NITRIC OXIDE FACILITATES NUCLEAR FACTOR OF ACTIVATED T-CELL (NFAT) ACTIVITY THROUGH AKT INDUCED GLYCOGEN SYNTHASE KINASE-3BETA (GSK-3Beta) PHOSPHORYLATION

By

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To my wife, Tiffany Drenning, who sacrificed much for this degree

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Skeletal muscle is characterized by different fiber types including one slow (type 1/β), and three fast (IIa, IIx, and IIb). These various phenotypes display contractile and biochemical properties responsive to altered physiological demand. Activity induced increases in intracellular calcium transients facilitate slow phenotypic adaptations via activation of calcineurin and its downstream target, nuclear factor of activated t-cells (NFAT). Nitric Oxide (NO) is an important signaling molecule in skeletal muscle and is produced enzymatically by nitric oxide synthases (NOS). Our lab has recently shown that NO facilitates NFAT activity through the NO-cGMP driven inactivation of glycogen synthase kinase-3β (GSK-3β) in C2C12 myotubes.

While NOS activity seems to be necessary for the calcium induced effects on fiber type change in C2C12s, it is unknown whether NOS is necessary for changes in adult skeletal muscle. Further, the pathway by which NO-cGMP activity results in GSK-3β phosphorylation has not been clearly elucidated. These experiments tested the central hypothesis that NO facilitates NFAT function by AKT-induced GSK-3β phosphorylation both in the C2C12 cell line, and in a genetic model. We tested this postulate by addressing two integrated specific aims: 1) we

determined that cultured myotubes and plantaris muscle from nNOS and eNOS knockout mice display altered NFAT function, and 2) we determined that AKT-induced GSK-3β phosphorylation explains a mechanism by which the NO-cGMP pathway affects NFAT in C2C12 myotubes. We investigated the role of NOS activity in NFAT function by ex vivo and in vitro methods using both an animal model and a myogenic cell line.

Type II diabetes mellitus (DM2) is a growing disease population and is becoming increasingly costly due to associated health care costs. DM2 is characterized by insulin resistance and impaired glucose clearance which has been linked to reduced expression of slow-oxidative muscle fibers. Skeletal muscle is responsible for most insulin-mediated glucose oxidation as muscle contractile activity augments glucose clearance by improving insulin sensitivity. Slow, type I/ β muscle fibers, in particular, are characterized as insulin sensitive due to a proclivity for being metabolically active. Therefore, understanding the mechanism(s) that contribute to the activity-induced changes in muscle phenotype is important. Our proposed experiments can provide insight into potential therapeutic interventions for DM2 patients.

CHAPTER 1
INTRODUCTION

Skeletal muscle is responsible for most insulin-mediated glucose clearance in the body (DeFronzo *et al.* 1981; Devlin *et al.* 1987; Ivy & Holloszy, 1981; Katz *et al.* 1983; Larsen *et al.* 1997). There is considerable plasticity in skeletal muscle as chronic exercise results in increased expression of slow, oxidative genes giving muscle fibers an insulin sensitive and metabolically active phenotype. Given the importance of understanding the mechanism(s) that contribute to fiber type switching and the potential role of nitric oxide (NO) to regulate the slow gene transcription factor NFAT, we postulate that NO is necessary for NFAT function both in vivo and in vitro.

Previous work in our lab suggests that NO is an important signaling molecule in controlling skeletal muscle plasticity downstream of calcium (Drenning *et al.* 2008). NO is produced enzymatically by nitric oxide synthases (NOS). The isoforms eNOS and nNOS are calcium-sensitive and constituitively expressed in skeletal muscle. These enzymes synthesize NO at low levels associated with low frequency muscle activation. Our preliminary data shows that NOS activity results in inhibitory phosphorylation of GSK-3β (in a NO-cGMP dependent manner) and subsequently enhances NFAT activity but, the mechanism by which this occurs is unclear. Further, our data regarding NFAT is limited to C2C12 myotubes. Hence, these experiments will investigate NFAT function in vivo and in cultured myotubes from nNOS and eNOS knockout mice and examine the mechanism(s) by which NO inhibits GSK-3β in C2C12 myotubes. Our central hypothesis is that NO facilitates NFAT function by inhibiting GSK-3β in a cGMP/PI3K/AKT dependent manner.

Background

Adult vertebrate skeletal muscle consists of different fiber types, one slow (type I/β) and three fast (IIa, IIx, and IIb), which differ in their contraction speed, strength, fatigability, and insulin sensitivity. Skeletal muscle exhibits a high degree of plasticity with transformations in fiber type occurring in response to altered physiological demand and contractile load (Chin *et al.* 1998; Schiaffino *et al.* 2007). Tonic, low-frequency neural activity or electrical stimulation causes a shift from fast, glycolytic fibers toward the slow, oxidative phenotype (Liu *et al.* 2005; Pette, 2001). The pathway by which low frequency muscle activation induces transcription of slow-twitch genes involves sustained calcium levels sufficient to stimulate calcineurin phosphatase activity (Dunn *et al.* 2001; Jiang *et al.* 2006; Wu *et al.* 2000). Dephosphorylation of the nuclear factor of activated t-cells (NFAT) transcription factors by calcineurin promotes its translocation from the cytoplasm to the nucleus, where it will bind to a nucleotide recognition sequence and stimulate the transcription of target, slow-twitch genes (Chin *et al.* 1998; Kubis *et al.* 2003;). Although this pathway explains activity-induced activation of NFAT, overall transcriptional activity, and therefore fiber type change, is determined by the balance between activation and deactivation of this transcription factor (Abbott *et al.* 1998; Delling *et al.* 2000). Recent studies suggest that GSK-3β synergistically regulates nuclear export of NFAT in skeletal muscle fibers by phosphorylation of its serine residues (Jiang *et al.* 2006; Shen *et al.* 2007).

Nitric Oxide (NO) is a ubiquitous signaling molecule produced enzymatically by nitric oxide synthases (NOS). Recently, it has been reported that NO is required for NFATc3 nuclear accumulation in mouse cerebral arteries in response to increased intravascular pressure, and that this effect was dependent upon inhibition of NFAT nuclear export (Gonzalez-Bosc *et al.* 2004). NFAT has been shown to be an important transcription factor in skeletal muscle as its calcium/calcineurin induced nuclear translocation and accumulation stimulate the expression of

slow genes, particularly MHC I/β (Meissner *et al* 2007). Further, our recent data confirm the role of NO in NFAT function as NOS activity has been shown to be necessary for NFAT translocation and transcription (Drenning *et al* 2008).

Problem Statement

A better understanding of how habitual physical activity can lead to changes in skeletal muscle gene expression has expanded our knowledge of the benefits of exercise. However, additional research is needed to provide a more comprehensive understanding of how chronic exercise improves fitness and decreases the risk of diabetes. In addition, more studies aimed at exploring the calcium-regulated signaling pathways and their molecular targets are needed. Work by numerous authors confirms that the transcription factor, NFAT is integral to calcium/calcineurin-induced fiber type changes in skeletal muscle. Given the importance of NO as an important signaling molecule capable of mediating NFAT in C2C12 myotubes, we propose that NO is essential to NFAT function ex vivo and in vivo as well. We postulate that removal of the NOS isoform in mice will result in altered NFAT nuclear translocation and fiber type aberration. NO could exert its effects on NFAT by activating AKT in a cGMP/PI3K dependent manner, thereby allowing NFAT nuclear accumulation due to AKT induced GSK-3β phosphorylation (inactivation). Discovery of the mechanism(s) that regulate exercise-related fiber type changes could lead to therapies with broad clinical application.

Variables in Study

Independent variables: Genetic manipulation of NOS expression will be achieved by purchasing homozygous mice harboring a targeted mutation of either the nNOS or eNOS gene. Knockout of the nNOS or eNOS protein, respectively, was confirmed in skeletal muscles of these mice compared to control mice from the parent strain. Cultured myotubes will be exposed

to various pharmacological agents in the treatment medium (supplementing with A23187, L-NAME, PAPA-NO, SNAP, ODQ, YC-1 and LY29004).

Dependent variables: We will measure NFAT nuclear accumulation and translocation, GSK-3β phosphorylation, AKT phosphorylation, MHC I/β mRNA, protein phosphatase activity and muscle fiber type.

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. However, this should not affect chronic satellite cell/myotube cultures or the stable phenotype of the plantaris muscle.

Specific Aims and Hypotheses

- **Question 1:** Do mice with targeted mutation of nNOS and eNOS have altered NFAT function in primary cultured myotubes and aberrant fiber type phenotype in the plantaris muscle?

- **Hypothesis 1:** nNOS and/or eNOS knockout mice display altered NFAT function compared to wild type (WT) mice. In addition, AKT and GSK-3β phosphorylation is reduced, MHC I/β mRNA activity is lessened, and fiber type expression is aberrant in NOS knockout mice.

- **Question 2:** Does the NO-cGMP pathway inhibit GSK-3β by activating the PI3K/AKT pathway?

- **Hypothesis 2:** Low levels of NO induce phosphorylation of AKT, which inhibits GSK-3β in a cGMP/PI3K-dependent manner.

- **Question 3:** Does NO have the capacity to inhibit protein phosphatases?

- **Hypothesis 3:** The NO-cGMP pathway inhibits protein phophatase activity subsequently leading to AKT activation.

List of Terms

A23187 (calcimycin): calcium ionophore known to upregulate calcineurin and NFAT activity

Calcineurin: protein phosphatase which dephosphorylates NFAT and induces nuclear translocation

Calcium (Ca2+): essential element for cellular and molecular signaling in muscle

Cyclic Guanosine Monophosphate (cGMP): synthesis of cGMP catalyzed by guanylate cyclase (GC); activated by, and often associated with NO in the NO-cGMP pathway

Endothelial Nitric Oxide Synthase (eNOS): NOS isoform present at low levels in all skeletal muscle fibers, co-localizing with mitochondrial markers and closely related to intracellular calcium levels and calmodulin binding

Glycogen Synthase Kinase-3β (GSK-3β): kinase which phosphorylates NFAT exporting it from the nucleus

L-NAME (N (G)-nitro-L-arginine methyl ester): NOS inhibitor capable of abrogating expression of all three NOS isoforms

LY294002 (2-(4-Morpholino)-8-phenyl-4*H*-1-benzopyran-4-one): potent inhibitor of PI-3K/AKT pathway

Muscle Fiber Type: slow- twitch (Type I) fibers characterized by slow contraction time, high resistance to fatigue and displaying insulin sensitivity; fast-twitch (Type II) fibers identified by quick contraction time, low resistance to fatigue and insulin resistant

Myosin Heavy Chain I/β: (MHC I/β): fatigue-resistant isoform most responsible for contracile force in skeletal muscle

Neuronal Nitric Oxide Synthase: isoform located in the sarcolemma and closely related to intracellular calcium levels and calmodulin binding

Nitric Oxide (NO): small, highly diffusible molecule synthesized by the enzyme nitric oxide sythase (NOS) from the conversion of L-arginine to L-citrulline

Nuclear Factor of Activated T-Cells (NFAT): important in skeletal muscle as transcription factor which contributes to the induction of slow genes

ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one): highly selective, irreversible, heme-site inhibitor of soluble guanylyl cyclase

PAPA-NO (1-[N-(3-Ammoniopropyl)-N-(n-propyl)amino]diazen-1-ium-1,2-diolate): rapidly degraded NO donor with half life of release of 15 minutes

Phosphoinositide-3 kinase (PI-3K): kinase which activates AKT

Protein Kinase-B (AKT): kinase known to be capable of phosphorylating GSK-3β at serine-9

Type 2 Diabetes Mellitus (DM2): metabolic disorder primarily characterized by insulin resistance, relative insulin deficiency and hyperglycemia often managed by engaging in exercise

YC-1 (3-(5'hydroxymethyl-2'furyl)-1-benzyl indazole): NO independent activator of soluble guanylyl cyclase

Limitations/Delimitations/Assumptions

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

Delimitations: Gender, age and species differences may exist in regard to signaling pathways and muscle fiber type. In our animal model, we have chosen to study young, male C57 mice.

Assumptions: It is assumed that the specific NOS isoform deleted in each of the respective knockout mice is not expressed. Previous experiments have confirmed that nNOS and eNOS knockout mice do not express the genetically removed NOS isoform.

Significance of the Study

Type 2 diabetes mellitus is modulated therapeutically by regular exercise as muscle cells undergo phenotypic changes resulting in insulin sensitive, metabolically active skeletal muscle. Inactivity results in a shift toward an insulin resistant, metabolically inactive phenotype. Thus, there is a need for individuals susceptible to DM2 to remain active throughout life.

This research will improve our knowledge of the mechanisms underlying the changes in skeletal muscle phenotype with chronic exercise. We seek to better understand the signaling pathways responsible for these phenotypic changes, and to explore the role of Nitric Oxide in skeletal muscle plasticity. This study will provide insight into clinical therapies designed to improve skeletal muscle metabolic activity and provide potential solutions for DM2 mediation.

CHAPTER 2
LITERATURE REVIEW

Many studies have shown that skeletal muscle is responsible for most insulin-mediated glucose oxidation (DeFronzo *et al.* 1981; Devlin *et al.* 1987; Ivy *et al.* 1981; Katz *et al.* 1983; Larsen *et al.* 1997). Exercise improves muscle glucose clearance due to the chronic effect of activity on fiber type expression (Shiaffino *et al.* 2007). While the calcium/calcineurin related pathways have been well established as contributing to the shift in fiber type toward a slow twitch, metabolically active phenotype, (Chin *et al.* 1998; Kubis *et al.* 2003; Naya *et al. 2000*) the role NO plays is unclear. To further understand the function of NO in fiber type related calcium signaling, this project examined the hypothesis that NOS activity promotes NFAT by inhibiting GSK-3β in a cGMP/PI3K/AKT dependent manner. The background section of this proposal will discuss the importance of the proposed work and develop ideas behind our hypothesis based on our prior research and the work of others. The preliminary data section will provide evidence of the feasibility of our proposed experiments.

Overview of Skeletal Muscle Fiber Type

The cells that make up skeletal muscle are known as myofibers. They are large multinucleated cells that often extend the entire length of individual muscles. These individual myofibers are expressed as different types and vary in size, metabolic activity, and contractile function. These "types" are generally categorized into two groups. Slow fibers are characterized as type I, and fast fibers as type IIa, IIb, and IId/x. Thus, skeletal muscle is comprised of numerous fiber types with different structural and functional properties (Kraus *et al.* 1994; Williams & Kraus, 2005; Pette 2001).

Fiber Type Characteristics

Type I fibers are also known as slow oxidative fibers and contain large numbers of oxidative enzymes and are enveloped by more capillaries than type II fibers. This phenotype also contains higher concentrations of myoglobin than fast fibers. All of these characteristics allow for type I fibers to have a large capacity for aerobic metabolism and a high resistance to fatigue (Williams & Kraus, 2005). Further, these types of cells have been shown to be insulin sensitive which contributes to the interest in understanding the mechanism(s) underlying the therapeutic effectiveness of endurance exercise in mediating DM2 (Shiaffino *et al.* 2007).

