

REDUCED SENSITIVITY TO NITRIC OXIDE LIMITS SATELLITE CELL ACTIVITY ON  
CULTURED MYOFIBERS FROM AGED MICE

By

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To my husband, Chad Betters, who never doubted my capabilities, even when I did

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## TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS .....	4
LIST OF TABLES .....	7
LIST OF FIGURES .....	8
ABSTRACT .....	9
 CHAPTER	
1 INTRODUCTION .....	11
Background.....	12
Problem Statement.....	13
Variables in Study.....	14
Specific Aims and Hypotheses .....	14
Limitations/Delimitations/Assumptions .....	16
Significance of the Study .....	16
2 LITERATURE REVIEW .....	17
Satellite Cell Activation.....	17
Hepatocyte Growth Factor (HGF).....	18
Nitric Oxide (NO).....	18
Stretch.....	20
Recovery from Atrophy.....	21
Aging and Muscle Satellite Cell Activity .....	23
Satellite Cell Numbers with Age .....	23
Aging and Muscle Regeneration .....	25
Notch Signaling and Satellite Cell Activation.....	27
Nitric Oxide and HGF/c-Met in Aged Muscle .....	30
Summary .....	31
3 MATERIALS AND METHODS .....	33
Experimental Designs .....	33
Animals.....	34
<i>In Vitro</i> Electrical Stimulation and Measurement of Nitric Oxide Production .....	34
Fiber Isolation .....	36
Immunohistochemistry .....	37
Asymmetric Dimethylarginine (ADMA) ELISA .....	38
Western Blotting .....	38
Statistical Analysis.....	39

4	RESULTS .....	40
	Muscle Atrophy in Male C57 Mice .....	40
	Nitric Oxide Production from Contracting Muscle .....	40
	Nitric Oxide Synthase Protein Expression Is Not Reduced.....	41
	Asymmetric Dimethylarginine Can Inhibit Satellite Cell Activity .....	41
	Asymmetric Dimethylarginine Is Not Elevated in Aged Muscle and Blood .....	42
	Satellite Cell Response to HGF Is Not Altered with Age .....	42
	L-Arginine and a Nitric Oxide Donor Can Rescue Aged Satellite Cell Activity .....	42
5	DISCUSSION .....	56
	Main Findings.....	56
	Reduced Satellite Cell Activation in Response to Mechanical Perturbation.....	56
	L-Arginine and a Nitric Oxide Donor Can Rescue Aged Satellite Cell Activity .....	57
	Nitric Oxide Production and NOS Protein Expression from Aged Muscle .....	58
	Satellite Cell Response to HGF Is Not Altered with Age .....	60
	Asymmetric Dimethylarginine Is Not Elevated in Aged Muscle and Blood but Does Inhibit Satellite Cell Activity .....	60
	Response to Nitric Oxide.....	62
	Limitations and Future Directions .....	63
	Conclusions.....	64
	LIST OF REFERENCES .....	67
	BIOGRAPHICAL SKETCH .....	72

## LIST OF TABLES

<u>Table</u>	<u>page</u>
4-1 Body mass, gastrocnemius mass, muscle mass/body mass ratios, and total protein for young, adult, and old mice.....	44
4-2 Asymmetric dimethylarginine (ADMA) concentration ( $\mu\text{mol/L}$ ) in serum and muscle of young and old mice.....	45
4-3 Quantification of satellite cell activation as measured by (A) cell emanation and (B) BrdU incorporation from young, adult, and old myofibers. ....	46

## LIST OF FIGURES

<u>Figure</u>	<u>page</u>
4-1 Nitric oxide (NO) production as quantified by 4,5-diaminofluorescein (DAF) fluorescence. ....	47
4-2 Representative immunoblot of neuronal nitric oxide synthase from young, adult, and old plantaris muscle. ....	48
4-3 Representative immunoblot of endothelial nitric oxide synthase from young, adult, and old plantaris muscle.....	49
4-4 Representative images of an emanating satellite cell and a BrdU <sup>+</sup> nucleus. ....	50
4-5 Satellite cell emanation and BrdU incorporation of fibers following exposure to ADMA. ....	51
4-6 Representative image of a fiber stained for pax7 immediately following isolation and plating. ....	52
4-7 Satellite cell emanation and BrdU incorporation of fibers following exposure to HGF.....	53
4-8 Satellite cell emanation and BrdU incorporation of fibers following exposure to L-arginine and DETA-NO. ....	54
5-1 Overview of the steps leading to satellite cell activation and the potential location of the aging deficit.....	66



Abstract of Dissertation Presented to the Graduate School  
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REDUCED SENSITIVITY TO NITRIC OXIDE LIMITS SATELLITE CELL ACTIVITY ON  
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By

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Although aging-induced deficits in satellite cell proliferation and muscle regeneration are well-characterized, our investigation provides novel evidence of an age-related deficit in satellite cell activation, independent of proliferation, that is intrinsic to the muscle fiber. Mechanical activation of muscle fibers induces enzymatic production of nitric oxide (NO) resulting in proteolytic release of hepatocyte growth factor (HGF) from its extracellular tethering, and subsequent activation of satellite cells. We hypothesized that aging would reduce *in situ* satellite cell activation, and that L-arginine or nitric oxide (DETA-NO) supplementation would improve satellite cell activity in old muscle. Single intact myofibers were isolated from the gastrocnemius muscles of young (2 mo), adult (10 mo), and aged (22 mo) male C57 mice. Fibers were centrifuged to stimulate satellite cell activation and incubated in serum-free media with 0.002% 5-bromodeoxyuridine (BrdU) and either 2 mM L-arginine, 10  $\mu$ M DETA-NO, or control media for 48 h. The total number of satellite cells per fiber (i.e., pax7<sup>+</sup>) was unchanged with aging. Nevertheless, the number of activated satellite cells (BrdU<sup>+</sup>) following centrifugation was reduced in the aged fibers. L-arginine or DETA-NO treatment to the aged fibers restored satellite cell activation to young-control levels. However, these treatments also increased satellite cell activation in young and adult fibers, such that an age-dependent deficit in satellite cell activity

persisted within treatment groups. Expression of neuronal and endothelial nitric oxide synthase (NOS) proteins in the plantaris muscles did not differ between age groups. Further, contraction-induced NO release from the extensor digitorum longus (EDL) did not differ between adult and old mice. These data suggest that impairment of NO production does not account for reduced satellite cell activity in aged muscle fibers. Also, treatment with exogenous HGF was equally effective in activating satellite cells across age groups, indicating that events downstream of HGF release are intact in the aged muscle. Therefore, these data imply a reduced sensitivity of aged muscle to NO. Insight gained from these experiments will augment therapeutic strategies to maintain skeletal muscle mass in the elderly, and to enhance muscle regeneration in this population following disuse or acute injury.

## CHAPTER 1 INTRODUCTION

Sarcopenia, the gradual decline of skeletal muscle mass and strength, is a hallmark of aging. Sarcopenia is generally responsible for the age-related decline in physical performance, and ultimately for physical disability in many elderly people. The prevalence of sarcopenia, defined as appendicular skeletal muscle mass (kg)/height<sup>2</sup> (m<sup>2</sup>) being less than two standard deviations below the mean of a young reference group, ranges from 13 to 24% in those 65 to 70 years old, and is over 50% for those greater than 80 years of age (6). Another associated characteristic of aging is a decline in ability of skeletal muscle to repair itself following damage. Age-associated muscle atrophy likely results, in part, from the reduced ability of muscle to regenerate following repeated micro-trauma occurring during the course of daily activities (e.g., eccentric contraction-induced injury).

Mature skeletal muscle fibers consist of post-mitotic and terminally differentiated nuclei. Thus, the addition of nuclei to skeletal muscle fibers during hypertrophy or regeneration must come from sources other than myonuclei. Satellite cells represent the muscle “stem” cells that provide these additional nuclei. Satellite cells are quiescent precursor cells residing in a “satellite” position alongside muscle fibers (37). The ability of these muscle precursor cells to increase muscle mass and repair muscle following injury makes them essential to skeletal muscle function. However, with advanced age, there is a reduction in the population of satellite cells (5, 12, 28, 31, 45, 48, 51) and their ability to activate/proliferate, and repair muscle (19, 20). Thus, the ability of aged muscle to increase mass or repair damage is poor compared to young or adult muscle. The mechanisms underlying the age-related deficit in satellite cell function warrant exploration.

## Background

Muscle atrophy during aging likely results, in part, from inefficient muscle repair consequent to injury. Skeletal muscle regeneration following immobilization-induced atrophy is lacking in aged rats, even after 77 days of recovery (17). However, direct administration of IGF-1 onto atrophied muscles restores the *in vivo* regenerative capacity of old muscle and *in vitro* satellite cell proliferation. This finding suggests that satellite cells from aged muscle do not lose their inherent ability to proliferate, but simply are not activated sufficiently to regenerate following an injury. Thus, it is important to isolate the process of activation from subsequent proliferation. Old satellite cells may remain inactive due to the lack of some unknown growth factor/mitogen, or the failure to respond to the levels of endogenous growth factors.

Satellite cells are known to be activated by several stimuli, such as hepatocyte growth factor (HGF) (4, 53, 54), mechanical stretch (54, 59) and exercise (23, 24, 32). Activation of satellite cells refers to entry into the cell cycle from quiescence, including DNA synthesis, and mobilization. Other growth factors, such as IGF-1 and basic FGF, can stimulate proliferation of satellite cells (7, 8, 9, 10, 51, 61) but thus far, only HGF affects activation.

Nitric oxide (NO) appears to be an important signaling molecule mediating satellite cell activation via HGF release (4). Inhibition of NO production inhibits HGF release, c-met/HGF co-localization, and satellite cell activation (2). In addition, mechanical stretch of rat satellite cells (54) and isolated mouse myofibers (59) identified the importance of NO in stretch-induced satellite cell activation. Similarly, HGF is crucial for stretch-induced satellite cell activation, and its release is dependent on NO (54).

Satellite cell proliferation and cell fate determination are regulated by the Notch signaling pathway. This pathway is triggered by the rapid upregulation of Delta, the Notch ligand, following injury. Recently, Conboy et al. (19) demonstrated deficiencies in Notch signaling that

affected proliferation and regeneration of old mouse muscle. They showed that old muscle fails to upregulate Delta in response to an injury. Forced activation of Notch improved regeneration in old muscle, while Notch inhibition decreased repair in young muscle. When old cultured satellite cells were exposed to young serum, satellite cells upregulated Delta and activated Notch (20). In addition, parabiotic pairing of a young and an old mouse, such that they shared a circulatory system, restored regenerative potential following an injury in the old mouse muscle. These experiments demonstrate that Notch activation is necessary for regeneration in young muscle and sufficient to stimulate effective repair in old muscle. Plus, components in young serum are capable of reversing the age-induced decline in activation/proliferation of satellite cells. Thus, satellite cells do not lose their innate ability to repair muscle when aged, but modifications of the old systemic environment prevent their proper functioning.

### **Problem Statement**

A better understanding of sarcopenia is essential since a significant proportion of the population is living long enough that muscle atrophy becomes a major issue. In addition, physical rehabilitation following an injury requires functional satellite cells. Aging is associated with an impaired ability to repair muscle following damage, and an increased amount of muscle wasting. Advanced age reduces satellite cell numbers (5, 12, 28, 31, 45, 48, 51) and satellite cell activation/proliferation (19, 20). Work by Conboy and colleagues (20) has shown that aged satellite cells can repair muscle when exposed to a young systemic environment. This suggests that extrinsic signals regulate aged satellite cell activity, and that the possibility exists that the loss of muscle regenerative capacity can be restored if the satellite cell environment more closely resembles that of a young animal. Given the importance of NO as the initiating signal for satellite cell activation, we propose that one key age-related modification in the muscle environment is a decrease in NO bioavailability. We postulate that NO production is reduced

with aging and may compromise satellite cell function. NO production could be reduced due to a decrease in the cationic amino acid transporter (CAT) expression responsible for transporting the NOS substrate L-arginine into the cell, a reduction in NOS expression, or an increase in asymmetric dimethylarginine (ADMA), an endogenously produced metabolite which inhibits NOS activity. Discovery of the age-associated factors that regulate satellite cell behavior will be instrumental for designing therapies targeted to augment the regenerative potential of muscle and thus maintain muscle mass in elderly individuals.

### **Variables in Study**

Independent variables: We will manipulate the age of the animals, electrical stimulation of incubated muscles, and the isolated myofibers' treatment medium (supplementing with L-arginine, DETA-NO, HGF, or ADMA).

Dependent variables: We will measure satellite cell activation via BrdU incorporation. We will also measure nNOS, eNOS, and iNOS protein content in plantaris muscle, CAT1/2 mRNA levels, and stain for Pax7 and BrdU in isolated myofibers. We will measure nitric oxide production in extensor digitorum longus (EDL) muscles electrically stimulated *in vitro*.

Control variables: Only male C57 mice will be studied, so gender is purposely excluded from this study.

Extraneous variables: We will not control prior activity level or food and water intake of the mice.

### **Specific Aims and Hypotheses**

This study is designed to answer the following questions:

Question 1. Is nitric oxide production reduced in old muscle?

Hypothesis 1: NO production (quantified by DAF fluorescence) is reduced in old muscle following *in vitro* electrical stimulation of the EDL. In addition, NOS protein expression and CAT mRNA are reduced in old muscle.

Question 2. Is there a deficit in satellite cell activation with aging in response to exogenous HGF?

Hypothesis 2. Aged satellite cells will respond to increasing doses of HGF by incorporating BrdU into DNA similar to young satellite cells, indicating that the deficit is upstream of HGF release.

Question 3. Is asymmetric dimethylarginine (ADMA) elevated in the muscle and/or blood of aged animals?

Hypothesis 3: ADMA, as measured by a commercially available ELISA kit, is elevated in both the serum and muscle of aged animals.

Question 4. Can ADMA inhibit satellite cell activation of young fibers?

Hypothesis 4: Physiologically relevant concentrations of ADMA will reduce satellite cell activation of young fibers in response to mechanical perturbation (centrifugation).

Question 5a. Is there a deficit in satellite cell activation with aging in response to mechanical stress?

Hypothesis 5a: Aged satellite cells will have decreased activation (cell emanation as well as BrdU incorporation) of satellite cells in response to mechanical perturbation (centrifugation).

