NITRIC OXIDE SYNTHASE ACTIVITY MEDIATES ACTIVATION OF AMP-ASSOCIATED PROTEIN KINASE IN ISOLATED MOUSE SKELETAL MUSCLE

By

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To my family for their unwavering support and all the teachers and professors who taught and mentored me along the way.

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Abstract of Thesis Presented to the Graduate School
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Nitric oxide (NO) production and signaling has an important role in exercise-induced metabolic and biochemical adaptations. Current research indicates that low levels of NO produced enzymatically by the nitric oxide synthases (NOS), regulates 5'-AMP-activated protein kinase (AMPK) in cell culture. The physiological significance of this regulatory mechanism in adult skeletal muscle is unknown. **Goal:** This research was designed to determine if NOS activity is necessary for induction of AMPK phosphorylation in skeletal muscles isolated from adult mice. **Hypotheses:** Incubation of isolated extensor digitorum longus (EDL) and soleus muscles with the non-isoform specific NOS inhibitor, L-NMMA, will prevent activating phosphorylation of AMPK, and phosphorylation of the AMPK substrate, acetyl CoA carboxylase (ACC). **Approach:** EDL and soleus muscles were surgically isolated bilaterally from young adult mice and incubated in micro tissue baths. AMPK was activated by 10 min of contractile activity or a 20-min incubation with the AMP analog, AICAR. The EDL and soleus muscle from the contralateral limb was treated identically, except for the addition of L-NMMA to the incubation media throughout the experiment. **Results:** The main findings of this study are: 1) electrical stimulation or AICAR incubation increases the phospho-AMPK/total AMPK ratio; 2) NOS inhibition prevents this increase; 3) AMPK activation by electrical stimulation and AICAR treatment is greater in the fast twitch EDL muscle

than the slow twitch soleus muscle; and 4) L-NMMA treatment tends to increase AMPK phosphorylation status, independent of electrical stimulation or AICAR effects. **Significance:** AMPK is an important regulator of skeletal muscle metabolism and the acute and chronic adaptations to exercise training. Activation of AMPK, pharmacologically or by exercise, increases oxidative capacity and improves insulin sensitivity leading to a metabolically active phenotype in skeletal muscle that effectively counters the metabolic syndrome. Understanding how AMPK is regulated in adult muscle fibers and the potential role of nitric oxide in mediating AMPK activation is important for development of more potent and efficient treatments for metabolic disorders.

CHAPTER 1
INTRODUCTION

The metabolic syndrome, which manifests itself in pathophysiological conditions such as insulin resistance, hypertension and dyslipidemia, is increasing at astounding rates throughout the United States and developing countries due in large part to physical inactivity. These conditions are coupled with increased morbidity and mortality rates and place an excessive burden on healthcare systems. Physical activity has long been hailed as a necessary component for a healthy lifestyle. Skeletal muscle is an exceptionally plastic tissue with a remarkable capacity to adapt to external stresses by making fine metabolic adjustments. Contracting skeletal muscle acutely increases insulin sensitivity in both healthy individuals and in people with Type 2 diabetes, and regular physical exercise is a cornerstone in the treatment of the disease. Exercise training alters the biochemical signaling cascades that regulate the metabolic properties of skeletal muscle. At present, the exact molecular mechanisms for these adaptations are poorly understood.

Background

During exercise skeletal muscle is placed under enormous stress in order to maintain energy levels and contractile function. 5'-AMP-activated protein kinase (AMPK) is a cellular regulator that responds to changes in the AMP-to-ATP ratio. AMPK is activated allosterically by AMP binding to the enzyme, and by phosphorylation of the catalytic α subunit by upstream AMPK kinases (AMPKK). AMPK has an important role in numerous biochemical pathways and is responsible for regulating energy metabolism processes. Chronic AMPK activation is an important signal for upregulation of glucose transporter molecules. Recent research has suggested that AMPK activation may be influenced by nitric oxide (NO), produced

enzymatically by the nitric oxide synthases (NOS). At present, it is not known if NO is necessary for AMPK activation in adult skeletal muscle.

Problem Statement

The benefits of exercise in metabolic disease prevention and therapy have been well-documented in literature but the specific mechanistic signaling pathways that exert the protective effects remain elusive. The aim of this study was to determine whether the presence of NO is required for AMPK activation and whether AMPK activation differs between skeletal muscle fiber types.

Variables in Study

Independent variables: We will simulate contractile load to isolated extensor digitorum longus (EDL) (primarily fast twitch muscle) and soleus muscles (primarily slow twitch muscle) by means of electrical stimulation in vitro, and by contraction-independent stimulation of AMPK via 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) administration in vitro. NOS activity will be inhibited via N(G)-monomethyl-L-arginine (L-NMMA) administration to determine the necessity of NO production for AMPK activation.

Dependent variables: We will measure phosphorylation of metabolic proteins AMPK and acetyl CoA carboxylase (ACC).

Control variables: Sex and age were controlled as all animals used within the study were young female adult mice (~4 months old) from the ICR strain. Animals were brought to the lab at least 12 hours prior to surgery to allow acclimatization to the new environment. All surgeries were conducted at the same time of day. Isolated muscles were set to their optimal length using a micromanipulator and individual isometric twitches.

Extraneous variables: Individual differences in daily activities such as ambulation and feeding were not controlled.

Hypotheses

We hypothesize that:

1. Ten minutes of electrical stimulation, in vitro, will induce phosphorylation of AMPK and ACC in the isolated EDL and soleus muscles.
2. Twenty minutes of AICAR treatment, in vitro, will also induce phosphorylation of AMPK and ACC in these muscles.
3. Addition of L-NMMA to the incubation media will significantly decrease NO biosynthesis in muscles at rest and during electrical stimulation, decreasing AMPK phosphorylation.
4. AMPK phosphorylation will be higher in fast twitch muscles than slow twitch muscles.

Definition of Terms

5'-AMP-activated protein kinase (AMPK). A heterotrimeric protein kinase that regulates cellular energy homeostasis and is largely responsible for exercise-induced skeletal muscle adaptations to endurance training.

5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR). AMP mimetic drug capable of activating AMPK.

Acetyl CoA carboxylase (ACC). Rate-limiting enzyme involved in fatty acid synthesis, and a known target of AMPK activity.

AMP-activated protein kinase kinase (AMPKK). A collective group of enzymes responsible for phosphorylating AMPK.

Electrical stimulation. A technique that uses platinum electrodes to generate a field current in an electrolyte buffer-filled chamber for the purpose of directly activating an isolated mouse skeletal muscle, suspended in the buffer solution.

Optimal length (L_o). The length at which maximum tetanic tension is generated by skeletal muscle.

N(G)-monomethyl-L-arginine (L-NMMA). A nonspecific competitive inhibitor of NOS activity.

Nitric oxide (NO). A short-lived endogenous mediator that acts as a signaling molecule and plays a role in a variety of biological reactions in virtually all mammalian tissues under certain physiological or pathophysiological conditions.

Nitric oxide synthase (NOS). A family of isozymes responsible for NO production. Three forms have been identified in mammalian skeletal muscle: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS).

Limitations/Delimitations/Assumptions

Limitations: Due to the invasive nature of this research, the use of humans as experimental subjects was not possible. Instead, a mouse model was chosen due to genetic similarity to provide results for the researcher that would mimic those seen in humans.

The *in vitro* experimental design allows isolation of intrinsic muscle responses. It is recognized that extrinsic responses to contractile activity, which are not considered in our design, likely contribute to skeletal muscle responses *in vivo*.

Delimitations: The study is restricted to female mice.

Assumptions:

1. L-NMMA, at the dose used, specifically inhibits NOS activity in skeletal muscle, and has negligible non-specific effects.
2. AMPK activity correlates well with ACC activity.

Significance of the Study

Whilst genetic predisposition for the metabolic syndrome cannot be modified by behavior, obesity, the largest risk factor, is largely preventable. Failure to curb increased obesity lies in large part with our lack of understanding of metabolic government in healthy and disease states. To maintain metabolic function, intracellular ATP concentrations must remain within acceptable limits. AMPK preserves ATP levels by managing energy utilization for metabolic pathways based on necessity. AMPK can also regulate exercise responsive genes leading to long-term metabolic stability. Therefore, it is clear that a dysfunction in AMPK signaling can induce rapid and contrary responses that directly affect metabolic function. The AMPK pathway is of particular interest within the clinical community because of its potential in treating and preventing the metabolic syndrome. Understanding the mechanism by which muscle contractions induce AMPK activity will aid in development of new strategies to prevent or treat metabolic disorders.