Type 2 fibers, or fast glycolytic fibers have a smaller number of mitochondria, limited capacity for aerobic metabolism and are less fatigue resistant than type I fibers. In contrast to slow oxidative fibers, fast glycolytic fibers are less metabolically active and more insulin resistant (Pette, 2001). However, it should be noted that type IIa fibers can be viewed as an intermediate between type I and type IIb fibers. Chronic exercise or tonic neural stimulation induces an increase in the oxidative capacity of IIa fibers to the extent that their oxidative capacity reaches levels close to that of type I fibers. Type IIb and type IId/x fibers are less efficient than the other fibers most likely due to high ATPase activity leading to greater energy expenditure per unit of work performed (Liu *et al.* 2005).

Nerve Activity

Skeletal muscle is a plastic tissue in the sense that it undergoes phenotypic changes based on the stress under which it is placed. Nerve activity has been shown by both nerve cross union and electrical stimulation studies to be able to induce fiber-type switching (Williams & Kraus, 2005). Phasic, high-frequency electrical stimulation causes a shift from slow oxidative fibers to a fast, glycolytic fiber. Motor neurons innervate skeletal myofibers and determine the timing, intensity, and duration of each myofibrillar contraction (Shiaffino, 2007). The fiber-type profile

of different muscles is largely influenced by the pattern of nerve activity induced by the motor neuron (Williams & Kraus, 2005). This is important in exercise-related research since different patterns of nerve activity can result in changes in muscle fiber type. The "tonic" pattern of contractions associated with endurance exercise has been shown to be helpful in reducing the risk of a number of diseases associated with inactivity, including diabetes (Shiaffino, 2007).

Fiber Type Switching

A number of studies have described fiber type switching based on the frequency of electrical stimulation. High frequency stimulation results in a shift in the direction: I→IIa→IId/x→IIb (Shiaffino, 2007) and is similarly induced by inactivity. Fast to slow transformations (IIb→IIx→IIa→I) occur by tonic low-frequency stimulation characteristic of the pattern induced by slow motoneurons (Pette, 2001; Kraus *et al.* 1994).

Ausoni *et al.* (1990) has shown that there are limitations to these transformations in rat skeletal muscle. Particularly, they observed that fast fibers have the ability to shift from IIb↔IIx↔IIa. Slow fibers also seem to be limited as the same researchers saw adaptability in the range of I↔IIa↔IIx.

Conflicting data with regard to the range of myofiber plasticity were published by Caiozzo *et al.* (1998). This group found that changes in the thyroid state may extend the range of fiber type changes. They showed that MHC IIb can be enhanced in type I fibers by multiple factors. Specifically, both the effect of hyperthyroidism and inactivity resulted in a shift from type I to type IIb MHC. Earlier data published by Kirshbaum *et al.* (1990) confirms the possibility of an expanded range with regard to IIb→I fiber type changes. These researchers found that hypothyroidism in conjuction with chronic low frequency stimulation resulted in a shift from type IIb to type I fibers.

Fiber type changes seem to be influenced by the duration of stimulation as evidenced by looking at long term (2-4 mo) low frequency stimulation. Termin *et al.* (1989) stimulated fast twitch muscles in rats for 2 mo and did not observe a significant increase in MHC I expression. However, Windisch *et al* (1998) performed a similar experiment, stimulating fast twitch muscles in rats for 4 mo and saw a fast to slow transformation. Further, type I fibers tend to disappear after long periods of inactivity.

Expanding our understanding of how skeletal muscle responds to repeated bouts of activity can help in the prevention of diabetes and other chronic diseases. Particularly, a greater knowledge of the molecular signaling pathways which serve as the mechanisms for the aforementioned plastic nature of muscle could lead to important biotechnological advances, potentially providing an alternative to physical activity. The molecular events pertinent to this study will be discussed below.

Nuclear Factor of Activated T-Cells and Skeletal Muscle

Nuclear factor of activated T-cells (NFAT) is a general name applied to a family of transcription factors shown to be expressed in a number of cells in the body. The NFAT transcription factor family consists of five members: NFATc1, NFATc2, NFATc3, and NFATc4. All the NFAT isoforms are regulated by calcium signaling and stimulated by the protein phosphatase calcineurin (Rao *et al.* 1997).

Activation of Calcineurin/NFAT Pathway

Calcium signaling pathways dependent on nerve activity play a major role in the maintenance and modulation of muscle fiber-type (Crabtree, 1999; Naya *et al.* 2000; Pette & Vroba, 1999). These molecular pathways have been studied extensively. Our laboratory has examined specifically the calcineurin/NFAT pathway.

Tonic patterns of motor nerve activity promote changes in intracellular calcium that result in the activation of numerous molecular signaling pathways (Williams & Kraus, 2005). These pathways link changes in nerve activity to changes in gene expression which establish myofiber diversity (Shiaffino *et al.* 2007). The calcineurin/NFAT pathway is an important mechanism which has been shown to affect fiber-type plasticity (Chin *et al.* 1998; Schultz & Yutzey, 2004; McCullagh *et al.* 2004; Fenyvesi *et al.* 2004), Rao *et al.* 1997; Ryder *et al.* 2001; Wu *et al.* 2000; Yan *et al.* 2001; Meissner *et al.* 2007; Fielder *et al.* 2002). This pathway is largely dependent on increases in intracellular calcium activity. Calcium signaling is critical to NFAT activation because calmodulin, a well known calcium sensor protein, activates calcineurin. Calcineurin is a calcium-dependent, serine/threonine protein phosphatase also known as protein phosphatase-2B (PP2B). The signaling cascade modulated by calcineurin results in the nuclear translocation of the transcription factor, NFAT. Activated calcineurin dephosphorylates the serine rich region and SP-repeats in the amino termini of NFAT, resulting in a conformational change that exposes a nuclear localization signal resulting in NFAT nuclear import (Meissner *et al.* 2007). Generally, nuclear import of NFAT is opposed by maintenance kinases in the cytoplasm and export kinases in the nucleus. Export kinases such as GSK-3β must be inactivated for NFAT nuclear retention. Accordingly, a number of studies have shown that an increases in calcium transients phophorylates GSK-3β, rendering it inactive and thus allowing for NFAT nuclear accumulation (Jiang *et al.* 2006; Shen *et al.* 2007). The details of the relationship between NFAT and GSK-3β will be addressed in the section entitled, "Role of GSK-3β in NFAT Function" below.

Nuclear Factor of Activated T-Cell Signaling

The importance of the transcription factors of the NFAT family as nerve activity-dependent mediators in skeletal muscle has been demonstrated by numerous studies. Liu *et al.* (2001) has observed differences in nuclear and cytoplasmic NFATc1 localization based on

muscle fiber type and neural stimulation in mice. Specifically, an NFATc1-GFP fusion protein expressed in isolated fibers in the flexor digitorum brevis (FDB) muscle was found to be predominantly cytoplasmic when unstimulated. The FDB typically displays fast-twitch glycolytic fibers and thus this study shows evidence for these type of myofibers to have less nuclear NFAT particularly when inactive. However, in the same study, when exposed to a low-frequency pattern of neural stimulation, the same NFAT-c1-GFP did indeed translocate to the nucleus demonstrating the plasticity of myofibers. In a similar study, Tothova *et al* (2006) used an in vivo model to demonstrate that NFATc1-GFP is largely cytoplasmic in the fast twitch, tibialis anterior (TA) muscle. Further, they observed a predominantly nuclear localization of NFATc1 in the soleus, a slow twitch muscle. Additional experiments by this group demonstrated a rapid nuclear import of NFATc1 via low-frequency stimulation in the TA.

Additional studies using NFAT reporters to monitor NFAT transcriptional activity have shown similar results. NFAT transcriptional activity is higher in slow twitch muscles and lower in fast twitch muscles. Also, when denervated, slow twitch muscles have attenuated NFAT activity (Parsons *et al.* 2003). McCullagh *et al* (2004) observed an ability for denervated, slow twitch muscles to respond to stimulation by increasing NFAT activity. However, this response could only be induced by continuous, low-frequency stimulation. Phasic, high-frequency firing patterns characteristic of fast motoneurons did not cause an increase in NFAT activity.

Other approaches to studying NFAT used a constituitively active form for the purpose of transfection experiments. Particularly, constituitively active NFATc1 has been shown to increase MHC I mRNA in regenerating, denervated muscle (McCullagh *et al.* 2004). This phenomenon was limited to the soleus though, as regenerating extensor digitorum longus muscles did not show the same induction of MHC I expression. Interestingly, NFAT knockout

mice have not been shown to have altered fiber type make-up. However, to date only NFATc2 and NFATc3 knockouts have been studied (Horsely *et al.* 2001; Kegley *et al.* 2001), strengthening the conclusion that the NFATc1 isoform is primarily responsible for fiber type regulation.

Nuclear Factor of Activated T-Cell Interaction with Other Transcription Factors

Work has shown that NFAT transcriptional activity is dependent on interaction with other transcription factors. Meissner *et al* (2007) has recently described the assembly of a transcriptional complex including NFATc1, MyoD, MEF2D, and the co-activator p300. This group observed that all these transcriptional factors assemble on the MHC I promoter region in response to calcium ionophore treatment, stimulating the induction of the MHC I gene.

The potential for NFAT interacting with AP-1 has been studied as well. AP-1, which is a co-factor known to interact with NFAT in the induction of the immune response, may play an important role in working with NFAT in skeletal muscle. Kramer *et al.* (2007) have recently shown that ERK1/2 are activated during exercise and during contraction of isolated muscle. ERK1/2 has shown a propensity for regulating AP-1 and has been shown to induce MHC I expression in rat soleus muscle. Therefore, ERK1/2 and AP-1 may both interact with NFAT to allow for the induction of slow genes.

Role of GSK-3β in NFAT Function

Nuclear NFAT concentrations are dependent on a balance between import and export (activation or deactivation). Kinases are known to phosphorylate NFAT (Shen *et al.* 2007), cause its nuclear export (Gonzolez-Bosc *et al.* 2004), inhibit DNA binding (Jiang *et al.* 2006), and blunt its transactivating potential (Shen *et al.* 2007). In skeletal muscle, a number of studies have shown that GSK-3β is most likely the kinase responsible for NFAT nuclear export.

The protein kinase GSK-3β was originally discovered as a suppressor of glycogen synthase (Embi *et al.* 1980). GSK-3β has been shown to be involved in various metabolic and signaling pathways (Frame & Cohen, 2001). Recently, GSK-3β has been implicated as a negative regulator of both cardiac and skeletal muscle hypertrophy (Haq *et al.* 2000; Rommel *et al.* 2001) as well as muscle differentiation (van der Velden *et al.* 2007).

Regulation of NFAT in skeletal muscle seems to be dependent on GSK-3β activation as Chin *et al.* (1998) demonstrated that phosphorylation of GSK-3β resulted in greater NFAT nuclear translocation. Other research groups have shown that active GSK-3β masks the nuclear localization signal, resulting in NFAT nuclear effusion and a subsequent decrease in gene transcription (Beals *et al.* 1997; Neal & Clipstone, 2001).

Overexpression of GSK-3β in avian skeletal muscle promotes nuclear export of NFAT while inhibition of GSK-3β augments NFAT transactivating potential and enhances MHC I/β expression (Jiang *et al.* 2006). We have shown that an NO donor induces inhibitory phosphorylation of GSK-3β (Drenning *et al.* 2008). Further, our lab has demonstrated that inhibition of GSK-3β by lithium chloride (LiCl) causes nuclear accumulation of NFATc1 and stimulates NFAT dependent transcription (Drenning *et al.* 2008). Additionally, these experiments have shown that the effects of LiCl on NFAT are not attenuated by the NO inhibitor L-NAME, suggesting that GSK-3β inhibition occurs downstream of NOS activity. However, the kinase involved in NO-dependent GSK-3β phosphorylation is unknown. Therefore, this study will focus, in part, on the pathway responsible for NO mediated GSK-3β phophorylation as discussed in the following section.

Nitric Oxide

This section will detail the role nitric oxide (NO) may play in affecting the calcineurin/NFAT pathway. First, the basis for the selection of NO as an important skeletal

muscle signaling molecule to be studied will be discussed. Subsequently, the contribution of NO to the control of the calcineurin/NFAT pathway will be examined in detail.

Introduction to NO

NO is modulated biosynthetically by the family of enzymes known as NO synthases (NOS) which are homodimers. The generation of NO by these enzymes requires L-arginine, nicotamide adenine dinucleotide phosphate (NADPH) and oxygen, as well as, five other cofactors (flavin adenine nucleotide, flavin mononucleotide, tetrahydrobiopterin, heme, and calmodulin) (Reid, 1998).

There are three known isoforms expressed by skeletal muscle including, nNOS, eNOS and iNOS. The particular isoform of nNOS in skeletal muscle is tissue specific, and is an alternatively spliced isoform sometimes referred to as nNOSμ. This isoform is targeted by the dystrophin-associated protein, α_1-synrophin, and thus is located in the sarcolemma (Kaminski & Andrade, 2001). eNOS is also present at low levels in all skeletal muscle fibers, co-localizing with mitochondrial markers (Kaminski & Andrade, 2001). The activity of nNOS and eNOS is closely related to intracellular calcium levels and calmodulin binding. iNOS also displays a sarcolemmal localization and its activity varies in skeletal muscle depending on disease state and species investigated (Reid, 1998). Increases in cytokines often provide the stimulus for iNOS upregulation, and thus iNOS typically exerts an antimicrobial action (Reid, 1998)

Nitric Oxide and Skeletal Muscle

NO has been found through numerous studies to be an important signaling molecule in muscle (Reid, 1998, Stamler & Meissner, 2001; Sugita *et al.* 2005; Nisoli *et al.* 2004). Endogenous production of NO via calcium-calmodulin-dependent NOS may play a role in skeletal muscle phenotypic plasticity. Also, stimulation of soluble guanylate cyclase (sGC) and the resultant accumulation of cGMP mediates many of the signaling functions of NO and

regulates complex signaling cascades through downstream effectors (Kelly *et al.* 2004; Lucas *et al.* 2000). We recently reported that NOS activity is necessary for overload-induced expression of MHC I/β mRNA in the rat plantaris (Sellman *et al.* 2006). Further data from our laboratory confirms these findings in C2C12 myotubes (Drenning *et al.* 2008). Additionally, Gonzalez-Bosc *et al.* (2006) demonstrated that NO is required for NFATc3 accumulation in vascular tissue. Our preliminary data indicates that indeed NFAT function is enchanced by the NO-cGMP pathway in mouse myotubes (Drenning *et al.* 2008). However, further study of the mechanism by which NO affects skeletal muscle plasticity is needed. Therefore, it is important to understand the molecular pathway controlling NFAT activity downstream of NO. Further, similar ex vivo and in vivo experiments in nNOS and eNOS knockout mice are necessary to substantiate our previous findings related to NO and NFAT.

Role of Nitric Oxide and AKT

As has been mentioned, numerous studies have provided evidence which suggest various molecular signaling pathways are in control of changes in skeletal muscle plasticity. Signaling molecules such as phosphatidylinositol 3-kinase (PI3K) and AKT have been studied mainly in the context of catabolic and anabolic processes (Stitt *et al.* 2004; Bodine *et al.* 2006). However, recently some of these molecules have been implicated as being involved in skeletal muscle metabolism as well (Jensen *et al.* 2007). Specifically, AKT and its downstream target GSK-3β have been examined (Jensen *et al.* 2007). AKT is known to phosphorylate GSK-3β at Ser9 rendering it inactive. Interestingly, it has been shown recently that high, S-nitrosylation-like levels of NO can inactivate AKT (Bouallegue *et al.* 2007). However, low levels of NO in muscle may have the opposite effect on AKT.

