The Effect of AICAR Treatment on Sarcoplasmic Reticulum Function and Possible Links to Skeletal Muscle Fatigue

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ABSTRACT

A compelling mystery in the study of exercise is mechanisms of skeletal muscle fatigue. Broadly described, muscle fatigue is the uncomfortable sensation that particular muscle groups are shutting down and muscle force production cannot continue. More specifically, muscle fatigue is defined as an activity-induced inability to continue to produce a desired level of force. Several groups suggest that a major cause of force loss during fatigue is reductions in the rates of sarcoplasmic reticulum (SR) calcium (Ca^{2+}) release and uptake. These changes result in diminished contractile machinery activation, reduced force production and slowed relaxation.

During exercise, adenosine 5'-triphosphate (ATP) is the energy currency that is used to support force production. As a result of ATP hydrolysis and re-synthesis, adenosine diphosphate (ADP) and adenosine monophosphate (AMP) levels rise. AMP kinase (AMPK) is an enzyme that becomes activated as a result of increased AMP levels. It is thought to function as a metabolic "master switch" within the muscle and plays a major role in carbohydrate and fat metabolism. Once AMPK is activated it regulates several ATP consuming and producing pathways. The overall objective of this project was to determine if increased metabolism during exercise contributes to SR Ca^{2+} dysfunction during fatigue. If this is true, artificial activation of AMPK via 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) should induce changes in SR function that are qualitatively similar to those caused by fatigue.

In study 1, mice were injected with 0.85 mg/kg AICAR (or saline solution) and both gastrocnemius muscles were removed one hour later. In study 2, EDL muscles were placed in a muscle bath and incubated in AICAR (4mM) or stimulated to fatigue. Glycogen, glucose-6-phosphate (G-6-P), ATP, ADP, and phosphocreatine (PCr) were examined in all groups of muscles. Alterations in SR calcium uptake and release rates due to the presence of AICAR were also studied as a likely cause of muscle fatigue.

AICAR treatment in vivo did not alter muscle glycogen, glucose, ATP, ADP or PCr concentrations. However, G-6-P levels were increased by 137%. This was accompanied by a 36% reduction in the SR Ca^{2+} uptake rate and a 42% reduction in Ca^{2+}-stimulated Ca^{2+} ATPase activity as well as 13-15% reductions in the rates of Ca^{2+} release. These changes were not associated with SR Ca^{2+} pump content. Administration of AICAR in vitro also increased G-6-P content (200%) without altering the concentrations of glycogen, glucose, G-6-P, ATP, ADP or PCr. AICAR decreased SR Ca^{2+} uptake rate by 28% and the rate of Ca^{2+} release by 16%. For comparison, fatiguing stimulation reduced the rates of SR Ca^{2+} uptake and release by 31 and 41%, respectively.

Taken together, these results indicate that when administered to skeletal muscle both in vivo and in vitro, AICAR evokes metabolic stress. More importantly, activation of AMPK alters skeletal muscle SR function to an extent that is qualitatively similar to that caused by fatiguing activity. At present, it is not clear how AMPK activation causes changes in SR function. However, the present finding is consistent with the notion that metabolic stress caused by exercise, affects SR function. This, in turn, leads to force loss but reduces energy demand and protects the cell from ATP depletion during maximal contractile activity.

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INTRODUCTION

INTRODUCTION

Muscle fatigue. These two words can spell disaster for any endurance or sprint athlete. A large athletic community exists today. The members range in age, ability and level of commitment. For those who choose to pursue a longer, more intense exercise regime, such as marathoners and triathletes, they sign on for much more in several ways. More attention needs to be given to dietary needs, perfected training, consistency and recovery. All of these are limiting factors that can determine success. As a result, much research has been directed to such areas to allow the athlete to have all of the latest information regarding factors affecting performance. However, despite the scientific and medical breakthroughs that allow professional and high endurance athletes to compete and be successful at high levels, there still remains an important factor that needs to be considered.

Muscle fatigue is a transient reduction in the ability to produce force. Its particular cause and cascade of events is a complex phenomenon and has been the focus of much research. Basically, muscle fatigue develops as the result of brief, high intensity exercise, as well as endurance exercise. The use of adenosine triphosphate (ATP) becomes greater than the skeletal muscles' ability to produce it. Although ATP is not the only reason for fatigue, it likely leads to several alterations within the cell allowing for the reduced ability of the skeletal muscle to produce the desired force.