Question 5b. Can L-arginine and/or a nitric oxide donor (DETA-NO) rescue satellite cell activation in old muscle?

Hypothesis 5b: Both L-arginine and DETA-NO treatment for 48 hours will return satellite cell activation of old fibers to young levels.

### **Limitations/Delimitations/Assumptions**

Limitations: The invasive nature of this study negates the use of human subjects. A mouse hindlimb model has been selected because of the similarities in structure and function of mouse and human skeletal muscle.

Delimitations: Gender and species differences may exist in regard to satellite cell function. We have chosen to study only male C57 mice.

Assumptions: It is assumed that satellite cells remain quiescent during the myofiber isolation procedure. Previous experiments have confirmed the quiescent nature of satellite cells during isolation using this technique.

### **Significance of the Study**

Normal daily activity subjects skeletal muscle to injuries, necessitating a means of repair to maintain muscle. Thus, there is a constant demand for satellite cells throughout life. A decrease in satellite cell abundance and activity with age may contribute to the age-associated deficiency in muscle repair, and ultimately to muscle loss.

This research will improve our knowledge of the mechanisms underlying the deficit in satellite cell function with aging. We seek to identify aging-associated deficiencies in signaling pathways leading to satellite cell activation, and to reverse these deficiencies. This study will provide insight into clinical therapies to attenuate muscle loss and improve muscle regeneration in the elderly.



## CHAPTER 2 LITERATURE REVIEW

Skeletal muscle is a multi-nucleated tissue capable of generating force that is essential for locomotion and breathing. It is continually subjected to injury and micro-trauma, resulting from mechanical stress, including weight bearing and exercise. Thus, skeletal muscle requires a renewable source of cells for muscle repair. Satellite cells provide the additional nuclei to muscle fibers in times of growth or regeneration.

Satellite cells are located adjacent to the muscle fiber and beneath the basal lamina (37). They are believed to be the committed stem cells of adult skeletal muscle (57). While normally quiescent, they are activated when needed for repair or to mediate muscle hypertrophy.

Aging is associated with a decreased satellite cell number (5, 12, 28, 31, 45, 48, 51) and marked impairments in regeneration following injury or atrophy (17, 19, 20, 43). This project seeks to understand the molecular mechanisms of satellite cell aging that lead to loss of tissue regenerative capacity in old muscle.

### **Satellite Cell Activation**

Satellite cell activation is defined as entry into G1 phase from quiescence, and mobilization (2). To date, the only known factors to activate satellite cells include hepatocyte growth factor (HGF) and nitric oxide (NO). Perturbations such as stretch and exercise act via NO and HGF release to activate satellite cells. In addition, the activating factor in crushed muscle extract (CME), a known activating treatment, is HGF (53). Other growth factors, such as insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF), cannot directly activate satellite cells, but do improve proliferation (7, 8, 9, 10, 61).

## **Hepatocyte Growth Factor (HGF)**

HGF is synthesized in an inactive form as a single chain of ~90 kDa, and is proteolytically cleaved into an active, disulfide-linked heterodimer of 60 kDa (alpha-chain) and 30 kDa (beta-chain) by serine proteases (40, 41). Tatsumi and Allen (52) showed that HGF is present in the extracellular domain of uninjured rat skeletal muscle as active heterodimeric HGF. Significant amounts of pro-HGF were only found in rat muscle when the integrity of the membrane was compromised. Thus, active HGF is present in the extracellular matrix (ECM) and can be released following injury. This is important because proteolytic conversion of pro-HGF to active HGF is therefore not a rate-limiting step in the release of active HGF from the ECM.

HGF is released from its tethering to the ECM by the action of matrix metalloproteinases (MMP's) (62). When released, HGF rapidly co-localizes with its receptor on satellite cells, the c-met tyrosine kinase receptor, and triggers activation from quiescence (53). Once activated, satellite cells synthesize both HGF and c-met (3, 53).

Treating muscle with CME causes dose-dependent activation of satellite cells (11, 53). CME increases expression of proliferating cell nuclear antigen (PCNA), an indicator of activation (DNA synthesis), in three week-old and nine month-old rat satellite cells (30). The factor in CME causing activation was later identified as HGF (53). Treating satellite cells with CME in the presence of anti-HGF neutralizing antibodies eliminated the CME-induced proliferation, indicating that HGF is the factor in CME responsible for stimulating satellite cell activity. Therefore, HGF is an important growth factor to skeletal muscle because of its role in activating satellite cells and thereby contributing to repair and regeneration.

## **Nitric Oxide (NO)**

Work has shown that HGF release from the extracellular matrix is NO-dependent, and inhibition of nitric oxide synthase (NOS) prevents that release (2, 4, 50, 52). Anderson and

colleagues have demonstrated that NO synthesis is implicated in the activation of satellite cells in crush injured skeletal muscle (2) and resident satellite cells on isolated myofibers cultured *in vitro* (4). *In vivo*, NOS inhibition prevents HGF/c-met colocalization and satellite cell activation (2). Activation of satellite cells on single myofibers was found to be dependent on the levels of NO (related to the amount of substrate, L-arginine), and HGF in the media (4). In contrast, NOS inhibition (L-NAME) prevented this activation.

Anderson and Pilipowicz (4) characterized the series of events leading to satellite cell activation on isolated single myofibers. CME, HGF, and L-arginine activated satellite cells, while L-NAME significantly blocked the potent activation by CME, as well as spontaneous control activation. These results suggest that NO plays a role in spontaneous and HGF-dependent activation. Since spontaneous activation was decreased by NOS inhibition but restored by HGF, the activation signaling by HGF appears to occur after NO-mediated signaling events.

Tatsumi and Allen (52) furthered our understanding of NO-mediated HGF release by showing that the NO donor, sodium nitroprusside (SNP, 300  $\mu$ M), caused release of active HGF from the extracellular matrix of rat EDL muscle and did not involve proteolytic cleavage of pro-HGF. The addition of protease inhibitors to the incubation medium did not increase the amount of pro-HGF released, demonstrating that the NO-stimulated mechanism of active HGF release does not require proteolytic cleavage of pro-HGF. In addition, Yamada and colleagues (62) provided evidence of the role of MMPs in the NO-dependent release of HGF from the extracellular matrix.

More recently, Wozniak and Anderson (58) demonstrated that NO concentration regulates the balance between satellite cell quiescence and activation on isolated fibers. A “normal” concentration of NO maintains quiescence, while higher or lower levels of NO induce activation.

This was shown by stretching normal fibers and increasing NO concentration and satellite cell activation. Likewise, satellite cell activation was increased in models of reduced NO availability, namely fibers from mdx mice and NOS-I<sup>-/-</sup> mice. These experiments highlight the importance of NO for satellite cell activity.

### **Stretch**

Mechanical stretch of cultured adult rat satellite cells was shown to cause release of HGF and accelerated entry into the cell cycle (54). Conditioned media from cultures of stretched satellite cells stimulated activation of unstretched cultures, and anti-HGF neutralizing antibodies in conditioned media eliminated this response. Additionally, conditioned media from cells stretched with L-NAME did not cause activation of unstretched satellite cell cultures, and HGF was not released into the media. Media from unstretched cells cultured in the presence of an NO donor was able to increase activation of control cultures, and HGF was detected in this media. NOS activity assays indicated that NOS was stimulated when muscle satellite cells were stretched. This data indicates that stretch stimulates intracellular events involving NO synthesis and subsequent HGF release leading to satellite cell activation.

Mechanical stretch of single fibers has been used to activate satellite cells associated with myofibers (59). Satellite cells on stretched fibers were significantly activated (incorporated BrdU) at 30 minutes and 2 hours after initiation of stretch. It was speculated that these two peaks may indicate that the same satellite cells entered proliferation after 30 minutes and 2 hours of stretch and that each cell requires more than one stimulus to activate. Or, that the two peaks symbolize two or more populations of satellite cells entering proliferation after differing stretch stimuli (30 minutes and 2 hours). The latter explanation suggests that different regulatory events may exist among various subpopulations of satellite cells.

This same study (59) also examined c-met expression in control, quiescent fibers. The number of quiescent c-met<sup>+</sup> cells/fiber, determined using *in situ* hybridization with c-met mRNA, was found to be approximately 1.4, with a range of 0-6 per fiber, in non-stretched cultures. There are a number of c-met<sup>+</sup> cells that are not activated (BrdU-negative) by an activating stimulus (HGF, CME, or stretch), indicating that the entire population of satellite cells is not uniformly activated. This may be due to a subset of satellite cells that remain quiescent to maintain the precursor pool. The use of stretch of single myofibers has the potential to further elucidate the mechanisms of stretch-induced activation of satellite cells in their resident position and from a quiescent state.

Mechanical stretch activates satellite cells on fibers (59) and in single cell cultures (54). NOS activity is stimulated by stretch, and activation is NO-dependent (54). Likewise, HGF is critical for activation and its stretch-induced release is prevented with NOS inhibition. Therefore, NO appears to be an important signal for stretch-induced HGF release and satellite cell activation.

### **Recovery from Atrophy**

Skeletal muscle atrophies greatly in response to unloading. Two weeks of hindlimb suspension resulted in a 36% reduction in myonuclear number in the mouse soleus (38). Two weeks of recovery restored myonuclear number and muscle mass, suggesting that muscle precursor cells can proliferate and fuse with myofibers. However, local gamma-irradiation, which inhibits muscle precursor cell proliferation, prevented the recovery in muscle mass and myonuclear number following unloading. Growth was normal in the first week of recovery, but inhibited in the second week. These data indicate that proliferating muscle precursor cells are required to completely restore myonuclear number and muscle mass after atrophy.

This same group showed that hindlimb suspension of two weeks reduced (by 14%) the total number of cells isolated from atrophied muscles, and the number of satellite cells associated with isolated myofibers compared to control (39). Atrophied myofibers produced fewer myoD<sup>+</sup>/desmin<sup>+</sup> cells per myofiber compared to non-atrophied fibers. To assess whether proliferation was affected by hindlimb suspension, isolated precursor cells were labeled with BrdU. A 25% decrease in BrdU<sup>+</sup>/desmin<sup>+</sup> cells was found in atrophied muscles, suggesting the ability to activate and/or proliferate was impaired. The ability of isolated cells from atrophied muscles to differentiate and form myotubes was adversely affected by hindlimb suspension, suggesting that differentiation and fusion were also affected. Conversely, recovery from atrophy (normal ambulation) reversed all the effects of atrophy. Total cell number and myoD<sup>+</sup>/desmin<sup>+</sup> cells per myofiber were increased. More BrdU<sup>+</sup>/desmin<sup>+</sup> cells were associated with recovered muscles, and formation of large myotubes from muscle precursor cells was restored. Therefore, no permanent changes occur in the muscle precursor pool with atrophic conditions. Upon cessation of the atrophic stimulus, all changes were reversible.

Similarly, casting muscle for extended periods of time and then allowing for recovery causes muscle wasting followed by muscle repair. Although a decline in satellite cell number is evident with immobilization in the adult rat, remobilization leads to complete recovery of muscle mass and myofiber regeneration (13). While protein synthesis increases during the recovery phase, muscle precursor cell proliferation also increases to provide additional nuclei (35). The satellite cells of adult animals are therefore able to activate and proliferate to promote recovery of atrophic muscle.

Skeletal muscle has the unique ability to repair and regrow, or to hypertrophy with appropriate stimuli. However, these actions cannot occur without the addition of new nuclei to

existing myofibers, or the fusion of myoblasts to form new myofibers. The source of these new nuclei is the muscle precursor pool of satellite cells. Satellite cell activation is therefore an indispensable step in muscle repair and adaptations to increased load. To date, HGF is the only growth factor able to directly activate satellite cells, and its release is dependent on NO.

### **Aging and Muscle Satellite Cell Activity**

Sarcopenia is the loss of skeletal muscle mass and strength with age. The age-related muscle atrophy has been attributed to marked satellite cell quiescence (25). In aged muscle, fewer satellite cells are present (5, 12, 28, 31, 45, 48, 51) and they exhibit a long latent period between the activating stimulus and proliferation (53, 61). However, data suggest that aged satellite cells retain their intrinsic myogenic potential, but the environmental stimuli are altered (20, 51).

### **Satellite Cell Numbers with Age**

Animal studies indicate that the satellite cell population declines with aging (5, 12, 28, 51). However, results from human skeletal muscle are less harmonious. Many researchers report a reduced proportion of human satellite cells with aging (31, 45, 48), while others report no changes compared to young muscle (29, 47). Renault et al. (45) found the proportion of satellite cells in the biceps brachii and masseter muscles was significantly lower in elderly men (mean age, 74 years-old) compared to younger men (mean age, 23 years-old). Kadi et al. (31) studied moderately active men and women, and found the elderly (age range, 70-83 years-old) had a significantly lower number of satellite cells and proportion of satellite cells compared to young subjects (age range, 20-32 years-old). Also, Sajko et al. (48) analyzed autopsy samples of vastus lateralis muscle from young (mean age, 28.7 years-old) and old (mean age, 70.8 years-old) and determined that numbers of satellite cells per fiber length and proportion of satellite cells were

significantly lower in the old muscles. These studies involving human subjects support the hypothesis that the satellite cell fraction declines with age.

In contrast, Hikida et al. (29) sampled young (mean age, 22.5 years-old) and elderly (mean age, 65 years-old) men before and after a period of strength training, and found no significant differences in the proportion of satellite cells between the age groups. Similarly, Roth et al. (47) found no significant differences between the satellite cell population of young (20-30 years-old) and old (65-75 years-old) men and women. Several factors may contribute to the opposing results reported by various researchers. First, the number of individuals sampled and their ages may affect the results. In general, age-related changes are clearly visible after age 70 (34). In the study by Renault et al. (45), the mean age was 74 and 4 of the 6 subjects were 78 years of age or over. In contrast, the mean age of the elderly group was only 65 years in the study by Hikida et al. (29). Other variables that may affect the differing results include different muscles sampled, and the training status of individuals.

