NITRIC OXIDE SYNTHASE ACTIVITY AFFECTS GENE EXPRESSION IN OVERLOADED SKELETAL MUSCLE

By

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To my parents, Richard and Rebecca Sellman, for their unconditional love and limitless support
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Nitric oxide is a mechanically sensitive signal in skeletal muscle. Inhibition of nitric oxide synthase (NOS) activity in vivo impedes hypertrophy in the overloaded rat plantaris. We investigated the mechanism for this effect by examining early events leading to muscle growth after 5 days of functional loading. We also tested the hypothesis that NOS activity is necessary for functional overload-induced upregulation of growth factors, myogenin, and contractile gene mRNAs in the rat plantaris muscle.

Twenty-four female Sprague-Dawley rats (~250 g) were randomly divided into three groups (n=8/group): Control, N-nitro-L-arginine methyl ester (L-NAME: 100 mg/kg/d), or 1-(2-trifluoromethyl-phenyl)-imidazole (TRIM: 10 mg/kg/d). Unilateral removal of synergists induced chronic overload (OL) of the right plantaris for 5 days. Sham surgery was performed on the left hindlimb, which served as a normally loaded (NL) control. No group differences were observed among NL muscles. Real-time PCR analyses showed elevated (p<0.05) mRNA expression for insulin-like growth factor-1 (IGF-1), mechano-
growth factor (MGF: load-sensitive splice variant of IGF-1), hepatocyte growth factor (HGF), and myogenin and reduced (p<0.05) total VEGF mRNA expression in the OL muscle compared to NL. Neither L-NAME nor TRIM affected HGF, VEGF, or myogenin responses. However, OL-induction of IGF-1 and MGF mRNA was greater (P<0.05) in the TRIM group compared to the Controls. Conversely, overload-induction of phosphorylated p70 S6 kinase (p70\(S6K\)) was prevented in the TRIM group. Type I (slow) myosin heavy chain (MHC) and skeletal \(\alpha\)-actin mRNAs were increased in the Control/OL muscle (an effect that was completely prevented in both NOS-inhibitor groups). Therefore, nNOS activity is necessary for overload-induction of Type I (slow) MHC and skeletal \(\alpha\)-actin mRNA and p70\(S6K\) phosphorylation. Further, the inhibition of nNOS causes a compensatory increase in IGF-1 expression during overload.
CHAPTER 1
INTRODUCTION

Skeletal muscle is an extremely plastic tissue. Variations in external load result in structural, biochemical, and morphological adaptations in muscle fibers. Specifically, alterations in mitochondrial number, enzymatic profile, structural protein expression and content, and capillary angiogenesis accompany both muscle atrophy and hypertrophy. Chronic overload causes dramatic muscle growth. This hypertrophy response involves the activation and later fusion of satellite cells to the muscle fibers (57, 58), and increased synthesis of structural and contractile proteins (28, 76). Concurrently, capillary angiogenesis is induced to support growing muscle (54). The increased fiber recruitment associated with muscle overload also causes an up-regulation of slow fiber type-specific genes. Molecular regulation of growth factors and transcription factors that govern muscle growth is poorly understood. Therefore, discovering the molecules responsible for signaling this coordinated response is vital for understanding load-induced adaptive changes in skeletal muscle.

Background

Loss of skeletal muscle mass is a serious clinical problem in disease states (such as cancer and AIDS) and in conditions such as prolonged bed rest and spaceflight. Preserving muscle mass by attenuating muscle loss and/or stimulating muscle growth can be vital to decreasing recovery time and increasing the patient’s ability to ambulate freely and enjoy independence. Muscle growth in response to increased load is a complex event that requires the transcription of many different factors to contribute to the changing
phenotype; therefore, it is important to understand the underlying mechanisms responsible for the transduction of mechanical forces into chemical signals. Nitric oxide (NO) is an important signaling molecule, playing a role in various physiologic processes. These effects are broad and far-reaching, as NO has been implicated in preserving endothelial function and may be necessary for the slow fiber-type transition associated with overload-induced hypertrophy. A family of nitric oxide synthases (NOS), all of which can be expressed in adult skeletal muscle, governs its synthesis. Neuronal NOS (nNOS) is the most abundant isoform in skeletal muscle, being associated with the dystrophin complex (39, 71). The activity of this enzyme is induced by contractile activity (70).

Problem Statement

Increased muscle load results in increased levels of growth/regulatory factors and contractile proteins, and greater nNOS activity. Therefore, we postulate that the events are related. We suggest that NO acts to coordinate the events associated with muscle growth. We tested the effects of NOS inhibition on short-term responses to chronic muscle overload.

Variables in Study

Independent variables. We manipulated load to the plantaris and NOS inhibition by administering N-nitro-L-arginine methyl ester (L-NAME) and 1-(2-trifluoromethyl-phenyl)-imidazole (TRIM).

Dependent variables. We measured expression of growth factors (insulin-like growth factor-1 (IGF-1), mechanogrowth factor (MGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF)); a myogenic regulatory factor,
myogenin (MGN); and contractile proteins, skeletal α-actin and Type I (slow) myosin heavy chain (MHC)

**Control variables.** Gender was excluded from the study: we used only female Sprague-Dawley rats. Aging effects were also excluded: we restricted observations to young adult rats (~4 months old).

**Extraneous variable.** We did not control for differences in the activities of the animals. Since some animals may ambulate more freely than others, the load frequency may differ among animals. However, preliminary experiments show a robust and reproducible hypertrophy of the plantaris in response to unilateral ablation of its synergists. Acute IP injections have the potential to induce a stress response that then results in a cascade of proteins that could confound the study. To control for this, each animal in the study was given an IP injection, either of TRIM or vehicle.

**Hypotheses**

1. Five days of functional overload will increase the local expression of growth/regulatory factor mRNA’s (IGF-1, MGF, HGF, VEGF, and MGN) and the local expression of skeletal α-actin and Type I (slow) MHC.

2. NOS inhibition will prevent increased local expression of the growth/regulatory factors, skeletal α-actin, and Type I (slow) MHC.

3. Specific nNOS inhibition will prevent increased local expression of growth/regulatory factors, skeletal α-actin, and Type I (slow) MHC to the same extent as non-isoform specific systemic NOS inhibition.

**Definition of Terms**

1-(2-trifluoromethyl-phenyl)-imidazole (TRIM): a selective inhibitor of the neuronal isoform of NOS (nNOS).

**Hepatocyte growth factor (HGF):** an autocrine/paracrine factor that binds to the *c-met* receptor. In skeletal muscle, HGF coordinates the activation and proliferation of
satellite cells during muscle regeneration and growth. This factor has been identified as
the component of crushed muscle extract that is capable of activating quiescent satellite
cells \textit{in vitro} and \textit{in vivo} (67).

\textbf{Hypoxanthine guanine phosphoribosyl transferase (HPRT):} an enzyme that
plays a role in purine biosynthesis. HPRT was chosen in this model as a normalizing
gene in Real-Time PCR because of its constant expression with our manipulations
(overload and NOS inhibition).

\textbf{Insulin-like growth factor-1 (IGF-1):} produced by the liver and skeletal muscle,
this growth factor is an important autocrine/paracrine factor capable of stimulating
proliferation and differentiation of activated satellite cells. IGF-1 also activates protein
synthesis in skeletal muscle fibers via Akt/mTOR activation of protein translation.

\textbf{Mechanosensitive growth factor (MGF):} a splice variant of IGF-1 that is
particularly sensitive to load and injury in skeletal muscle.

\textbf{Myogenin (MGN):} a myogenic regulatory factor that directs muscle
differentiation. MGN expression in adult muscle fibers plays a role in directing the slow-
oxidative phenotype.

\textbf{N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME):} a non-isoform selective
competitive inhibitor of nitric oxide synthase activity.

\textbf{Nitric oxide (NO):} rapidly diffusing signaling molecule synthesized by a family
of isozymes, the nitric oxide synthases. Its responses are largely mediated by \textit{S}-
nitrosylation of cysteine residues or by interactions with iron and copper. It has been
implicated in many skeletal muscle processes, including gene expression.
**Nitric oxide synthase (NOS):** a complex enzyme responsible for the production of NO. It acts on molecular oxygen, arginine, and NADPH to produce NO, citrulline, and NADP+. The process requires five cofactors (FAD, FMN, Heme, calmodulin, and tetrahydrobiopterin) and two divalent cations (calcium and heme iron). Three distinct forms of NOS have been identified: nNOS, eNOS, and iNOS. All NOS isoforms are expressed in mammalian skeletal muscle.