Skeletal muscle AKT activity increases in response to numerous stimuli, including hormones such as insulin-like growth factor (IGF-1) and insulin (Kimball *et al.* 2002; Bodine *et*

al. 2006). IGF-1 binding to its receptor leads to the activation of its downstream target, PI3K, which facilitates the recruitment of AKT (Bodine *et al.* 2006). Recently, the necessity of NOS activity on IGF-1 receptor induced PI3K/AKT activation has been studied (Chung *et al.* 2004). While this research group showed the possibility of NO activating IGF-1 receptor expression and subsequent PI3K/AKT activity in neurons, this pathway has not been explored in skeletal muscle.

Another possible explanation for the effect of NO on AKT could be the role NO may play in inhibiting protein phosphatase activity. Mdx mice, which are known to have aberrant nNOS expression, exhibit high protein phosphatase-1 (PP1) and GSK-3β activity (Villa-Moruzzi *et al.* 1996). Tokui *et al.* 1996) has demonstrated that protein phosphatase inhibitor-1 (PPI-1) is a potent inhibitor of PP1 when phosphorylated by cGMP dependent kinase. Additionally, Ugi *et al.* (2004) has recently shown that protein phosphatase 2A (PP2A) is capable of inhibiting AKT. These findings all suggest that the NO-cGMP pathway may be affecting AKT by decreasing the activity of certain protein phosphatases, thereby removing their inhibitory effect on AKT.

Summary

The continued expansion of the type II diabetes mellitus (DM2) epidemic can be greatly aided by researching exercise-related changes in skeletal muscle glucose tolerance and insulin sensitivity. By understanding the mechanisms behind why exercise is therapeutically successful in terms of DM2, intervention strategies can be better implemented. In the regard, preliminary work in our laboratory has demonstrated that the signaling molecule NO is integral to the molecular adaptations experienced during exercise induced nerve activity in skeletal muscle (Sellman *et al.* 2006; Lira *et al.* 2007; Drenning *et al.* 2008). Since NO can facilitate NFAT function, determining the mechanisms by which this occurs is important. Similarly, exploring this model in vivo is integral to potential therapeutic strategies aimed at alleviating DM2.

CHAPTER 3
MATERIALS AND METHODS

Experimental Designs

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

Question 1. Do mice with nNOS and eNOS genetically silenced have altered NFAT function?

Experiment 1. Cultured myotubes from wild-type (WT), nNOS and eNOS knockout mice were treated for as follows: 1) Control (DMSO), 2) A23187 (0.4μM), or 3) PAPA-NO (1μM). MHC I/β mRNA (24h treatment), NFAT nuclear accumulation (4h treatment), NFAT translocation, AKT phosphorylation (1h treatment) and GSK-3β phosphorylation (1h treatment) were measured.

Table 3-1. Experiment 1.

Animals	Control	A23187 (0.4μM)	PAPA-NO (1μM)
WT	n=4	n=4	n=4
nNOS KO	n=4	n=4	n=4
WT	n=4	n=4	n=4
eNOS KO	n=4	n=4	n=4

(n=number of cultures for each treatment)

Experiment 2. Western blots for AKT and GSK-3β phosphorylation were run using muscle homogenate from the plantaris muscle of each animal. Also, the plantaris was used for immunohistochemical staining for detecting fiber type differences.

Table 3-2. Experiment 2.

Animals	Number of animals
WT	n=3
nNOS KO	n=3
WT	n=3
eNOS KO	n=3

(n=number of muscles from each group)

Question 2. Does the NO-cGMP pathway inhibit GSK-3β by activating the PI3K/AKT pathway?

Experiment 3. C2C12 myotubes were cultured with varying concentrtations of the NO donor SNAP (1μM-1mM) for 1h. Western blots were run to measure AKT phosphorylation.

Table 3-3. Experiment 3.

Cell type	Control	1μM	10μM	100μM	500μM	1mM
C2C12 myotubes	n=6	n=6	n=6	n=6	n=6	n=6

(n=number of cultures for each group)

Experiment 4. C2C12 myotubes were cultured with the NO donor, PAPA-NO, the sGC inhibitor ODQ, and the sGC enhancer, YC-1 for 1h. Western blots were run to measure AKT phosphorylation.

Table 3-4. Experiment 4.

Cell type	Control	PAPA-NO 1μM	PAPA-NO 10μM	ODQ	YC-1 200μM
C2C12 myotubes	n=6	n=6	n=6	n=6	n=6

(n=number of cultures for each group)

Experiment 5. C2C12 myotubes were cultured with the NO donor, PAPA-NO, the sGC enhancer YC-1, and the PI-3K/AKT inhibitor LY294002 for 1h.

Table 3-5. Experiment 5.

Cell type	Control	PAPA-NO 1μM	PAPA-NO LY29 1mM	YC-1 200μM	YC-1 LY29 1mM	LY29
C2C12 myotubes	n=6	n=6	n=6	n=6	n=6	n=6

(n=number of cultures for each group)

Question 3. Does NO have the capacity to inhibit protein phosphatases?

Experiment 6. C2C12 myotubes were cultured with the calcium ionophore A23187, the NO inhibitor, L-NAME, the NO donor, SNAP, the sGC inhibitor ODQ, and the sGC enhancer, YC-1. A PnPP assay was used to detect protein phosphatase activity.

Table 3-6. Experiment 6.

Cell type	Control	A23187 0.4μM	A23 LNAME	LNAME	SNAP Dose 1μM-1mM	SNAP Dose ODQ 10μM	A23 ODQ	ODQ	YC-1 Dose 200μ-1mM
C2C12 myotubes	n=8	n=8	n=8	n=8	n=8	n=8	n=8	n=8	n=8

(n=number of cultures for each group)

Animals

Young C57 wild type, as well as, eNOS and nNOS knockout mice were used for experiments one and two. The animals were approximately 3-4 weeks old at the time of sacrifice. Hindlimb skeletal muscle, excluding the plantaris, was pooled from 3 animals for each satellite cell isolation. All animals were housed at the University of Florida Animal Care Services Center according to guidelines set forth by the Institutional Animal Care and Use Committee.

Protocol for Experiments 1 and 2

Myogenic cultures were prepared in parallel from WT and nNOS/eNOS knockout mice (C57), using 3 mice per isolation. Cells were isolated from soleus, gastrocnemius, tibialis anterior (TA), and quadriceps muscles after careful dissection of the muscles to minimize connective tissue contribution. Collected muscles were enzymatically digested, satellite cells released, and single cells cultured. Isolated cells were re-suspended from a pellet into serum-rich growth medium consisting of Dulbecco's minimum essential medium (DMEM) supplemented with 25% fetal bovine serum (Hyclone, Logan, UT), 10% horse serum (Hyclone), 1% chicken embryo extract, and antibiotics. Cells were plated at a density of 105 cells per plate using 35-mm plates pre-coated with 2% gelatin. Myoblasts were trypsinized and passed to 6-well plates at 60% confluency. Cultures were maintained in a standard tissue culture, with fresh growth medium replaced following the first 3 days in culture and every 2 days thereafter, and were

harvested, measured for protein content and western blots run according the cell culture and western blot methods described below.

Transient Transfections

C2C12 myotubes were terminally differentiated and transfected with the .4μg of the NFAT promoter plasmid, NFAT-GFP (2). The NFAT-GFP construct was prepared by fusing three tandem NFAT-binding sites with enhanced GFP cDNA. (Addgene plasmid 11107). The plasmid was complexed with Lipofectamine reagent (Invitrogen) and exposed to myotubes in serum-free DMEM for 24h. After transfection, cells were placed in 2% HoS media and cultures visualized by fluorescent microscopy before and during treatment with the calcium ionophore, A23187, A23187 and the NOS inhibitor L-NAME, and L-NAME alone.

Immunohistochemistry

Histochemistry was done on serial cross-sections of frozen muscles that will be collected on glass coverslips. Sections of the plantaris were of 10μM muscle thickness. Fiber types were determined by immunohistochemical analysis of serial sections using monoclonal antibodies specific for IIB [BF-F3 (53)], IIA [SC-71 (53)], and type I [A4.840 (64)] myosin heavy chains. Type IIX fibers were identified on the basis of their lack of reaction with these three antibodies. A variable percentage of muscle fibers were hybrid or intermediate types that contain more than one myosin isoform. Any IIB/IIX or IIX/IIA intermediate fibers were counted as IIB and IIA fibers, respectively, on the basis of their reaction with the IIB- and IIA-specific antibodies, so the IIX fiber type excludes intermediate types. Hybrid fibers reacting with both type IIA and type I myosin antibodies were typed as IIA fibers.

Chemicals and Reagents

N(G)-L-nitro-arginine methyl ester (L-NAME), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 3-(5'-hydroxymethyl-2'furyl)-1-benzyl indazole (YC-1), diethylenetriamine-NONO

(DETA-NO), methylamine hexamethylene methylamine-NONO (MAHMA-NO), 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) and 3-(2-Hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine-NONO (PAPA-NO) were obtained from Cayman Chemical (Ann Arbor, MI).

Cell Culture

Mouse C2C12 myoblasts were obtained from American Type Culture Collection (Manassas, VA) and cultured at 37°C in 5% CO2 and 95% atmospheric air.

Myoblasts were plated on 6-well collagen-coated plates and proliferated in Dulbecco's Modified Eagle's Medium (DMEM) growth media (GM) containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. For all Western Blots and RNA isolation, C2C12 myotubes were grown to 70-80% confluency, and differentiation induced by switching to medium containing 2% horse serum for 7 days. Myotubes were treated with one or more of the following chemicals in media containing 2% serum: LY294002, L-NAME, PAPA-NO, ODQ, YC-1. Whenever treatments were used in combination, inhibitors of NOS (L-NAME), PI3K (LY294002) and guanylate cyclase (ODQ) were added 30 minutes prior to other treatments. Control groups were exposed to treatment vehicles in concentrations equal to experimental groups. When harvesting for total protein extracts, cells were washed twice in ice-cold PBS and harvested in non-denaturing lysis buffer (NDL) containing 1% v/v Triton X-100, 0.3M NaCl, 0.05M TRIS-Base, 5mM EDTA, 3.1μM NaN3, 95mM NaF, 22μM Na3VO4. For isolation of nuclear proteins, cells were harvested in ice-cold PBS containing 1μM Na3VO4 and 0.05% v/v protease inhibitors (catalog #p-8340) and 0.5% v/v phosphatase inhibitors (catalog #p-5726) from Sigma (Saint Louis, MO), centrifuged, and the resulting pellets treated with NE-PER nuclear and cytosolic extraction reagents according to the manufacturer´s procedures (Pierce Biotechnology Inc., Rockford, IL). Both NDL and NE-PER buffers contained 0.1% v/v protease

inhibitors and 1% v/v phosphatase inhibitors from Sigma. For RNA, cells were harvested in Trizol Reagent (Life Tech, Carlsbad, CA) according to manufacturer's instructions.

Ribonucleic Acid Expression by RT-PCR

Concentration and purity of the extracted RNA were measured spectrophotometrically at A260 and A280 in 1X TE buffer (Promega, Madison, WI). Reverse transcription (RT) was performed using the SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Reactions were carried out using 5μg of total RNA and 2.5μM oligo(dT)20 primers. First strand cDNA was treated with two units of RNase H and stored at -80□ C. Primers and probes for MHC I/β (GenBank NM_012751,1m Assay # Rn00562597_m1) were obtained from the ABI Assays-on-Demand service and consist of Taqman 5' labeled FAM reporters and 3' nonfluorescent quenchers. Primer and probe sequences from this service are proprietary and therefore, are not reported. Primer and probe sequences also consisting of Taqman 5' labeled FAM reporters and 3' nonfluorescent quenchers for hypoxanthine guanine phosphoribosyl transferase (HPRT) were obtained from Applied Biosystems (Assays-by-Design) and are: Forward, 5'-GTTGGATACAGGCCAGACTTTGT-3'; Reverse, 5'-AGTCAAGGGCATATCCAACAACAA -3'; Probe, 5'-ACTTGTCTGGAATTTCA-3'.

Quantitative real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (ABI, Foster City, CA). Each 25μl PCR reaction will contain 1 □l of cDNA reaction mixture. In this technique, amplification of the fluorescently labeled probe sequence located between the PCR primers was monitored in real-time during the PCR program. The number of PCR cycles required to reach a pre-determined threshold of fluorescence (CT) was determined for each sample. Samples were quantified relative to the CT (using the 2 -ΔΔCT method, where

CT is threshold cycle) (20) for a normalizing gene (HPRT) determined separately in the same sample.

Western Blotting

Protein concentrations were measured using the DC Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Aliquots of cell lysates (8-15μg) were run in 4-20%, and 12% SDS-PAGE gels for phospho-AKT (p-AKT), total AKT (AKT), phospho-GSK-3β (p-GSK-3β), total GSK-3β (GSK), NFATc1, β-actin and histone. Nuclear extracts (11μg) were run in 12% SDS-PAGE gels for NFAT blots. Protein was transferred to nitrocellulose membrane and blocked with Odyssey blocking buffer for 1 hour. The primary antibodies used were: p-AKT1/2/3 (Ser-473): sc-7985-R (rabbit), 1:1000 dilution, AKT1/2 (N-19): sc-1619 (goat), 1:500 dilution, (Santa Cruz Biotechnology, Santa Cruz, CA) p-GSK-3β (Ser-9): sc-11757 (goat), 1:1000 dilution, GSK-3β (H-76): sc-9166 (rabbit), 1:1000 dilution, (Santa Cruz Biotechnology, Santa Cruz, CA), NFAT c1 (F-1) sc-8405 (mouse), 1:500 dilution, (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (mouse), 1:4000 dilution, (Abcam, Cambridge, MA) and anti-histone H2B, (rabbit), 1:5000 dilution (Upstate, Lake Placid, NY). The membranes were incubated at 4°C overnight in primary antibody diluted with Odyssey blocking buffer (LI-COR Biosciences, Lincoln NE), TBS and 0.01% Tween-20, then washed with TBS-T four times and incubated for 35 minutes in secondary antibody, Odyssey blocking buffer and TBS-T. Secondary antibodies used were: IRDye 800CW rabbit anti-goat, (LI-COR, 1:5000); IRDye 680 mouse anti-rabbit (LI-COR, 1:2500); IRDye 680 rabbit anti-mouse, (LI-COR, 1:5000). Membranes were washed four times with TBS-T and once with TBS before being scanned and detected using the Odyssey infrared imaging system (LI-COR).

Statistical Analysis

Data were analyzed by a two-way ANOVA with Tukey's HSD post hoc test. Significance was established a priori at $p<0.05$. Values reported are means ± SEM.