CHAPTER 2
LITERATURE REVIEW

Nitric oxide (NO) is a short-lived endogenous mediator that acts as a signaling molecule and plays a role in a variety of biological reactions in virtually all mammals under certain physiological or pathophysiological conditions. Since its declaration as a cardiovascular homeostatic mediator, NO has emerged as a fundamental signaling device regulating intracellular processes and cell-cell interactions. NO production is primarily dependent on a group of heme-containing proteins known as nitric oxide synthases (NOS). NO regulates processes in skeletal muscle that include force production, autoregulation of blood flow, mitochondrial biogenesis, mitochondrial respiration and glucose transport (6,15, 25, 29, 45, 57). AMPK is a key metabolic enzyme that has emerged as a metabolic master switch. AMPK activity is involved in many of the training adaptations acquired with chronic exercise. Recent studies provide evidence that AMPK activation is dependent upon NO (8, 34). At present, a complete understanding and grasp of NO participation in homeostatic functions and pathophysiological conditions has not yet been reached. This chapter will review the recent literature concerning nitric oxide and AMPK effects in skeletal muscle and potential interaction between these two important signaling molecules.

Enzymatic Production of Nitric Oxide(NO)

Nitric Oxide Synthase(NOS) Isoforms

Three isoforms of NOS produce NO from the amino acid, L-arginine (L-arg), and require oxygen and NADPH as substrates with citrulline as a by-product. Nitric oxide is synthesized in normal skeletal muscle by neuronal NOS (nNOS) and endothelial NOS (eNOS). NOS is constitutively expressed in rodent skeletal muscle (56, 66). Localization of the nNOS isoform varies greatly among cell types and is directly associated with the dystrophin complex in skeletal

muscle. It is located in close proximity of the sarcolemma, and primarily in fast-twitch fibers of rodents. nNOS expression in humans appears to be similar, except for a more widespread distribution across fiber types (63). eNOS is uniformly distributed in muscle fibers and localized in the mitochondria and also in vessel walls (63). nNOS and eNOS are highly regulated by Ca^{2+} and calmodulin. The third isoform, inducible NOS (iNOS), is not produced at significant levels in skeletal muscle. Its expression is regulated by transcription in response to cytokines and other inflammatory agents and is largely Ca^{2+} independent.

Substrate Availability

NOS activity varies between muscles. Generally, higher activity of NOS is measured in fast twitch fibers (56). The availability of cofactors and co-substrates as well as subcellular localization can influence the identity of the enzymatic product (57). It has been hypothesized that cellular supply of L-arginine is a rate-limiting step for NOS activity. Ogonowski and colleagues suggest that L-arginine transport by y^+ transporters may be the limiting step (41). McConell et al (37) performed a study that infused L-arginine into humans while cycling at 72% VO_2 Max. They reported improved glucose disposal with arginine infusion during an exercise protocol. This group theorized that L-arginine supplementation upregulated NO production during skeletal muscle contraction and increased GLUT4 translocation.

Calcium-Calmodulin

Calmodulin binding is an essential requirement for NO production by all NOS isoforms. iNOS is tightly bound to calmodulin in an almost irreversible fashion (21, 56), therefore, the activity of this isoform is essentially independent of calcium. nNOS and eNOS, however, bind to calcium-calmodulin and, therefore, rely on a calcium signal for upregulation of enzymatic activity. Upon release of calcium from the sarcoplasmic reticulum during contraction, calcium concentration increases and facilitates the interaction of nNOS and eNOS with calmodulin.

Jagnandan et al. (21) illustrated the calcium-dependence of eNOS by limiting access to calcium and measuring NO production. Calcium blockade essentially eliminated NO production by eNOS but had no significant effect on NO production by iNOS.

Physical Activity

Increasing research findings show that NO is an important metabolic regulator during exercise. Furthermore, physiological adaptations such as improved functional ability and cardioprotection result from chronic physical activity. At rest skeletal muscle produces relatively small amounts of NO. However, there are significant changes in NO production in response to exercise and physical activity. Exercise upregulates NOS activity and nNOS gene expression (3, 29).

NO Signaling and Skeletal Muscle

Mechanisms of Action

Because NO is a short-lived free radical with a half life of 2 to 5 seconds (13), regulation of signaling occurs largely at the level of NO biosynthesis. It can act locally on the cell that it is produced within or it can permeate cell membranes of adjacent cells. Research has shown that NO is involved in such diverse activities as vasodilation, metabolic regulation, immune function, cellular signaling, contractile regulation and glucose homeostasis, to name a few (3, 45, 55). There are a number of mechanisms whereby NO effects are mediated.

sGC and cGMP. Activation of soluble guanylate cyclase (sGC), a heme-containing heterodimer, is a primary mechanism mediated by NO. NO binds to the heme increasing the catalytic activity of cGC several hundredfold increasing the levels of the second messenger cyclic guanylyl monophosphate (cGMP) (54). The resulting increase in cGMP concentration serves to signal a multitude of complex physiological operations. cGMP can directly activate its downstream effectors including Protein Kinase G (PKG), Cyclic Nucleotide Gated Channels

(CNG) and Cyclic nucleotide phosphodiesterases, all of which govern the operation of many proteins involved in physiological functioning (35).

Protein nitrosylation. Following production, NO translocates to nearby cells and interacts with the interior of the cell at metal sites within proteins. Arguably the most influential mechanism of action is nitrosylation, the reaction of NO with cysteine residues in proteins or by interactions with heme or non-heme copper and iron. A continuum between nitrosative and oxidative stress must be maintained to reduce hazardous levels of stress which has been connected to muscle fatigue and cell injury (57).

Acute Effects

Glucose transport. Exercise acutely increases glucose transport in both healthy individuals and those with Type 2 diabetes. NO signaling markedly increases glucose transport in isolated skeletal muscle fibers (5). Translocation of intracellular GLUT4, the primary glucose transporter isoform found in skeletal muscle, to the plasma membrane is the underlying mechanism by which exercise causes increased glucose transport (23). Recent studies have shown a link between NO and AMPK responsible for exercise-induced glucose clearance.

Contractile function. At present, complete functional significance of the NOS isoforms and skeletal muscle remains elusive. However, NO does play a pivotal role in maintenance of skeletal muscle contractile ability. This regulation appears to be mediated through cGMP-dependent and –independent mechanisms. Skeletal muscle fibers exposed to NO have shown reduced actin-myosin cross-bridge cycling in vitro (18, 26). Lau et al (32) provided evidence of the linkage between NO and cGMP. When electrically stimulated, the cGMP content of wild type mouse extensor digitorum longus muscle cGMP content increased approximately 250%.

It has recently been discovered that colocalization of cGMP and nNOS at the sarcolemma inhibits excitation-contraction coupling in skeletal muscle by impaired Ca^{2+} activation of thin

filaments (26). NO also interferes with contraction by inhibiting creatine kinase, and the sarcoplasmic reticulum Ca^{2+}-ATPase in fast-twitch and slow-twitch muscle fibers.

Mitochondrial respiration. NOS activity has been associated with inhibition of mitochondrial respiration. Identified respiration targets of NO include cytochrome-*c* oxidase, creatine kinase and Ca^{2+}-ATPase in skeletal muscle (57). NO binds to cytochrome-*c* oxidase, inhibiting comples IV of the electron transport chain and mitochondrial oxygen consumption. This disruption in cellular respiration can result in alterations of calcium flux. Disruptions in mitochondrial function can drastically alter energy levels. Redox balance is necessary for homeostasis and optimal functioning of numerous physiological interactions.

Satellite cell activation Muscular injury due to exercise, mechanical stretch or blunt force mobilizes satellite cells from quiescence to serve as antecedents for new muscle formation. At present NO and hepatocyte growth factor (HGF) are the only known activators of satellite cells. Satellite cell activation is defined as mobilization and entrance into the G1 interphase of the cell cycle (1). Studies have shown that release of HGF from the extracellular matrix is NO-dependent and inhibition of NOS activity reduces HGF release and satellite cell activation (1).

Chronic Adaptations

Mitochondrial biogenesis. In arterioles, NO vasodilates smooth muscle and increases blood flow and O_2 delivery to the tissues. NO contributes to the regulation of mitochondrial respiration by inhibiting cytochrome-c oxidase at complex III of the electron transport chain. This leads to decreased levels of cellular ATP and increase in levels of ADP, AMP, GDP and P_i (13).

Adult skeletal muscle experiences a transformation from fast to slow fiber type with exercise training. eNOS and neuronal nNOS isoforms may be differentially involved in the regulation of mitochondrial biogenesis in skeletal muscle (66). Evidence to support this was

presented by Wadley et al. NO donor experiments in rodents lead to increased expression of peroxisome proliferator-activated receptor coactivator 1 (PGC-1), nuclear respiratory factor 1 (NRF-1), and mitochondrial transcription factor A (mtTFA), all of which are markers of mitochondrial biogenesis (66).