**Normal load (NL):** the contralateral (left) plantaris serves as an internal control since the gastrocnemius, plantaris, and soleus remain intact. It is assumed that the work done by the NL plantaris mimics the normal, physiologic condition.

**Overload (OL):** the right plantaris serves as the unit that receives a higher external load due to removal of the muscles synergistic to it, the gastrocnemius and the soleus.

**Polymerase chain reaction (PCR):** a technique that creates copies of specific DNA fragments by supplying gene-specific primers, a nucleotide pool, and DNA Taq polymerase.

**Reverse transcription (RT):** a process that synthesizes DNA from an RNA template (isolated total cellular RNA) using the enzyme reverse transcriptase as a catalyst.

**Satellite cells:** normally quiescent, satellite cells are committed stem cells of adult skeletal muscle. Residing between the basal lamina and the sarcolemmal surface of adult muscle fibers, they are activated by conditions such as increased load and injury. They help repair skeletal muscle and mediate muscle growth by differentiating into myocytes.

**Vascular endothelial growth factor (VEGF):** a regulator of blood vessel synthesis induced by IGF-1 during muscle growth.
Limitations/Delimitations/Assumptions

**Limitations.** The invasive nature of this research negates the use of humans as subjects. A rat model was chosen due to genetic homogeneity that allows the researcher to draw conclusions about a population based on the sample studied. Also, rat skeletal muscle shows similarities in phenotype and genetic makeup to human muscle.

Surgically induced overload provides a dramatic response in determining changes in gene expression and alterations in protein synthesis. Therefore, this model provides ease with which the researcher is able to uncover the signaling pathways responsible for the changes associated with hypertrophy. However, unilateral removal of synergists may not guarantee the animal utilizes each leg uniformly during post-surgical ambulation. Therefore, it is possible that the contralateral limb serving as the NL control may experience a small hypertrophic response should the animal favor that leg. To control for this, we performed a sham surgery to the left leg by isolating the synergists without transection. Therefore, the animal would perceive injury to both legs that would allow the ambulation to better mimic the normal, physiologic condition.

Similar to previous studies using L-NAME administered in the animal’s drinking water, our animals showed an initial decrease in the water consumed. However, after an adjustment period, the L-NAME animals did not consume liquid at a different rate than either the TRIM or the Control groups.

Finally, TRIM administered intraperitoneally (IP) may have induced an undesired stress response in the animal. Therefore, the L-NAME and Control animals received IP injection of phosphate-buffered saline (PBS) vehicle. TRIM inhibits both nNOS and, to a lesser extent, iNOS. Therefore, to assess the possible potentiation effects of iNOS
inhibiton, we ran Western blots on NO and OL control plantaris to measure iNOS protein levels.

**Delimitations.** Gender and species differences may exist in the hypertrophic response to the functional overload model. We have chosen to study female Sprague-Dawley rats.

**Assumptions.** Since the animals were housed in pairs, water consumption was assumed to be equal between cage mates. It is also assumed that the animal did not favor the contralateral limb during the OL period. Our lab previously showed that functional overload induced by synergist ablation was a sufficient stimulus to show significant differences between the overloaded plantaris and the contralateral control.

**Significance of the Study**

Attenuating muscle wasting is a pertinent clinical goal in combating pathologies and conditions that lead to loss of function and impairment of ambulation. Aging and disease lead to impaired skeletal muscle regrowth and regeneration, resulting in loss of muscle mass and strength. Recovery from neurodegenerative disorders, spinal cord lesions, and muscle unloading due to spaceflight, injury, or extended bed rest will be more rapid if therapeutic strategies are discovered and implemented. Therefore, it is critical to determine the signaling mechanisms responsible for the load-induced phenotypic adaptations in skeletal muscle. Elucidating the biochemical pathways controlling muscle growth and the phenotypic changes that support recovery from injurious assault can lead to therapeutic strategies that prevent or possibly reverse muscle atrophy. Progression in this basic science may aid in identifying targets for anti-atrophy drugs.
CHAPTER 2
LITERATURE REVIEW

Adult skeletal muscle is highly responsive to changes in functional demands and, therefore, serves as an excellent model in elucidating the molecules that serve as links between mechanical stimuli and gene expression. Researchers employ clinically relevant in vivo models as well as in vitro models due to the ease with which the mechanical effects can be controlled and isolated. In whole animals, chronic overload causes structural and phenotypic alterations in muscle fibers, resulting in dramatic muscle growth. Likewise, myotubes subjected to stretch experience growth, proliferation and associated fiber type changes in culture. The pathways governing these changes, however, are poorly understood. Nitric oxide synthase (NOS) activity may be essential during increased contractile activity, suggesting nitric oxide (NO) may act as a coordinating master signal during muscle growth. The purpose of this study was to determine if NOS activity inhibition would attenuate the increased expression of muscle growth/regulatory factors and proteins associated with the contractile apparatus during overload-induced hypertrophy. This chapter provides a critical review of the scientific literature related to the proposed project along with interpretations where possible.

Skeletal Muscle Adaptations to Load

Adult skeletal muscle is extremely adaptable to changes in mechanical loading. The adaptations to chronic overload involve many coordinated steps that lead to structural and phenotypic changes within the muscle fibers. The resulting growth involves the activation and fusion of satellite cells, the increased synthesis of structural
and contractile proteins, and subsequent capillary angiogenesis. Each of these steps is controlled by specific growth and/or regulatory factors. Hepatocyte growth factor (HGF) initiates satellite cell activation, insulin-like growth factor-1 (IGF-1) governs cell proliferation and fusion, and activates protein synthesis, vascular endothelial growth factor (VEGF) regulates angiogenesis, and myogenin (MGN) is involved in differentiation and expression of the slow phenotype. Each of these factors and its involvement in skeletal muscle growth following bouts of overload and/or stretch is described below.

**Hypertrophy and Fiber Type Shifts with Loading**

Growth of skeletal muscle fibers in response to an increased mechanical load is a complex event marked by large-scale remodeling of fiber architecture. The process is associated with injury, immune cell infiltration, and regeneration (69), and involves the activation and subsequent fusion of satellite cells to the muscle fibers (48, 57, 58), and increased synthesis of structural and contractile proteins (28, 76). Increased protein synthesis causes the accumulation of contractile and structural proteins resulting in the addition of myofibrils and an accompanying increase in fiber cross-sectional area. The increase in fiber cross-sectional area reflects an increase in muscle fiber diameter without an increase in the number of muscle fibers (26, 72). Finally, the fiber type changes associated with increased muscle recruitment are mediated via calcium-dependent signaling, including calcium-calmodulin-dependent protein kinase and calcineurin. These pathways interact with others to transcriptionally regulate expression of the myogenic regulatory factor, myogenin (77). Myogenin, in turn, plays a major role in activation of the myogenic program in differentiating satellite cells, as well as regulating the slow-oxidative phenotype in adult myofibers (34).
Skeletal Muscle Hypertrophy and Insulin-Like Growth Factor (IGF-1) Signaling

IGF-1 signaling cascade. IGF-1 is an important regulator molecule in muscle physiology, particularly important for maintenance and growth of adult skeletal muscle mass (3). Although the major part of the circulating fraction of total IGF-1 is produced in the liver, IGF-1 is also locally expressed in the muscle (1). This endogenous IGF-1 acts in a paracrine/autocrine manner by binding to skeletal muscle IGF-1 receptor (IGFR). IGF-1 binding to IGFR, leads to activation of the insulin receptor tyrosine kinase and subsequent activation of several cytosolic substrates, such as calcineurin, the mitogen-activated protein kinase/extracellular signal-related protein kinase (MAPK/ERK) and the phosphoinositide 3’-kinase (PI3K). Interestingly, these pathways have distinctive and possibly opposing end results. Calcineurin is a phosphatase that targets a family of transcription factors, the nuclear factors of activated T-cells (NFAT). Once dephosphorylated, cytosolic NFAT translocates to the nucleus to increase the transcription of IGF-1-sensitive genes, specifically Type I (slow) myosin heavy chain (MHC). Interestingly, IGF-1 also activates MAPK/ERK, a powerful myoblast proliferative cascade that inhibits differentiation, and PI3K, whose activity ultimately leads to increased protein synthesis and differentiation. However, IGF-1 is a more powerful and potent activator of PI3K. Once activated, PI3K phosphorylates Akt/PKB and initiates a protein kinase cascade that includes mammalian target of rapamycin (mTOR), p70s6 kinase and increased protein synthesis. As a result, IGF-1 plays a major role in the growth and maintenance of adult skeletal muscle and is a potent stimulator of anabolic, mitogenic, and myogenic processes in skeletal muscle (51, 74).