CHAPTER 4
RESULTS

Nuclear Factor of Activated T-Cell Activity Is Attenuated in Cultured Myotubes from NOS$^{-/-}$ Mice

Immunoblots confirmed that nNOS and eNOS protein was undetectable in plantaris muscle of nNOS$^{-/-}$ and eNOS$^{-/-}$ mice, respectively (Figure 4-1). We found evidence of reduced NFAT function in cultured myotubes from both nNOS$^{-/-}$ and eNOS$^{-/-}$ mice. Specifically, baseline (i.e. in untreated control cultures) MHC I/β mRNA (Figure 4-2), NFAT nuclear accumulation (Figure 4-3), GSK-3β phosphorylation (Figure 4-4), and AKT phosphorylation (Figure 4-5) were all significantly lower in both transgenic models compared to WT mice. This was unexpected as we anticipated either nNOS$^{-/-}$ or eNOS$^{-/-}$ mice to display attenuated NFAT activity, but not both. Measurement of MHC I/β mRNA via RT-PCR showed that 24h treatment with the calcium ionophore, A23187 (0.4μM) rescued the blunted NFAT effect in the NOS$^{-/-}$ mice (Figure 4-2). However, A23187 did not enhance abrogated AKT or GSK-3β phosphorylation after 1h of treatment (Figures 4-4 and 4-5) or NFAT nuclear accumulation after 4h of treatment (Figure 4-3) in either nNOS$^{-/-}$ or eNOS$^{-/-}$ mice. Further, cultured myotubes from WT mice showed responsiveness to A23187. Treatment with the NO donor, PAPA-NO (1μM), resulted in significant changes in MHC I/β mRNA, NFAT nuclear accumulation, GSK-3β phosphorylation, and AKT phosphorylation in both groups of WT mice, as well as, in both nNOS$^{-/-}$ and eNOS$^{-/-}$ mice, as anticipated. Our findings suggest that both nNOS and eNOS are necessary for NFAT activity, and may collaborate to produce physiologically significant levels of NO. However, the blunted effects from loss of either isoform can be rescued by extended exposure (24h) of myotubes to A23187, and by treatment with PAPA-NO (1h, 4h, and 24h).

Nitric Oxide Synthase-$^{-/-}$ Mice Display Aberrant NFAT Function in Vivo

Immunohistochemical staining of serial cross-sections of the plantaris muscle for specific fiber types revealed that both nNOS$^{-/-}$ and eNOS$^{-/-}$ had significantly less Type I myofibers per cross sectional area (μm^2) (Table 4-1 and Figure 4-6). Also, plantaris homogenate from all four groups was used for western blotting to observe potential deficits in GSK-3β (Figure 4-7) and AKT phosphorylation (Figure 4-8). Indeed, we did see deficits in NOS$^{-/-}$ mice compared to WT controls. Our findings suggest that both NOS isoforms found constitutively in skeletal muscle contribute to enhancing NFAT activity and are necessary for normal fiber type distributions.

Effect of NO on AKT Is Dose Dependent

C2C12 myotubes treated with varying concentrations of the NO donor, SNAP (1μM, 10μM, 100μM, 500μM, and 1mM) for 1h showed that low levels of NO (1μM and 10μM) cause phosphorylation (activation) of AKT (Figure 4-9). Conversely, high, S-nitrosylation-inducing levels of NO (500μM and 1mM) result in less phosphorylation of AKT. These results are in agreement with previously reported data by Boullegue *et al.* (2007) showing a similar dose-dependent effect of NO in vascular smooth muscle.

Nitric Oxide -cGMP Pathway Phosphorylates AKT

Our lab has shown that NO is capable of inducing phosphorylation of GSK-3β in a cGMP dependent manner. In addition, our preliminary data indicate that AKT is necessary for NO-cGMP-induced GSK-3β phosphorylation (unpublished data). Therefore, we sought to determine if the NO-cGMP pathway is responsible for the NO-induced AKT phosphorylation shown in experiment 3. Treatment of C2C12 myotubes with the NO donor, PAPA-NO, increased the ratio of phopho-/total-AKT by 2 fold (Figure 4-10). Co-treatment with the guanylate cyclase inhibitor, ODQ, completely prevented this effect. YC-1, which activates soluble guanylate

cyclase (sGC) independent of NO (Kelly *et al.* 2004), also increased the ratio of phospho-/total AKT by 2-fold demonstrating that activation of sGC is sufficient for this effect.

Nitric Oxide -cGMP Induced AKT Phosphorylation Is PI-3K-Dependent

Next, we sought to determine if the NO-cGMP-induced effects on AKT, seen in experiment 4, were PI-3K-dependent. A common stimulator of AKT activity is PI-3K (Stitt *et al.* 2004). These kinases are often associated, acting as a mediator of insulin or IGF-1 signaling. We found that treatment of myotubes with PAPA-NO, ODQ, and YC-1 produced effects similar to the previous experiment. Also, co-treatment of myotubes with PAPA-NO and the PI-3K inhibitor LY294002 resulted in no increase in phosphor-/total AKT. (Figure 4-11). Thus, our findings indicate that the NO-cGMP pathway activates the PI-3K/AKT pathway, and that these pathways work in conjunction, providing a possible mechanism for the effect of NO on NFAT function seen in our previous work.

Nitric Oxide Inhibits Protein Phosphatase Activity

We hypothesized that NO may play a role in inhibiting protein phosphatase activity. Given the unknown mechanism by which the NO-cGMP pathway activates the PI-3K/AKT pathway, and the evidence in previous studies for NOS activity being capable of inhibiting several protein phosphatases, we sought to determine if NO is a general protein phosphatase inhibitor (Tokui *et al.* 1996; Ugi *et al.* 2004; Villa-Moruzzi *et al.* 1996). Further, PP2A has been shown to have an inhibitory effect on AKT. We performed a general protein phosphatase assay on protein lysates from C2C12 myotubes treated with several pharmacological agents intended to determine if NOS and GC activity limit protein phosphatase activity. Our findings demonstrated that indeed NO does inhibit protein phosphatase activity, and does so through cGMP (Figure 4-12). Myotubes treated with the calcium ionophore showed a significant increase in enzyme activity, while co-treament with the NO inhibitor, L-NAME prevented this effect. Low levels of

the NO donor, SNAP (1μM and 10μM) resulted in an inhibitory effect on protein phosphatase activity below control levels. As concentrations of SNAP increased, the inhibitory effect lessened, demonstrating that high levels (500μM and 1mM) were similar to untreated myotubes. The sGC inhibitor, ODQ attenuated the inhibitory effect of SNAP. In addition, YC-1, at low levels, had a similar concentration-dependent effect as SNAP indicating a NO-cGMP-dependent mechanism by which NO inhibits protein phosphatase activity.

Table 4-1. Plantaris fiber type morphology for nNOS WT, nNOS -/-, eNOS WT, and eNOS -/- mice as measured by percentage.

animals	% type I	% type IIa	% type IIb/x	% non-contractile tissue
nNOS WT	11±1.1	30±1.4	40±2.3	19±1.1
nNOS -/-	5±0.9*	32±2.0	42±1.3	21±1.3
eNOS WT	13±1.4	29±2.2	36±0.8	22±2.1
eNOS -/-	6±1.9#	31±2.1	43±1.4	20±0.8

Values represent mean ± SEM. *Significantly different from nNOS WT. #Significantly different from eNOS WT.

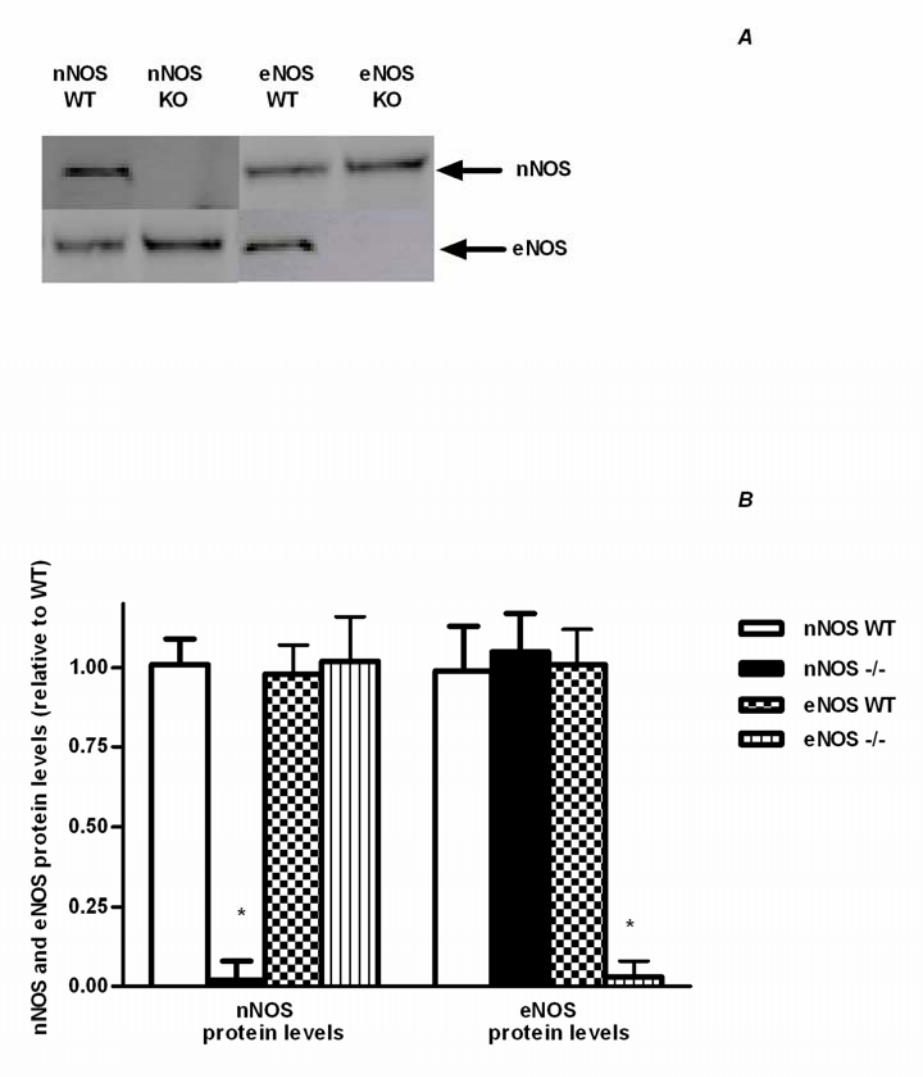
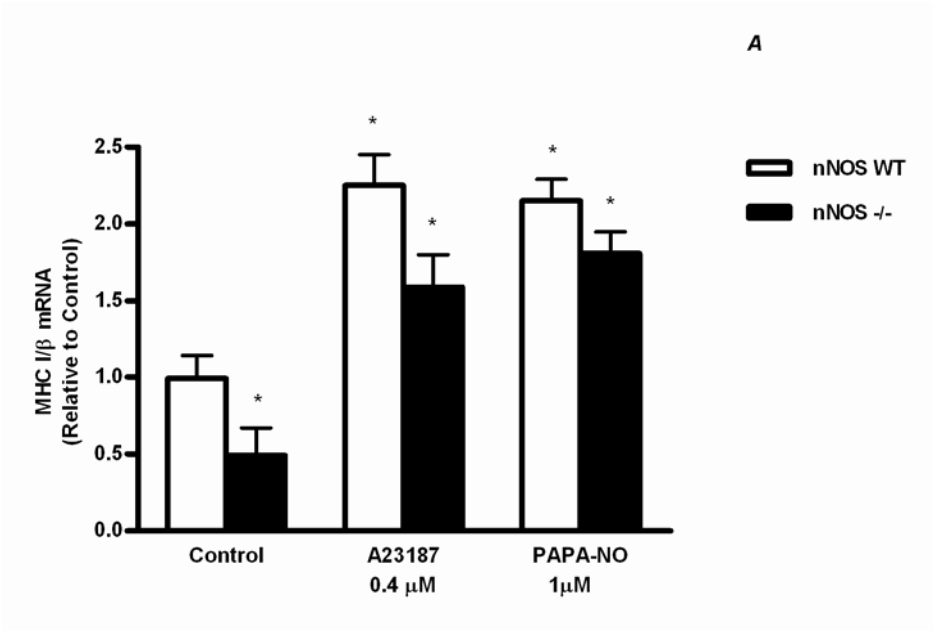


Figure 4-1. Protein expression of neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS). (A) Representative immunoblots from nNOS WT, nNOS-/-, eNOS WT, and eNOS-/- primary myotubes. (B) Quantification of immunoblots for nNOS and eNOS. nNOS-/- were compared to nNOS WT and eNOS-/- to eNOS WT. Values represent mean ± SEM. *Significantly different from WT.



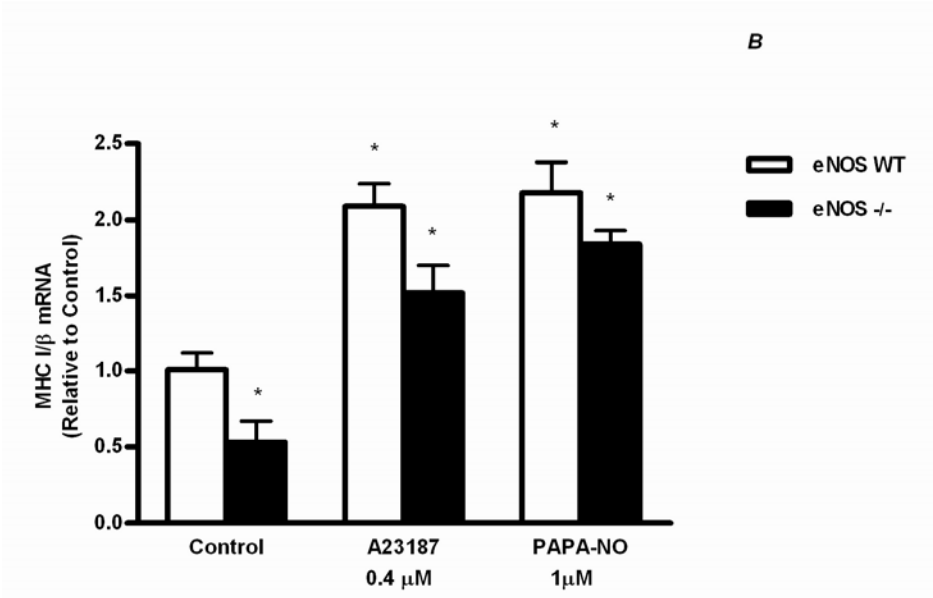


Figure 4-2. MHC I/β mRNA expression in $NOS^{-/-}$ mice. (A) Quantitative RT-real time PCR analysis of MHC I/β mRNA in total RNA isolated from $nNOS^{-/-}$ and WT mouse myotubes (24h treatment). (B) Quantitative RT-real time PCR analysis of MHC I/β mRNA in total RNA isolated from $eNOS^{-/-}$ and WT mouse myotubes (24h treatment). Values represent mean ± SEM. * = significant difference from nNOS WT control and eNOS WT control respectively