Skeletal muscles are initially activated by α-motor neurons. Acetylcholine is released into the neuromuscular junction and binds to acetylcholine receptors on the surface of the muscle cell. The cell membrane becomes depolarized as sodium ions flow into the cell signaling an action potential. The action potential propagates along the membrane surface and eventually down the T-tubule, ultimately reacting with the dihydropyridine receptor

(DHPR). The DHPR communicates with the ryanodine receptor (RyR) on the terminal cisternae of the sarcoplasmic reticulum (SR). This causes the release of calcium which enters the myoplasm. Calcium binds to troponin C on the actin filament and causes tropomyosin to move away from the myosin binding sites on actin. Myosin cross bridges then engage the actin molecule and force development occurs (Figure 1).

A key component in the contraction of a muscle is release of calcium ions (Ca^{2+}) from the SR. As the concentration of free myoplasmic calcium ions increases, muscle contraction is triggered (Westerblad et al., 2000). Williams and Klug (1995) report that changes in the rates of SR Ca^{2+} uptake and release by isolated SR occur following various types of activity ranging from prolonged exercise to short-term electrical stimulation. Force production is ultimately dependent on the amount of free calcium present in the myoplasm. Thus, any reduction in the release or uptake of these ions from the SR is an important component of the fatigue mechanism.

ATP drives nearly every process in the body that requires energy. Energy is produced when the ATP bond is broken by hydrolysis forming ADP (adenosine diphosphate) or AMP (adenosine monophosphate). In healthy cells, a proper ratio of these nucleotides is maintained allowing body processes to function normally. During metabolically stressful situations, such as exercise, as ATP is broken down, sufficient amounts of AMP begin to accumulate in the muscle activating adenosine monophosphate-activated kinase (AMPK) (Myburgh, 2004).

AMPK appears to play a key role in several metabolic processes. Winder and Hardie (1999) report that AMPK phosphorylates many key proteins which control the movement through metabolic pathways such as cholesterol synthesis, lipogenesis, and skeletal muscle

fatty acid oxidation. They also state recent evidence points to AMPK being responsible for triggering the stimulation of glucose uptake that is induced by muscle contraction. Other potential roles of AMPK include the phosphorylation of numerous proteins that result in an increase in fat oxidation and glucose metabolism which buffer the ATP concentration in response to a low cellular energy charge (Hardie, 2004). Activation of this enzyme, during exercise, plays a role in mechanisms that adjust the supply of ATP to more effectively balance the demand (Myburgh, 2004). Hardie and Hawley (2001) summarize there are many downstream effects of AMPK activation. One of these is the increase in glucose uptake by GLUT-4 translocation and GLUT-1 activation (Hardie and Hawley, 2001; Wright et al., 2004).

Exercise and insulin stimulate glucose transport (Wright et al., 2004). Wright and colleagues state that early studies have shown an increase in cyctosolic Ca^{2+} during muscle contractions initiates the process that ultimately leads to increased glucose transport. They conducted a study to more clearly determine that Ca^{2+} and AMPK activation are both responsible for the increased glucose transport during contractile activity. Their results proved conclusive that AMPK activation along with Ca^{2+} are responsible for the increase in muscle glucose transport during muscle contractile activity.

Additional studies have indicated an intriguing link between AMPK and certain diseases such as Type 2 diabetes (Winder and Hardie, 1999; Hardie and Hawley, 2001). Diabetes is a result of an abnormally high blood glucose level. Type 2 diabetes is not insulin deficiency but reduced insulin sensitivity of the tissue resulting in elevated blood glucose. For example, muscle of Type 2 diabetics shows lowered insulin sensitivity. Consumption of high calorie foods, lack of exercise, and an ever-increasing obesity issue in this country have

all contributed to the rise of Type 2 diabetes among Americans (Hardie and Hawley, 2001). Based solely on the data presented regarding the actions of AMPK, it might be thought that some of the Type 2 diabetes metabolic abnormalities may be reversed with AMPK activation. However, it should be noted that Type 2 diabetes has many mechanisms that lead to reduced insulin sensitivity and to conclude that this is solely the result of a deficient AMPK system may prove inaccurate. Winder and Hardie (1999) present evidence that AMPK influences numerous metabolic processes, many of which become disrupted in diabetes.

