from four young healthy males right after a meal, and after 24h or 48h of fasting (study 4). B) FFA levels were measured in overnight fasting plasma sampled from 22 healthy overweight males (BMI 25-32) before and after 25 days of subsisting on a protein-enriched formula diet that provided 2.1 MJ/d (study 5). Error bars represent SEM. * = significantly different according to Student's T-test (p<0.05).
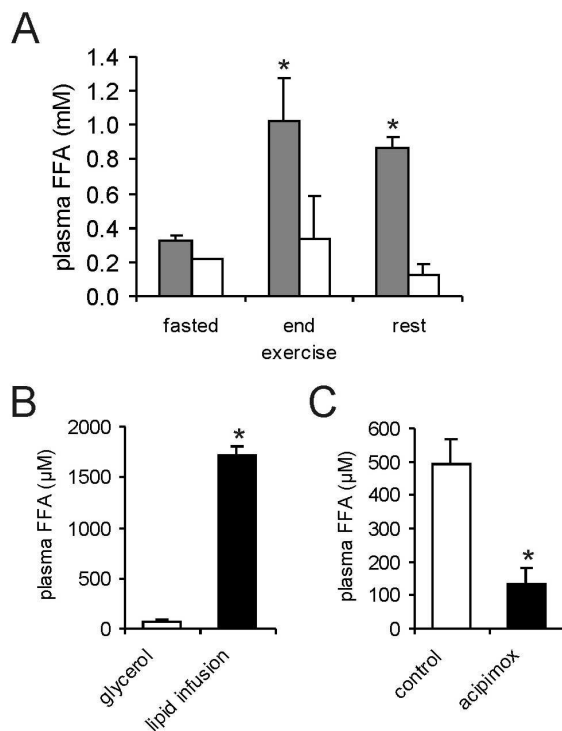
Supplemental Figure II: Plasma FFA levels increase during exercise in fasted but not fed state. A) After an overnight fast, seven subjects underwent a protocol of 2 hours of endurance exercise followed by 4 hours of post-exercise recovery either in the fasted state (grey bar) or with glucose ingestion (white bar) (study 6). Blood was samples at various time points for assessment of plasma FFA. B) Plasma FFA levels were determined in nine healthy lean male subjects after undergoing a 6-h hyperinsulinaemic–euglycaemic clamp with simultaneous infusion of either glycerol or a lipid emulsion (study 7). C) Plasma FFA levels were determined in nine lean males after receiving a 3-h infusion of salbutamol with or without simultaneous administration of acipimox (study 8). Error bars represent SEM. * = significantly different according to Student's T-test (p<0.05).