Most animal studies report declines in satellite cell numbers with age (5, 12, 28, 51). Muscle-derived cell yield was significantly reduced in old rat muscle compared to young (5). Bockhold and colleagues (12) reported a decline in the number of myogenic cells with aging in the mouse using cells that migrated from single fibers in culture. Shefer and colleagues (51) used molecular markers to characterize quiescent satellite cells and their progeny as they proliferate, differentiate, and replenish the reserve pool in mouse muscle. The results of this study (51) show that resident satellite cell numbers decline with age in myofibers from both fast- and slow-twitch muscles. Enrichment of the culture medium with FGF2 enhances the regenerative capacity of aged satellite cells, indicating the myogenic potential of aged satellite cells is not decreased as abundance declines.



In contrast, Yablonka-Reuveni et al. (61) found that myofibers cultured from old rat muscle retained a greater number of proliferating myoD<sup>+</sup> cells than young rat muscle. However, the ages of old rats in this study were 9-11 months-old, and were compared to young (3 weeks-old) rats in the rapidly growing phase. Conflicting data from various groups may result from differences in species used, ages sampled, and techniques to identify satellite cells.

### **Aging and Muscle Regeneration**

Following limb immobilization, young muscle has the ability to completely regain muscle mass (13). In contrast, old muscle exhibits remarkably little regrowth following limb immobilization (17, 43). The gastrocnemius of old rats showed no significant muscle regrowth, even after 77 days of recovery. In addition, the soleus muscle of old rats fails to show any regrowth after 30 days of recovery (43), whereas the soleus of young muscle recovers 37 and 40% of mass after 6 and 15 days of reload, respectively, after the same atrophy stimulus (18).

Chakravarthy et al. (17) administered insulin-like growth factor-1 (IGF-1) directly onto atrophied muscle of 25-30 month-old rats, and found that *in vivo* skeletal muscle regrowth and *in vitro* satellite cell proliferation were stimulated. They further showed that IGF-1 improves proliferation by decreasing the cell cycle inhibitor p27Kip1 via the PI3kinase/Akt pathway (16). These data prompted the hypothesis that satellite cells were in sufficient quantity in old muscle, but the systemic environment lacked appropriate growth factors/stimuli, or that old satellite cells were less responsive to stimuli.

Age-related muscle loss could partially result from repeated episodes of incomplete repair and regeneration (5). Muscle-derived cells (mdc) from young rats started to proliferate sooner than adult and aged mdc. Cell cycle duration was faster in old cells compared to young, which could lead to a more rapid exhaustion of replicative potential. Serum stimulation significantly induced c-met and PCNA expression in young mdc, but the increase was much lower in old mdc.

When measured on crushed muscle extracts, plasminogen activators (PAs) and matrix metalloproteinase 2-9 (MMP 2-9) enzyme activities were significantly lower with aging. These modifications in events associated with satellite cell activation and proliferation may lead to repeated bouts of incomplete repair and regeneration and to the loss of skeletal muscle mass with aging.

The hypothesis of incomplete repair by Barani et al. (5) is supported by others (26, 27). Gallegly and colleagues (27) found evidence of ongoing regeneration in old rat muscle. Centrally located nuclei were elevated in the soleus of aged (32 month-old) compared to young (6 month-old) and adult (20 month-old) rats. Myonuclear domain size was decreased with age since soleus muscle atrophy was not accompanied by a change in myonuclear number. Total BrdU<sup>+</sup> nuclei were decreased with age, but BrdU<sup>+</sup> myonuclei were increased. These results suggest that muscle atrophy with age is coincident with increased myofiber nuclear density from proliferation and fusion of satellite cells, resembling ongoing regeneration. Likewise, Edstrom and Ulfhake (26) found no lack of regenerative drive in aged rat muscle. Aged soleus muscle had an accumulation of nuclei, a wide distribution of fiber sizes, and an increase in embryonic MHC staining compared to young muscle. The gastrocnemius muscle had high levels of MyoD and myogenin mRNA expression compared to young. The regenerative drive is apparently intact in aged muscle, but the efficiency or outcome of regeneration is poor.

In young rats, intermittent reloading for one hour/day during 7 or 14 days of hindlimb suspension was sufficient to attenuate soleus muscle atrophy (27). However, this intervention did not attenuate atrophy in aged (32 month-old) rats. Intermittent reloading was only associated with an increase in BrdU<sup>+</sup> nuclei in young rats. This altered response of the soleus to an atrophy-reducing stimulus likely results from altered satellite cell activation with age.

Conflicting data concerning myonuclear domain were published by Brack et al. (14). This group found that loss of nuclei per unit of fiber length with age is exacerbated in larger fibers, resulting in an increased domain size in these fibers, whereas Gallegly et al. (27) reported a decrease in nuclear domain size. This loss of nuclei reported by Brack and colleagues (14) can be accounted for by either excessive loss, or insufficient replacement. These researchers hypothesize that muscle atrophy occurs to restore the nuclear domain size of a fiber to its set-point after a transient increase in domain size due to nuclear loss/inadequate replacement. Thus, the debate seems to be whether nuclei are lost prior to fiber atrophy, or vice versa.

Gene regulation during recovery from atrophy is altered in aged muscle (1, 27, 36, 43). In particular, MyoD remained elevated in old muscle after regeneration, whereas its levels were restored to normal in young animals (36). Plus, aging reduced the increase in MyoD protein levels during hindlimb suspension (1). With advancing age, the increase in MyoD and myogenin mRNA with hindlimb suspension in young rats was absent (27). Therefore, it is highly probable that genes regulated by these transcription factors are dysregulated. Pattison et al. (43) also demonstrated altered gene regulation with recovery from atrophy in aged muscle. A growth factor (amphiregulin) was not upregulated, while a cell death-associated protein (clusterin) remained upregulated in the old versus the young rat soleus muscle.

### **Notch Signaling and Satellite Cell Activation**

Conboy and Rando (21) utilized an isolated myofiber model to study the role of Notch-1 and its antagonist Numb in satellite cell activation. Muscle injury (tibialis anterior) led to increased expression of activated Notch-1 in myofibers isolated 72 hours after injury. Interestingly, Numb immunostaining indicated an asymmetric distribution in myofiber-associated mononucleated cells. Numb and pax3, a premyoblast marker, were virtually mutually exclusive, whereas Numb mostly co-expressed with myf-5, pax7, and desmin. Therefore, Numb

cells have a less committed muscle precursor phenotype, while Numb<sup>+</sup> cells have a more committed myogenic phenotype.

Expression of constitutively active Notch-1 in muscle progenitor cells increased pax3 expression and decreased myf-5, myoD, and desmin (21). Constitutively active Notch also enhanced myoblast proliferation while reducing embryonic myosin heavy chain (MHC) expression and the production of multinucleated myotubes. In contrast, overexpressing Numb decreased pax3 expression and increased myf-5 and desmin protein levels. High levels of Numb led to myotube formation. Taken together, these data indicate that Notch-1 activity promotes myogenic cell proliferation, while inhibition of Notch-1 activity by Numb leads to exit from the cell cycle, expression of muscle regulatory factors (MRFs), and myogenic differentiation.

The Notch-1 ligand, Delta, increases in response to muscle injury in satellite cells of young (2-3 month-old) and adult (5-7 month-old) mice, but fails to do so in satellite cells of old (23-24 month-old) mice (19). Increased Delta expression in young and adult mice is coincident with decreased Numb expression and increased proliferation. Satellite cells from old mice have similar Notch-1 levels, but consistently lower levels of activated Notch than young mice. Following muscle injury, Delta expression is upregulated in satellite cells from young mice, but old satellite cells failed to do so. There was approximately one-fourth the number of activated satellite cells in old muscle as in young and adult after injury. When Jagged-Fc, an inhibitor of Notch activation, was introduced at injury sites, young muscle regeneration was ineffective, similar to that seen in old muscle. Direct activation of Notch, using an antibody to its extracellular domain, markedly improved *in vivo* regeneration in old muscle, making it similar to young muscle. Therefore, Notch activation is not only necessary for young muscle regeneration but is also sufficient to induce muscle regeneration in old muscle.

To further support these experiments, Conboy et al. (20) performed a series of parabiotic pairings between young (2-3 month-old) and old (19-26 month-old) mice. These parabiotic pairings allowed for a shared circulatory system, exposing old mice to factors present in young serum and vice versa. After 5 weeks of parabiosis, the hindlimb muscles were injured and injected with BrdU. Five days after injury, young muscle of both isochronic (young-young) and heterochronic (young-old) pairings had regenerated. In contrast, old muscle from isochronic pairings regenerated poorly. However, pairing with a young mouse significantly enhanced muscle repair in old partners. This regeneration was due almost exclusively to activation of resident aged satellite cells and not to infiltration of cells from the young partner. Satellite cells from the aged partner in heterochronic pairings showed marked Delta upregulation, whereas this induction was lacking in old isochronic pairings. There was a slight inhibition of Delta upregulation in young satellite cells from heterochronic pairings compared to young isochronic pairs.

The researchers also studied the effects of heterochronic parabiosis *in vitro* (20). Exposure of satellite cells cultured from old muscle to serum collected from young mice improved Delta upregulation and Notch activation compared to old satellite cells cultured in old serum. Young serum also significantly increased myofiber-associated satellite cell proliferation of old cultures. Young satellite cells exposed to old mouse serum exhibited reduced Delta upregulation. When Notch signaling was inhibited, proliferation was decreased in both young and old satellite cells cultured in young mouse serum. Taken together, these *in vivo* and *in vitro* data suggest that factors in old serum negatively influence satellite cell activation, proliferation, and muscle repair. However, components in young serum alone are sufficient to restore satellite cell function in aged muscle. This implies that the old systemic environment is altered such that there is a

decrease in a positive factor found in young serum, an increase in a negative or inhibitory factor, or both.

### **Nitric Oxide and HGF/c-Met in Aged Muscle**

Neuronal nitric oxide synthase (nNOS) activity and percentage of NOS-containing fibers were significantly reduced in muscles from aged rats compared to young (46). In young (8 month-old) animals, nNOS activity was highest in the EDL, which also had the greatest percentage of NOS-containing fibers. Activity was lowest in the soleus, and intermediate in the diaphragm. NOS activity was significantly reduced in all muscles of the 24 month-old animals, with EDL still having the greatest activity. In contrast to these results, Capanni and colleagues (15) reported an increase in nNOS expression in 24 month-old rat gracilis muscle. However, this group measured NOS expression via immunoblots and immunohistochemistry, whereas Richmonds and colleagues (46) measured functional NOS activity. It is possible that NOS enzymatic activity is inhibited even with an upregulation of nNOS protein expression.

Asymmetrical dimethylarginine (ADMA) is a likely candidate for the inhibition of NOS activity with aging. ADMA is an endogenous inhibitor of NOS, and is elevated in the serum of aged animals compared to young (60). The role of ADMA in endothelial dysfunction has been studied (22), and by blocking NO generation, ADMA promotes processes leading to atherogenesis, plaque progression, and plaque rupture. There are associations between elevated levels of ADMA and many cardiovascular risk factors, which are also associated with advancing age. Xiong et al. (60) measured ADMA levels in the serum of young (6 month-old) and old (20 month-old) rats. ADMA levels were significantly increased in the serum of the aged animals compared to the young. Serum L-arginine levels were similar between the two ages, so the L-arginine/ADMA ratio was lower in old rats compared to young. Lastly, serum concentrations of nitrite were significantly decreased in old animals. Thus, it may be that ADMA levels increase

with age, and inhibit NO signaling in old muscle. To date, no studies have measured ADMA levels in young and old skeletal muscle.

NO is important for triggering the release of HGF in the extracellular matrix to activate satellite cells (2, 4, 50, 52). Barani and colleagues (5) reported a lower increase in the HGF receptor, c-met, expression in cultured muscle-derived cells (mdc) of old rats with serum stimulation compared to young. Young (3 week-old) and adult (9 month-old) mdc cultures increased relative c-met expression by ~190% with serum stimulation. In contrast, old (24 month-old) mdc increased c-met expression by just 130% with serum stimulation. Cell surface density of the c-met receptor was increased to the same extent in the three ages with serum exposure.

In conclusion, muscle wasting associated with aging is well documented. Deficiencies in satellite cell activation/proliferation, and incomplete repair of old muscle are also well recognized. Several groups have identified strategies to improve muscle regeneration in aged animals, including supplemental IGF-1 (17), forced Notch activation (19), and exposure to young serum (20). A decrease in c-met expression and nNOS activity, and an increase in the NOS inhibitor ADMA may contribute to the age-associated reduction in satellite cell activation.

### **Summary**

Nitric oxide production triggering HGF release is an essential step in the activation of satellite cells from a quiescent state. With aging, nNOS activity is decreased (46) and serum levels of the NOS inhibitor ADMA are increased (60). It appears that aged satellite cells retain the intrinsic ability to activate and proliferate when exposed to a young systemic environment (21). This young environment both exposes aged satellite cells to young systemic factors, and reduces their exposure to old systemic factors. Identifying those factors that affect the activity of endogenous satellite cells will be crucial to improving the regenerative capacity of aged muscle.

It is possible that if satellite cell activation and proliferation can be maintained with age, skeletal muscle loss due to sarcopenia will be prevented.



## CHAPTER 3 MATERIALS AND METHODS

### Experimental Designs

This project was designed to answer the following questions with the accompanying experimental designs:

**Question 1.** Is nitric oxide (NO) production reduced in old muscle?

**Experiment 1.** Extensor digitorum longus (EDL) muscles from young, adult, and old mice were incubated in small tissue baths and NO production was measured using DAF fluorescence in the presence and absence of electrical stimulation.

**Question 2.** Is there a deficit in satellite cell activation with aging in response to HGF?

**Experiment 2.** Young, adult, and old single myofibers were isolated from the gastrocnemius muscle and cultured with different concentrations of HGF (1-50 ng/ml) to activate satellite cells.

**Question 3.** Is asymmetric dimethylarginine (ADMA) elevated in the muscle and/or blood of aged animals?

**Experiment 3.** Blood was collected and serum isolated from young, adult, and old mice for measurement of ADMA. Also, plantaris muscles from the three age groups were harvested and homogenized for measurement of ADMA.

**Question 4.** Can ADMA inhibit satellite cell activation of young fibers?

**Experiment 4.** Gastrocnemius myofibers were isolated from young animals and exposed to different concentrations of ADMA (0.1-50  $\mu$ M).