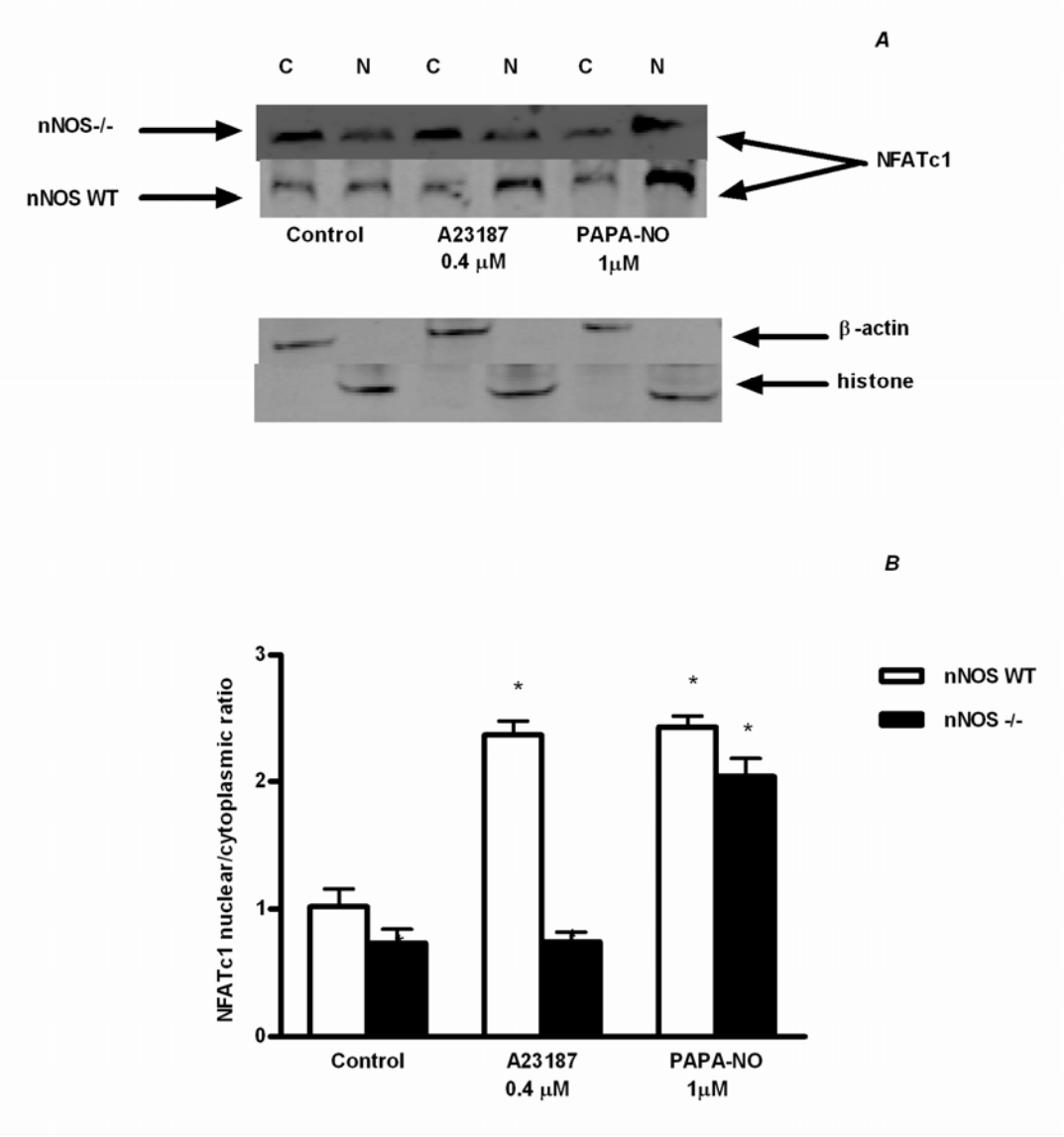


Figure 4-3. Protein expression of NFAT nuclear/cytoplasmic ratio. (A) Representative immunoblot of nuclear/cytoplasmic NFAT from nNOS$^{-/-}$ and WT mouse myotubes treated for 4h. (B) Quantification of immunoblots for NFAT normalized to nNOS WT control. (C) Representative immunoblot of nuclear/cytoplasmic NFAT from eNOS$^{-/-}$ and WT mouse myotubes treated for 4h. (D) Quantification of immunoblots for NFAT normalized to eNOS WT control. Values represent mean ± SEM. * = significant difference from nNOS WT control and eNOS WT control respectively.

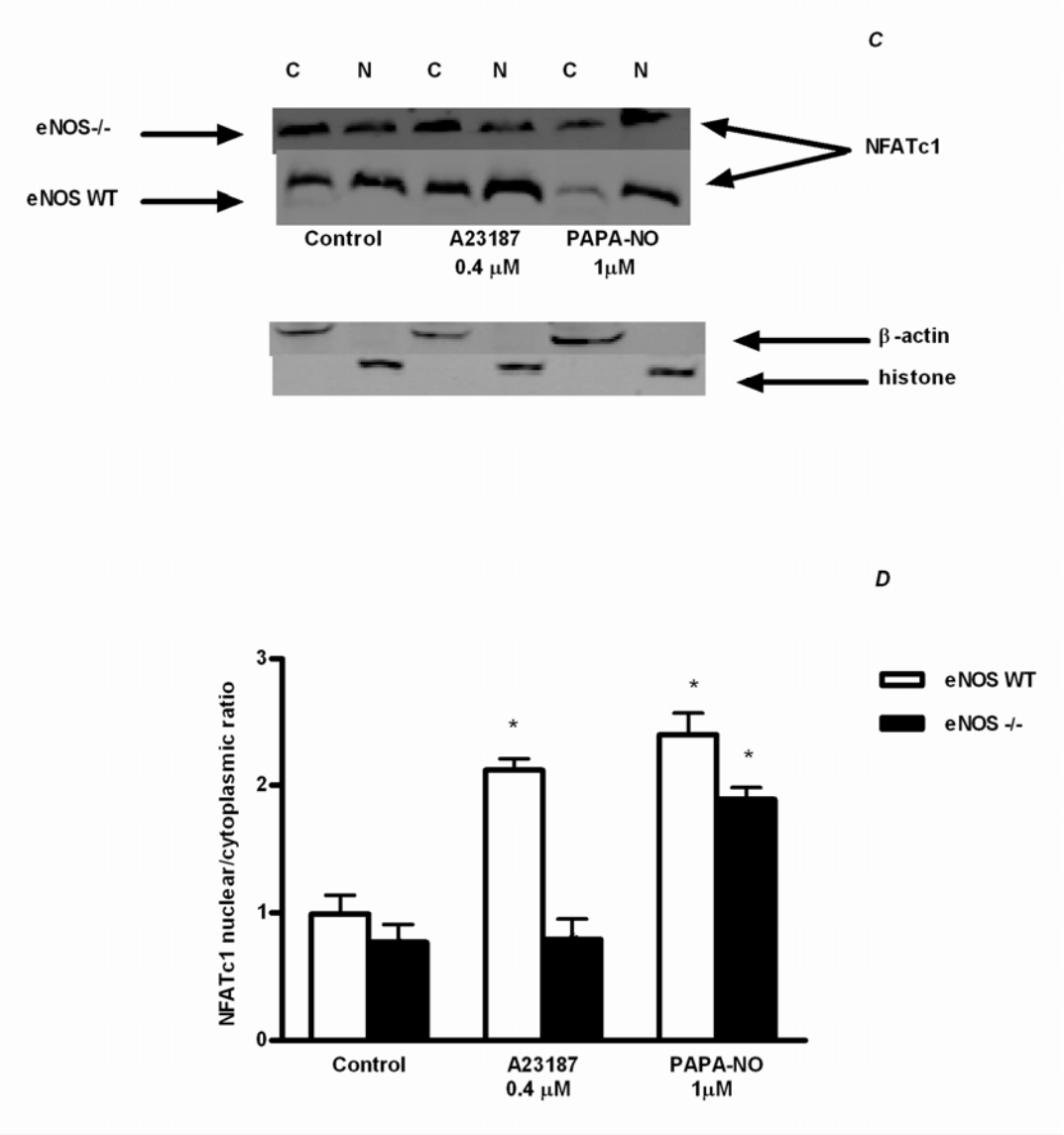


Figure 4-3 Continued.

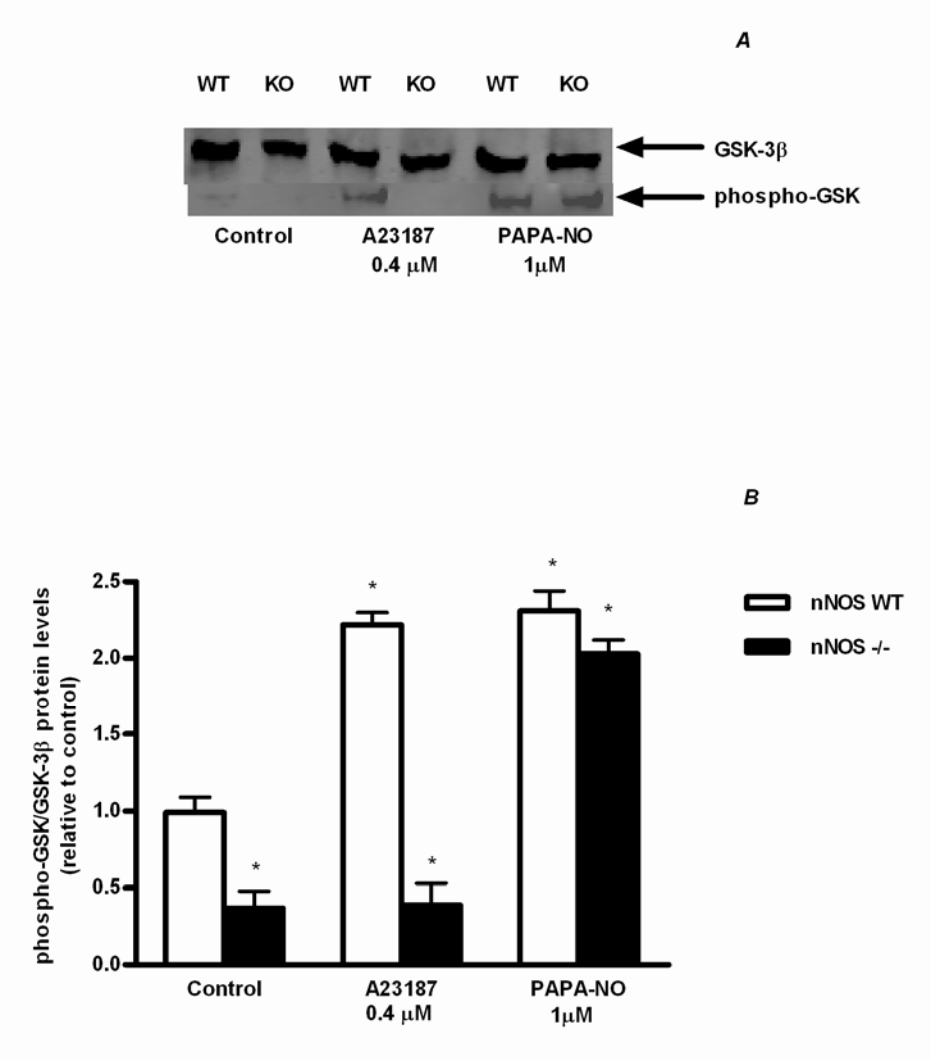


Figure 4-4. Protein expression of phospho/total GSK-3β. (A) Representative immunoblot of GSK-3β from nNOS$^{-/-}$ and WT mouse myotubes treated for 1h. (B) Quantification of GSK-3β immunoblots normalized to nNOS WT control. (C) Representative immunoblot of GSK-3β from eNOS$^{-/-}$ and WT mouse myotubes treated for 1h. (D) Quantification of GSK-3β immunoblots normalized to eNOS WT control. Values represent mean ± SEM. * = significant difference from nNOS WT control and eNOS WT control respectively.

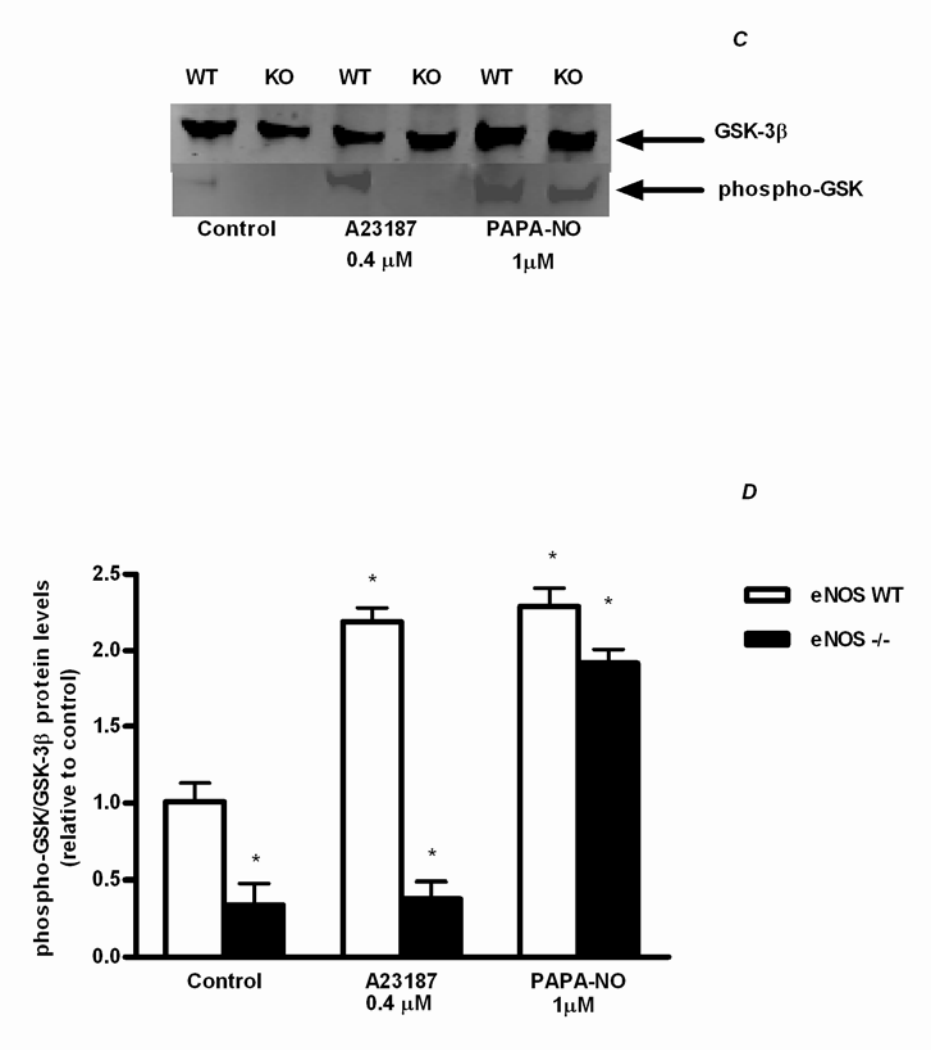


Figure 4-4 Continued.