GLUT 4 expression. Regular aerobic exercise is associated with biochemical changes to numerous metabolic genes. It is well established that chronic exercise is a cornerstone in the treatment and prevention of Type 2 diabetes due in large part to increased insulin sensitivity. Insulin stimulation leads to recruitment of the GLUT 4 transporter to the surface of the cell to transport glucose intracellularly (24). AMPK has recently been linked to upregulation of GLUT4 transporter gene expression.

Adenosine Monophosphate Activated Protein Kinase(AMPK) is a Key Metabolic Regulator in Skeletal Muscle

AMPK Structure and Activation

The AMPK molecule is a heterotrimeric protein kinase composed of a catalytic α subunit and regulatory β and λ subunits (61). The α and β subunits each have two genes and the λ subunit has three, yielding at least 12 possible heterotrimeric sequences (17). The presence of AMPK in primitive organisms suggests that this molecule has served an important genetic role throughout evolution (12).

AMPK is an exercise-responsive gene that acts to preserve cellular energy levels (22, 40, 61). AMPK is activated during times of cellular energy stress including hypoxia, glucose depletion and exercise. It is well established that exercise is characterized by heightened energy turnover. AMPK is activated during times of elevated metabolism and ATP consumption. Once activated, AMPK turns on catabolic processes that generate ATP while concurrently turning off anabolic processes such as cellular growth and proliferation. Two independent articles were

published in 1973 describing protein fragments that had the capacity to inactivate acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, 2 enzymes necessary for lipid synthesis. It was later discovered that the protein fragments were composed of protein kinases and that ACC kinase and HMG-CoA reductase kinase were activated by 5'-AMP. In 1987 Carling et al realized that the same kinase was responsible for activation of both functions (7). This molecule was named AMPK.

AMPK is activated by AMP in one of two ways: phosphorylation by upstream kinases or allosteric activation. Phosphorylation of AMPK by upstream kinases occurs within the catalytic α subunit at Thr^{172} (53). LKB1 and Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) have been identified as well-suited upstream kinases capable of activating AMPK (53, 61). Activation of AMPK during *ex vivo* electrical stimulation is dependent upon modification of the α-Thr^{172} subunit by AMP phosphorylation making it a desirable substrate for LKB1 (52). Direct activation of AMPK occurs allosterically by AMP binding to the λ subunit. This renders AMPK a more suitable substrate for upstream kinases and a less suitable substrate for phosphatases which can deactivate AMPK (61).

The level of metabolic stress placed on the body affects the level at which AMPK is activated (17). Interestingly, AMPK is activated during strength training in endurance athletes and during endurance training in strength athletes. It is reasoned, therefore, that AMPK is upregulated during training at intensity levels greater than the individual is adapted to.

AMPK Signaling Effects

AMPK was first discovered in the liver and evidence suggested that the enzyme was likely present in other tissues of the body. Protein expression analysis found mRNA levels of AMPK expression is greatest within skeletal muscle (64). Over the past years extensive research has been conducted with inconclusive results to decipher which particular subunits are expressed

in animal and human muscle. Within skeletal muscle, AMPK is known to exhibit regulatory effects on glucose transport, fatty acid oxidation and mitochondrial biogenesis as exhibited in Figure 2-1.

Glucose transport. Many of the downstream effects of AMPK homestatic-preservation were first discovered using the AMP mimetic drug 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (17). It was initially found by Merrill et al in 1997 that muscle incubated with AICAR exhibited increased glucose transport and AMPK activity (38). *Ex vivo* experiments with AICAR incubation in transgenic animals where AMPK signal is greatly decreased or nonexistent completely diminished glucose transport.

The mechanism for exercise-induced skeletal muscle glucose transporter (GLUT4) upregulation appears to be influenced by NO. The interaction between AMPK and NOS is not certain but is very intriguing. Acutely, AMPK influences glucose transport. Long term adaptations show AMPK to be a mediator of exercise-induced glucose transport by increasing GLUT4 concentrations at the cell surface (38). Balon and colleagues demonstrated that incubation of skeletal muscle with a NOS inhibitor decreased glucose transport (4). Recently, Lira et al reported that NO and cGMP were active partners in inducing GLUT4 expression in skeletal muscle. They also proposed the novel idea that NOS activity is required upstream and downstream of AMPK to induce GLUT4 expression (20).

Fatty acid oxidation. Accumulation of fatty acids within the endothelium is a major contributor to atherosclerosis and, therefore, the metabolic syndrome. Fatty acid clearance is necessary for normal endothelial function and accumulation can cause excess production of damaging radicals. It has been shown that fatty acid oxidation accounts for approximately 40% of ATP production by endothelial cells (10). Both resting and active skeletal muscle metabolize

fatty acids to produce energy. Several studies have indicated that AMPK activation can augment fatty acid oxidation. AMPK is responsible for inhibitory phosphorylation of acetyl-CoA carboxylase (ACC). ACC is responsible for fatty acid synthesis and when inactivated reduces accumulation of triglycerides by stimulating mitochondrial uptake of fatty acids. AMPK activity is markedly reduced in obese animals and activation of AMPK reduces intracellular accumulation of triglyceride levels (40).

Mitochondrial biogenesis. Chronic endurance exercise is associated with increased mitochondrial oxidative capacity. One of the mechanisms responsible for mitochondrial enzyme gene transcription involves upregulation of AMPK during times of skeletal muscle contraction. AMPK promotes mitochondrial biogenesis by increasing PGC-1α and NRF expression. In an experiment by Winder et al. (69) 28 days of chronic AICAR treatment in rats resulted in increased AMPK activation as well as levels of citrate synthase, succinate dehydrogenase, malate dehydrogenase and cytochrome c, all mitochondrial enzymes. AMPK clearly effects mitochondrial regulation during times of energy deprivation by promoting expression of mitochondrial enzymes and biogenesis markers.

Inhibition of muscle growth. Mammals have two distinct signaling cascades responsible for exercise-induced adaptations: one which is responsible for increases in cardiovascular endurance and another which signals protein synthesis and muscular growth. These two pathways are exhibited in Figure 2.2. Increases in cardiovascular endurance are primarily signaled through AMPK. Muscle hypertrophic response to exercise is signaled through the mammalian target of rapamycin (mTOR) pathway due to its ability to stimulate protein translation and synthesis. Protein synthesis begins with insulin stimulating the PI 3-kinase/Akt pathway leading to increased mTOR activity (11). Inhibition of mTOR immediately before

muscle overload prevents protein synthesis, (31) suggesting that this pathway is necessary for normal muscle hypertrophy. AMPK is a negative regulator of the mTOR pathway. The α2 and λ3 subunits of AMPK have been identified as the complexes responsible for inhibiting mTOR activity (11). These two subunits have the ability to phosphorylate and thus deactivate the mTOR signaling cascade.

Interaction Between NO and AMPK

AMPK-Induced Phosphorylation of nNOS and eNOS

The interaction between NOS and AMPK is not fully understood. It is known that AMPK has the ability to phosphorylate and activate eNOS and nNOS isoforms (8, 9). In addition, the AMP mimetic drug 5-aminoimidazole-4carboxamide-1-β-D-ribofuranoside (AICAR) upregulates NOS activity in H-2K^b muscle cells (14). Altogether, the activation of AMPK in response to exercise or hypoxia is a critical mechanism required for normal metabolic regulation as well as nNOS and eNOS activity.

NO Facilitates AMPK Activation in Cell Culture

Current research indicates that NO produced enzymatically by the nitric oxide synthases regulates AMPK in cell culture. Lira et al (34) found that NOS activity was necessary for AMPK-induced upregulation of the GLUT4 transporter gene expression. L6 myotubes were treated with AICAR or AICAR and the non-specific NOS inhibitor L-NAME. Cotreatment with AICAR and L-NAME ablated 70% of the AICAR effect on GLUT4 mRNA, suggesting that NO is necessary downstream of AMPK for normal function. They also suggest that NO is necessary upstream of AMPK. Low concentrations of SNAP, an NO donor, at 1 and 10 μM increased GLUT4 mRNA expression and AMPK phosphorylation; in contrast, high concentrations of SNAP at 100 and 300 μM did not affect AMPK or GLUT4 mRNA expression. Furthermore, they propose the idea of a positive feedback system between NOS and AMPK enzyme activity.

Conclusions

The metabolic syndrome, characterized by the clustering of cardiovascular risk factors that leads to Type 2 diabetes, is increasing at astounding rates throughout the United States and developing countries due in large part to physical inactivity. NO production and signaling has an important role in exercise-induced metabolic and biochemical adaptations. AMPK is an important regulator of skeletal muscle metabolism and the acute and chronic adaptations to exercise training. Activation of AMPK, pharmacologically or by exercise, increases oxidative capacity and improves insulin sensitivity leading to a metabolically active phenotype in skeletal muscle that effectively counters the metabolic syndrome. Understanding how AMPK is regulated in adult muscle fibers and the potential role of nitric oxide in mediating AMPK activation is important for development of more potent and efficient treatments for metabolic disorders.