**Question 5a.** Is there a deficit in satellite cell activation with aging in response to mechanical stress?

**Question 5b.** Can L-arginine and/or a nitric oxide donor (DETA-NO) rescue satellite cell activation in old muscle?

**Experiments 5a and b.** Isolated gastrocnemius myofibers from young, adult and old mice (4 mice per age group) were mechanically stimulated by centrifugation and activated satellite cells were quantified by cell emanation as well as BrdU incorporation.

### **Animals**

The subjects were young (~2 mo old, n=13), adult (~10 mo old, n=8), and old (~22 mo old, n=6) male C57 mice. All were housed in the J. Hillis Miller Animal Science Center in specific pathogen-free housing. Animals consumed the same diet (mouse chow and water ad libitum), and were maintained on a 12 h light:12 h dark photoperiod. Mice were maintained in the facility for at least one week prior to experiments. C57 mice are an accepted rodent model of aging and were selected since our lab and others have successfully used them as subjects for single fiber and satellite cell isolation. All procedures were approved by the University of Florida's Animal Care and Use Committee.

### ***In Vitro* Electrical Stimulation and Measurement of Nitric Oxide Production**

We validated a technique for quantifying the release of nitric oxide from isolated mouse skeletal muscles at rest and immediately following contractions, using the cell impermeable fluorescent detector of nitric oxide, 4,5-diaminofluorescein (DAF-2, Cayman Chemical Company, Ann Harbor, MI). This method was first reported as a reliable method of quantifying NO release from cultured endothelial cells (33). Addition of the NO-donor, MAHMA-NO (0-100 nM), to oxygenated buffer containing DAF-2 (0.1  $\mu$ M) was used to establish the relationship between fluorescence intensity and NO concentration ( $R^2=0.98$ , data not shown).

The EDL muscle from one leg was electrically stimulated for 10 min to quantify activity-related nitric oxide production. The contra-lateral EDL was incubated without stimulation for the same amount of time. Animals were anesthetized with inhaled isoflurane (2–5%) with oxygen as the carrier gas. Both EDL muscles were removed and mounted in separate micro-tissue baths

between two platinum electrodes positioned 8 mm apart and surrounding the central part of the muscle. Baseline tension was set for each muscle at 2.0 g of tension. During the experiment, muscles were incubated in oxygenated Krebs-Henseleit bicarbonate buffer (KHB buffer: 1.2 mM  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ , 118.5 mM  $\text{NaCl}$ , 4.7 mM  $\text{KCl}$ , 0.25 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , pH 7.4). The incubations were carried out at constant temperature (30°C) and under continuous gassing with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ .

After setting baseline tension (2.0 g), muscles were incubated for 10 min. Muscle viability was indirectly assessed by testing whether muscles were able to contract in response to a specific electrical stimulation protocol (1 train/sec, train duration of 250 ms, 50 Hz, 10 ms delay, 10 ms duration, 30 V). Muscles were then electrically stimulated at 10 Hz, 50 V, 10 ms pulses with 90 ms delays for 10 min. while the contralateral EDL from each animal served as a non-contracting control. Buffer was refreshed 3 times at the end of the contraction period, and muscles were incubated with the cell impermeable fluorescent detector of nitric oxide, 4,5-diaminofluorescein (DAF-2, 0.02  $\mu\text{M}$ , Cayman Chemical Company, Ann Harbor, MI) in the dark for 10 min. Aliquots of buffer were analyzed for DAF fluorescence using a spectrofluorometer with excitation wavelength at 485 nm and emission wavelength at 538 nm. Absorbances were normalized to EDL wet weight.

To test for DAF-2 specificity, a separate experiment was conducted using both EDL and soleus muscles from the same animal. In this experiment both EDL muscles were electrically stimulated, while the soleus muscles were not. The protocol consisted of an initial 10 min incubation, after which the inhibitor of NOS, L-NMMA (1 mM), was added to the buffer of one EDL and one soleus. Muscles were incubated with L-NMMA for 10 min, followed by 10 min of contraction (EDL muscles) or resting (soleus muscles). Buffer was refreshed 3 times afterwards,

followed by a 10 min incubation with DAF-2. Aliquots of buffer were analyzed spectrophotometrically for DAF fluorescence. We measured an ~10% increase in background fluorescence in the soleus muscles induced by L-NMMA, and a total prevention of the contraction-induced fluorescence in the EDL muscles with L-NMMA. Thus, DAF-2 specificity was confirmed in this model.

### **Fiber Isolation**

The gastrocnemius complex, consisting of the plantaris, soleus, and medial and lateral heads of the gastrocnemius, was carefully removed from both hindlimbs. The plantaris and soleus were removed, weighed, and frozen in liquid nitrogen for further analyses. The remaining gastrocnemius muscle was rinsed in PBS, cut down the middle, weighed, and incubated in DMEM containing 0.02% collagenase, type I (Worthington, Lakewood, NJ). Both gastrocnemius muscles per animal were incubated in a 15-ml Falcon tube for 1.5 h at 37°C with gentle shaking. After this incubation, the digest was carefully triturated three times, and incubated an additional 10 min. The collagenase was then diluted by allowing the digest to settle for approximately 5 min. Most of the media was removed and fresh media without collagenase was added. This was repeated for a total of 3 times. The resulting digested muscles were decanted into p-100's containing DMEM. Intact, isolated fibers were selected using a pipet. Only fibers that were free of other cellular debris were selected and plated. These viable fibers were plated in 24-well plates with growth factor-reduced matrigel (BD Biosciences, Bedford, MA) and serum free media. Occasionally, emanating cells of non-muscle origin (non-spindle-shaped) were evident and fibers containing these cells were excluded from analyses.

For the HGF experiment, fibers were cultured with 0.002% BrdU and HGF in serum free media for 48 h, and fixed for satellite cell emanation quantification, and BrdU immunostaining. For the ADMA experiments, fibers were incubated with ADMA at concentrations of 0.1-50  $\mu$ M

for 24 h in serum free media. Then, 0.002% BrdU was added and the plates were centrifuged at 1500 x g for 30 min to induce satellite cell activation. The fibers were cultured for an additional 24 h and then fixed for quantification and BrdU immunostaining. For the final fiber experiment, L-arginine and DETA-NO were added to the media with 0.002% BrdU immediately before centrifuging at 1500 x g for 30 min to induce satellite cell activation. Fibers were cultured for 48 h and fixed for cell emanation quantification and BrdU immunostaining. A subset of fibers with emanating cells was stained for myoD to confirm that emanating cells were of muscle origin. Fibers stained for pax7 were plated in 24-well plates and fixed immediately.

### **Immunohistochemistry**

To measure satellite cell activation, the number of cells staining positive for BrdU (BrdU<sup>+</sup>) were counted. Fixed fibers were incubated in 1 M HCl for 45 min at 45°C. This was neutralized with 0.1 M borate buffer (pH 8.5) for 10 min. Fibers were blocked in 5% goat serum for 1 h, followed by incubation in primary anti-BrdU antibody (1:75, Megabase Research, Lincoln, NE) for 2 hours. They were then incubated in secondary anti-rabbit-Texas Red antibody (1:100, Vector Laboratories) for 1 hour and stained with DAPI-containing mounting media (Vector Laboratories). Fibers stained for pax7 were permeabilized with 0.1% Triton-X for 5 min. and blocked in 5% goat serum for 1 h. Primary anti-pax7 (R&D Systems, Minneapolis, MN) was applied for 1 h at 1:50 dilution. Secondary anti-mouse-Texas Red (1:100) was incubated for 1 h. Fibers were mounted with DAPI-containing mounting media. All fibers were viewed on a Zeiss microscope with rhodamine, FITC, and DAPI filters.

To confirm that the spindle-shaped emanating cells (i.e. mononuclear cells growing on the substratum adjacent to myofibers 48 h after centrifugation) were of muscle origin, a subset of cultures was stained for myoD using the Tyramide Signal Amplification (TSA) kit (Perkin Elmer, Boston, MA). Fixed cultures were incubated in 3% H<sub>2</sub>O<sub>2</sub> to quench endogenous

peroxidases. Fibers were blocked in TNB blocking buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.5% Blocking Reagent, pH 7.5) for 1 h, followed by incubation in primary anti-myod antibody (1:200, Novo Castra, UK) for 2 h. They were then incubated in secondary anti-mouse-biotinylated antibody (1:400, Pierce) for 1 h. Cultures were incubated with streptavidin-HRP for 30 min, followed by fluorophore tyramide working solution for 5 min. Finally, cultures were mounted with DAPI-containing mounting media (Vector Laboratories). Greater than 95% of these emanating cells were myoD<sup>+</sup>, confirming that the spindle-shaped cells adjacent to the myofibers were emanating cells from those fibers.

### **Asymmetric Dimethylarginine (ADMA) ELISA**

ADMA was measured in serum and muscle (plantaris) homogenate samples using an ELISA kit (Alpco Diagnostics, Salem, NH) according to the kit instructions. The kit is a more convenient method to measure ADMA than existing techniques, such as HPLC, liquid chromatography-mass spectrometry, and gas chromatography-mass spectrometry, but correlates well with these other methods (49).

### **Western Blotting**

Plantaris muscle samples were homogenized in 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 50 mM sodium phosphate, 15% glycerol, 1mM benzamidine, 10 mg/ml pepstatin A, 5 µg/ml leupeptin, 1 mM PMSF, 2 µM DTT, and 1 µg/ml aprotinin. Homogenates were centrifuged for 10 min at 1,000g to remove connective tissue and cellular debris and protein content was quantified in the supernatant using the DC Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Forty micrograms of total protein were fractionated by SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked for 1 h in Tris-buffered saline -Tween 20 (TBS-T) (Tris•HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk at room temperature, and then incubated with

primary antibody (nNOS 1:500, eNOS 1:500) overnight at 4°C. Membranes were washed three times in TBS-T, 10 min each, followed by incubation with peroxidase-labeled anti-rabbit or anti-mouse antibodies (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Reactions were developed by using the enhanced chemiluminescence detection reagents (ECL Plus; Amersham Biosciences, Buckinghamshire, UK), according to the manufacturer's instructions, and protein levels were determined by densitometry (Scion Image software). Blots were stripped (Restore Stripping Buffer, Pierce Chemical) and reprobbed for  $\beta$ -actin (1:8000, Abcam) to control for loading errors.

### **Statistical Analysis**

Data were analyzed by a two-way ANOVA (age x treatment) with Tukey's HSD post hoc test. Data from Experiment 4 (ADMA treatment of young fibers only), western blots, and pax7 data were analyzed by a one-way ANOVA. Significance was established a priori at  $p < 0.05$ . Values reported are means  $\pm$  SEM.

## CHAPTER 4 RESULTS

### **Muscle Atrophy in Male C57 Mice**

We found evidence of both maturation and senescence effects when comparing muscle masses and the ratio of muscle mass to body mass between our three age groups (Table 4-1). Specifically, muscle masses for the gastrocnemius, plantaris, soleus, and tibialis anterior (TA) significantly increased between ages 2 and 10 months, representing maturation effects. The muscle mass to body mass ratio for the gastrocnemius, plantaris, and TA significantly decreased between these ages. Senescence effects were noted as a significant decrease in gastrocnemius, plantaris, and soleus masses between ages 10 and 22 months. In addition, the muscle mass to body mass ratio of the soleus significantly decreased between ages 10 and 22 months. The total protein per plantaris muscle was significantly increased from 2 month to 10 months, while total protein per gram of wet weight was not different between ages.

### **Nitric Oxide Production from Contracting Muscle**

Since nitric oxide (NO) is a primary signal for satellite cell activation (2, 4, 50, 52), we sought to determine whether NO production was reduced from aged muscle and whether this could account for the previously reported deficits in satellite cell activity (53, 61) and muscle repair (17, 19, 20, 43). NO production, as quantified by DAF fluorescence immediately following a 10-min contraction protocol, was significantly lower from adult and old EDL muscles as compared to young muscles (Figure 4-1). NO production did not differ between adult and old EDL muscles. This finding was unexpected since deficits in satellite cell activity are not evident in the adult muscles, compared to young. All ages exhibited a significant increase in NO production following contraction, and the fold change over resting was not significantly reduced



in aged and adult EDL muscles compared to young, suggesting that NO production is not limiting in adult or aged muscles.

### **Nitric Oxide Synthase Protein Expression Is Not Reduced**

Next, we sought to determine whether protein expression of nitric oxide synthases (NOS) was decreased in aged muscle. Both constitutively active NOS isoforms, nNOS and eNOS, were expressed equally in plantaris homogenate from young, adult, and aged muscle ( $p = 0.907$  for nNOS and  $p=0.976$  for eNOS, Figures 4-2 and 4-3, respectively). Thus, a reduction in NOS protein content cannot explain the decreased NO production from adult and aged muscle.

### **Asymmetric Dimethylarginine Can Inhibit Satellite Cell Activity**

We found that inhibition of NOS activity with the competitive inhibitor, asymmetric dimethylarginine (ADMA), prevented satellite cell activation, as quantified by both satellite cell emanation (Figure 4-4A) and BrdU incorporation (Figure 4-4B), from young mouse myofibers (Figure 4-5A-D). In all isolated myofibers experiments, satellite cell activation was assessed by quantifying emanating cells, and positive immunostaining for BrdU. In a subset of fibers with emanating cells, greater than 95% of these cells were myoD<sup>+</sup>, confirming that the spindle-shaped cells adjacent to the myofibers were emanating from those fibers. ADMA doses of 0.1-50  $\mu$ M reduced satellite cell activation of young myofibers exposed to the inhibitor for 24 h prior to mechanical stimulation. Both 10 and 50  $\mu$ M ADMA significantly reduced satellite cell emanation from young fibers (Figure 4-5A), while 50  $\mu$ M ADMA significantly reduced BrdU incorporation compared to control (Figure 4-5C). However, even the lowest dose (0.1  $\mu$ M) negatively affected satellite cell activation on young fibers. Our findings suggest that inhibition of NOS activity with the competitive inhibitor, ADMA, is sufficient to attenuate satellite cell activation from young fibers exposed to mechanical stress.

### **Asymmetric Dimethylarginine Is Not Elevated in Aged Muscle and Blood**

Next, we measured the levels of endogenous asymmetric dimethylarginine (ADMA) in the serum and plantaris homogenate of young and old animals. ADMA levels in the muscle and serum did not differ between young and old mice (Table 4-2), thereby eliminating an accumulation of ADMA as a potential cause of decreased NO production with age.