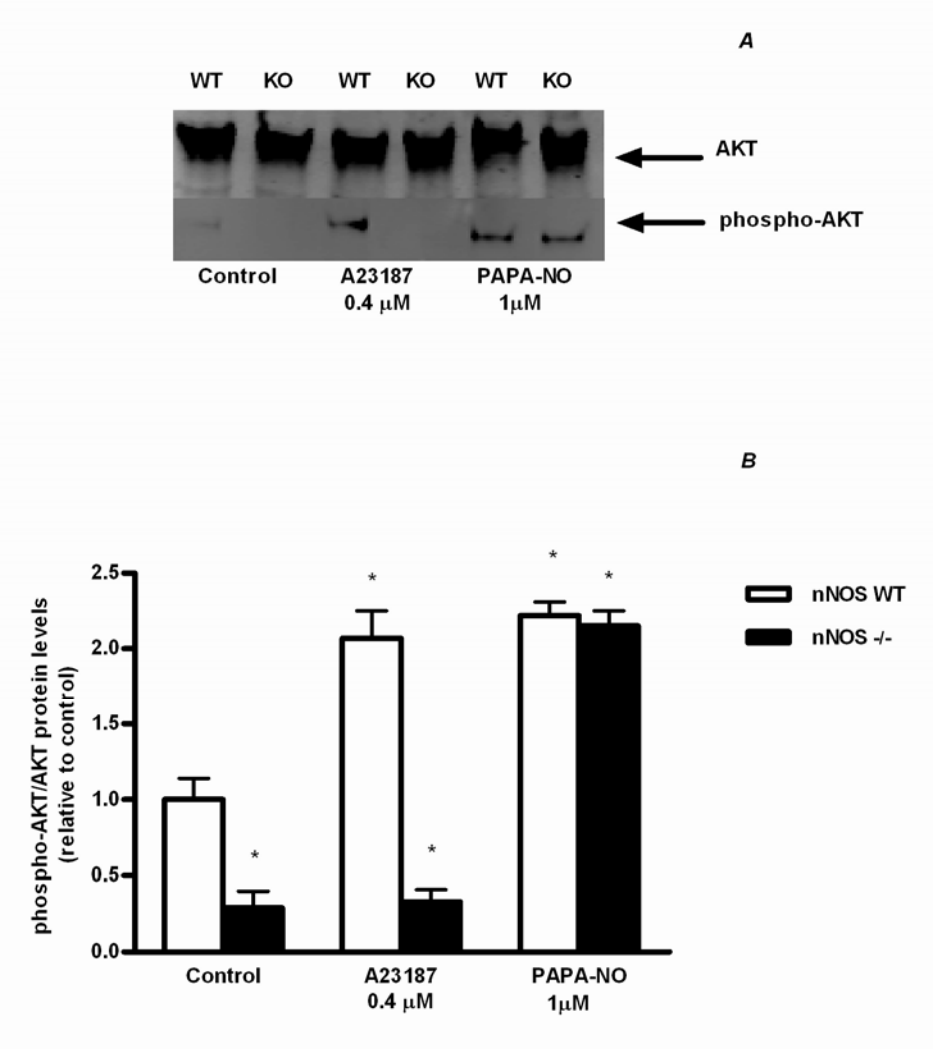


Figure 4-5. Protein expression of phospho-/total AKT. (A) Representative immunoblot of AKT from nNOS$^{-/-}$ and WT mouse myotubes treated for 1h. (B) Quantification of AKT immunoblots normalized to nNOS WT control. (C) Representative immunoblot of AKT from eNOS$^{-/-}$ and WT mouse myotubes treated for 1h. (D) Quantification of AKT immunoblots normalized to eNOS WT control. Values represent mean ± SEM. * = significant difference from nNOS WT control and eNOS WT control respectively.

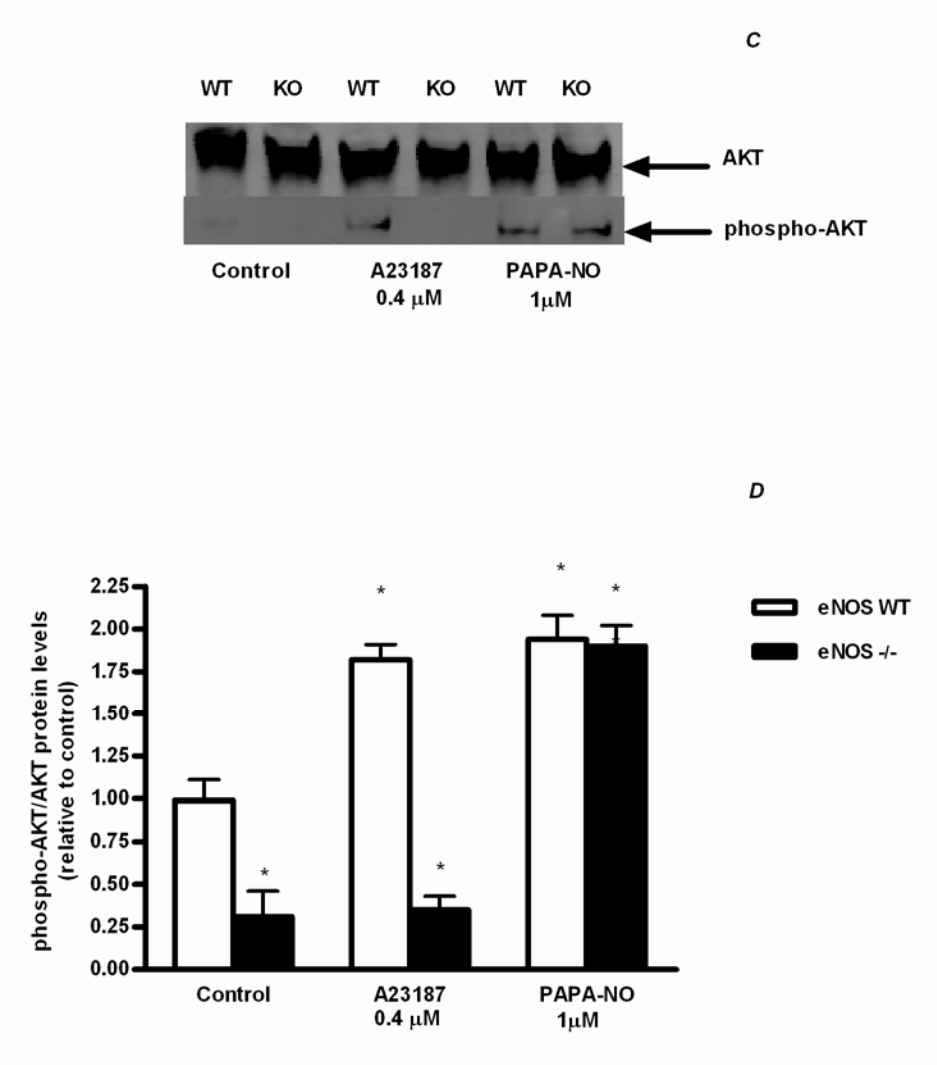


Figure 4-5 Continued.

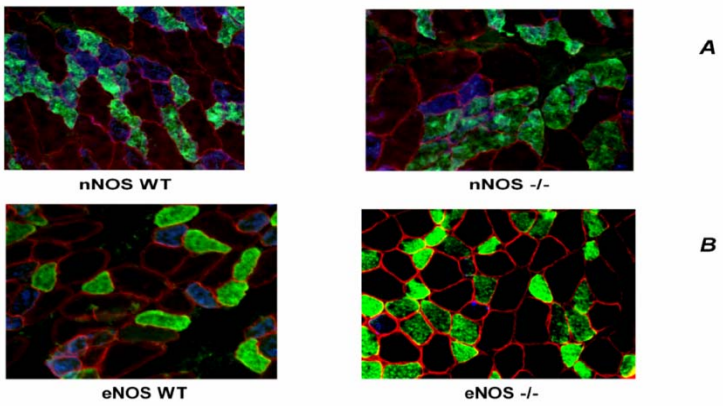


Figure 4-6. Immunohistochemical measurement of fiber type. (A) Representative cross section of $nNOS^{-/-}$ mouse plantaris muscle compared to WT. (B) Representative cross section of $eNOS^{-/-}$ mouse plantaris muscle compared to WT.

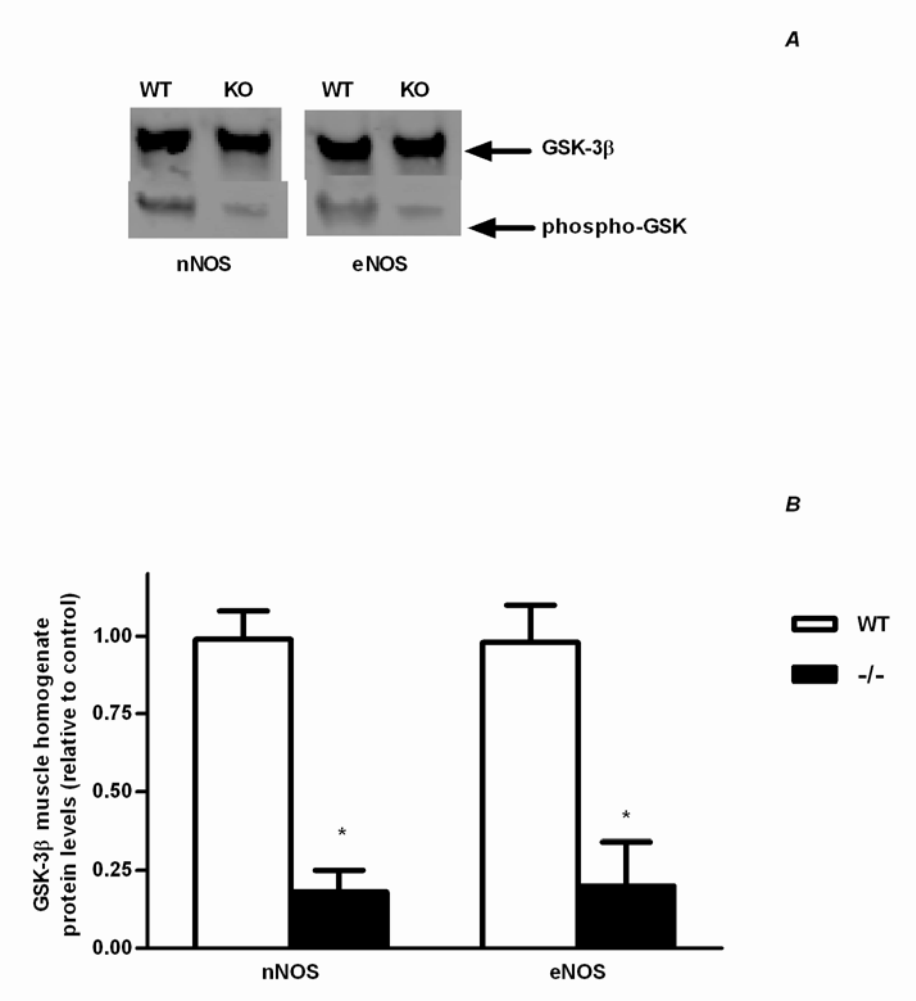


Figure 4-7. Plantaris homogenate protein expression of phospho-/total GSK-3β. (A) Representative immunoblot of GSK-3β from $nNOS^{-/-}$ and $eNOS^{-/-}$ compared to respective WT mice. (B) Quantification of immunoblots for GSK-3β from $nNOS^{-/-}$ and $eNOS^{-/-}$ compared to WT control mice. Values represent mean ± SEM. * = significantly different than respective WT control.

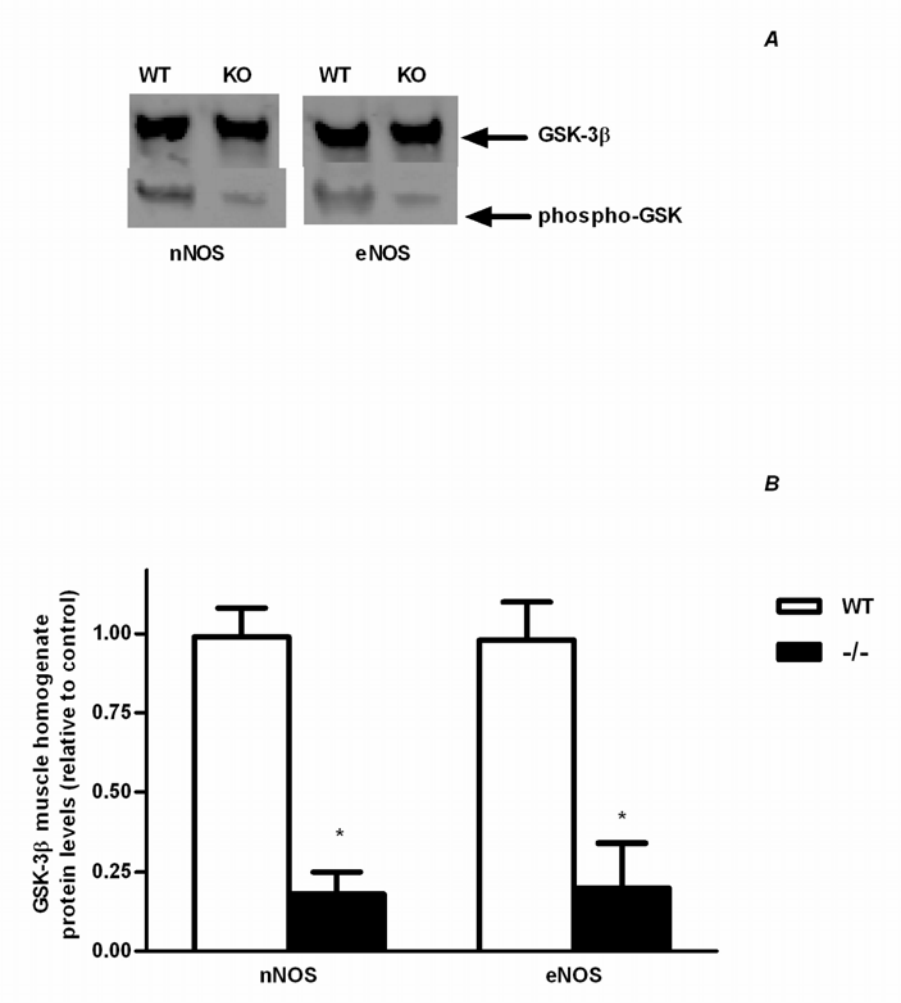


Figure 4-8. Plantaris homogenate protein expression of phospho/total AKT. (A) Representative immunoblot of AKT from $nNOS^{-/-}$ and $eNOS^{-/-}$ compared to respective WT control. (B) Quantification of immunoblots for AKT from $nNOS^{-/-}$ and $eNOS^{-/-}$ compared to WT control mice. Values represent mean ± SEM. * = significantly different than respective WT control.