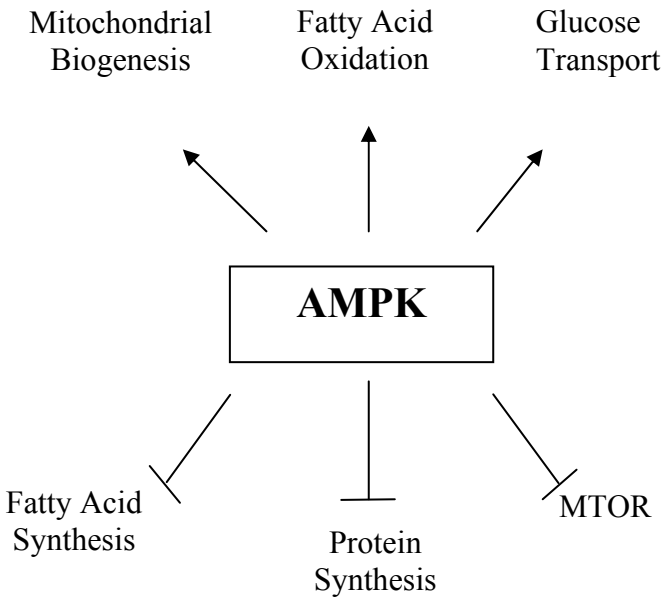


Figure 2-1. Metabolic processes regulated by AMPK. Arrows indicate activation of pathways, whereas lines with a bar at the end indicate inhibition of pathways.

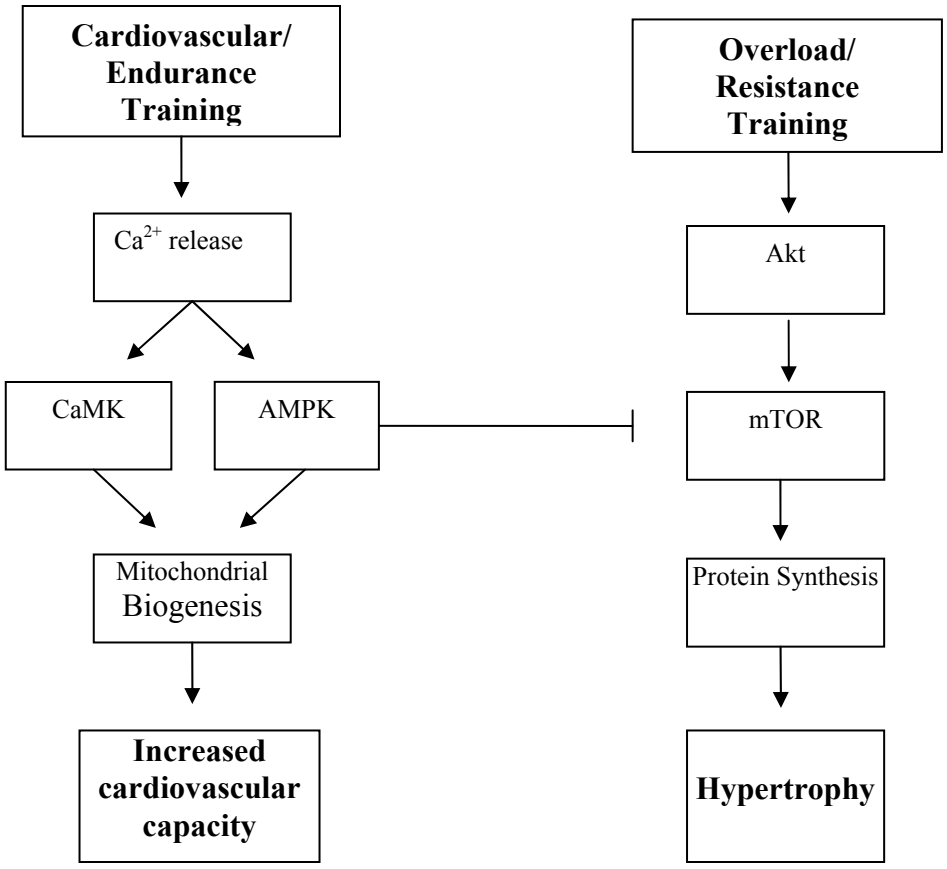


Figure 2-2. Exercise-induced signaling cascades. Arrows indicate the signaling events for adaptations to occur whereas a line with a bar at the end indicates processes inhibited.

CHAPTER 3
METHODS

Experimental Design

Extensor digitorum longus (EDL) and soleus muscles were dissected from young mice for in vitro bath manipulation. One EDL and one soleus muscle were subjected to 10 minutes of electrical stimulation or exposed to 5-aminoimidazole-4-carboxamide-1-β-D ribonucleoside (AICAR) for 20 minutes. Contralateral EDL and soleus muscles of the mice were treated with N(G)-monomethyl-L-arginine (L-NMMA) to inhibit NOS activity. The experimental design protocol is depicted in Figure 3-1.

Animals

The University of Florida Institutional Animal Care and Use Committee approved the protocol of this study. The subjects were young (~2 months old) female ICR mice purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). All animals were housed in the J. Hillis Miller Animal Science Center and fed the same diet (chow and water *ad libitum)* throughout the experiment. They were kept on a 12 hr light:dark photoperiod. Animals were brought to the lab approximately 12 hr prior to surgery to allow acclimatization to their new environment. Animals were divided into 8 treatment groups as illustrated in Figure 3-2.

Anatomical Dissection

Surgical removal of the extensor digitorum longus (EDL) and soleus muscles was necessary for *ex vivo* manipulation. The mice were anaesthetized with 2-5% isoflurane with oxygen as the carrier gas. Once anaesthetized, both hindlimbs were skinned and the Achilles tendon of the right leg cut. Because of its oxidative nature, the soleus was removed first to avoid hypoxia and tissue death. An incision was made along the fascia of the gastrocnemius muscle beginning at the distal end up to the posterior aspect of the fibula. The gastrocnemius/soleus

muscle complex was reflected to expose the deep soleus muscle and the proximal tendon. The proximal tendon was cut and the soleus carefully maneuvered away from the gastrocnemius. The distal tendon was then cut and the muscle immediately placed in the oxygenated buffer. The same surgical procedure was repeated on the left leg.

The distal ends of the tibialis anterior (TA) and EDL tendons were located and cut from the phalanges. An incision was made along the fascia of the TA up to the proximal end of the tendons at the fibula. The TA/EDL complex was removed as a whole and the EDL carefully maneuvered away from the TA and immediately transferred to the oxygenated buffer.

Experimental Protocol

Soleus and EDL muscles were surgically removed from fed anaesthetized mice and immediately transferred to a bath containing Krebs-Henseleit (KH) buffer supplemented with 25 mM sodium bicarbonate, 5 mM Hepes, 2.54 mM calcium chloride and 100 uM L-arginine (pH 7.15) continuously bubbled with 95% O2, 5% CO2. The tendons of each muscle were then clamped in micro tissue clamps and suspended separately between platinum field electrodes in water-jacketed micro tissue baths containing oxygenated buffer solution and maintained at 29ºC. After a 30 min equilibration period, each muscle was set to its optimal length (Lo) by repeated isometric twitches while gradually increasing muscle length. After determining Lo the buffer was washed from the baths and reloaded and equilibrated for an additional 10 min. One EDL and one soleus muscle of each animal was subjected to electrical stimulation or 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) treatment. Following the equilibration period, contraction was induced by electrical stimulation delivered at 10 Hz, 13 V for 10 min. For AICAR treatment, 25 mM AICAR solution was added to the baths with buffer for an incubation of 20 min. The contralateral EDL and soleus muscles served as non-contracted or non-AICAR treated controls. Muscles from half of the mice were treated with 1mM N(G)-

monomethyl-L-arginine (L-NMMA) to inhibit NOS activity during electrical stimulation or AICAR treatment. Immediately following the experimental treatment, muscles were blotted and frozen for subsequent protein isolation and quantification.