### **Aged Myofibers Have Equal Numbers of Pax7<sup>+</sup> Satellite Cells**

We quantified the number of pax7<sup>+</sup> nuclei/fiber by immunostaining myofibers immediately fixed after isolation and plating (Figure 4-6). There were no significant differences between the mean number of pax7<sup>+</sup> nuclei per fiber for any age group (Young =  $3.16 \pm 0.296$ , Adult =  $3.08 \pm 0.384$ , Old =  $2.79 \pm 0.321$ ). This is important because it confirms that any deficits in satellite cell activation are not due to fewer satellite cells present initially on aged fibers.

### **Satellite Cell Response to HGF Is Not Altered with Age**

To determine whether the deficit in aged satellite cell activation is upstream of HGF release, we examined the response of satellite cells on isolated myofibers to various doses of HGF. Adult and aged satellite cells reacted similarly to young satellite cells in response to HGF (Figure 4-7A-D). Regardless of age, 10-20 ng/ml of HGF significantly increased satellite cell activation (as quantified by satellite cell emanation and BrdU incorporation 48 h post-plating). Fifty ng/ml HGF did not significantly increase BrdU incorporation above control levels for any age. This finding implies that there is an upper limit to HGF's effects on satellite cell activation. However, satellite cells from adult and aged fibers respond to HGF in the same manner as young satellite cells, suggesting that the aging deficit lies upstream of HGF release.

### **L-Arginine and a Nitric Oxide Donor Can Rescue Aged Satellite Cell Activity**

The similar response of satellite cells to an HGF dose response suggests the aging deficit lies upstream of HGF release. Since NO triggers HGF release, we sought to determine whether

the NOS substrate, L-arginine, or a NO donor (DETA-NO) could “rescue” satellite cell activation from mechanically stimulated aged myofibers. Myofibers were treated with L-arginine (2 mM) or DETA-NO (10  $\mu$ M) and centrifuged to stimulate activation of satellite cells. Adult satellite cell activation (satellite cell emanation and BrdU incorporation 48 h post-plating) mirrored young satellite cell activity with both treatments. Satellite cell activation from aged myofibers was improved with L-arginine and DETA-NO (Table 4-3 and Figure 4-8 A-D). However, both treatments increased satellite cell activity in young and adult fibers as well. Importantly, young, adult, and old control fibers, in the absence of mechanical stress, were not significantly different when comparing satellite cell emanation or BrdU incorporation. There was a trend for centrifugation to stimulate satellite cell emanation in young ( $p=0.074$ ) and adult ( $p=0.065$ ) fibers, while this was not true for old fibers ( $p=0.788$ ).

To confirm these results were due to NO production and not indirect effects of the parent compound, we tested a dose response of NO-depleted DETA (5-50  $\mu$ M) and found no increase in satellite cell activation above control levels (data not shown). In sum, both the NOS substrate and the NO donor were able to rescue satellite cell activity from aged muscle to above young control levels, but were not able to normalize aged satellite cell activity to levels measured in young myofibers with the same treatment.

Table 4-1. Body mass, gastrocnemius mass, muscle mass/body mass ratios, and total protein for young, adult, and old mice. Values represent mean  $\pm$  SEM. Total protein measurements were made for plantaris muscle. \*Significantly different from Young. #Significantly different from Adult.

	Young (2 mo)	Adult (10 mo)	Old (22 mo)
Body Mass (g)	24.77 $\pm$ 0.58	34.63 $\pm$ 1.38*	29.17 $\pm$ 0.95* <sup>#</sup>
Gastrocnemius			
Mass (mg)	126.92 $\pm$ 2.45	160.40 $\pm$ 2.14*	131.05 $\pm$ 2.887 <sup>#</sup>
Mass/Body Mass	5.13 $\pm$ 0.069	4.67 $\pm$ 0.117*	4.50 $\pm$ 0.063*
Plantaris			
Mass (mg)	17.88 $\pm$ 0.49	21.58 $\pm$ 0.50*	18.21 $\pm$ 0.46 <sup>#</sup>
Mass/Body Mass	0.72 $\pm$ 0.016	0.63 $\pm$ 0.026*	0.63 $\pm$ 0.013*
Protein (mg/g w.w.)	4.58 $\pm$ 0.143	5.06 $\pm$ 0.236	5.00 $\pm$ 0.349
Total Protein (mg)	0.86 $\pm$ 0.046	1.07 $\pm$ 0.055*	0.93 $\pm$ 0.088
Soleus			
Mass (mg)	10.02 $\pm$ 0.32	13.55 $\pm$ 0.45*	9.61 $\pm$ 0.34 <sup>#</sup>
Mass/Body Mass	0.40 $\pm$ 0.011	0.40 $\pm$ 0.018	0.33 $\pm$ 0.014* <sup>#</sup>
Tibialis anterior			
Mass (mg)	49.98 $\pm$ 1.64	59.28 $\pm$ 0.86*	55.68 $\pm$ 1.61*
Mass/Body Mass	2.02 $\pm$ 0.060	1.73 $\pm$ 0.057*	1.92 $\pm$ 0.067

Table 4-2. Asymmetric dimethylarginine (ADMA) concentration ( $\mu\text{mol/L}$ ) in serum and muscle of young and old mice. Values represent mean  $\pm$  SEM. Muscle ADMA was measured in plantaris homogenate.

	Young (2 mo)	Old (22 mo)
Serum	$1.46 \pm 0.14$	$0.82 \pm 0.15$
Muscle	$6.18 \pm 0.87$	$5.00 \pm 0.44$

Table 4-3. Quantification of satellite cell activation as measured by (A) cell emanation and (B) BrdU incorporation from young, adult, and old myofibers 48 h post-plating. Values represent mean  $\pm$  SEM for n = ~50-90 fibers/group. Centr.=Centrifuged for 30 min at 1500g. \*Significantly different from Young Control and Young Centrifuged. <sup>#</sup>Significantly different from Young Centrifuged + Arginine. <sup>†</sup>Significantly different from Young Centrifuged + DETA-NO. <sup>‡</sup>Significantly different from Age-Matched Centrifuged. <sup>ω</sup>Significantly different from Adult Control. <sup>£</sup>Significantly different from Adult Centrifuged. <sup>¥</sup>Significantly different from Adult Centrifuged + Arginine. <sup>§</sup>Significantly different from Adult Centrifuged + DETA-NO. <sup>κ</sup>Significantly different from Old Control.

A.	Young (2 mo)	Adult (10 mo)	Old (22 mo)
Control	0.18 $\pm$ 0.059	0.16 $\pm$ 0.056	0.19 $\pm$ 0.088
Centrifuged	0.42 $\pm$ 0.068	0.42 $\pm$ 0.069 <sup>#,†</sup>	0.24 $\pm$ 0.072 <sup>#,†,¥,§</sup>
L-Arg + Centr.	1.04 $\pm$ 0.101 <sup>*,ω,κ</sup>	1.18 $\pm$ 0.115 <sup>*,‡,ω,κ</sup>	0.74 $\pm$ 0.103 <sup>*,#,†,‡,ω,£,¥,§,κ</sup>
DETA-NO + Centr.	1.30 $\pm$ 0.101 <sup>*,#,ω,κ</sup>	1.38 $\pm$ 0.111 <sup>*,#,‡,ω,κ</sup>	0.73 $\pm$ 0.091 <sup>*,#,†,‡,ω,£,¥,§,κ</sup>
B.	Young (2 mo)	Adult (10 mo)	Old (22 mo)
Control	0.21 $\pm$ 0.08	0.26 $\pm$ 0.11	0.22 $\pm$ 0.09
Centrifuged	0.28 $\pm$ 0.061	0.27 $\pm$ 0.065 <sup>†</sup>	0.19 $\pm$ 0.058 <sup>#,†,¥,§</sup>
L-Arg + Centr.	0.60 $\pm$ 0.079	0.56 $\pm$ 0.084	0.44 $\pm$ 0.087 <sup>†</sup>
DETA-NO + Centr.	0.79 $\pm$ 0.085 <sup>*,ω,κ</sup>	0.73 $\pm$ 0.089 <sup>*,‡,ω,κ</sup>	0.60 $\pm$ 0.083 <sup>‡</sup>

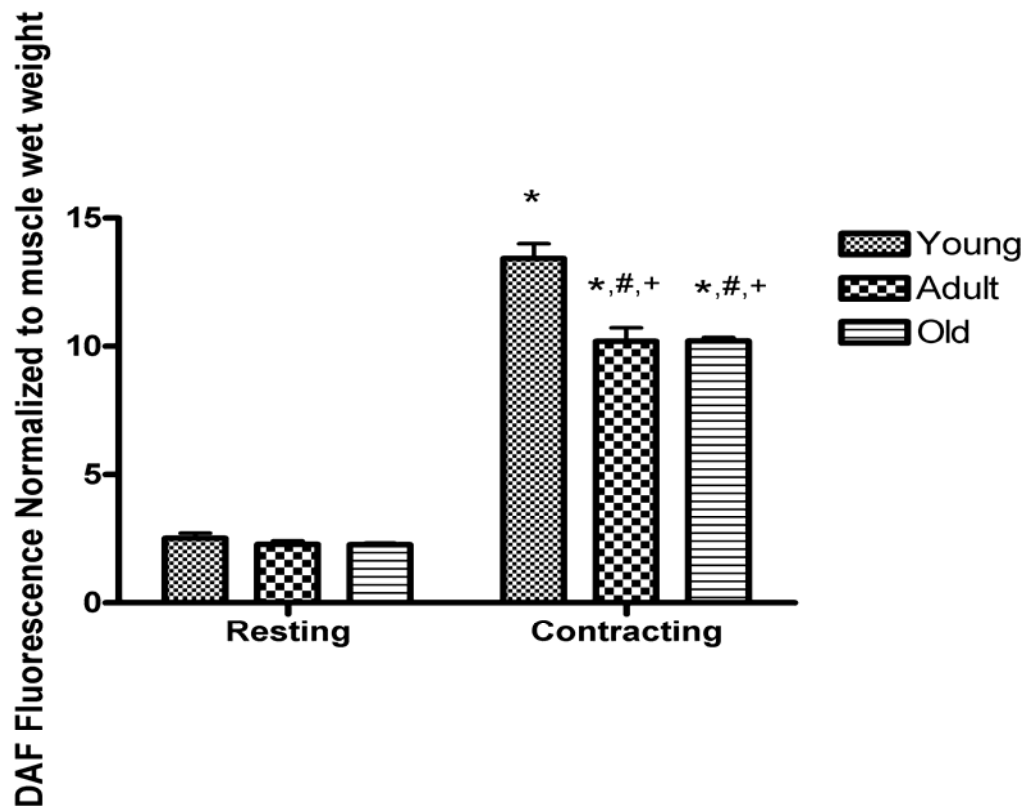


Figure 4-1. Nitric oxide (NO) production as quantified by 4,5-diaminofluorescein (DAF) fluorescence following 10 min of *in vitro* EDL muscle contraction. Values are normalized to EDL muscle wet weight. Values represent mean  $\pm$  SEM. \*Significantly different from Young Resting,  $p < 0.05$ . #Significantly different from Age-Matched Resting,  $p < 0.05$ . +Significantly different from Young Contracting,  $p < 0.05$ .

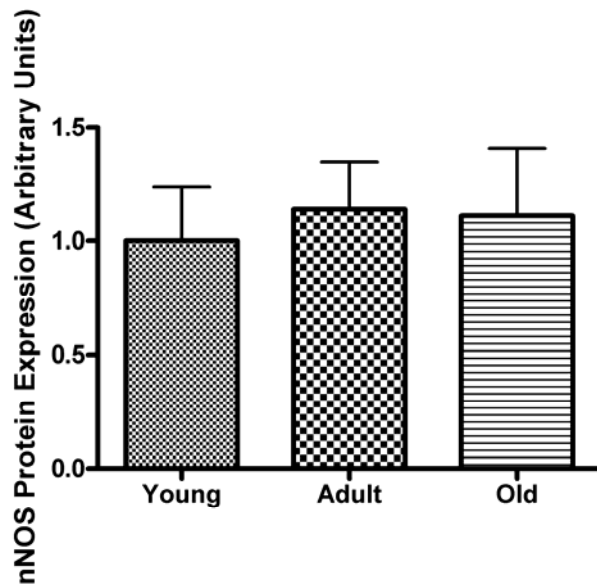
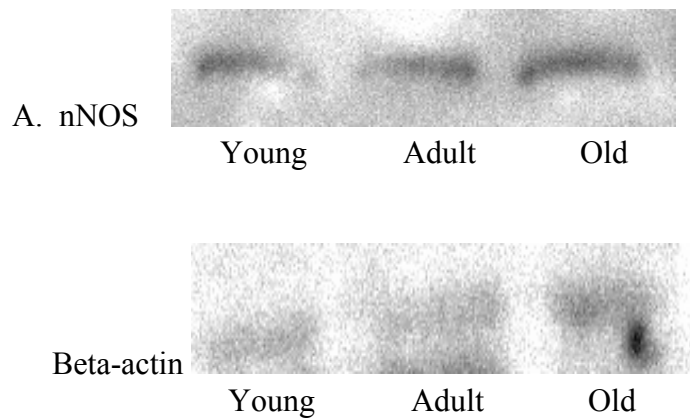


Figure 4-2. Protein expression of neuronal nitric oxide synthase (nNOS). (A) Representative immunoblot of nNOS from young, adult, and old plantaris muscle. Beta-actin served as a loading control. (B) Quantification of immunoblots for nNOS normalized to beta-actin. Values represent mean  $\pm$  SEM.