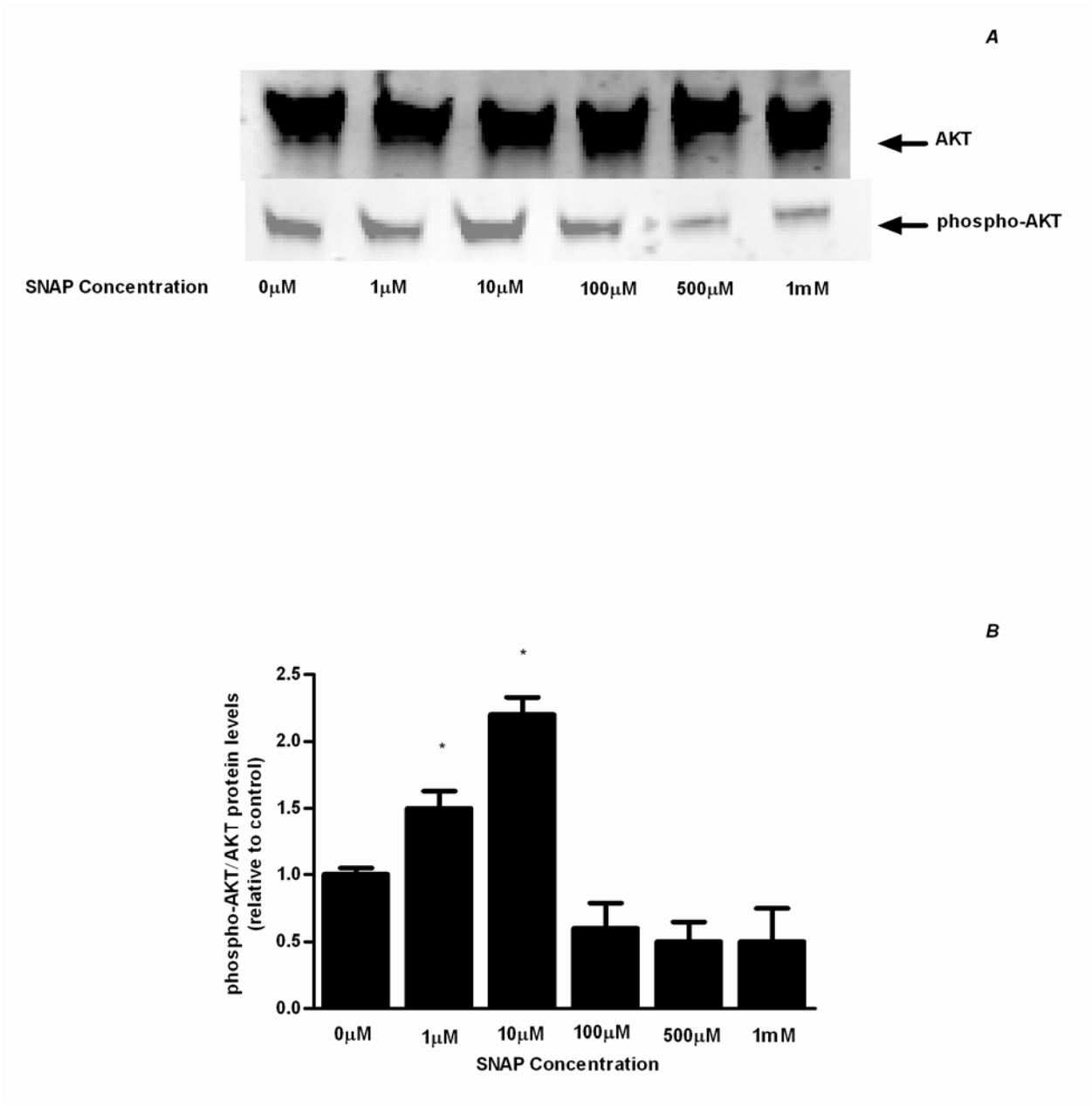


Figure 4-9. Protein expression of AKT. (A) Representative immunoblot of phospho/total AKT from C2C12 myotubes treated with varying does of the NO donor, SNAP. (B) Quantification of immuoblots for phosphor/total AKT normalized to control. Values represent mean ± SEM. * = significantly different from control (0μM).

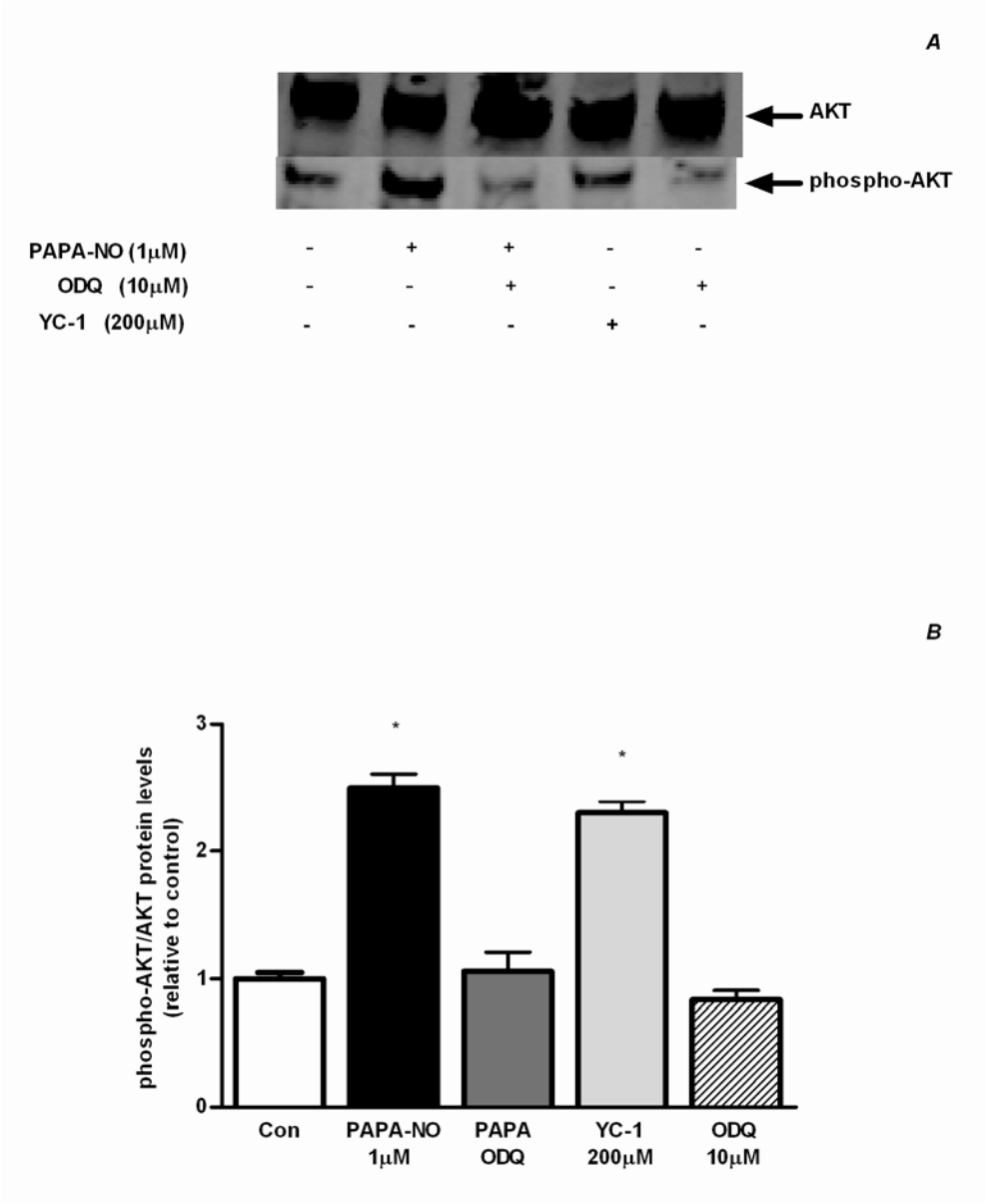


Figure 4-10. Protein expression of AKT. (A) Representative immunoblot of phospho/total AKT from C2C12 myotubes treated with the NO donor, PAPA NO, the sGC inhibitor ODQ, and the sGC enhancer YC-1. (B) Quantification of immunoblots for phospho/total AKT normalized to control. Values represent mean ± SEM. * = significantly different from control (Con).

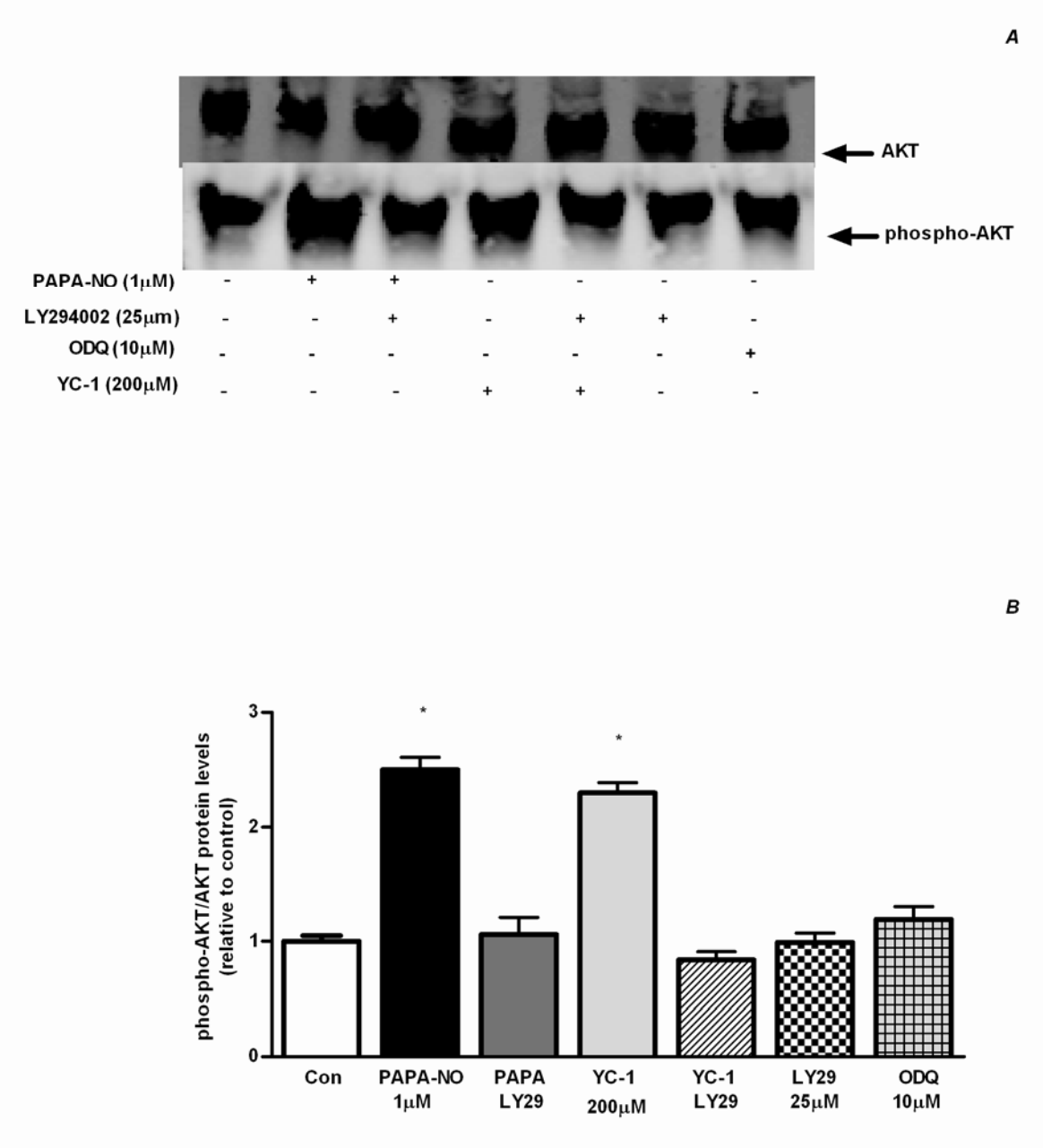


Figure 4-11. Protein expression of AKT. (A) Representative immunoblot of phospho/total AKT from C2C12 myotubes treated with the NO donor, PAPA-NO, PI3K inhibitor LY29, sGC enhancer YC-1, and sGC inhibitor ODQ. (B) Quantification of immunoblots for phospho/total AKT normalized to control. Values represent mean ± SEM. * = significantly different than control (Con).

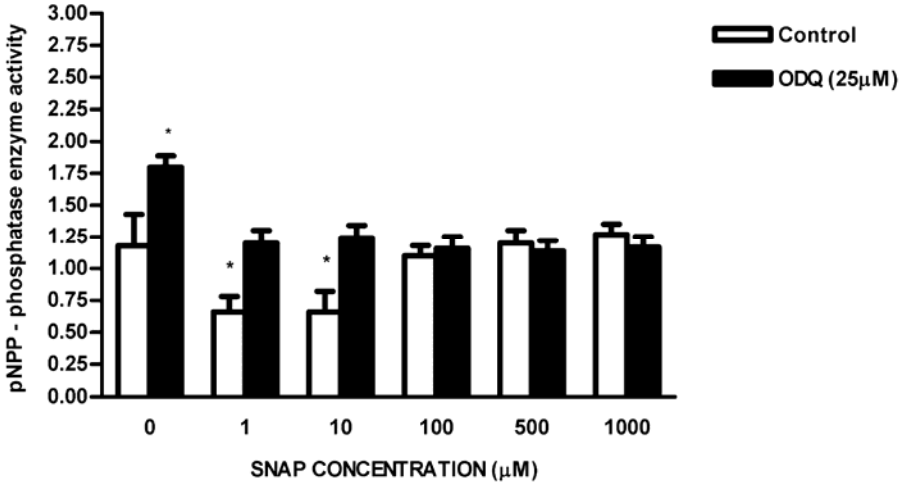


Figure 4-12. pNPP protein phosphatase activity assay. C2C12 myotubes were treated with or without the sGC inhibitor, ODQ, and with the NO donor, SNAP. at varying concentrations. Bars are representative of enzyme activity normalized to protein content. Values represent mean ± SEM. * = significantly different than control (0μM).

CHAPTER 5
DISCUSSION

Main Findings

Although our previous work with regard to NO-cGMP mediated NFAT activity via GSK-3β phosphorylation was novel, we sought to extend our data from a myogenic cell line to a transgenic animal model, and to better understand the mechanism(s) by which the NO-cGMP pathway affects GSK-3β. We used commercially available $nNOS^{-/-}$ and $eNOS^{-/-}$ mice (The Jackson Laboratory (Bar Harbor, ME) to determine both the ex vivo and in vivo effects of NO on NFAT function. Also, experiments in this study aimed at furthering our understanding of how NO inhibits GSK-3β were done in C2C12 myotubes. The main findings of this study are: 1) Both nNOS and eNOS are necessary for the ex vivo facilitation of NFAT activity, as evidenced by attenuated MHC I/β mRNA, NFAT nuclear accumulation, GSK-3β phosphorylation and AKT phosphorylation in $nNOS^{-/-}$ and $eNOS^{-/-}$ mice; 2) With regard to NFAT activity, cultured myotubes from $nNOS^{-/-}$ and $eNOS^{-/-}$ mice did not show responsiveness to the calcium ionophore, A23187 (0.4μM) at 1h and 4h. Nevertheless, MHC I/β mRNA is induced in $nNOS^{-/-}$ and $eNOS^{-/-}$ cultures after 24h of ionophore treatment. Further, the NO donor, PAPA-NO (1μM) is capable of enhancing NFAT function, GSK-3β and AKT phosphorylation, and MHC I/β mRNA expression in myotube cultures from $nNOS^{-/-}$ and $eNOS^{-/-}$ mice. 3) nNOS and eNOS are both necessary for NFAT activity and fiber type regulation in vivo as demonstrated by significant differences in Type I fibers, GSK-3β phosphorylation, and AKT phophorylation in $nNOS^{-/-}$ and $eNOS^{-/-}$ mice as compared to WT control mice. 4) NO inhibits GSK-3β in a cGMP/PI3K/AKT dependent manner, and the interaction of the NO-cGMP and PI3K/AKT pathways may be mediated by NO-dependent inhibition of protein phosphatases.