Western Blotting

Protein levels of total AMPK, phosphorylated AMPK, and phosphorylated acetyl CoA carboxylase (ACC) were determined by standard immunoblotting technique. All muscles were homogenized using glass-on-glass technique in 2X homogenizing buffer: 20 mM Tris (pH7.5), 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 containing 1% vol/vol phosphatase inhibitor (p-5726) from Sigma. Protein concentrations were measured using the DC Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Aliquots of muscle homogenates (16–78μg) were run in SDS-PAGE gels for phospho-ACC, phospho- and total αAMPK blots. The primary antibodies used are as follows: rabbit anti-αAMPK and anti-phospho-αAMPK (1:1000 dilution; Cell Signaling), rabbit anti-phosphoACC (1:500 dilution; Upstate). Ponceau stain and β-actin blots were used to control for loading. Reactions were developed by using the enhanced chemiluminescence detection reagents (ECL Plus; Amersham Biosciences, Buckinghamshire, UK), and protein levels were determined by densitometry (Kodak 1D Image Analysis Software version 3.6).

Statistical Analysis

Treatment main effects were analyzed by 2-way ANOVA (E-stim or AICAR vs. L-NMMA) with repeated measure on E-stim or AICAR. Individual group differences were assessed by paired t-tests with Bonferroni's correction for multiple tests applied.

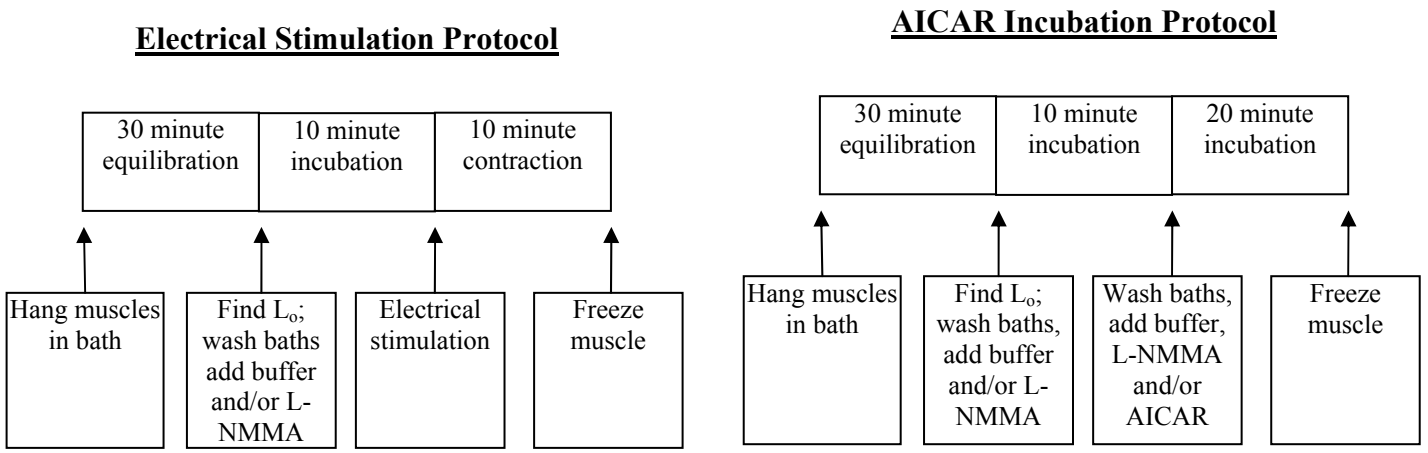


Figure 3-1. Experimental design flowchart. Electrical stimulation muscles were subjected to 10 minutes of stimulation; half of the muscles were treated with N(G)-monomethyl-L-arginine (L-NMMA) to inhibit NOS activity. 5-aminoimidazole-4-carboxamide-1-β-D ribonucleoside (AICAR) muscles were subjected to 20 minutes of AICAR treatment; half of the muscles were co-treated with L-NMMA to inhibit NOS activity.

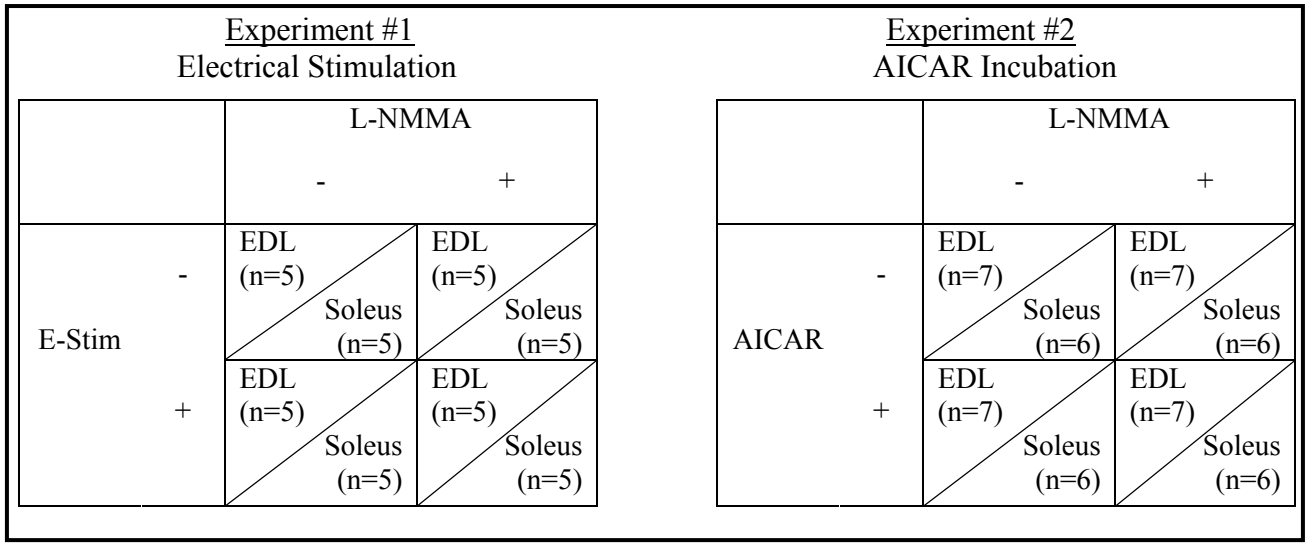


Figure 3-2. Experimental groups chart. Animals were divided based on electrical stimulation, AICAR treatment or L-NMMA treatment.

CHAPTER 4
RESULTS

Electrical Stimulation

Phospho-/Total-AMPK Ratio

EDL. To examine the effects of electrical stimulation and NOS inhibition on AMPK activation, a 2-way repeated measures ANOVA was conducted. The main effect of electrical stimulation on the ratio of phospho-to-total AMPK was near significant ($p = .082$) in the EDL. Further, we observed a trend of L-NMMA increasing the phospho-AMPK/total AMPK ratio at rest in the EDL though there was not a significant main effect of NOS inhibition. Comparison of individual groups revealed that electrical stimulation caused a significant, ~ 4 fold, increase in the phospho-AMPK/total AMPK ratio, while NOS inhibition by L-NMMA abrogated the electrical stimulation effect in the muscle (Figure 4-1).

Soleus. Analysis of the soleus revealed a significant main effect of electrical stimulation on phospho-AMPK/total AMPK ratio; however, a minimal increase in AMPK activation with electrical stimulation alone was observed (Figure 4-2). There was not a statistical main effect of L-NMMA on AMPK activation ($p = .065$). Interestingly, individual group comparison revealed that L-NMMA treatment during electrical stimulation increased AMPK activation above L-NMMA treatment alone.

Total-AMPK, Phospho-AMPK, and Phospho-Acetyl Co-A Carboxylase(ACC)

Quantification of phospho-AMPK, total AMPK and phospho-ACC data (means ± SEM) normalized to the control mean for the EDL and soleus is provided in Table 4-1.

Electrical stimulation and NOS inhibition of the EDL did not produce significant main effects on these proteins, although phospho-AMPK was elevated in the electrical stimulation, L-NMMA, and electrical stimulation plus L-NMMA groups, compared to control levels.

In the soleus, electrical stimulation and NOS inhibition did not have a significant effect on total AMPK levels. In contrast, there was a significant effect of electrical stimulation and NOS inhibition on phospho-AMPK. Although no changes in phospho-ACC reached statistical significance in the electrical stimulation study, the electrical stimulation main effect on phospho-ACC in the soleus was nearing significance ($p = .051$).

5-aminoimidazole4-carboxamide-1-β-D-ribofuranoside(AICAR)

Phospho-/Total-AMPK Ratio

EDL. To examine the effects of AICAR treatment and NOS inhibition on AMPK activation a 2-way repeated measures ANOVA was conducted. There was a significant main effect of AICAR treatment on phospho-AMPK/total-AMPK ratio ($p = .013$) in the EDL. There was not a significant main effect of L-NMMA on AMPK activation. AICAR treatment caused a significant, ~ 2 fold increase in the phospho-AMPK/total AMPK ratio. However, NOS inhibition by L-NMMA incubation blunted the AICAR effect (Figure 4-3).