The utilization rate of ATP increases during exercise thus allowing for repeated contractions. When the ATP replenishment rate lags behind utilization, alterations in the muscle contraction process must occur to prevent intracellular ATP depletion and, ultimately, cell death. The SR is the organelle responsible for intracellular Ca^{2+} movement that leads to muscle contraction and relaxation. Indirectly, it controls energy use by the cell during periods of activity. Alterations in its function seem to cause fatigue. This raises the question, does metabolic stress cause changes in SR function that lead to fatigue? More specifically, does activating the AMPK system cause changes in SR function?

SPECIFIC AIMS

The overall goal of this project is to determine if metabolic stress induces changes in SR function that are qualitatively similar to those caused by fatiguing activity. Specifically, this project will determine if:

- Activation of AMPK by acute administration of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) alters the metabolic state of the muscle. This will be

indicated by alterations in intracellular concentrations of glycogen, glucose, G-6-P, ATP, ADP and PCr.

- Activation of AMPK by AICAR reduces the rates of SR Ca^{2+} uptake and release and diminishes Ca^{2+} ATPase activity. This will be indicated by alterations in SR Ca^{2+} transport using muscle homogenate fractions.

These results should shed light on the interaction between increased metabolism during exercise and the development of fatigue.

HYPOTHESES

1. In vivo treatment with AICAR will not alter the metabolic content of the muscle (intracellular concentration of ATP, ADP, glycogen, glucose, G-6-P and PCr).
2. In vivo treatment with AICAR will not alter the peak rates of SR Ca^{2+} release, uptake and Ca^{2+} ATPase activity.
3. In vitro treatment with AICAR will not alter the metabolic content of the muscle (intracellular concentration of ATP, ADP, glycogen, glucose, G-6-P and PCr).
4. In vitro treatment with AICAR will not alter the peak rates of SR Ca^{2+} release, uptake and Ca^{2+} ATPase activity.

LIMITATIONS

The results of these experiments are limited to:

1. Swiss-Webster mice.
2. The gastrocnemius and EDL muscles.

3. Characterization of SR function by the peak rates of Ca^{2+} uptake and Ca^{2+} release using muscle homogenate fractions.
4. Single drug concentrations administered in vivo and in vitro.
5. Specific times post-administration.
6. Metabolic changes characterized by measurements of glycogen, glucose, G-6-P, ATP, ADP, and PCr.

ASSUMPTIONS

The following assumptions were made:

1. All mice were healthy and disease-free.
2. All mice were well-fed and hydrated.
3. AICAR was effective in activating AMPK activity.

CHAPTER TWO:

REVIEW OF LITERATURE

INTRODUCTION

All exercise requires attention to specific areas of preparation. Nutrition, to fuel the body properly, is an important aspect to consider. Providing the proper nutrients to the body to prepare and recover from competition can play a large role in the athlete's success. Training, if not done correctly and specifically, can have detrimental effects. The athlete's goal is to prepare nutritionally, physically, and mentally the best that he or she can to be better than the opponent. However, fatigue, an obstacle that even the best endurance athletes must battle with, plays the biggest role in an athlete's success.

Skeletal muscle, when activated repeatedly during intense or prolonged exercise such as marathons and triathlons, typically becomes functionally impaired. This is manifest in reductions in the desired level of force output. This is also known as fatigue (Westerblad et al., 2000). General fatigue can be described as the inability of a physiological system to function at a desired level. However, muscle fatigue is the term used to imply the inability of the muscle to produce force and is a result of severe metabolic stress placed on the activated muscles. An increased level of activation is required to maintain a desired force. This type of fatigue can be extremely debilitating for an athlete.

ATP, a cell's energy currency, is produced very rapidly to match energy demand. This also results in the accumulation of metabolites within the cell, such as lactate and inorganic phosphate (P_i). As the body continues to meet the energy demand, ATP production slows. As a result, the rate at which the muscle contracts and relaxes also slows. The primary underlying mechanism that causes this cascade of events is reduced SR calcium release. However, the cause of fatigue is multi-faceted so other mechanisms, such as the function of the contractile machinery, as well as nervous activation, may also be involved

(Westerblad et al., 2000). Also, metabolic components such as glycogen, phosphocreatine, and ATP depletion, along with inorganic phosphate and ADP accumulation, are all disturbances that can potentially lead to muscle fatigue (Leppik et al., 2004). Thus, the exact causes of muscle fatigue remain a mystery. There are a variety of schools of thought. One possible idea is the "accumulation theory" that suggests the accumulation of metabolic byproducts disrupts contractile performance. The second is the "depletion theory" whereby depletion of key energy substrates leads to reductions in [ATP] and reduced force production. Finally, the "Ca^{2+} exchange" hypothesis suggests that Ca^{2+} handling within the SR may be compromised. This, in turn leads to reduced contractile apparatus activation and lowered force output.