The IGF-1 response in functionally overloaded skeletal muscle. While circulating IGF-1 is important to these skeletal muscle responses, it is clear that the
autocrine/paracrine actions of endogenously produced IGF-1 are critical for muscle hypertrophy (1). Perhaps the most convincing evidence that IGF-1 mediates adult skeletal muscle hypertrophy comes from muscle-specific overexpression of IGF-1 in a transgenic mouse model (15), and local infusion of IGF-1 to the rat tibialis anterior muscle (3). In the first study, Criswell and colleagues discovered that the mice overexpressing IGF-1 demonstrated a higher absolute muscle mass in both the gastrocnemius and the tibialis anterior (TA) compared to their wild-type counterparts. In the second study, the authors implicated IGF-1 in acting directly to stimulate protein synthesis and satellite cell proliferation, resulting in skeletal muscle hypertrophy. Further, these conclusions were supported by the observed increase in absolute weight of the TA injected with IGF-1. Additionally, the total protein and DNA content of these muscles increased. Both of these models produced dramatic muscle hypertrophy without any change in loading pattern, indicating the sufficiency of IGF-1 for skeletal muscle hypertrophy.

Not only is IGF-1 necessary for skeletal muscle growth, its expression is increased during periods of increased load. Functionally overloaded rat plantaris muscles experienced a significant increase in IGF-1 mRNA as well as the compensatory increases in muscle size and fiber cross-sectional area (CSA) when compared to the contralateral control plantaris (78). Interestingly, these rats were hypophysectomized to decrease the levels of pituitary-derived growth hormone, illustrating the inherent importance of IGF-1’s autocrine and paracrine properties to muscles during overload-induced hypertrophy. Regenerating skeletal muscle also shows an increase in the IGF-1 signal. Rat TA muscles recovering from bupivicaine injection demonstrated temporal and maximal expressions of IGF-1 mRNA at days 5 and 10, respectively (43). Further, Yang and
colleagues (79) showed that IGF-1 is strongly correlated with the phenotypic adaptations associated with stretch and overload. Therefore, IGF-1 signaling is important for both skeletal muscle growth and the phenotypic changes associated with this growth. Adams and Haddad (2) suggested that the increases in muscle IGF-1 preceded the phenotypic changes in the functionally overloaded rat plantaris. These IGF-1 mRNA increases peaked at 3 days of overload with a maximal increase of 4 times normal levels. Finally, McCall and colleagues (44) showed that IGF-1 is transcriptionally regulated in vivo during hypertrophy as evidenced by the induction of the endogenous IGF-1 pre-mRNA during functional overload of the rat plantaris.

**IGF-1 induction in cell culture.** In vitro studies have also been used to elucidate the pathways that participate in and activate muscle growth and the phenotypic changes associated with the growth. The research designs most commonly used are myotubes subjected to stretch or electrical stimulation. Media depleted of mitogens is commonly used to activate differentiation, as it induces the expression of localized IGF-1 (83). This induction overrides the negative control of serum mitogens on differentiation. Electrical stimulation (ES) produces changes in metabolic enzyme and contractile protein gene expression, and stretch potentiates the ES-induced changes, including harvesting a more robust IGF-1 response (46). Bayol et al. (6), suggest that proteins associated with the IGF-1 axis, in part, mediate these changes. Addition of IGF-1 to culture media stimulates both MAPK/ERK and PI3K/Akt signaling pathways during differentiation, but not to the same degree, suggesting the necessity of both pathways during proliferation and the induction of differentiation (22, 63). IGF-1 enhanced in vitro muscle growth through increased satellite cell proliferation via the PI3K/Akt pathway (12). Finally, repetitive
stretch/relaxation of differentiated muscle cells stimulated the acute release of IGF-1 during the first 4 hours of stretch (51). \textit{In vivo} and \textit{in vitro} models suggest that IGF-1 uniquely stimulates both muscle cell proliferation and differentiation.

\textbf{MGF acts as a mechanically sensitive splice variant.} Although IGF-1 is essential to muscle growth and maintenance, recent evidence suggests a splice variant of the growth factor that may be particularly responsive to loading and/or injury. Goldspink and colleagues (81) have identified and cloned this splice variant of IGF-1, called mechano growth factor (MGF). MGF, produced by active muscle, controls local tissue repair, maintenance, and remodeling through induction of local protein synthesis, and prevention of apoptosis. The alternative splicing of the IGF-1 mRNA results in a 52 base pair insert in the E domain that likely causes it to bind to a different protein. The reading frame shift associated with the MGF peptide also causes it to have a different C terminal sequence, inducing differential protein/receptor affinities. Therefore, the MGF peptide may mediate slightly different processes. \textit{In vitro}, MGF inhibits terminal differentiation of C2C12 cells and increases cell proliferation by processes that are independent of the IGF-1 receptor (82). MGF expression increases with high resistance exercise in humans (30), following acute local damage in rat muscle (32), and with \textit{in vivo} electrical stimulation and stretch (13). The peak in MGF expression is approximately on the third day of differentiation. Therefore, local production and post-transcriptional processing of IGF-1 is critical for adaptations in muscle mass in response to altered loading. Nevertheless, the signal(s) linking mechanical load and skeletal myofiber IGF-1 gene expression remain a mystery.
Skeletal Muscle Hypertrophy and Vascular Endothelial Growth Factor (VEGF)

Skeletal muscle hypertrophy is temporally coupled to the induction of angiogenesis and is closely linked to blood vessel recruitment and capillary angiogenesis, such that capillary density is maintained during the early stages of hypertrophy (54). Regular contractile activity induces an increase in capillarization. Along with basic fibroblast growth factor (bFGF), VEGF regulates angiogenesis, and is highly induced in growing and hypertrophying skeletal muscle (66). VEGF is a potent mitogen of endothelial cells. VEGF binds to two primary receptors on endothelial cells (VEGFR1 and VEGFR2). In addition to inducing endothelial cell proliferation, migration and differentiation, activation of VEGFR2 leads to NO production via eNOS. NO production can be vital to VEGF signaling, as inhibiting NO production diminishes the angiogenic response (7, 41). Recently, expression of VEGF in skeletal muscle cells has been shown to be under the control of IGF-1-dependent Akt signaling (66) and its expression is increased via an AMPK-p38 MAPK signaling cascade.

VEGF expression and skeletal muscle overload. Increased angiogenesis is necessary to support a growing muscle as its metabolic demands increase. In humans (56), dogs (31), and rats (80), increased metabolic demands associated with an increased exercise paradigm caused enhanced remodeling of capillaries local to the muscle, and increased muscle contraction in rats caused an upregulation of VEGF (4). Although many models have induced angiogenesis by way of increases in blood flow and accompanying capillary shear stress, few have investigated the isolated role of muscle contraction and overload on growth factor expression and release, and on endothelial cell proliferation.
Skeletal muscle overload is a potent stimulus for capillary growth *in vivo*. Rat extensor digitorum longus muscles exhibited an increase in capillary-to-fiber ratio after removal of the synergist tibialis anterior muscle (19), thereby implicating the importance of capillary angiogenesis during synergist ablation and the resulting overload. Degens and colleagues (17) found that VEGF mRNA expression increased after 2 weeks of compensatory overload following functional elimination of synergists to the plantaris, and this expression precedes capillary proliferation. This timepoint coincides with the half time for increases in capillary supply, suggesting VEGF plays a preparatory role in capillary angiogenesis and may be elevated during the week of recovery post surgery. Additionally, NO donors increase VEGF mRNA *in vivo*. Benoit and colleagues (7) found that injecting rats with nitroprusside, an NO donor, resulted in elevated levels of the growth factor in the gastrocnemius muscle. Further, another NO donor, S-nitroso-N-acetylpenicillamine (SNAP) induced angiogenesis *in vivo* and *in vitro* (41). Endothelial cells treated with SNAP demonstrated increased cell migration and differentiation into capillaries. Therefore, there is strong evidence that NO acts as a potential coordinator of capillary angiogenesis via transcriptional regulation of VEGF.