Soleus. Analysis of the soleus revealed no significant main effects of AICAR treatment or NOS inhibition on AMPK phosphorylation status. However, it is important to note that the AICAR main effect was nearing significance ($p = .107$). (Figure 4-4).

Total-AMPK, Phospho-AMPK, and Phospho-ACC

Quantification of phospho-AMPK, total AMPK and phospho-ACC data (means±SEM) normalized to the control mean is provided in Table 4-2 for the EDL and soleus. AICAR or L-NMMA treatment did not have a significant effect on these proteins in the EDL or soleus.

Table 4-1. Quantification of phospho-AMPK, total AMPK and phospho-ACC/β actin levels for the EDL and soleus for electrical stimulation experimental groups. Data were normalized to the mean of the control group. Values represent mean ± SEM. EDL=extensor digitorum longus, E-stim=10min of in vitro electrical stimulation (see text for details), L-NMMA=L-N^G-monomethyl Arginine citrate. *Significantly different from control.

	Control	E-stim	L-NMMA	E-stim + L-NMMA
EDL				
p-AMPK	1.000 ± .754	5.877 ± 1.939*	2.566 ± .754	3.169 ± 1.939
t-AMPK	1.000 ± .201	1.054 ± .250	1.108 ± .201	1.334 ± .250
p-ACC	1.217 ± .409	1.113 ± .44	.841 ± .366	1.534 ± .393
Soleus				
p-AMPK	1.000 ± .305	.899 ± .192	1.328 ± .341	2.162 ± .215
t-AMPK	1.000 ± .295	.965 ± .300	1.376 ± .330	1.388 ± .335
p-ACC	1.000 ± .253	.844± .370	.659 ± .326	2.322 ± .478*

Table 4-2. Quantification of phospho-AMPK, total AMPK and phospho-ACC/β actin levels for the EDL and soleus for the AICAR experimental groups. Data were normalized to the mean of the control group. Values represent mean ± SEM. AICAR = 5-aminoimidazole-4-carboxamide-1-ß -D-ribofuranoside (1 mM).

	Control	AICAR	L-NMMA	AICAR + L-NMMA
EDL				
p-AMPK	1.009 ± .318	1.289 ± .269	1.668 ± .318	1.673 ± .269
t-AMPK	1.000 ± .105	.862 ± .114	.935 ± .105	.897 ± .114
p-ACC	1.000 ± .273	1.741 ± .392	1.319 ± .273	1.193 ± .392
Soleus				
p-AMPK	1.000 ± .827	3.176 ± 1.193	2.547 ± .906	3.117 ± 1.307
t-AMPK	1.000 ± .474	1.804 ± .517	1.588 ± .520	1.482 ± .567
p-ACC	.965 ± .555	2.826 ± 1.634	2.076 ± .555	3.661 ± 1.634

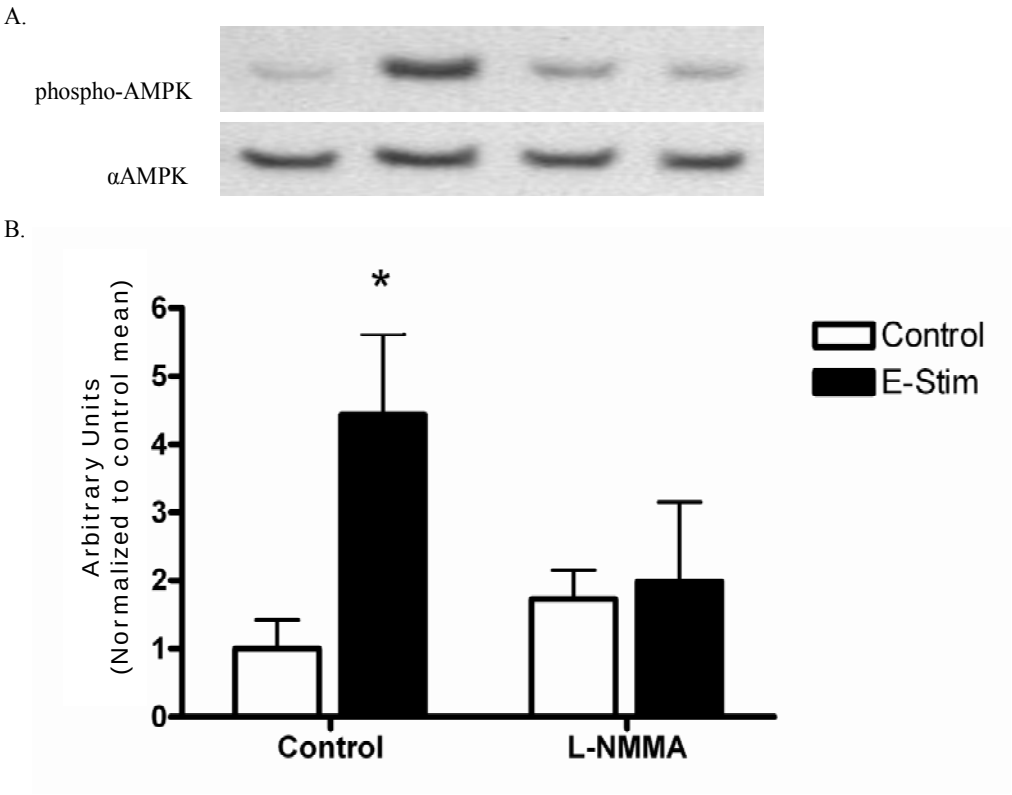


Figure 4-1. EDL phospho-AMPK/αAMPK ratio for electrical stimulation experiment. (A) Representative immunoblot from control, electrical stimulation, L-NMMA and L-NMMA + electrical stimulation for phospho-AMPK and total(α) AMPK. (B) Quantification of immunoblots for phospho-AMPK/total AMPK ratio. Values represent mean ± SEM. L-NMMA = L-monomethyl Arginine citrate. E-stim = electrical stimulation. *Significantly different from control.

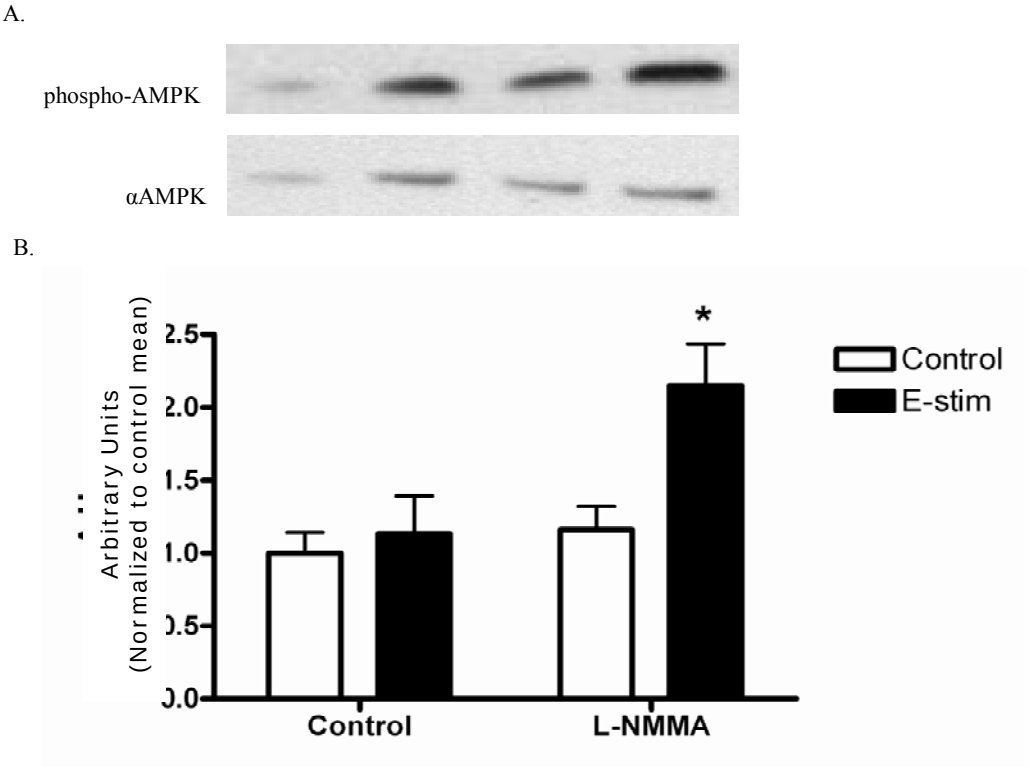
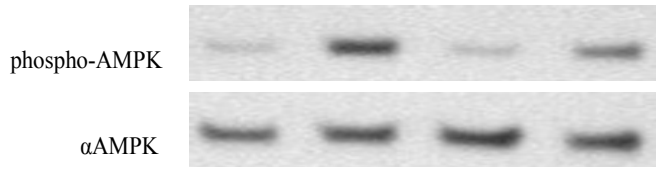


Figure 4-2. Soleus phospho-AMPK/αAMPK ratio for electrical stimulation experiment. (A) Representative immunoblot from electrical stimulation, L-NMMA and L-NMMA + electrical stimulation for phospho-AMPK and totalAMPK. (B) Quantification of immunoblots for phospho-AMPK/total AMPK ratio. Values represent mean ± SEM. *Significantly different from control.