While many theories and questions exist, many external factors play a role. Other factors include energy level, genetics, nutrition, altitude, fitness level, training regime, and temperature as well as the duration and intensity of the exercise. Another possibility to consider is central fatigue.

CENTRAL FATIGUE

The fatigue that results from physical exercise is thought to derive from two causes. The first is metabolic changes in the muscle while the second is modifications in the central nervous system (CNS) (Fernstrom and Fernstrom, 2006). Central fatigue begins with the brain. The CNS is where decisions are made; the decision to do or not do something or to set a limit to what is done. This adds another dimension to the goal of truly defining the source of muscle fatigue. The activity of the CNS involves several components and if any of these

becomes impaired at any moment, muscle force production cannot occur to its full potential. For example, a lack of motivation is enough to impair the central nervous system.

Findings by Ikai and Steinhaus (1961), suggest it is a psychological factor, not a physiological factor that limits a maximal voluntary effort. Their study demonstrated that muscle force was increased with a simple gunshot preceding the lift or a shout during the effort. This promotes the idea that maybe the maximal effort of the muscle is not physiologically set but psychologically. If the person does not think or does not want to produce the force, there is a small chance it will occur because mentally the limit has been set.

EXCITATION-CONTRACTION COUPLING

Excitation contraction coupling (ECC) is the process by which muscles are activated by electrical activation (see Figure 1). This cascade of events begins with the activation of an α-motor neuron and the generation of an electrical signal, the action potential. The action potential then travels down the axon and arrives at the nerve terminal. The influx of sodium (Na^+) into the cell causes the cell to depolarize. Acetylcholine (Ach) is released into the synaptic cleft of the neuro-muscular junction. Acetylcholine binds to acetylcholine receptors on the muscle cell membrane (sarcolemma) surface. As a result, the sarcolemma becomes more permeable to Na^+. The influx of Na^+ causes the cell to depolarize, initiating a sarcolemmal action potential. The action potential continues down the muscle fiber to the transverse tubules (T-tubules). T-tubules run alongside the terminal cisternae of the SR. One tubule and two cisterae form a triad junction that is joined to the SR. At the SR-tubule junction are Ca^{2+} release channels known as the ryanodine receptors (RyR). When an action

potential reaches the T-tubule, the dyhydropyradine receptor (DHPR) activates the RyR in the SR causing a release of Ca^{2+}. Calcium then binds to troponin C. This removes the troponin I inhibition over the actin filament which results in the actin-myosin complex to shift from a state of weak binding to strong binding. The myosin head contains myosin ATPase. The hydrolysis of ATP to ADP + P_i releases energy, causing the myosin molecule to undergo a conformational change. This, in turn, "pulls" the thin filament across the thick and the muscle contracts. This process is the sliding filament theory and continues until there is no longer an action potential and the SR actively removes Ca^{2+} from the sarcoplasm. Without an action potential, no Ca^{2+} can be released to the sarcomere.

SARCOPLASMIC RETICULUM DYSFUNCTION

The SR is a structure found in striated and smooth muscle. It is a reservoir for Ca^{2+} and allows for Ca^{2+} uptake and release to regulate myoplasmic free concentration. Release occurs through the RyR located on the SR while ATP hydrolysis powers the SR Ca^{2+}-ATPase to re-sequester the released Ca^{2+}. The level of free Ca^{2+} ultimately controls force production during muscle contraction. SR Ca^{2+} uptake accounts for approximately one third of the total energy cost required for muscle contraction in mammals (Stienen et al., 1995). The other two thirds are required by the contractile apparatus. Recent estimates suggest that the SR Ca^{2+} pump may account for as much as 80% of total energy consumption during contraction (Zhang et al., 2006). Research shows that Ca^{2+} release and uptake rates are decreased in isolated SR preparations from exercised muscles compared to those in rested muscle (Luckin et al., 1991; Belcastro et al., 1993; Favero et al., 1993; Ward et al., 1998; Williams et al., 1998, Ørtenblad et al., 2000). Such changes have a two-fold effect. First,

activation of the contractile apparatus is reduced resulting in force loss, and, more importantly, reduced energy consumption. Second, the slowed rate of uptake prolongs relaxation but reduces the rate of ATP utilization. Thus, while force production is compromised, energy consumption is reduced to a level that more closely matches the rate of replenishment.