**VEGF and skeletal muscle cells.** In addition to playing an important role in capillary angiogenesis, VEGF has also been implicated in stimulating skeletal muscle fiber regeneration in vivo. Arsic and colleagues (5) delivered VEGF using an adeno-associated virus vector to animals subjected to unilateral ischemia and muscle injury. As a result, VEGF delivery significantly reduced the damaged area and increased the number of regenerating fibers when compared to control muscle. The same investigators also found that VEGF increased the number, length, and nuclear content of
differentiated C2C12 cells. VEGF decreased the rate of proliferation and promoted
differentiation, a result that paralleled VEGF’s ability to protect the differentiating cells
from apoptotic death. Taken together, these data suggest the importance of investigating
VEGF as a target for a possible therapeutic role in muscular disorders.

VEGF receptors are members of a family of tyrosine kinase receptors that are
expressed by a variety of tissues. Initially described on endothelial cells, recent data has
shown the presence of VEGF receptors on hematopoietic and vascular smooth muscle
cells. More important to the present study, others present evidence of VEGF receptor
expression on cultured myoblasts and myotubes, and on skeletal myofibers isolated in
culture. This helps to explain the close association of myocyte remodeling with
angiogenesis.

**Hepatocyte Growth Factor (HGF) and Muscle Growth**

**Satellite cells are skeletal muscle progenitor cells.** Skeletal muscle satellite cells
are myogenic stem cells residing between the sarcolemma and the basement membrane in
postnatal skeletal muscle. Normally quiescent, they are activated in response to
mechanical changes in the muscle. They actively participate in muscle regeneration
following injury and are required for load-induced hypertrophy. Inactivation of satellite
cells by gamma irradiation prevents muscle hypertrophy following compensatory
overload. Once activated, the satellite cells migrate to the area of injury or growth to
supply myonuclear precursors to the expanding fibers, preserving the myonuclear domain
inherent to adult skeletal muscle. In response to stretch or compensatory overload,
satellite cells are activated, enter the cell cycle and subsequently proliferate. Schiaffino
and colleagues (61) have shown that this proliferative activity begins at ~3 days after
compensatory overload. Activated satellite cells fuse into preexisting fibers or form new fibers (45).

Although other growth factors have the capacity to stimulate satellite cell proliferation and/or differentiation (IGFs and fibroblast growth factors (FGFs)), it appears that hepatocyte growth factor (HGF) initiates satellite cell activation. It is active as a heterodimer that results from proteolytic cleavage of the inactive, single chain pro-form. Present in the liver, kidney, lung, and spleen, HGF message and protein have also been localized in adult, uninjured skeletal muscle sequestered to the extracellular matrix (62, 67). The signaling receptor for HGF is the c-met receptor, and both its message and protein have been found in satellite cells in vitro and in vivo. Muscle stretch or injury releases HGF from the extracellular matrix to bind the c-met receptor, thereby inducing the satellite cells to enter the cell cycle and proliferate. Cultured satellite cells also express HGF, which acts in an autocrine fashion to induce proliferation (62).

Additionally, chronic low frequency stimulation of rat muscle in vivo leads to the activation and proliferation of satellite cells. Further, this increase in satellite cell progeny paralleled the increase in myonuclear content (55). However, the link between changes in external mechanical and electrical stimuli and chemical signaling is poorly understood.

**HGF release and compensatory overload.** Overloaded plantaris muscle shows an increase in HGF message. After unilateral removal of the synergists to the plantaris, HGF mRNA expression increased at 3, 7 and 21 days when compared to the contralateral control (78). Plantaris hypertrophy was closely associated with the upregulation of HGF and FGF mRNA, even when the plantaris muscles were denervated during the overload
period. This suggests that HGF expression may be involved in overload-induced hypertrophy.

**HGF and stretch.** HGF release from the extracellular space and subsequent satellite cell activation appears to depend on external load. Cultured quiescent satellite cells subjected to stretch entered the cell cycle earlier than those in control conditions, and conditioned media from these stretched cells activated unstretched satellite cells (68). Additionally, blocking HGF in stretched cultures prevented satellite cell activation. *In vitro* experiments suggest that acute satellite cell activation is mediated by release of local HGF already present in skeletal muscle. NO serves as the link between mechanical stretch in vitro and HGF release from satellite cells. Tatsumi et al. (67) found that NOS activity is increased in cyclically stretched cells and the resulting NO mediates HGF release.

HGF is synthesized and secreted by satellite cells in vitro, suggesting HGF possesses both autocrine and paracrine properties (62). Further, it is suggested that isolated satellite cells also express and secrete an HGF activator to ensure the active form of HGF will be available for autocrine action. Other progenitor cells, specifically fibroblasts, do not express HGF message or protein, lending support to the idea that satellite cells are the cells responsible for increasing HGF levels upon activation. Based on these data, it seems likely that sustained hypertrophic activity may require upregulation of HGF expression in activated satellite cells and/or muscle fibers.

**Myogenin (MGN) and Control of Adult Muscle Phenotype**

The myogenic regulatory factors (MRFs), including MGN, MyoD, myf-5, and MRF-4, belong to the basic helix-loop-helix superfamily of transcription factors. These proteins form heterodimers with ubiquitous E-proteins and bind to DNA at consensus E-
box domains, present in many muscle-specific gene promoters and drives the
differentiation process (52). During muscle development, the MRFs are expressed in a
highly regulated temporal pattern, with MGN appearing late in differentiation
corresponding to the beginning of myoblast fusion.

MGN continues to be expressed in adult skeletal muscle fibers and is believed to
control the slow-twitch fiber phenotype. MGN is an important regulatory factor during
overload-induced hypertrophy. Its expression in activated satellite cells corresponds to
terminal differentiation of these cells and fusion with existing muscle fibers, a process
that is required for normal hypertrophy of adult muscle fibers.

**Hypertrophy and Nitric Oxide**

The exquisite coordination of the complex response to skeletal muscle overload
suggests a common regulator. Smith et al. (64) reported that inhibition of nitric oxide
synthase (NOS) activity prevents the normal hypertrophy and fiber type adaptations to
chronic skeletal muscle overload. Further, the release of HGF during skeletal muscle
loading is nitric oxide synthase-dependent (68), and endurance exercise increases VEGF
expression in skeletal muscle via a nitric oxide-dependent mechanism (24). Together,
these data suggest that nitric oxide may be acting as a common master signal. Therefore,
we hypothesized that inhibition of NOS activity would prevent the increased local
expression of growth/regulatory factor mRNAs (IGF-1, MGF, HGF, VEGF, and
myogenin) and Type I (slow) myosin heavy chain (MHC) expected during the early
stages of functional overload in the rat plantaris.

**NOS activity and fiber type composition.** Adult skeletal muscle is a mosaic of
four different fiber types, one slow (I), and three fast (IIa, IIb, IId/x). These differ with
respect to specific biochemical and morphological characteristics. Skeletal muscle
possesses a high degree of plasticity, allowing it to respond and adapt to altered physiological demands by switching between fiber types. The adaptation to functional demands is a well-documented process, known as fast-to-slow or slow-to-fast transformation. A fast-to-slow transformation is induced by muscle growth (hypertrophy), and results in an increased oxidative capacity and a greater resistance to fatigue. Conversely, a slow-to-fast fiber transformation characterizes muscle wasting (atrophy) and heightens a muscle’s fatigability and glycolytic capacity. Expression of the different fiber isoforms is determined by the type of nerve (8), the level of physical activity (60), and the amount of passive stretch (59). There has been extensive research in uncovering the pathways or molecules that are responsible for directing the fiber type transition, including NO.

The metabolic phenotype of skeletal muscle fibers, including fiber type, is controlled primarily by calcium-dependent signaling. The tonic activity of slow motor neurons produces a chronic low-level of cytoplasmic calcium in slow-twitch fibers. This activates calcium-dependent signals. Most notably, the calcium-dependent phosphatase, calcineurin, and the calcium-calmodulin-dependent protein kinases (CaMK). The constitutive NOS enzymes are also calcium-calmodulin dependent and may play a regulatory or facilitative role in the control of muscle fiber type. Smith and colleagues (64) demonstrated the involvement of NOS in skeletal muscle adaptations to overload. As in previous research, overload induced hypertrophy and a fast-to-slow fiber type shift. Treatment with the NOS inhibitor, L-NAME, however, attenuated the overload-induced hypertrophy and prevented the increase in type I fibers with hypertrophy. This suggests that NO is necessary for changes in skeletal muscle phenotype in vivo. Additionally, NO
covalently modifies thiol groups on the ryanodine receptors associated with calcium channels of the sarcoplasmic reticulum. This modification results in a release of calcium into the cytosol (21). Therefore, NO could facilitate activation of calcium-dependent pathways. Planitzer et al. (53) discovered that fast-oxidative fibers carry the highest concentration of NOS-1, possibly facilitating the fiber type transitions through a slower phenotype. Future research on NOS activation and the associated changes in phenotype is important in elucidating the complex signaling pathways associated with hypertrophy and muscle fiber type.