A.



B.

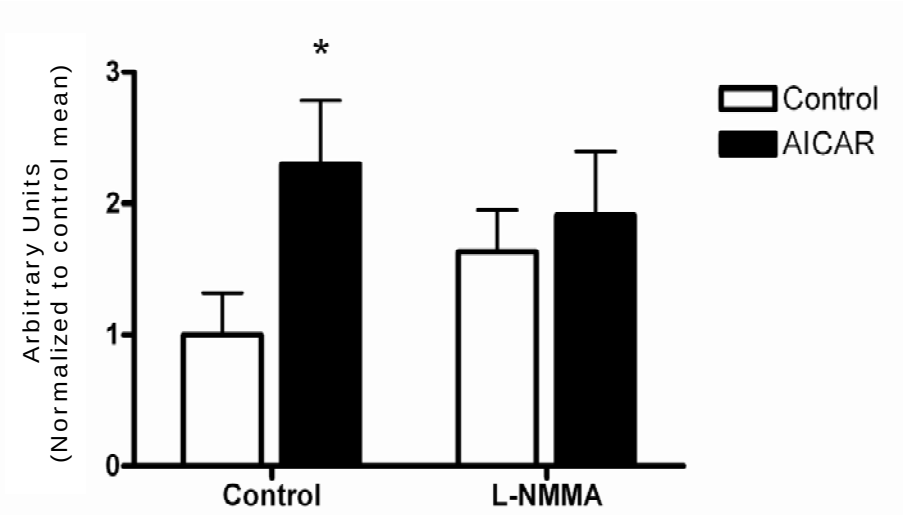


Figure 4-3. EDL phospho/total AMPK ratio for AICAR experiment. (A) Representative immunoblot from control, AICAR, L-NMMA and L-NMMA + AICAR for phospho-AMPK and total AMPK. (B) Quantification of immunoblots for phospho-AMPK/total AMPK ratio. Values represent mean ± SEM. AICAR =5-aminoimidazole-4-carboxamide-1-β-D ribonucleoside (1 mM). *Significantly different from control.

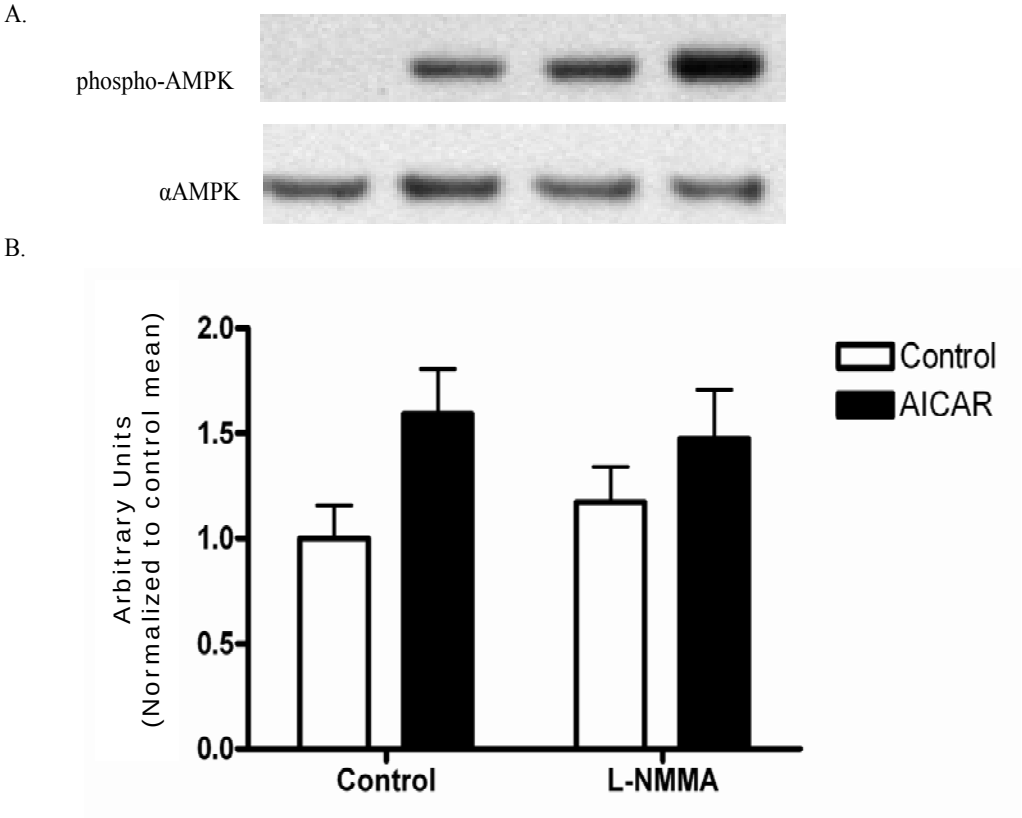


Figure 4-4. Soleus phospho/total AMPK ratio for AICAR experiment. (A) Representative immunoblot from control, AICAR, L-NMMA and L-NMMA + AICAR for phospho-AMPK and total(α). (B) Quantification of immunoblots for phospho-AMPK/total AMPK ratio. Values represent mean ± SEM. AICAR =5-aminoimidazole-4-carboxamide-1-β-D ribonucleoside (1 mM). *Significantly different from control.

CHAPTER 5
DISCUSSION

Main Findings

Our observations are based on relatively short-term exposure of isolated skeletal muscles to AMPK-activating stimuli. The data support our hypotheses that electrical stimulation or AICAR treatment is sufficient to increase AMPK activation, in vitro, in the fast-twitch EDL muscle, and that NOS activity is necessary for this affect. Inhibition of NOS abrogated electrical stimulation- and AICAR-induced activation of AMPK in the EDL; however interpretation is complicated by a trend for L-NMMA treatment to induce phosphorylation of AMPK. The main findings of this study are: 1) electrical stimulation or AICAR incubation increases the phospho-AMPK/total AMPK ratio; 2) NOS inhibition prevents this increase; 3) AMPK activation by electrical stimulation and AICAR treatment is greater in the fast twitch EDL muscle than the slow twitch soleus muscle; and 4) L-NMMA treatment tends to increase AMPK phosphorylation status, independent of electrical stimulation or AICAR effects.

Electrical Stimulation and AICAR Treatment Induces AMPK Activation

Activation of AMPK in response to exercise or hypoxia is a critical mechanism required for normal metabolic regulation as well as nNOS and eNOS activity (8, 9, 14). Our data, in agreement with several studies (14, 22, 38, 60) demonstrates that AMPK is an exercise-responsive gene, activated by increases in intracellular AMP/ATP concentrations. The greater the metabolic stress placed on skeletal muscle fibers, the greater the activation of AMPK (17). Our stimulation protocol induced greater AMPK activation in the EDL compared to the soleus. The level of metabolic stress placed on the oxidative fibers of the soleus was not great enough to induce a significant increase in AMPK activation.

NOS Inhibition Decreases Electrical Stimulation- and AICAR-Induced AMPK Activation

NOS inhibition decreased the level of AMPK activation in the EDL following electrical stimulation and AICAR treatment. We recently reported experiments in cultured L6 myotubes demonstrating that AICAR-induced AMPK phosphorylation is nitric oxide-dependent (34). Further, we found that nitric oxide donors are sufficient to induce AMPK phosphorylation (34). Our working hypothesis to explain these data involves the convergence of AMP binding to AMPK (induced experimentally be the AMP mimetic, AICAR) and NO-dependent activation of AMPK kinases. Both of these events seem to be required to induce AMPK phosphorylation and downstream signaling (Figure 5-1). The current experiments were designed to confirm that these mechanisms are functional in adult skeletal muscle. We predicted that L-NMMA treatment would prevent AMPK phosphorylation induced by either contractile activity or AICAR treatment in adult skeletal muscle, in vitro. The current data indicate that NO plays an important role in the regulation of AMPK phosphorylation in contracting or metabolically active muscle fibers.