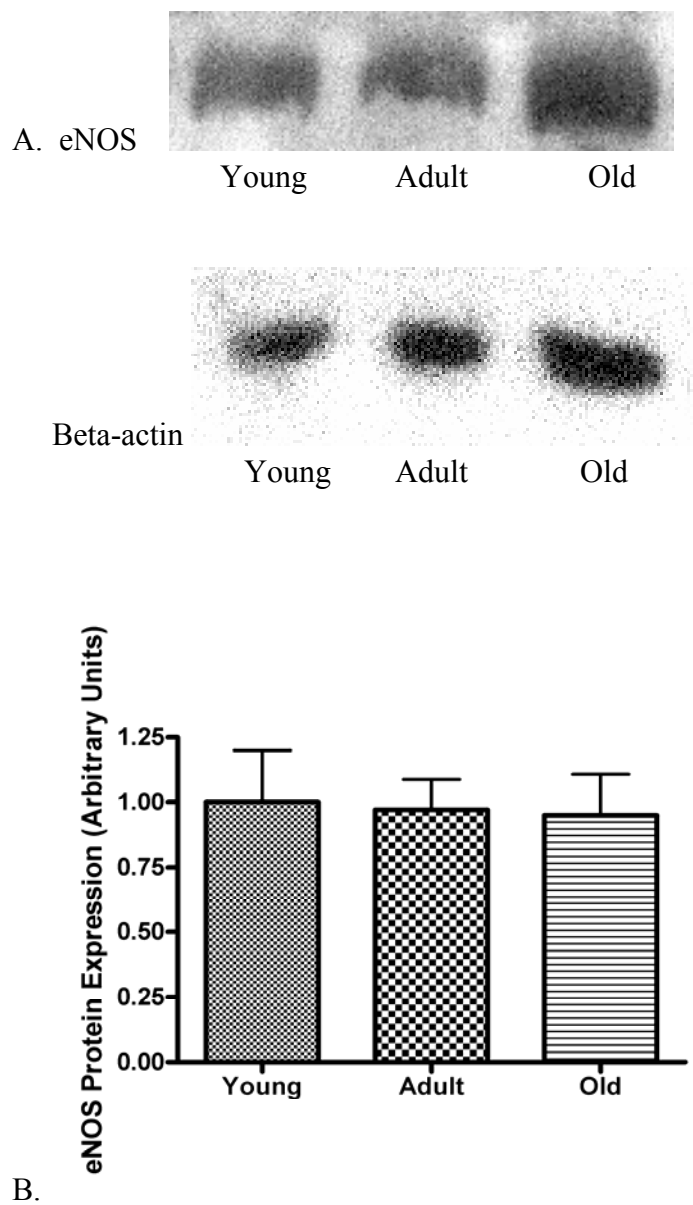


Figure 4-3. Protein expression of endothelial nitric oxide synthase (eNOS). (A) Representative immunoblot of eNOS from young, adult, and old plantaris muscle. Beta-actin served as a loading control. (B) Quantification of immunoblots for eNOS normalized to beta-actin. Values represent mean  $\pm$  SEM.

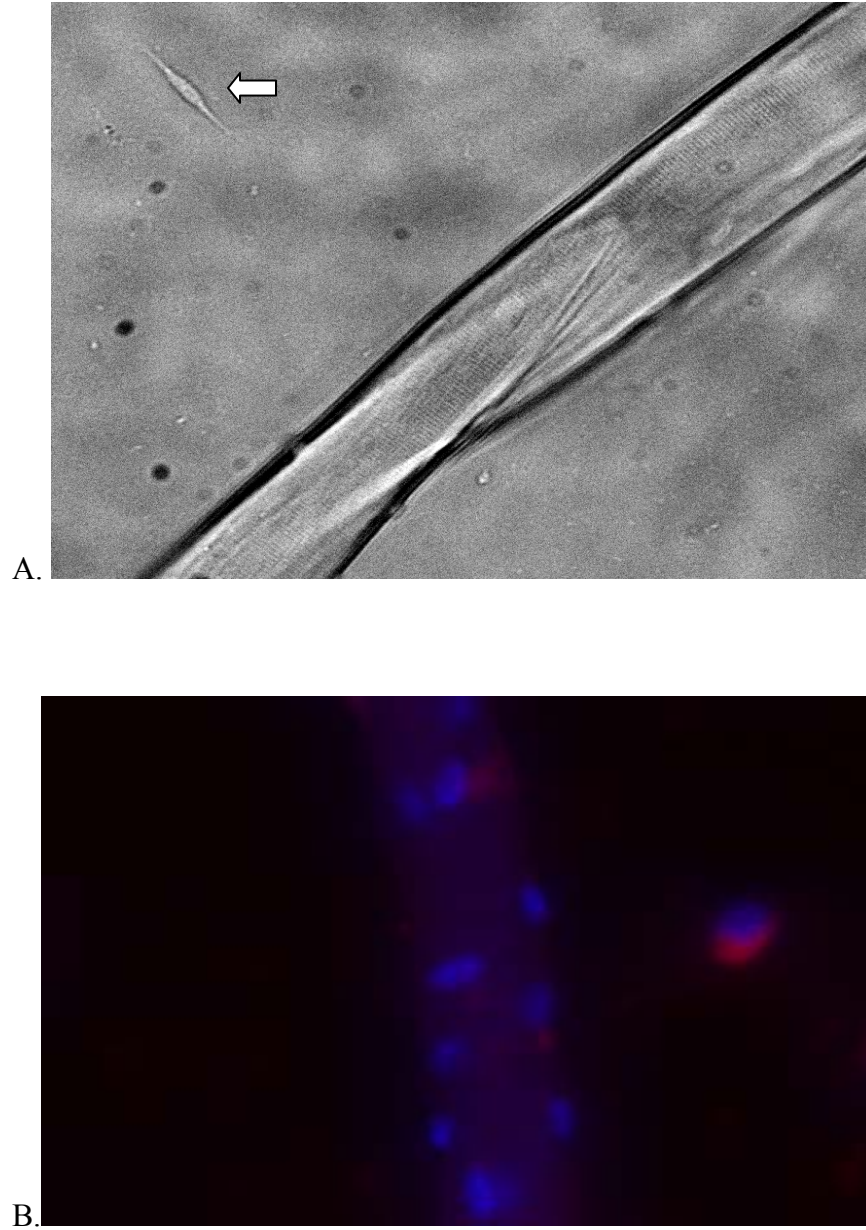
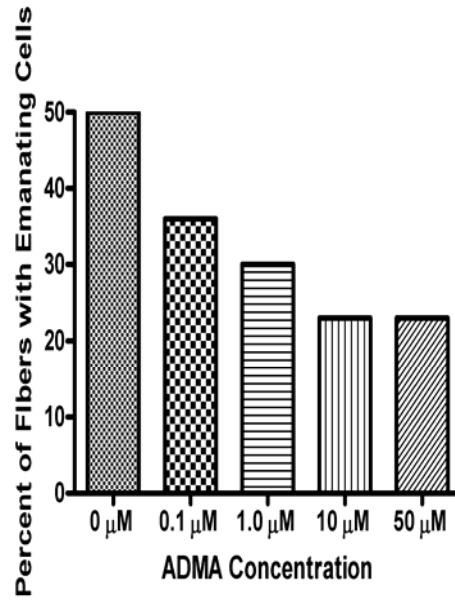
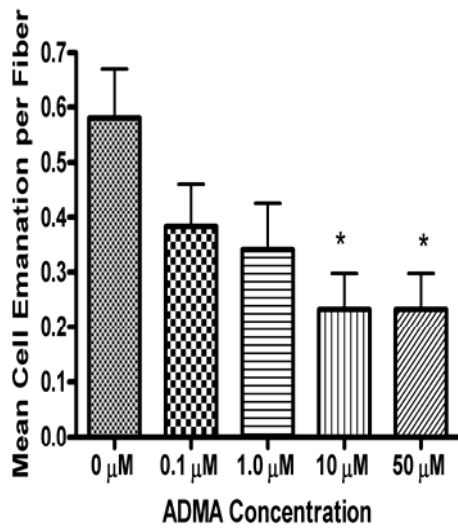
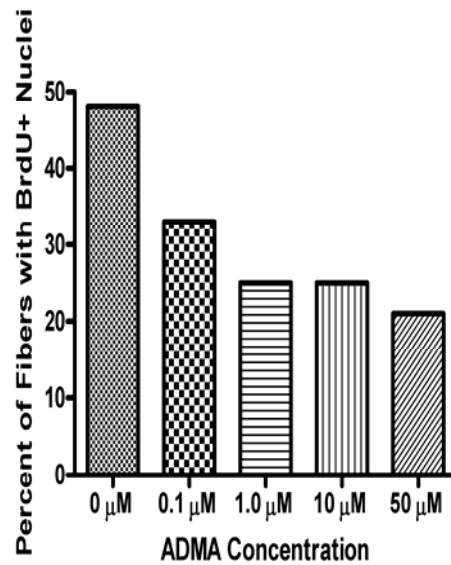
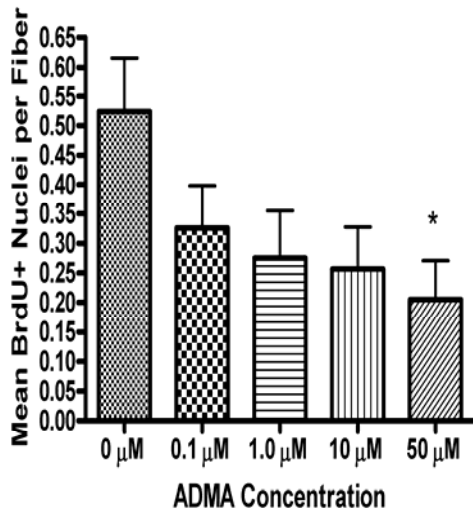


Figure 4-4. Representative images of activated satellite cells. (A) Representative light image of a satellite cell emanating from an isolated mouse myofiber indicated by white arrow (120x magnification). (B). Representative fluorescent image of a satellite cell staining positive for BrdU (red). Nuclei are stained blue with DAPI (60x magnification).



B.



D.

Figure 4-5. Response of fibers to asymmetric dimethylarginine (ADMA). (A) Mean satellite cell emanation from young fibers and (B) percentage of young fibers with emanating satellite cells following exposure to varying concentrations of ADMA 24 h prior to mechanical centrifugation (1500g, 30 min). (C) Mean BrdU<sup>+</sup> cells per fiber and (D) percentage of fibers with BrdU<sup>+</sup> nuclei following exposure to varying concentrations of ADMA 24 h prior to mechanical centrifugation. Values represent mean  $\pm$  SEM of  $\sim$ 50 fibers/group. \*Significantly different from 0  $\mu$ M ADMA.

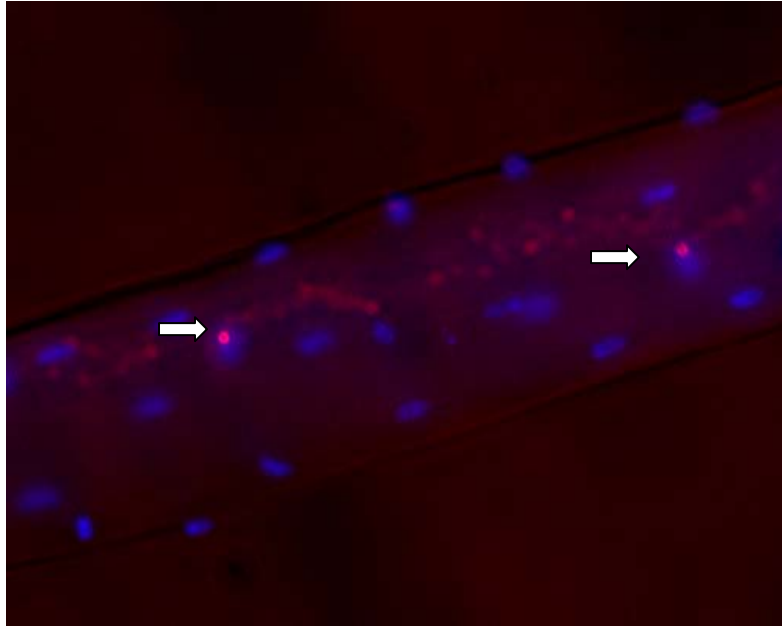
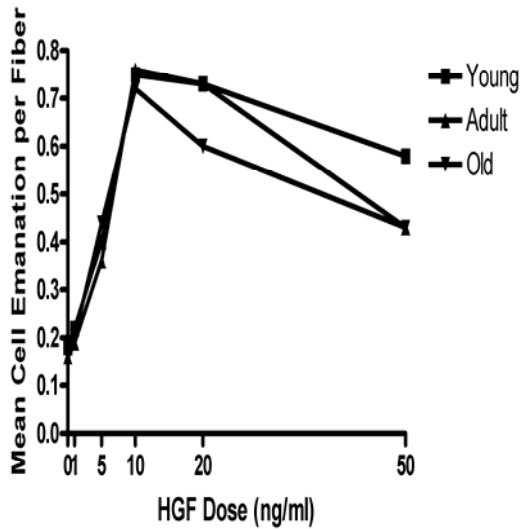
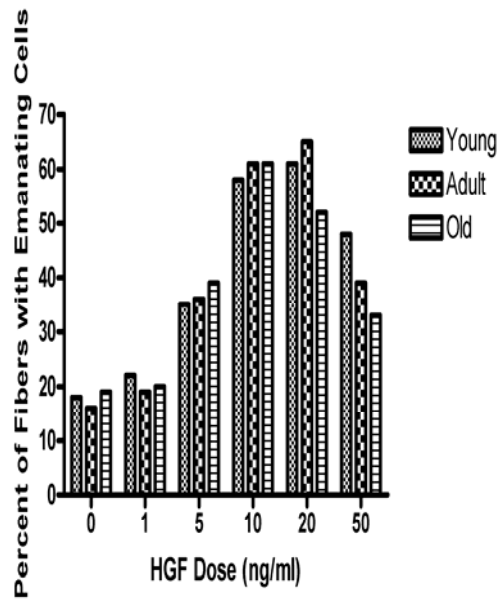


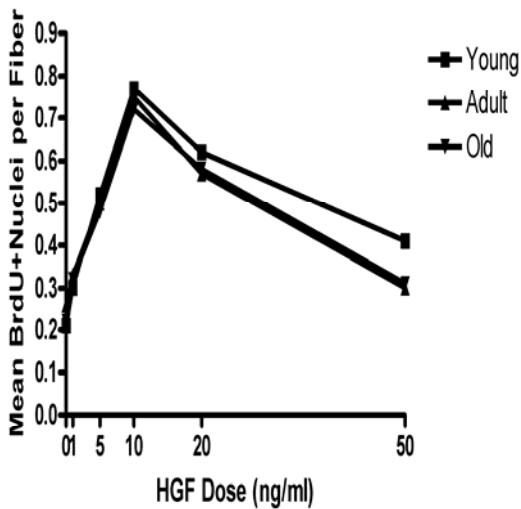
Figure 4-6. Representative image of a fiber stained for pax7 (red) immediately following isolation and plating (~30 fibers per age group). White arrows indicate pax7<sup>+</sup> nuclei. Blue = DAPI-stained nuclei, Red = pax7<sup>+</sup> nuclei (60x magnification).