**Summary**

Disease states and conditions such as spaceflight and extended bed rest unload skeletal muscle and cause muscle atrophy. The characteristic phenotypic changes associated with muscle wasting are a serious clinical concern and modalities to prevent these changes should be investigated. Defining the mechanisms by which intracellular and extracellular signaling molecules control skeletal muscle growth shows promise for developing novel therapeutics to combat muscle wasting. The rat model of unilateral synergist removal provides a robust hypertrophic response, making this model a valuable tool in elucidating the regulatory pathways. Nitric oxide is a ubiquitous signaling molecule, located in essentially every tissue in the body. Inhibition of nitric oxide synthase (NOS) activity interferes with the muscle growth response. However, NO’s specific role in muscle hypertrophy remains a mystery. Previous research implicates NO signaling in the fiber type transition associated with muscle growth. Further, NO plays a role in satellite cell activation and proliferation. Therefore, NO may act as a coordinating molecule in the many different growth steps of skeletal muscle. This project will
determine whether blocking NOS activity can attenuate the expression of various growth/regulatory factors during functional overload of the plantaris muscle.
CHAPTER 3
METHODS

Figure 3-1. Experimental design flowchart. Animals were given the drugs starting 2 days before the surgeries. Tissue extraction was on day 5.

**Animals**

The subjects were adult (~4 month-old) female Sprague-Dawley rats (~250 g). All were housed in the J. Hillis Miller Animal Science Center and fed the same diet (rat chow and water *ad libitum*) throughout the experiment. They were maintained on a 12 h light:dark photoperiod (light 0700 to 1900h). All procedures followed NIH guidelines.
and were approved by the University of Florida’s Institutional Animal Care and Use Committee.

**Inhibition of NOS Activity**

After an acclimation period of one week after arrival, the rats were randomly divided among the three experimental groups. The pharmacological inhibition of nitric oxide synthesis was achieved by administering the competitive non-isoform-specific NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical) or the nNOS-selective inhibitor, 1-(2-trifluoromethyl-phenyl)-imidazole (TRIM, Cayman Chemical) during the treatment period. L-NAME (1mg/ml) was added to the drinking water to maintain a dose of ~90 mg/kg/d. TRIM was dissolved in phosphate buffered saline (PBS) and injected intraperitoneally (IP) at a concentration of 10 mg/kg/d. To control for the possible confounding influences of a daily IP injection, the Control and L-NAME animals were injected daily with a volume of PBS equal to the volume injected into the TRIM animals.

**Synergist Ablation Surgery**

Chronic overload of the plantaris was induced by surgical, unilateral removal of the synergist muscles to the plantaris. The rats were anaesthetized with inhaled isoflurane (2-5%) with oxygen as the carrier gas. Using aseptic technique, a midline incision was made in the skin of the right hind limb, from the popliteal fossa to the Achilles tendon region. A second longitudinal incision was made through the hamstrings exposing the distal gastrocnemius and Achilles tendon region. The gastrocnemius tendon was carefully separated from the plantaris tendon, and the gastrocnemius muscle sectioned. The distal two-thirds of the gastrocnemius was removed, taking care not to disturb the plantaris
nerve and blood supply. Next, the soleus muscle was carefully isolated and removed. The hamstring incision was closed with 4-0 vicryl absorbable suture. The overlying skin was closed with sterilized metal wound clips and treated with a topical antibiotic cream to avoid infection.

A sham operation was performed on the left leg of each rat. In this procedure, the same incisions were made as above and the gastrocnemius tendons were isolated without transecting the muscles. The animals were allowed to fully recover from the anesthetic before returning to their cages. The rats were examined daily for signs of infections or wound openings, which were promptly treated, if found.

**Experimental Protocol**

Two days before the surgery, L-NAME was added to the drinking water of the appropriate group, and daily injections of TRIM (TRIM group) or PBS (Control and L-NAME groups) were begun. Forty-eight hours after the start of drug treatments, the animals underwent the ablation surgeries. All animals received the unilateral synergist ablation surgery along with the sham surgery on the contralateral limb. After a brief recovery period, the rats were group-matched, housed in pairs, and allowed to ambulate freely. Water was replaced each day, and body mass and water consumption were recorded daily throughout the experimental period. The dose of L-NAME was calculated for each rat. On day 5 post surgery, the animals were anesthetized with inhaled isoflurane and sacrificed by exsanguination. The plantaris muscles were immediately and bilaterally removed, trimmed of excess connective tissue and fat, weighed on an analytical scale, and flash-frozen in liquid nitrogen. Frozen muscles were powdered using a liquid nitrogen-cooled mortar and pestle. The powdered muscle was divided into separate tubes and stored at -80° C for subsequent biochemical analyses.
Nitric Oxide Production

To determine the efficacy of the pharmaceutical NOS blockers, L-NAME and TRIM, serum nitrate/nitrite levels were measured. Whole blood (~ 4 mL) collected from the animals at the time of sacrifice was incubated for 30 minutes at room temperature to allow for clotting. The samples were centrifuged 20 minutes at 5000g to separate the serum from the cellular fraction. The serum was removed and stored at -80°C until further analysis. Using a pre-designed kit (Cayman Chemical), the serum samples were analyzed for nitrate/nitrite levels as per the manufacturer’s specifications.

Reverse Transcription and Real-Time Quantitative PCR

Total RNA was isolated using Trizol Reagent (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. The amount of total RNA was evaluated by spectrophotometry and the integrity checked by gel electrophoresis. Total RNA (5 µg) was reverse transcribed using the Superscript III First-Strand Synthesis System (Life technologies, Carlsbad, CA) using oligo(dT)20 primers and the protocol outlined by the manufacturer. One µL of cDNA (5 µL for MGF) was added to a 25 µL PCR reaction for real-time PCR using Taqman chemistry and the ABI Prism 7000 Sequence Detection System (Applied Biosystems (ABI), Foster City, CA). The comparative Ct method (ABI User Bulletin #2) was employed for the relative quantitation of gene expression. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as the normalizer. The enzyme is important in purine biosynthesis, and it was chosen based on initial experiments showing that our manipulations did not affect the expression of the transcript. Five-fold dilution curves were assayed on selected samples to confirm the validity of this quantitation method for each gene. IGF-1(GenBank NM_178866), HGF
(GenBank NM_017017), skeletal $\alpha$-actin, Type I (slow) MHC, and myogenin (GenBank NM_017115) mRNA transcripts were assayed using pre-designed rat primer and probe sequences commercially available from Applied Biosystems (Assays-on-Demand). MGF (5’-CACTGACATGCCCCAAGACTCA (forward) and 5’-CTTTGCAGCTTTTCTTTGT (reverse)) and HPRT (5’-GTTGGGATAAGGCAGACTTTGT (forward) and 5’-AGTCAAGGGCATAATCCAACACAA (reverse)) mRNA were assayed using custom made primers (Applied Biosystems, Assays-by-Design). The MGF reverse primer was custom designed to span the 52-base pair insert unique to the rat MGF cDNA.

**Semi-Quantitative RT-PCR**

Because the pre-designed rat primers and probes supplied by ABI did not differentiate among the four isoforms of VEGF, we chose to do semi-quantitative PCR to determine if there was differential expression between the isoforms associated with this model. Total RNA was isolated as described above and reverse transcribed using Ready-to-Go You-Prime First-Strand Beads (Amersham Biosciences, Buckinghamshire, UK) and a combination of random hexamers and oligo(dT)$_{20}$ primers. cDNA for the four isoforms of VEGF expressed in rat skeletal muscle were amplified simultaneously using published primer sequences and conditions (16), with expected sizes of 632-, 560-, 500-, and 428-bp PCR products for amplification of VEGF-188, VEGF-164, VEGF-144 and VEGF-120, respectively. 18S ribosomal cDNA was amplified simultaneously as an internal control for amplification efficiency (Ambion, QuantumRNA internal standards). PCR products were separated by electrophoresis on 1% agarose gels, stained with ethidium bromide, and band intensity was quantified by densitometry (Scion Image software).
Immunoblotting

It has been shown that TRIM has significant inhibitory effects on both nNOS and the inducible form of NOS, iNOS. Both nNOS and eNOS are constitutively active in skeletal muscle, while iNOS activity is normally absent. However, iNOS protein expression and activity can be induced in skeletal muscle by the inflammatory process. To assess the possibility of the potentiation effects of iNOS, we evaluated iNOS protein levels via Western blotting in the normal loaded (NL) and the overloaded (OL) plantaris muscles from the Control group. For all Western blots, the powdered muscle was homogenized in 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 1 mM PMSF, and 10 µg/ml aprotinin using a stainless steel blade. Homogenates were centrifuged for 10 min at 1000g to remove connective tissue and cellular debris and protein content quantified in the supernatant using the DC Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA, USA).