It appears that NOS inhibition caused a nonspecific effect on the phospho-AMPK/total AMPK ratio in the soleus. Understanding this interaction is complicated as NO is intricately involved in numerous physiological processes. The exact mechanisms whereby NO exerts its effects on skeletal muscle contractility and metabolism remain uncertain. It is well established that NOS inhibition increases force production by slowing cross bridge cycling, decreasing ATPase activity and AMP levels and increasing oxidative phosphorylation. Logically, this would suggest that basal AMPK activation would be reduced following treatment with a NOS inhibitor. Nevertheless, our results suggest the opposite effect. Interestingly, NOS inhibition decreases maximum shortening velocity in mixed fiber types but has no affect on the velocity of unloaded shortening (determined by fast fibers) suggesting the possibility of a fiber-type-specific, NO-

dependent augmentation of ATPase activity (2, 36, 39) In contrast, NO thiol nitrosylation can reduce ATPase activity and force production (28, 62).

Redox balance is necessary for homeostasis and optimal functioning of numerous physiological interactions and may be an underlying condition affecting our results. The role of NO as a signaling molecule has been well described in skeletal muscle (57). Most of the physiological actions of NO are the result of its ability to stimulate guanylate cyclase, thereby increasing the production of cGMP (57). Nevertheless, NO is a free radical capable of inducing oxidative stress. Although it is not highly reactive itself, it can react with other compounds to produce more toxic species (46, 70). Most notably, NO reacts with superoxide to produce peroxynitrite, a highly reactive species that can oxidize lipids, proteins and nucleic acids (46). Paradoxically, due to NO's low redox potential and its ability to combine with more reactive species and convert them to less reactive products, it is capable of acting as an antioxidant as well as a pro-oxidant (46, 70). The disparate effects of NO in tissue (i.e. positive signaling and antioxidant effects vs. negative pro-oxidant effects) appear to be primarily determined by concentration, and perhaps localization of the NO signal. The constitutive, calcium-dependent NOS isoforms (nNOS and eNOS) produce nanomolar concentrations of NO, which are sufficient for activation of guanylate cyclase, and produce antioxidant effects (46, 57, 70). Conversely, iNOS which is expressed by neutrophils or macrophages, or by muscle cells in response to oxidative stress, endotoxin, or inflammatory cytokine signaling, produces 100 to 1000 fold higher concentrations of NO leading to nitrosylation and oxidant stress (50, 71). We believe that NO concentration regulates the equilibrium between reduced and oxidized states within cells, similar to that proposed by other research teams in relation to satellite cell activation and NO concentration (1). At low and high concentrations of NO, the cell is placed in a state of oxidized

stress. An optimal concentration of NO exists creating an environment wherein skeletal muscle metabolism can transpire without placing excess stress on the cell. This idea could explain why lowering NO concentration by treatment with L-NMMA (a non-isoform-specific NOS inhibitor) tended to increase basal metabolic stress and induce AMPK phosphorylation. Meanwhile, activation of AMPK during contractile activity or elevated AMP concentration (mimicked by AICAR) requires NO.

ACC Phosphorylation

Our electrical stimulation and AICAR treatment protocols were not sufficient to induce significant main effects on phosphorylation of the AMPK target, ACC, in the soleus or EDL, most likely due to the relatively short incubation times (10 min for E-stim and 20 min for AICAR). The only significant group difference was observed between control and electrical stimulation groups in the L-NMMA-treated soleus muscles. Although contractile performance data was not recorded for this study, we consistently observed an increased force production during electrical stimulation plus L-NMMA treatment in the soleus (~50% increase) compared to electrical stimulation alone. Consistent with this greater force production, there were strong trends for AMPK and ACC phosphorylation to be elevated in these samples above L-NMMA alone ($p = .051$). Apparently, there is a fiber type-specific effect of NOS inhibition during electrical stimulation since only the soleus demonstrated this synergistic effect of contractile activity and NOS inhibition on AMPK activation. This could be due to a general mitochondrial dysfunction in the mitochondria-rich slow-twitch fibers of the soleus leading to exaggerated metabolic stress during contractile activity.

Limitations and Future Directions

The electrical stimulation protocol was not sufficient to cause significant changes in AMPK activation in the soleus most likely because of its oxidative phenotype. Stimulation for a

longer period of time most likely would have activated AMPK to a higher degree. Future studies need to increase the time of stimulation to see these changes.

Our limited ability to probe for oxidative enzymes and other proteins made it difficult to interpret our results. Data on ATPase, total ACC and cytochrome-c oxidase may have given additional insight as to what changes occurred as a result of our protocol.

A dose-response experiment needs to be performed to test our theory that NO concentration regulates a balance between reduction and oxidation in healthy cells. In addition, future studies should examine the effects of a high-intensity electrical stimulation protocol imitating a strength or power exercise to determine how exercise intensity affects AMPK activation.

Conclusions

NOS activity is involved in activation of AMPK during chronic electrical stimulation or AICAR treatment. To date, the full extent of NOS involvement in AMPK activation and exercise-induced metabolic adaptations is not known. Further studies concentrated on NOS activity and AMPK are needed to investigate this relationship.

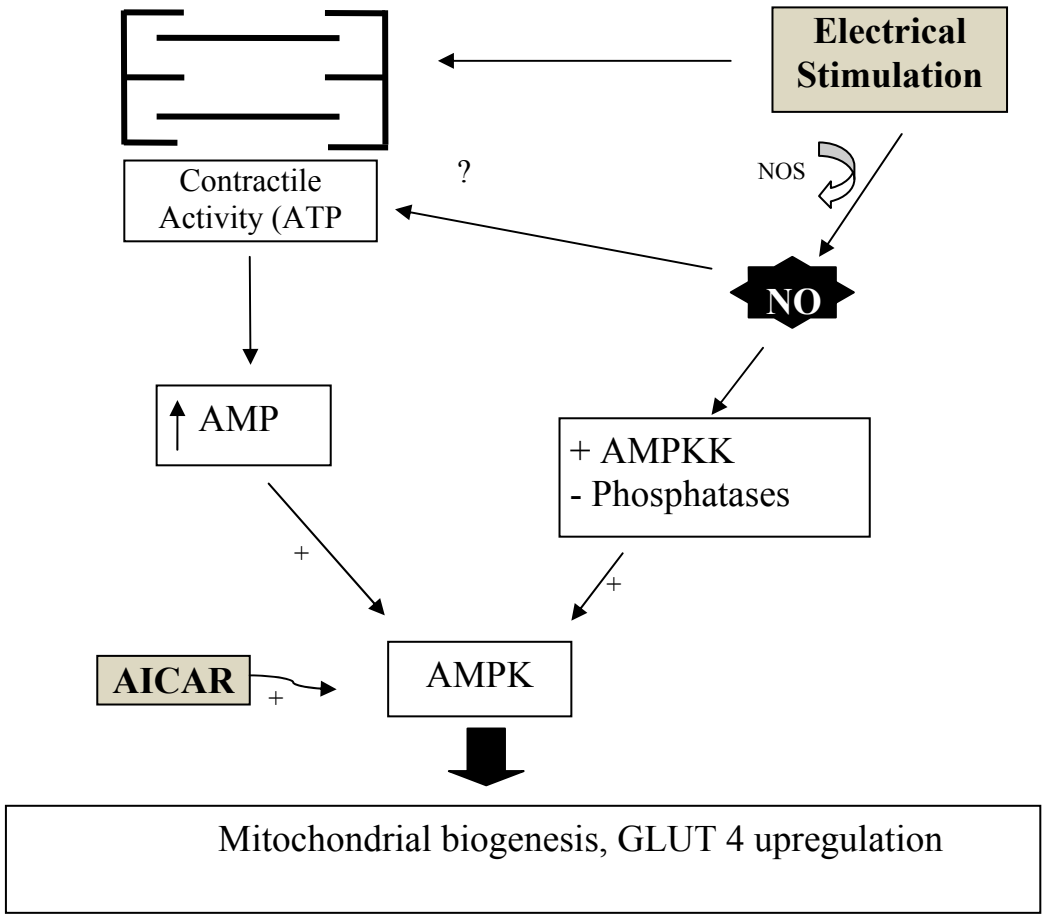


Figure 5-1. Proposed model illustrating potential role of nitric oxide in the activation of AMPK. NO may facilitate activation of AMPK by increasing AMPK kinases and/or decreasing phosphatases. Inhibiting NOs metabolic signal regulation may have affected our results.

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BIOGRAPHICAL SKETCH

Dana Brown was born in Fort Walton Beach, Florida, in 1984. She graduated in the top 3% of her high school in 2002. She received a Bachelor of Science degree in exercise and sport sciences in 2006 from the University of Florida, where she was a member of the Golden Key Honor Society and Social Entrepreneurship and an employee of UF's Department of Recreational Sports. Dana will begin an additional Master of Science degree in business entrepreneurship at the University of Florida beginning in the summer of 2008. After finishing school Dana plans to continue working in the field of health and fitness.