Neuronal NOS and eNOS Are Necessary for NFAT Function ex Vivo

Our first aim was directed at confirming the validity of our previously published data concerning NO and NFAT activity (Drenning *et al.* 2008). Also, we sought to determine which constituitive NOS isoform is responsible for the effects of NO on NFAT in skeletal muscle. Unexpectedly, we found that both $nNOS^{-/-}$ and $eNOS^{-/-}$ mice display attenuated NFAT function compared to WT control mice in cultured myotubes. Experiments directed at measuring MHC I/β mRNA, NFAT nuclear accumulation, GSK-3β phosphorylation and AKT phosphorylation (Figures 4-2 to 4-5) all displayed similar results suggesting that nNOS and eNOS are necessary collectively for NO-dependent NFAT activity.

Other studies have looked specifically at the effect of chronic exercise and nerve activity on nNOS and eNOS protein expression (Roberts *et al.* 1997; Balon & Nadler, 1997; Tidball *et al.* 2000). Both nNOS and eNOS are activated by interaction with calcium and calmodulin (Stamler & Meissner, 2001) and each have been shown to be increased with prolonged treadmill running in rats (Balon & Nadler, 1997; Tidball *et al.* 1998). However, most studies aimed at determining which NOS isoform is most responsive to nerve activity, and subsequent calcium transient influx, have concluded that nNOS is more dominant than eNOS in affecting the molecular signaling pathways associated with NO in skeletal muscle (Reiser *et al.* 1997; Roberts *et al.*; Reid *et al.* 1998; Stamler & Meissner, 2001). Interestingly, our data shows that both nNOS and eNOS are necessary for calcium-induced NFAT function. NO biosynthesis in skeletal muscle could be dependent on collaboration of the calcium-calmodulin dependent NOS isoforms (nNOS and eNOS) in generating an optimal amount of NO to induce the effects on NFAT function observed in these experiments. However, this is the first study to imply that both nNOS and eNOS play a major role in skeletal muscle plasticity; our findings may be due to a number of factors: 1) The eNOS isoform, while constituitive in skeletal muscle, has been

difficult to localize and study due to its low level of expression; thus, eNOS may exert its effects at very low, undetectable physiological levels, possibly explaining nNOS predominance in previous findings. 2) The NOS proteins are among the most tightly regulated enzymes (Christopherson & Bredt, 1997), and it is unknown how much NO needs to be produced to activate soluble guanylate cyclase optimally, and subsequently the PI3K/AKT/GSK-3β pathway facilitating NFAT nuclear accumulation and slow gene induction. Our data suggest that both isoforms work collectively to produce the optimal amount of NO needed for NFAT function. 3) nNOS has been shown to increase in mice with age (Chang *et al.* 1996); the mice used for this study were young (3-4 weeks) and may have lower levels of nNOS as compared to adult mice used in other studies, providing another explanation for the similar effects observed in $nNOS^{-/-}$ and $eNOS^{-/-}$ mice.

Nuclear Factor of Activated T-Cell Activity Can be Rescued With Pharmacological Manipulation in $NOS^{-/-}$ Mice

The NO donor, PAPA-NO (1μM) rescued the attenuated NO pathway in both $nNOS^{-/-}$ and $eNOS^{-/-}$ mice. Treatment with PAPA-NO resulted in increased MHC I/β mRNA, NFAT nuclear accumulation, GSK-3β phosphorylation, and AKT phosphorylation in WT control mice, and the $NOS^{-/-}$ mice (Figures 4-2 to 4-5). This correlates with our previous work (Drenning *et al.* 2008) which showed a significant effect of PAPA-NO on GSK-3β phosphorylation in C2C12 myotubes. Treatment of cultured myotubes with the calcium ionophore, A23187 (0.4μM) yielded conflicting results. Experiments aimed at studying GSK-3β and AKT phosphorylation from myotubes treated with A23187 for 1h were not responsive in $nNOS^{-/-}$ or $eNOS^{-/-}$ mice. Similarly, immunoblots for NFAT nuclear accumulation, showed an inability for A23187 to increase nuclear NFAT at 4h in the $NOS^{-/-}$ mice. However, myotubes from both $nNOS^{-/-}$ and $eNOS^{-/-}$ mice, treated for 24h, demonstrated a significant increase in MHC I/β mRNA. These

loss of function and rescue experiments demonstrate that NO is an important signal for calcium-induced nuclear translocation of NFAT. However, the effect of A23187 may be time sensitive. Further, calcium-dependent activation of NOS does not fully account for slow gene induction.

Neuronal NOS and eNOS Are Necessary for NFAT Activity in Vivo

To ensure that the effects of NO on NFAT function are not limited to in vitro measures, we sought to determine whether $NOS^{-/-}$ mice display aberrant NFAT activity in vivo. Our in vivo data confirms that both nNOS and eNOS are necessary for normal expression of Type I fibers as well as, GSK-3β and AKT phosphorylation in the plantaris muscle of WT and $NOS^{-/-}$ mice (Table 4-1 and Figures 4-6 to 4-8). As has been mentioned, the role of both isoforms as they pertain to NO-related signaling in skeletal muscle was unexpected. Interestingly, although other authors have suggested that basal NOS activity is involved in fiber type establishment (Stamler & Meissner, 2001), no one has reported fiber type differences in $nNOS^{-/-}$ mice compared to WT mice. Also, no previous studies have reported fiber type differences in $eNOS^{-/-}$ mice. Hirschfield et al. (2000) report that soleus and diaphragm muscles from 6-8 week-old eNOS knockout mice exhibit essentially normal contractile characteristics. This suggests that fiber type distribution may be normal at this age. However, several potentially important differences exist between our study and that of Hirschfield et al. For instance, we studied plantaris muscle of 3-4 week-old mice from a different transgenic strain (Jackson Labortory). We propose that either nNOS or eNOS ablation delays the development of slow-twitch fibers by interfering with NFAT signaling. However, this may not prevent the attainment of normal fiber type distribution as the animal reaches adulthood. Further study will be required to examine this possibility.

Low Levels of NO Induce Phosphorylation of AKT

In response to varying levels of NO, AKT phosphorylation was shown to be concentration dependent (Figure 4-9). These data confirm previous work from Bollegue *et al.* (2007)

demonstrating that low levels of NO activate, and high levels of NO inhibit AKT in vascular smooth muscle cells. Our study provides evidence of NOS-dependent AKT phosphorylation in C2C12 myotubes. Further, these data indicate a possible mechanism for the effect of NO on GSK-3β, as AKT is capable of phosphorylating GSK-3β (Ser-9).

Our data from the knockout mice imply that NOS isoform specificity may not play a role in NO/cGMP induced activation of the AKT. Previous work has established that both constituitive isoforms of NOS (nNOS and eNOS) sythesize NO at a low rate, resulting in nanomolar levels. Similarly, treatment with 1μM and 10μM of SNAP respectively, resulted in an increase in phosphor-total-AKT ratio. We did not measure NO concentration in the culture media during NO-donor treatments. However, based on SNAP concentration, the half-life of NO in solution, and the half-life of NO release from SNAP, we estimate that 1-10 μM SNAP produces steady-state NO concentrations in the nM range. In addition, low levels of NO production by constituitive NOS appears to be a calcium-dependent process (Reid, 1998), which is consistent with our findings of NOS involvement in calcium-dependent effects. Although we did not quantify which NOS isoform is responsible for the our previous data regarding NFAT in C2C12s, the effect of the calcium ionophore, A23187, on NFAT function in our previous work is in accordance with both nNOS and eNOS regulating physiological levels of NO downstream of calcium. iNOS activity in skeletal muscle is observed in response to an inflammatory challenge, and produces NO at micromolar levels. It seems likely that the treatment groups with high concentrations of SNAP (500μM and 1mM) could have caused S-nitrosylation-induced events similar to effects observed with iNOS induction, subsequently leading to a decrease in phosphor-total-AKT ratio.

Nitric Oxide-induced AKT Activity Is cGMP Dependent

Stimulation of sGC and the resultant accumulation of cGMP mediates many of the signaling functions of NO and regulates complex signaling cascades through immediate downstream effectors, including cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases, and cyclic nucleotide-gated ion channels (Lucas *et al.* 2000). Guanylate cyclases and cGMP-mediated signaling cascades play a central role in the regulation of diverse physiological processes (Kelly *et al.* 2004; Lucas *et al.* 2000). Previous studies have shown that the NO-cGMP pathway can affect the phosphorylation of AKT (Boullegue *et al.* 2007) and our preliminary work (unpublished data) indicated that AKT activity is necessary for NO-induced GSK-3β phosphorylation. In addition, our previous data show that the sGC inhibitor, ODQ, effectively blocks calcium-induced nuclear accumulation of NFATc1 and NFAT dependent transcription. Further, our lab has shown that GSK-3β phosphorylation is NO-cGMP-dependent in C2C12 myotubes. Therefore, understanding the role of the NO-cGMP pathway on AKT phosphorylation is important for this study as it provides a potential mechanism to explain our previous work (Drenning *et al.* 2008).

To better understand the effect of NO on GSK-3β and subsequently NFAT, we designed this experiment expecting to observe NO-cGMP-dependent phosphorylation of AKT. Indeed, phospho-total-AKT ratio was increased by the NO donor, PAPA-NO, while the sGC inhibitor, ODQ, abrogated this effect (Figure 4-10). Similar to our previous data, YC-1 induced phosphorylation of AKT. Taken together, these data indicate that low levels of NOS activate AKT through the NO-cGMP pathway.

The NO-cGMP Pathway Activates the PI-3K/AKT Pathway

PI 3-kinases (PI-3K) have been linked to an extraordinarily diverse group of cellular functions, including cell growth, proliferation, differentiation, motility, survival and intracellular

trafficking (Stitt *et al.* 2004). Many of these functions relate to the ability of class I PI 3-kinases to activate AKT. PI-3K is also a key component of the insulin signaling pathway. Hence, there is great interest in the role of PI 3-kinase signaling in DM2. AKT is activated as a result of PI3-kinase activity as AKT requires the formation of the PtdIns, P3 (or "PIP3") molecule in order to be translocated to the cell membrane (Stitt *et al.* 2004). At PIP3, AKT is then phosphorylated by another kinase called phosphoinositide dependent kinase 1 (PDK1), and is thereby activated. The PI-3K/AKT signaling pathway has been shown to be required for an extremely diverse array of cellular activities. Our data is novel in that this is the first study to show that the NO/cGMP pathway can activate the PI-3K/AKT pathway.

We hypothesized that PI-3K is necessary for the effect of the NO/cGMP pathway on AKT phosphorylation. Our data confirm that NO activates AKT via a cGMP/PI-3K dependent pathway (Figure 4-11). The drug, LY294002 was used as it has been shown to be a potent inhibitor of the PI-3K/AKT pathway. Consistent with data from the previous experiment, PAPA-NO induced an increase in phospho-total-AKT ratio. Myotubes treated with PAPA-NO and LY294002 demonstrated that PI-3K is necessary for NO induced AKT phosphorylation. These data provide a further understanding of our previous data, but it remains unclear how the NO-cGMP pathway activates the PI-3K pathway.

Nitric Oxide Inhibits Protein Phosphatase Activity

Although these data have provided insight into the mechanism(s) behind the role NO plays in mediating NFAT function, some questions remain. Particularly, the mechanism of activation of the PI-3K/AKT pathway by the NO/cGMP pathway is unclear. In skeletal muscle, the PI-3K/AKT pathway is induced in numerous ways. Some typical enhancers of this pathway include hormones such as insulin-like growth factor (IGF-1) and insulin (Bodine *et al.* 2006; Kimball *et al.* 2002). IGF-1 binding to its receptor leads to the activation of PI-3K which

subsequently recruits AKT (Bodine *et al.* 2006). Chung *et al.* (2004) has recently shown that NO is necessary for IGF-1R activity.

We proposed that NO is capable of inhibiting protein phosphatase activity. Previous studies have shown that NO can inhibit specific protein phosphatases including PP1 and PP2A (Tokui *et al.* 1996; Ugi *et al.* 2004; Villa-Moruzzi *et al.* 1996). We expected, in a general protein phosphatase assay, to see inhibition of phosphatase activity by NO in a cGMP-dependent manner. Our data confirms this hypothesis as pharmacological manipulation of C2C12 myotubes with a number of drugs showed that phosphatase activity was inhibited by the NO/cGMP pathway (Figure 4-12). Interestingly, dose responses were seen both by the NO-donor SNAP, and the sGC enhancer YC-1. Both drugs inhibited phosphatase activity at low levels, and were not significantly different than the untreated control group at high levels. This data implies again that low levels of NO have physiogical effects, and high levels do not.

Limitations and Future Directions

The selection of treatment times for cultured myotubes were based on optimal times reported in other studies. Also, we wanted to extend our previous studies in a myogenic cell line, to a transgenic animal model. Therefore, we chose to treat myotubes for 1h for the phosphorylation experiments, 4h for the NFAT nuclear accumulation experiment, and 24h for the MHC I/β just as we did in our previous work (Drenning *et al.* 2008). This did not affect our data regarding the NO donor, PAPA-NO, but we did see a discrepancy in the results from myotubes treated with A23187. Therefore, the effect of NOS ablation on myotube responses to calcium ionophore treatment may be time dependent.

We did not measure NO activity via DAF-FM fluorometric analysis in the $NOS^{-/-}$ or in the WT mice. Our results suggest that when both nNOS and eNOS are present, physiologically significant levels of NO are produced, which would be expected in the WT mice. However, we

are unsure of the difference in NO production in the $nNOS^{-/-}$ and $eNOS^{-/-}$ mice. Future studies should seek to understand better the details of the amount of NO needed to enhance NFAT function.

Lastly, we did not do any in vivo measurement in muscles other than the plantaris muscle. Since the plantaris is predominantly a type II muscle, we are unsure as to the levels of GSK-3β and AKT phosphorylation in other fiber types in $NOS^{-/-}$ mice. Also, our fiber type data is limited to the plantaris muscle, thus the fiber type differences observed may not be present in other muscles.

Conclusions

Although the present study does not provide evidence for the specific NOS isoform responsible for enhanced NFAT activity, we do demonstate that NO is necessary for NFAT function ex vivo and in vivo. Based on our data, both nNOS and eNOS may work together to produce an optimal amount of NO to exert its downstream molecular signaling effects. We conclude that the NO-cGMP pathway activates the PI3K/AKT pathway through protein phosphatase inhibition, and leads to GSK-3β phosphorylation, thus facilitating NFAT activity and leading to slow gene induction.

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BIOGRAPHICAL SKETCH

Jason Drenning was born in Roaring Spring, Pennsylvania, in 1975. He was an all-state baseball (pitcher) and football (quarterback) player at Northern Bedford High School. After finishing 9th in his graduating class of 1993, he received his bachelor's degree in exercise science from The George Washington University (GW) in Washington, D.C., in 1997. At GW, he was on the Athletic Director's Honor Roll (baseball) and won the Warren Fulton award for leadership in a team sport. Jason obtained his Master of Science in clinical exercise physiology in 2000 and started his own personal training business. He went on to pursue a Doctor of Philosophy degree from the University of Florida and worked in the Center for Exercise Science both in clinical and basic science research. After graduating, Jason intends to work in the medical device or pharmaceutical industry.