For iNOS protein, 80 µg of total protein were subjected to SDS-PAGE on 7% polyacrylamide gels. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes under cold conditions, which were stained with Ponceau S (0.1% w/v in 5% acetic acid) to verify equal loading. The membranes were subsequently blocked for 1h at room temperature in Tris-buffered saline-Tween (TBST; Tris–HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk and then incubated (4°C overnight) with primary antibody for iNOS (Transduction Laboratories) diluted in blocking solution. The membranes were washed three times in TBST, 10 minutes each, followed by treatment with horseradish peroxidase-labeled anti-mouse antibody (Vector
Labs) for 2h at room temperature. Reactions were developed using the Enhanced Chemiluminescence detection reagents (ECL; Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions, and protein levels were determined by densitometry (Scion Image software).

For p70$_{S6K}$, membranes were then blocked for 1 h in Tris-buffered saline–Tween (TBST; Tris–HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk and incubated with primary antibody for phosphor(Thr389)-p70$_{S6K}$ (Cell Signaling Technology, Beverly, MA) overnight at 4°C. Membranes were washed three times in TBST, 10 min each, followed by incubation with a peroxidase-labeled anti-rabbit antibody (Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h at RT. Reactions were developed using the Enhanced Chemiluminescence detection reagents (ECL; Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions, and protein levels were determined by densitometry (Scion Image software).

**Limitations**

Although our lab has had previous success in using the synergist ablation model to induce overload hypertrophy, the procedure is not without criticism. The invasive nature of the surgery could affect skeletal muscle gene expression independent of mechanical overload. Nevertheless, comparison of overloaded muscles to contralateral normally-loaded muscles (i.e. within-subject design) controls for potential effects of systemic factors, such as circulating cytokines, hormones, or inflammatory cells. Further, sham surgeries control for local surgical effects. It is impossible to determine the source of intramuscular NO in the present design. Future studies will be necessary to examine potential intrinsic and extrinsic sources of NO signaling during muscle overload.
Our model investigated the effects of 5 days of overload on gene expression of several growth factors, myogenin, and contractile proteins. This time point was chosen based on published accounts of overload-induced IGF-1 and MGN expression, protein synthesis, and RNA and DNA accumulation. All of these factors are significantly elevated in the overloaded plantaris at 5 days. However, the dynamics associated with HGF and VEGF induction is unknown, as is the time course of NO signaling. Therefore, more studies utilizing the functional overload model at different time points during the hypertrophic response may be necessary to elucidate possible differential gene expression.

**Vertebrate Animals**

Female Sprague-Dawley rats were used in this research. This study required the removal of hindlimb muscles synergistic to the plantaris via a non-terminal surgery, with subsequent ambulation. This invasive procedure prevented the use of human subjects. Sprague-Dawley rats were selected based on the large amount of preliminary data collected with this model in our lab and many others.

**Statistical Analysis**

This experiment was designed to test the hypothesis that NOS activity is necessary for the induction of growth factor and contractile protein expression during overload-induced hypertrophy. A 3 x 2 (treatment x loading condition) ANOVA with repeated measures on the loading condition was employed to determine main effects and interactions for each variable. Where significant differences were found, Tukey’s HSD test was implemented *post hoc* to determine individual group differences. Significance was established *a priori* at p<0.05.
CHAPTER 4
RESULTS

Systemic and Biological Responses to Treatment

Body mass did not change from pre- to post-overload treatment for any group. Further, body mass did not differ between groups at any time point (Table 4-1).

Consistent with a report from Adams and Haddad (2), no OL-induced changes in total protein were observed at 5 days of overload. Nevertheless, this model does cause muscle protein accumulation following 14 days of OL (64). This study focuses on the early signaling events leading to this protein accumulation. Water consumption in the L-NAME-treated group was reduced ~40% during the first 24h of L-NAME treatment, but then returned to normal (not different from the Control group; data not shown) for the remainder of the treatment period. The average L-NAME dose was 89.2 mg/kg/d.

L-NAME and TRIM treatments significantly lowered serum nitrate/nitrite levels (P<0.05), indicating successful systemic NOS inhibition. Mean (±SEM) serum nitrate/nitrite levels were: Control = 7.08±0.31 μM, L-NAME = 1.65±0.09 μM, and TRIM = 4.49±0.19 μM.

Myogenin mRNA Expression

Five days of plantaris overload caused an approximately 4-fold increase in myogenin mRNA expression. No significant differences were observed between treatment group (Table 4-2).
**Contractile Protein mRNA Expression**

Five days of OL resulted in a 90% increase in skeletal α-actin mRNA and a 140% increase in type I (slow) MHC (MHC-I) mRNA (Figure 4-1; Control NL vs. Control OL). However, these data indicate a complete inhibition of this OL-induced response in the NOS-inhibited groups. Skeletal α-actin and MHC-I mRNA levels did not differ between NO and OL muscle in either treatment group (L-NAME and TRIM). Further, skeletal α-actin and MHC-I transcript levels in L-NAME and TRIM muscles did not differ from that in control NL muscles.

**Growth Factor mRNA Expression**

Expression of mRNA for HGF, IGF-1, MGF, and the 120 amino acid splice variant of VEGF (VEGF-120) were all increased in the OL muscles (Table 4-2). IGF-1 mRNA was increased ~4 fold and MGF mRNA ~9 fold in Control/OL compared to Control/NL muscles (Figure 4-2). TRIM treatment did not affect IGF-1 or MGF mRNA expression in the NL muscle, but approximately doubled expression of both transcripts in the OL muscle compared to Control/OL (Table 4-2). HGF and VEGF-120 mRNAs were increased 15-20 fold in the OL muscles compared to NL, with no effect of either L-NAME or TRIM treatment (Table 4-2 and Figure 4-3). Transcripts for VEGF-188, VEGF-164, and VEGF-144 tended to be reduced in the OL muscles, but this did not reach statistical significance (p>0.05). Real-time PCR assessment of total VEGF mRNA showed a ~50% reduction in OL muscle, compared to NL. The discrepancy between real-time assessment of total VEGF mRNA (Table 4-2) and semi-quantitative assessment of VEGF isoform expression (Figure 4-3) is most likely due to the relatively small contribution of VEGF-120 to the total VEGF mRNA pool and the variability in the semi-
quantitative RT-PCR method, which failed to demonstrate a significant OL-related reduction in the more abundant VEGF isoforms.

**Phosphorylation of p70^{S6K}**

Expression of total p70^{S6k} protein was increased in OL muscles in all three treatment groups. Likewise, phosphorylated p70^{S6k} was increased in OL muscles compared to NL. The ratio of phosphorylated to total p70^{S6k} did not differ between Control NL and OL muscles. Conversely, the ratio of phospho to total p70^{S6k} was significantly elevated in OL muscles from L-NAME and TRIM animals, indicating a greater relative phosphorylation status (Figure 4-4) in these muscles.

**iNOS Protein Expression**

TRIM significantly inhibits nNOS and, to a lesser degree, iNOS. Therefore, we sought to determine if iNOS induction during overload could contribute to the observed nitric oxide-dependent effects. iNOS protein was not detected in either normally loaded or overloaded plantaris muscle (Figure 4-5), suggesting that the TRIM effects are due to inhibition of nNOS activity.