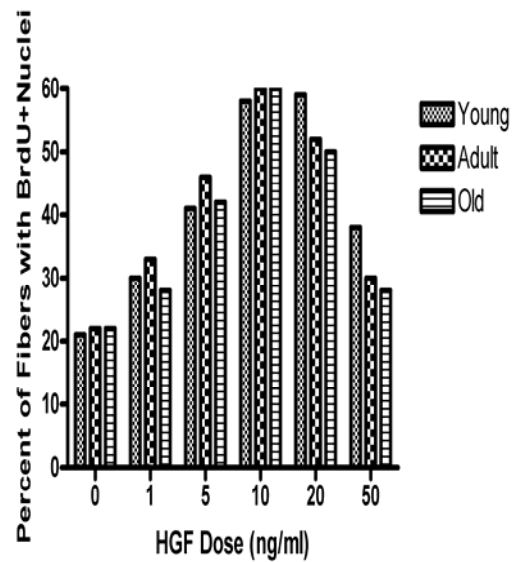
A.



B.



C.



D.

Figure 4-7. Response of fibers to hepatocyte growth factor (HGF). (A) Mean satellite cell emanation per fiber and (B) percentage of fibers with emanating satellite cells in response to varying concentrations of HGF. (C) Mean BrdU<sup>+</sup> cells per fiber and (D) percentage of fibers with BrdU<sup>+</sup> nuclei following exposure to varying concentrations of HGF. Approximately 50 fibers/group were analyzed.

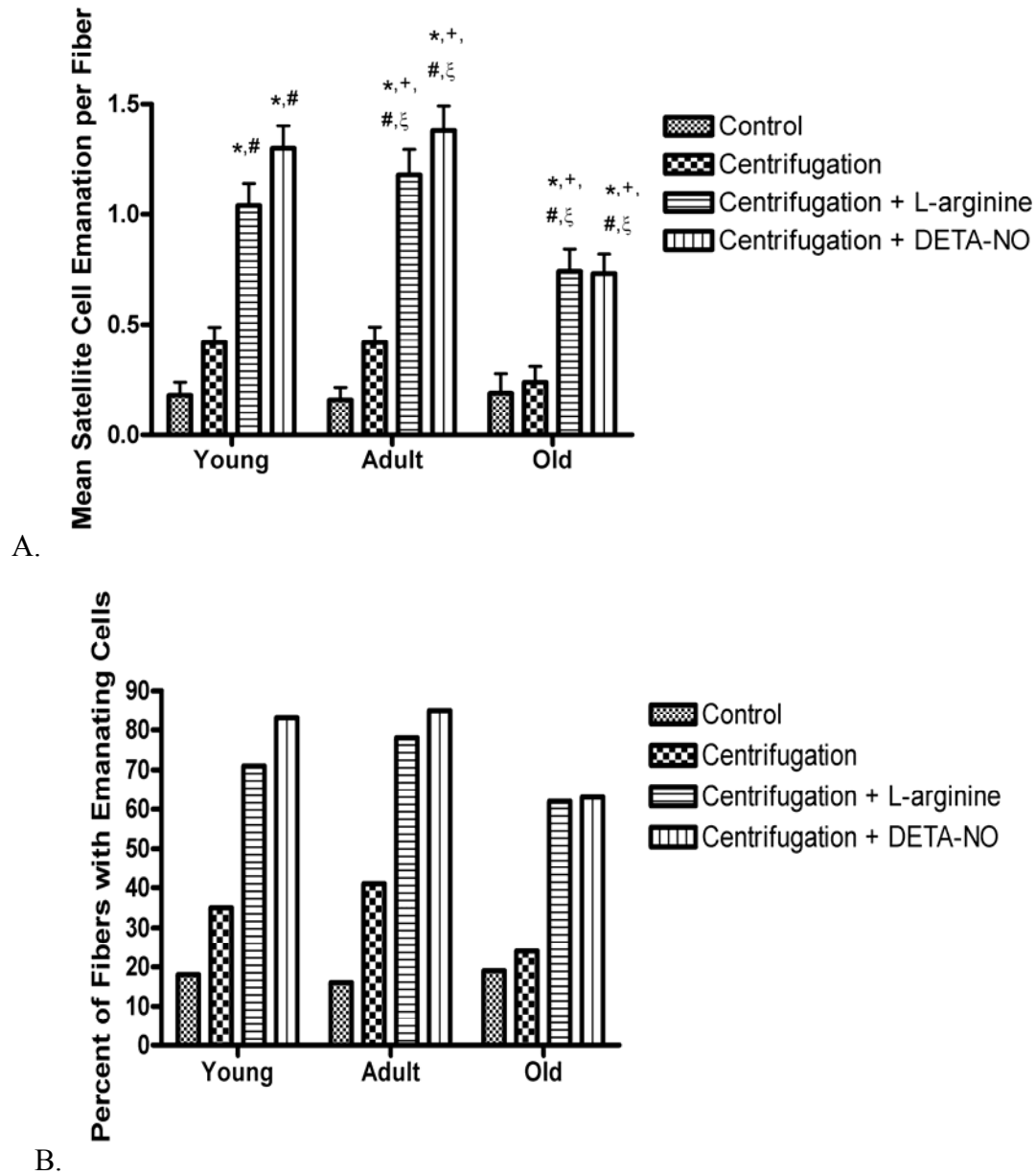
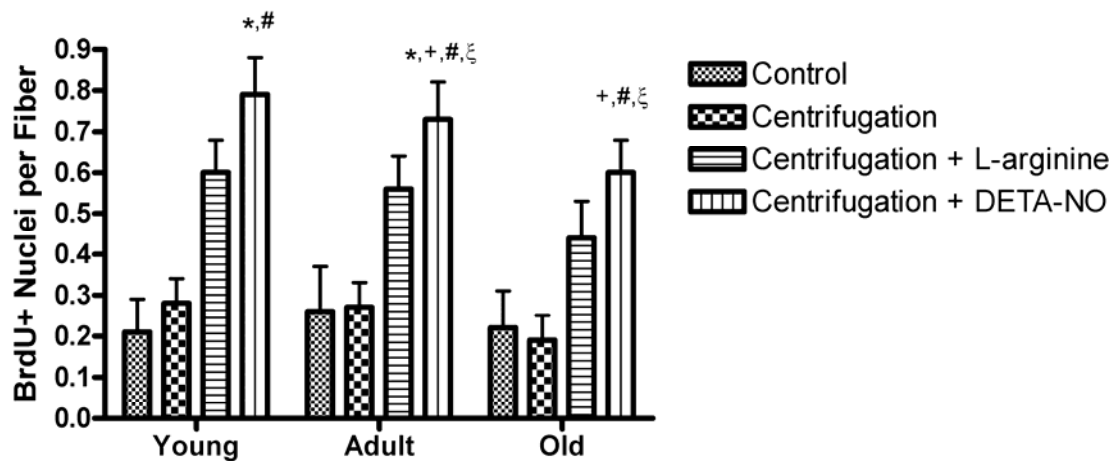
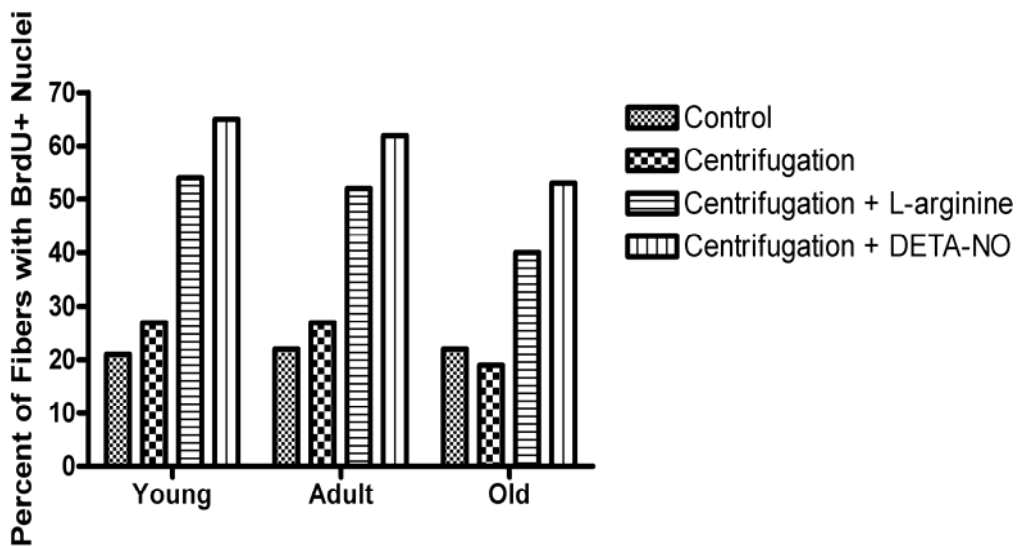


Figure 4-8. Response of fibers to L-arginine and DETA-NO. (A) Mean satellite cell emanation per fiber and (B) percentage of fibers with emanating satellite cells in response to treatment with 2 mM L-arginine or 10  $\mu$ M DETA-NO and centrifugation (1500g, 30 min). (C) Mean BrdU<sup>+</sup> cells per fiber and (D) percentage of fibers with BrdU<sup>+</sup> nuclei following exposure to L-arginine and DETA-NO and centrifugation. Approximately 90 fibers per group were analyzed. (A.) and (C.) represent mean  $\pm$  SEM. \*Significantly different from Young Control. <sup>+</sup>Significantly different from Age-Matched Control. <sup>#</sup>Significantly different from Young Centrifuged. <sup>ξ</sup>Significantly different from Age-Matched Centrifuged.

Figure 4-8 Continued.



C.



D.

## CHAPTER 5 DISCUSSION

### **Main Findings**

Although our old mice were in the early stages of senescence, based on survival curves for this strain, we found evidence of muscle atrophy in the gastrocnemius, plantaris, and soleus muscles. Single myofibers were isolated from the gastrocnemius muscles with resident satellite cells remaining. We studied the activation of these satellite cells, defined as myogenic cell emanation from the fiber and BrdU incorporation at 48 h post-plating. The main findings of this study are: 1.) Satellite cell activation in response to mechanical stimulation is reduced with age; 2.) Nitric oxide (NO) and L-arginine supplementation effectively increase satellite cell activation; however, an age-related deficit remains; 3.) *In vitro* NO production is reduced from adult and aged contracting muscle; and 4.) Aged fibers respond normally to exogenous hepatocyte growth factor (HGF). The data indicate that the aging deficit in satellite cell activation lies in the sensitivity of aged satellite cells to NO.

### **Reduced Satellite Cell Activation in Response to Mechanical Perturbation**

In response to a mechanical stress (centrifugation), the increase in dividing cells emanating from aged fibers was reduced compared to young, and fewer nuclei stained positive for BrdU (Table 4-3 and Figure 4-8 control vs. centrifuged, across supplement groups). These data depict the deficit in satellite cell activation from old muscle. Nevertheless, there were no differences in pax7<sup>+</sup> satellite cells present on young, adult, and old fibers fixed immediately after isolating, in agreement with several human (29, 47) and animal studies (42). In contrast, many animal studies indicate that the satellite cell population declines with aging (5, 12, 28, 51). Differences in muscles studied, ages of animals, and methods for identifying satellite cells may explain the differences in findings. Unlike most aging studies, our old group (22 mo) was younger than the



50% mortality age for this strain (27 mo). Therefore, it is possible that the decline in the quiescent satellite cell population reported by other authors occurs only during advanced senescence. Our animals, which were in early senescence, exhibit impaired satellite cell responsiveness to external stimuli, without a decline in the overall quiescent population.

Our data imply that there is a deficit in the signaling pathways leading to activation of resident satellite cells on old muscle. This pathway involves a sequence of events including: 1) mechanical perturbation, 2) nitric oxide release, 3) HGF release from the extracellular matrix, 4) HGF binding to the c-met receptor on satellite cells, and finally, 5) activation of DNA synthesis and migration of satellite cells (Figure 5-1). We studied the mechanism of age-associated satellite cell dysfunction by examining satellite cell activation in isolated myofibers following experimentally induced mechanical perturbation (centrifugation), exogenous NO treatment, or exogenous HGF application, in order to localize the aging deficit.

#### **L-Arginine and a Nitric Oxide Donor Can Rescue Aged Satellite Cell Activity**

The NOS substrate, L-arginine, or an NO donor, DETA-NO, “rescued” satellite cell activation in mechanically stimulated aged myofibers, in that satellite cell activation from old fibers exposed to these treatments exceeded the level of satellite cell activation in young control fibers (Table 4-3 and Figure 4-8). We confirmed that the DETA-NO effects were due to NO production and not indirect effects of the parent compound, DETA, by exposing fibers to NO-depleted DETA (5-50  $\mu$ M). These data suggest that an age-related reduction in NO production could contribute to reduced satellite cell activity. However, both L-arginine and DETA-NO treatments also increased satellite cell activity in young and adult fibers such that satellite cell activity in old fibers remained significantly lower than in young and adult fibers with the L-arginine or DETA-NO treatments. It appears that NOS activity/NO production may limit satellite

cell activation from all ages since exogenous NO supplementation increased satellite cell activity across age groups.

Our centrifugation protocol was a submaximal stimulus that did not induce 100% satellite cell activation. Consequently, the degree of satellite cell activation following a fixed stimulus (i.e., centrifugation or exposure to a fixed dose of DETA-NO or L-arginine) indicates the sensitivity of the system to that particular stimulus. Therefore, in spite of the potential therapeutic effects of augmenting NO production, we must conclude that age-related deficits responsible for compromised satellite cell activation lie downstream of this event.

Other groups have completely restored satellite cell activation/proliferation in old muscle with exposure to young serum (20), forced Notch activation (19), IGF-1 (17), or basic FGF2 (51). However, these researchers studied both activation and proliferation. Our experimental design, on the other hand, focused primarily on the activation step, since fibers were fixed before significant proliferation occurred. We conclude that the aging deficit in satellite cell activation lies in the sensitivity of aged satellite cells to NO.