**Table 4-1. Body mass, plantaris mass, and total protein data for the overloaded rats.**

<table>
<thead>
<tr>
<th></th>
<th>Body Mass (g)</th>
<th>Plantaris mass (mg)</th>
<th>Total Protein (mg/muscle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>340.4 ± 8.9</td>
<td>323.0 ± 21.1</td>
<td>38.8 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>NL</td>
<td>OL</td>
<td></td>
</tr>
<tr>
<td>L-NAME</td>
<td>334.4 ± 11.5</td>
<td>303.3 ± 6.1</td>
<td>40.9 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>NL</td>
<td>OL</td>
<td></td>
</tr>
<tr>
<td>TRIM</td>
<td>340.1 ± 9.8</td>
<td>324.5 ± 19.3</td>
<td>36.5 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>NL</td>
<td>OL</td>
<td></td>
</tr>
</tbody>
</table>

Definition of abbreviations: L-NAME = N^G-nitro-L-arginine methyl ester; TRIM = 1-(2-trifluoromethyl-phenyl)-imidazole; OL = 5-day overloaded plantaris; NL = contralateral normally loaded plantaris. Values represent means ± SEM.
Table 4-2. Real-time PCR quantification of mRNA transcripts for selected growth factors and a regulatory gene in the plantaris muscle. Transcripts are normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA and expressed relative to the Control, normally loaded (NL) value, using the comparative Ct method.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Control NL</th>
<th>L-NAME OL</th>
<th>TRIM OL</th>
<th>Control NL</th>
<th>L-NAME OL</th>
<th>TRIM OL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGN</td>
<td>1.38 ± 0.32</td>
<td>5.28 ± 0.81 a</td>
<td>1.09 ± 0.33</td>
<td>3.58 ± 0.39 a,b</td>
<td>0.89 ± 0.16</td>
<td>3.68 ± 0.79 a,b</td>
</tr>
<tr>
<td>IGF-1</td>
<td>1.12 ± 0.18</td>
<td>3.98 ± 0.28 a</td>
<td>1.06 ± 0.18</td>
<td>5.06 ± 0.59 a,b</td>
<td>1.22 ± 0.14</td>
<td>8.49 ± 0.86 a,b,c</td>
</tr>
<tr>
<td>MGF</td>
<td>1.07 ± 0.33</td>
<td>8.84 ± 0.78 a</td>
<td>0.73 ± 0.12</td>
<td>11.32 ± 3.05 a,b</td>
<td>0.99 ± 0.29</td>
<td>22.03 ± 6.31 a,b,c</td>
</tr>
<tr>
<td>HGF</td>
<td>1.18 ± 0.30</td>
<td>19.76 ± 4.1 a</td>
<td>2.45 ± 0.80</td>
<td>20.78 ± 2.7 a,b</td>
<td>1.85 ± 0.39</td>
<td>17.78 ± 0.62 a,b</td>
</tr>
<tr>
<td>total</td>
<td>1.22 ± 0.27</td>
<td>0.58 ± 0.09 a</td>
<td>1.68 ± 0.52</td>
<td>0.68 ± 0.14 b</td>
<td>1.94 ± 0.50</td>
<td>0.52 ± 0.08 a,b</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.27 ± 0.09 a</td>
<td>0.58 ± 0.52</td>
<td>0.14 b</td>
<td>0.52 ± 0.08 a,b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Definition of abbreviations: L-NAME = N\textsuperscript{G}-nitro-L-arginine methyl ester; TRIM = 1-(2-trifluoromethyl-phenyl)-imidazole; OL = 5-day overloaded plantaris; NL = contralateral normally loaded plantaris; MGN = myogenin; IGF-1 = insulin-like growth factor-1; MGF = mechanosensitive growth factor; HGF = hepatocyte growth factor; VEGF = vascular endothelial growth factor. Values represent means ± SEM.

\( a \) = Significantly different from Control NL, \( p<0.05 \).
\( b \) = Significantly different from corresponding NL (within group), \( p<0.05 \).
\( c \) = Significantly different from Control OL, \( p<0.05 \).
Figure 4-1. Real Time PCR assessment and quantification of contractile protein mRNA transcripts. A) skeletal α-actin mRNA level relative to HPRT mRNA in 5-day overloaded (OL) and contralateral normally loaded (NL) plantaris muscles of Control, L-NAME and TRIM-treated rats. B) Type I (slow) myosin heavy chain (MHC) mRNA level relative to HPRT mRNA in 5-day overloaded (OL) and contralateral normally loaded (NL) plantaris muscles of Control, L-NAME and TRIM-treated rats. Values represent means ± SEM. a = Significantly different from corresponding NL, p<0.05.
Figure 4-2. Real Time PCR quantification of insulin-like growth factor mRNA transcripts. A) Insulin-like growth factor-1 (IGF-1) mRNA level relative to HPRT mRNA. B) Mechano growth factor (MGF) mRNA level relative to HPRT mRNA. Values represent means ± SEM. a = Significantly different from Control/NL, p<0.05. b = Significantly different from Control/OL, p<0.05.
Figure 4-3. Semi-quantitative RT-PCR analysis of VEGF mRNA splice variant expression. A) Expression levels of VEGF-120 mRNA, relative to ribosomal 18S RNA in 5-day overloaded (OL) and contralateral normally loaded (NL) plantaris muscles of Control, L-NAME and TRIM-treated rats. B) Representative ethidium bromide stained 1% agarose gel illustrating PCR products following amplification of VEGF and 18S. See Methods for details of assay conditions. Values represent means ± SEM. \( a = \) Significantly different from Control/NL, \( p<0.05 \).
Figure 4-4. Western blot analysis of p70\textsuperscript{s6K}. A) Representative immunoblot for phosphor(Thr389)-p70\textsuperscript{s6K}, Total-p70\textsuperscript{s6K}, and beta-actin (loading control) in 5-day overloaded (OL) and contralateral normally loaded (NL) plantaris muscles of Control, L-NAME, and TRIM-treated rats. B) Quantification of phosphor(Thr389)-p70\textsuperscript{s6K} to total p70\textsuperscript{s6K} ratio. Values are means (±SEM) expressed relative to Control/NL mean. \textit{a} = Significantly different from corresponding NL p<0.05. \textit{b} = Significantly different from Control-OL, p<0.05.
Figure 4-5. Immunoblot assessment of iNOS protein expression in 5-day overloaded (OL) and contralateral normally loaded (NL) plantaris muscles of Control rats. iNOS expression was not detected in any of the samples.
CHAPTER 5
DISCUSSION

To our knowledge, this is the first study to investigate the effects of functional overload on the up-regulation of key signaling pathways leading to increased protein synthesis, angiogenesis, and satellite cell activation and proliferation. Our primary observations were focused on an early time point (5d of overload) when growth factor expression is at its peak (2). The data supports our hypothesis that NOS activity is important for up-regulation of contractile gene expression. Specifically, skeletal α-actin type I (slow) MHC up-regulation in the overloaded plantaris was prevented with both non-isoform-specific inhibition of NOS activity (L-NAME) and nNOS-specific inhibition (TRIM). Conversely, neither L-NAME nor TRIM treatment repressed the overload-related increase in skeletal muscle mRNA expression for myogenin and the growth factors: HGF, VEGF-120, IGF-1, and MGF. In fact, the TRIM-OL group expressed approximately double the MGF and IGF-1 transcripts compared to Control-OL. Consistent with increased IGF-1 signaling, NOS inhibition induced greater relative phosphorylation of p70\textsuperscript{s6K} in OL muscle. Although it appears that nitric oxide signaling is not necessary for the up-regulation of the growth factors we measured, the data suggest that nitric oxide may play a role in the transcriptional regulation of slow MHC, skeletal α-actin, the phosphorylation status of p70\textsuperscript{s6K}, and, perhaps, feedback control of IGF-1/MGF mRNA during skeletal muscle overload.

Employing *in vivo* models to investigate nitric oxide signaling, although physiologically meaningful, is not without consequence. Systemic non-isoform-specific
NOS inhibition (L-NAME) has physiological consequences throughout the body, and can lead to significant effects on hemodynamics (14), muscle contractility (20), and gene expression in non-muscle tissue (35). The majority of these systemic L-NAME effects are thought to be secondary to eNOS inhibition and the resulting effects on blood flow and blood pressure. In fact, the nNOS-specific isoform, TRIM, has been administered to rats *in vivo* with no reported systemic side-effects (39). Since nNOS is the most abundant NOS isoform in skeletal muscle (70), and is reportedly sensitive to muscle loading (71), we hypothesized that this isoform accounts for overload-induced nitric oxide signaling in the plantaris muscle. To test this possibility, and partially control for the systemic effects of L-NAME, we treated one group of rats with daily IP injections of TRIM. Since TRIM inhibits iNOS as well as nNOS, we confirmed by immunoblots that iNOS protein was not expressed in NL or OL plantaris muscles (data not shown). Our data support a role for the nNOS isoform in early adaptations to skeletal muscle overload.

**Skeletal α-Actin mRNA Expression**

Skeletal α-actin protein is an important component of the contractile apparatus, and is known to be transcriptionally up-regulated during skeletal muscle hypertrophy (9). Since adult skeletal muscle sarcomeric actin is derived from the single α-skeletal actin gene, rather than from multiple isoforms, the regulation of this gene serves as an index of overall contractile protein synthesis. Carson et al (11) have reported that transcriptional activity of the actin promoter is increased in skeletal muscle during *in vivo* stretch overload. This effect is mediated by serum response factor (SRF) binding to actin promoter (10). A recent paper (36) found that nitric oxide donors were sufficient to induce SRF binding to a myosin heavy chain promoter element and increase promoter
activity in cultured smooth muscle cells. Our data suggest that nNOS activity is important for induction of skeletal α-actin transcription during chronic overload.

**VEGF Expression**

The reduction in total VEGF mRNA level in the 5d-overloaded muscle was unexpected, given the known capillary angiogenesis occurring in overloaded rat muscle (54). However, the few studies reporting VEGF mRNA expression in hypertrophying skeletal muscle show mixed results. Degens et al. (17) found no significant changes in VEGF mRNA in hypertrophying quail muscle. Similarly, 4 wks of strength training in human subjects, even under hypoxic conditions, did not change skeletal muscle VEGF mRNA expression (23). On the other hand, overload of the rat plantaris for 2 wks (i.e. 2.8X longer than our 5d treatment) did increase VEGF mRNA by ~50% (18).

To further characterize the VEGF mRNA response, we examined expression of the four splice variant isoforms found in rat skeletal muscle (16) using semi-quantitative RT-PCR and published primer sequences (16). To our knowledge, ours is the first study to measure expression of specific VEGF mRNA splice variants in overloaded, hypertrophying skeletal muscle. Unlike aerobic exercise, which primarily induces the VEGF-164/5 isoform (29, 37), we found that the VEGF-120 isoform was induced in the 5d-overloaded plantaris muscle. This effect, however, was not influenced by L-NAME or TRIM treatments.

**IGF-1 Expression and Phosphorylation of p70<sup>66</sup> Kinase.**

These results are consistent with previous findings that endogenous IGF-1 mediates adult skeletal muscle hypertrophy, as our data shows a dramatic increase in the amount of transcript with OL. Additionally, we show that the MGF splice variant is particularly responsive to overload. These data support a role for nitric oxide that is either
independent of the IGF-1 axis, or downstream of IGF-1 transcription. The exaggerated expression of IGF-1 and MGF mRNA in the overloaded plantaris of the nNOS-inhibited TRIM group suggest the possibility of an nNOS-dependent negative feedback mechanism controlling the load-induced IGF-1 response. IGF-1/Akt signaling in the rat kidney is known to activate eNOS by phosphorylation and increase nitric oxide production (75). The phosphorylation status of the NOS isoforms in overloaded skeletal muscle is unknown, but it seems possible that IGF-1-dependent nNOS activation could produce a feedback signal to control IGF-1/MGF expression during muscle growth.

Activation of the key translational regulator, p70^{66K}, is correlated to increased protein synthesis in skeletal muscle, induced by phosphoinositide 3-kinase (PI3K) or mechanical stretch (33). Since NOS inhibition reduces protein accumulation in overloaded rat plantaris, we postulated that phosphorylation of p70^{66K} would be inhibited in the L-NAME and TRIM groups. On the contrary, we found that OL induced expression of total p70^{66K} protein in all groups, and that this corresponded to an increase in phosphorylated p70^{66K} in the OL muscles. However, the ratio of phosphor/total p70^{66K}, indicating the relative degree of activation of the pathway was elevated only in the OL muscles of the L-NAME and TRIM groups. This suggests that protein translation may have been elevated in partial compensation to reduced transcriptional activity. Further experiments are needed to directly measure effects of nitric oxide on skeletal muscle protein synthesis rates during hypertrophy.

**Future Directions**

Although many of our results are unremarkable, there remain many unanswered questions regarding the mechanisms underlying skeletal muscle hypertrophy. Therefore, there are other pathways yet to be investigated that may play an important role in
contributing to muscle growth. We will look at two possible contributors that deserve future attention and the possibility of interaction with nitric oxide signaling: calpain-mediated proteolysis and calcineurin-NFAT pathway.

**Calpain-Mediated Proteolysis.** The calpains are a ubiquitous family of calcium-dependent cytosolic cysteine proteases. Calpain proteolysis activity contributes to overall protein degradation. Specifically, the calpains target proteins that are important in linking cytoskeletal proteins together to the cell membrane (25). Nitric oxide has been implicated as a possible regulator of calpain activity. The nitric oxide donor, sodium nitroprusside, reversibly inactivates calpain activity via S-nitrosylation (47). Further, NOS activity has been shown to inhibit calpain protease activity in skeletal muscle (38). Therefore, it is also possible that protein accumulation during overload in the NOS-inhibited groups could be hindered by an increase in protein degradation. Preliminary data comparing protein content of cleaved α(II)-spectrin between the groups at 5 days of overload showed inconsistent results (data not shown). However, this could be due to a variety of factors, including length of time between tissue harvest and data collection in this instance. Nonetheless, calpain activity inhibition may represent an important mechanism by which nitric oxide production facilitates hypertrophy.

**Calcineurin-NFAT pathway and the slow MHC phenotype.** Calcineurin is a calcium-dependent protein phosphatase located in skeletal muscle cytoplasm that preferentially responds to intracellular calcium concentration. Once activated, the cascade of events leads directly to muscle growth and fiber type differentiation (65, 73). A target for calcineurin is a member of the nuclear family of activated T-cells (NFAT), which remains in the sarcoplasm in a phosphorylated state. Upon dephosphorylation, the
protein translocates to the nucleus and promotes transcription of genes which are involved in hypertrophy and fiber morphology shift (40, 50). Calcineurin has been implicated in both stimulation of type I gene expression and facilitation of type II to type I fiber type transition. Naya and colleagues (49) concluded that activated calcineurin induces type I gene expression via NFAT transcription. The c-Jun N-terminal kinase (JNK) branch of the mitogen-activated protein kinase (MAPK) signaling pathway has been implicated in the rephosphorylation and subsequent resequestering of NFAT to the cytoplasm. Interestingly, in cardiac muscle there exists a cross talk between JNK and calcineurin-NFAT signaling such that JNK activation acts to modulate calcineurin-NFAT signaling and inhibit cardiac growth(42). Further, nitric oxide increases NFAT nuclear accumulation indirectly via cGMP-dependent kinase (PKG) in smooth muscle (27). PKG, in turn, directly inhibits JNK activity, allowing NFAT to accumulate in the nucleus. Taken together, these data suggest additional pathways through which nitric oxide may be regulating the hypertrophy response in skeletal muscle and should be investigated further.

**Conclusions**

Up-regulation of type I (slow) MHC and skeletal α-actin mRNA (presumably via transcription) during chronic skeletal muscle overload is dependent upon nNOS activity. Conversely, induction of growth factors and activation of protein translation (p70s6k phosphorylation) are not dependent upon NOS activity. Nevertheless, nitric oxide production may provide feedback control of IGF-1 and MGF signaling in hypertrophying muscle.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Jeff Sellman was born in Kettering, Ohio, and grew up in Littleton, Colorado. He graduated summa cum laude from Heritage High School in 1991. After 2 years of undergraduate work at the University of Colorado at Boulder, Jeff took time off to pursue other interests. This led him to the Florida Army National Guard where he was a land combat missile system repair technician. During his time in the army, Sergeant Sellman was an expert M16 marksman, was designated an army physical fitness leader, and received his combat lifesaver certificate. He was awarded The Army Reserve Component Achievement Medal and The Army Achievement Medal. Jeff returned to school at the University of Florida, Gainesville, Florida, and graduated cum laude with a bachelor’s degree in exercise and sports sciences. He began a master’s program in applied physiology and kinesiology also at the University of Florida. He has worked for four years as a research assistant in the Molecular Physiology Laboratory within the Center for Exercise Science. Jeff has been accepted into the University of Florida College of Medicine Class of 2009 and will pursue an M.D. degree.