### **Nitric Oxide Production and NOS Protein Expression from Aged Muscle**

Nitric oxide is a primary signal for HGF release leading to satellite cell activation (2, 4, 50, 52). Therefore, we hypothesized that NOS expression and/or NO production would be reduced in aging skeletal muscle. Myofiber isolation required both gastrocnemius muscles, leaving insufficient tissue for additional measurements. Therefore, in vitro contraction-induced NO production was measured in the EDL, and NOS protein expression in the plantaris. These muscles were chosen since they resemble the gastrocnemius in fiber type (primarily type II) and location (hindlimb). NO production, as quantified by DAF fluorescence following muscle contraction, was significantly lower in aged and adult, compared to young EDL muscle (Figure 4-1). While all ages exhibited an increase in NO production following contraction, the young

EDL muscles produced significantly more NO following contraction than adult or aged muscles. However, the fold-change over resting values was not significantly reduced in adult or aged muscles, suggesting that contraction-induced NO production is not limiting in aged animals.

Ours is the first study providing evidence of a functional decrease in NO production from aged, as well as adult muscle. The reduced NO production from adult muscle was unexpected since no reduction in satellite cell responses were noted in adult fibers compared to young (Figure 4-8). We found no differences in nNOS and eNOS protein expression between young, adult, and old plantaris muscles (Figures 4-2, 4-3). Thus, down-regulation of NOS enzyme expression may not explain the reduced NO production from adult and old EDL muscles. We did not measure NOS activity, but Richmonds and colleagues (46) found a reduction in nNOS activity and percentage of NOS-containing fibers in muscles from aged rats (24 mon) compared to young (8 mon). In contrast, Capanni and colleagues (15) reported an increase in nNOS expression in 24 month-old rat gracilis muscle. However, this group measured NOS protein expression via immunoblots and immunohistochemistry, whereas Richmonds and colleagues (46) measured functional NOS activity. It is possible that NOS enzymatic activity is inhibited even with an upregulation of nNOS protein expression (15), or no change in NOS expression as we show.

While it is an interesting observation that NO production is decreased in adult and aged muscle compared to young, it does not appear that a significant loss of contraction-induced NO production by the EDL is associated with senescence (i.e., no difference between adult and old muscles). Therefore, reduced NO production is unlikely to explain age-related deficits in satellite cell activation in these muscles.

### **Satellite Cell Response to HGF Is Not Altered with Age**

We determined that the deficit in aged satellite cell activation lies upstream of HGF release since the response of aged satellite cells on isolated myofibers to a dose response of HGF was similar to young satellite cells. Regardless of age, 10-20 ng/ml of HGF significantly increased satellite cell activation (satellite cell emanation and BrdU incorporation 48 h post-plating), in agreement with previously published studies (55, 58). Fifty ng/ml HGF did not significantly increase BrdU incorporation above control levels for any age. This finding implies that there is an upper limit to HGF's effects on satellite cell activation.

The similar response of young, adult, and old satellite cells is important because it implies that all events downstream of HGF release, such as HGF/c-met colocalization and c-met signaling, are normal in aged muscle and satellite cells. In contrast, Barani and colleagues (5) reported a lower increase in the HGF receptor, c-met, expression in cultured muscle-derived cells (mdc) of old rats with serum stimulation compared to young. Young (3 week-old) and adult (9 month-old) mdc cultures increased relative c-met expression by ~190% with serum stimulation. In contrast, old (24 month-old) mdc increased c-met expression by just 130% with serum stimulation. However, the cell surface density of the c-met receptor was increased to the same extent in the three ages with serum exposure. Therefore, similar responses to a fixed dose of HGF, as we observed, are not surprising. These observations effectively localize the aging deficit in satellite cell activation to the mechanisms mediating HGF release in response to NO production.

### **Asymmetric Dimethylarginine Is Not Elevated in Aged Muscle and Blood but Does Inhibit Satellite Cell Activity**

To explain the aging deficit in satellite cell activation and the reduction in NO production with age, we studied the levels of ADMA, a competitive inhibitor of NOS, in the serum and

plantaris homogenate of old and young mice, and its effects on satellite cell activity. Conboy and colleagues (20) used a series of *in vivo* and *in vitro* experiments to show that factors in old serum negatively influence satellite cell activation, proliferation, and muscle repair. However, components in young serum alone are sufficient to restore satellite cell function in aged muscle. Their data imply that the old systemic environment is altered such that there is a decrease in a positive factor found in young serum, an increase in a negative or inhibitory factor, or both. We hypothesized that ADMA was that negative factor found at high levels in old serum. As a NOS inhibitor, it may potentially inhibit satellite cell activity.

We found that ADMA doses of 0.1-50  $\mu$ M negatively impacted satellite cell activation (Figure 4-5). Myofibers from young muscles were adversely affected by ADMA such that 10 and 50  $\mu$ M significantly reduced satellite cell emanation from fibers, while 50  $\mu$ M ADMA significantly reduced BrdU incorporation compared to control. Our findings provide further evidence of the importance of NO for satellite cell activation since ADMA is sufficient to attenuate satellite cell activity.

However, we did not measure an increase in ADMA in old animals. ADMA levels were similar in the serum and muscle homogenate of young and old animals (Table 4-2), suggesting that ADMA concentration and subsequent NOS inhibition is not elevated with age.

One group of researchers has reported an increase in ADMA in serum from aged (20 mo) rats compared to young (6 mo) animals (60). Serum L-arginine levels were similar between the two ages, so the L-arginine/ADMA ratio was lower in old rats compared to young. Lastly, serum concentrations of nitrite were significantly decreased in these old animals. While ADMA is an appealing candidate for the reduction in NO production with age, we were unable to provide evidence of an accumulation of ADMA in old mice. Nevertheless, our data showing that ADMA

inhibits satellite cell activity on fibers confirm the integral role of NO signaling for satellite cell activation from quiescence.

### **Response to Nitric Oxide**

Under conditions where satellite cells are kept quiescent, muscle produces NO in low level pulses (56, 58). On the other hand, mechanical stress, associated with satellite cell activation, produces a bolus of NO via nNOS acting as a mechanotransducer (56). NO is important for triggering the release of HGF from the extracellular matrix (ECM) to activate satellite cells (2, 4, 50, 52). Tatsumi et al. (54, 55) have demonstrated that release of HGF, in response to mechanical perturbation, is NO-dependent. When released, HGF rapidly co-localizes with its receptor on satellite cells, the c-met tyrosine kinase receptor, and triggers activation from quiescence (53). Once activated, satellite cells synthesize both HGF and c-met (3, 53). HGF is synthesized as a precursor protein requiring proteolytic cleavage to produce the active growth factor. However, Tatsumi and Allen (52) confirmed that active heterodimeric HGF is present in the ECM of uninjured rat muscle, indicating that proteolytic cleavage to activate HGF is not a rate-limiting step in satellite cell activation, but rather the steps leading to release of active HGF from its ECM tethering.

Yamada and colleagues (62) recently provided evidence of the involvement of matrix metalloproteinases (MMPs) in the NO-dependent release of HGF *in vitro*. Inhibition of MMPs along with treatments that induce satellite cell activation (stretch, NO supplementation) inhibited activation of cultured rat satellite cells. In addition, conditioned medium from satellite cells exposed to activating stimuli plus the inhibitor of MMPs did not activate cultured satellite cells and did not contain HGF. Importantly, the MMP inhibitor did not inhibit NOS activity. These data suggest that MMPs cleave HGF from the ECM and that this step is triggered by NO release.

Barani et al. (5) measured MMP 2-9 activity on crushed muscle extract (CME), and found that these activities were reduced with aging. MMP2-9 enzyme activity measured in initial muscle derived cells (mdc) was identical in young (3 week-old) and old (24 month-old) rats, and serum exposure increased activity uniformly in both ages. However, when measured on CME, MMP 2-9 enzyme activity was ~2-fold lower in old CME compared to young. This may explain the aging deficit in satellite cell activation and warrants further investigation. Another possible explanation for the aging deficit in satellite cell activation is a decrease in endogenous HGF levels with aging. If HGF production goes down with age, there would be less present to stimulate activation in response to NO signaling. Thus, we postulate that an age-related decline in MMP activity and/or a reduction in HGF expression decreases the sensitivity of satellite cells to NO, thus limiting satellite cell activation.

### **Limitations and Future Directions**

The age of the “old” mice (22 mo) may have limited the severity of the aging deficit in satellite cell activation in response to mechanical perturbation. According to the National Institute on Aging (NIA), the age of 50% mortality for male C57 mice is 27 months. Using mice 27 months or older may have amplified the effects of our treatments on satellite cell activity. On the other hand, the presence of a deficit in satellite cell activation during early senescence (i.e., 22 mo) highlights the potential clinical significance of this phenomenon in an aging population.

Higher doses of arginine and DETA-NO may have improved satellite cell activation from old fibers. These doses were selected based on dose responses in young muscle. It may be that higher doses were necessary to maximally stimulate satellite cell activation on old fibers. Plus, our mechanical stimulus (centrifugation, 1500g, 30 min) was a submaximal stimulus for activation. Using a more severe mechanical perturbation and/or higher doses of supplements may have activated more satellite cells and eliminated aging deficits. Nevertheless, the submaximal

stimuli chosen allows assessment of the sensitivity of the satellite cell activation system. Future studies should focus on physiologically-relevant activating stimuli, such as stretch or injury.

Isolated myofibers are extremely useful for studying satellite cells in their resident position on the myofiber. However, it is difficult to harvest fibers for such assays as Western blotting or PCR. Thus, we were limited to immunostaining of fibers to identify and count satellite cells. Future studies should include treatment of the whole animal with L-arginine or NO donors to allow for more robust measurements.

We did not measure NOS activity in the gastrocnemius to confirm that it is reduced with aging. NOS protein expression in the plantaris was not changed with aging, but NO production was decreased in contracting adult and aged EDL muscle. We cannot determine whether NO production by isolated gastrocnemius myofibers during centrifugation was reduced. Regardless, reduced NO production does not seem to be the cause of the aging deficit in satellite cell activation since this deficit persists with administration of a fixed amount of NO donor.

Lastly, we did not find a reduction in pax7<sup>+</sup> cells per fiber between our ages. However, due to the limitation of available fibers, only ~30 fibers from each age were stained and analyzed. Although there did not appear to be a trend for reduced satellite cells in the aging gastrocnemius, the variability between fibers within an age group, and even within one mouse, was high. Staining more fibers, such as 100 fibers per age group, may have shown a significant loss of satellite cells with age. Future studies need to confirm equal pax7<sup>+</sup> nuclei per fiber with this strain of mouse and these ages.

## **Conclusions**

We have successfully narrowed the aging deficit in satellite cell activation in response to mechanical perturbation to the steps between NO production and HGF release. Based on our data, NO production does not limit satellite cell activity in old muscle, and the steps downstream



of HGF are not impaired in old fibers. Figure 5-1 diagrams the signaling events leading to satellite cell activation from quiescence. We conclude that the deficit in activation lies between NO production and HGF release, in the sensitivity of old satellite cells to NO.

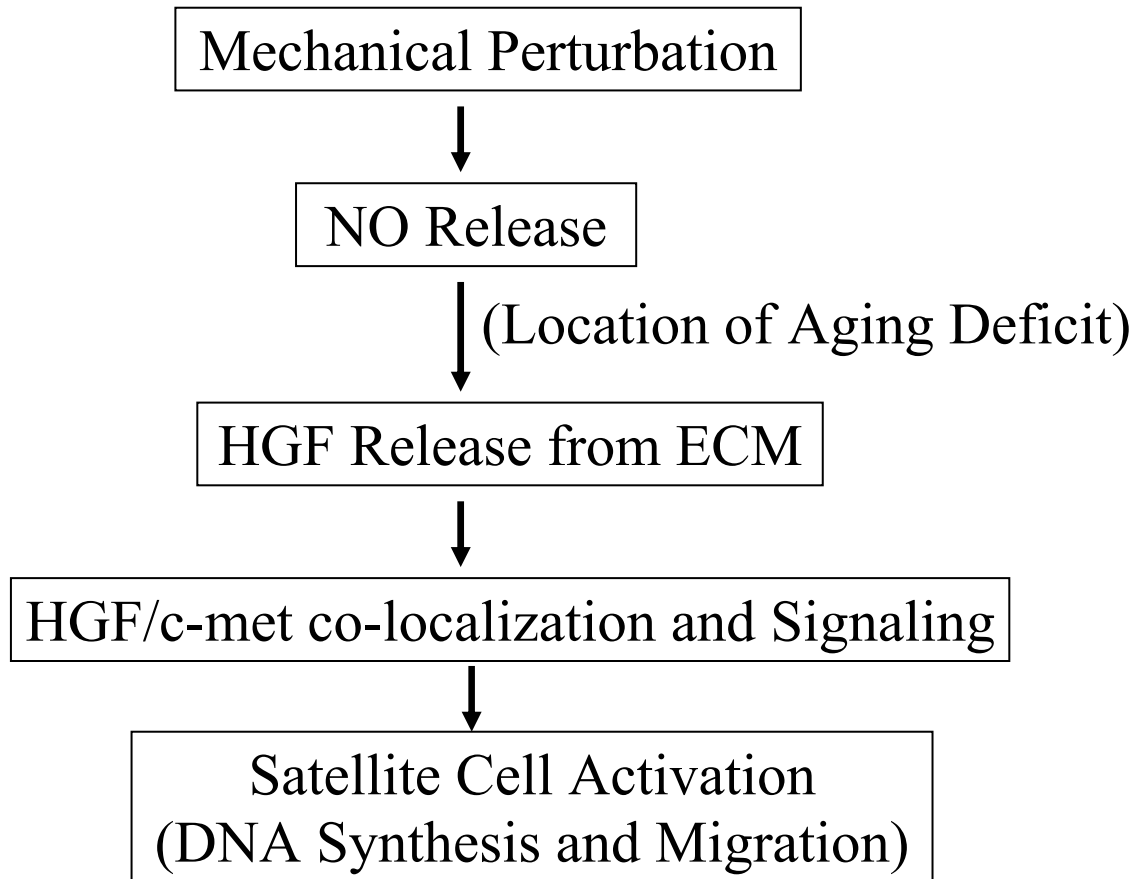


Figure 5-1. Overview of the steps leading to satellite cell activation and the potential location of the aging deficit.

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## BIOGRAPHICAL SKETCH

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