Most reports indicate that despite prolonged contractile activity, overall ATP levels do not decline to any large extent (<20%) (see Williams & Klug, 1995). There is still enough present to support force. The counter to increased ATP utilization is reduced energy demand, namely decreased SR Ca^{2+} release, and decreased force production. Thus, it would appear that increased metabolic demand and SR function are somehow linked. The loss of force is due to the utilization of ATP being greater than ATP synthesis.

Researchers have shown that there is a link between muscle metabolism and altered SR Ca^{2+} handling. This suggests the possibility that a decrease in SR function may reduce contractile machinery activation and lower the energy demand on the muscle fiber, thus protecting it from energy depletion (Williams and Klug, 1995). Lees et al. (2001) found a close physical association between muscle glycogen, glycogen phosphorylase (GP) and the SR. They showed that fatiguing activity reduces this depot of glycogen and speculated that there may be a functional link between the SR and the contractile machinery. Two recent studies support this link. Batts (1997, 2002) showed that a reduction in glycogen in rested muscle altered SR function similar to that caused by exercise. By injecting an experimental group of rats with epinephrine and the control group with saline, it was shown that the epinephrine animals had 23% less glycogen and nearly double the amount of glucose as the controls. The epinephrine group also had a depressed calcium uptake rate and a significant

decreased Ca^{2+} ATPase activity. Similar results were found when muscle glycogen levels were lowered using combinations of diet and exercise (Batts, 1997, 2002). These studies indicate that low glycogen levels associated with decreased SR function may account for reduced SR function and muscle force loss during exercise.

Williams et al. (1998) demonstrated a fatigue-induced reduction in SR Ca^{2+} handling. This study utilized frog semitendinosus in two different forms, skinned fibers with the SR intact, and a homogenate fraction disrupting the SR and then reassembling the SR into vesicles. Williams et al. (1998) demonstrated that fatigue development was associated with intrinsic alterations of the functional properties of the SR and contractile machinery. These changes play an important role in force production and energy consumption because the SR and the contractile machinery were altered. However, the exact contributing factors to fatigue development remain to be seen.

Allen and Westerblad (2001) stated there are three cellular mechanisms that control force. These include the Ca^{2+} concentration that surrounds the myofiliaments, the sensitivity of the myofilaments to the Ca^{2+} concentrations, and the force produced by the cross-bridges as characterized by the maximum Ca^{2+}-activated force. Therefore, fatigue may not be caused by one mechanism but possibly by a combination of mechanisms.

Batts (1997, 2002) showed there is considerable damage done to the SR and contractile proteins that ultimately lead to reduced force output. Reduced force output effects energy use by the muscle.

AMPK ACTIVITY

During exercise, or other metabolically stressful situations, ATP is broken down allowing a large amount of AMP to accumulate in the cell. As a result, the AMPK enzyme (adenosine monophosphate-activated kinase) becomes activated.

AMPK is expressed in all mammalian cells and contains α, β, and γ subunits (Aschenbach et al., 2002). The α subunit has two forms, contains a kinase domain, and contributes to the AMP binding site (Winder and Hardie, 1999). Form 2 is more AMP dependent than Form 1. The β subunit also has two forms while the γ subunit may possibly exist in multiple forms (Winder and Hardie, 1999). Its activity is stimulated by cellular stresses that deplete ATP and increase AMP, such as exercise (Hardie and Hawley, 2001) as well as elevated intracellular Ca^{2+} (Wright et al., 2005). Winder and Hardie (1999) state that AMPK activation is accomplished by four mechanisms, the first being an allosteric activation of AMPK by AMP. The second is the binding of AMP to AMPK making it a better substrate for AMP kinase kinase (AMPKK). The third mechanism is the binding of AMP to AMPK making it a worse substrate for protein phosphatases. The final mechanism is the allosteric activation of AMPKK by AMP. These mechanisms combined increase AMPK activation greater than 200-fold and make the enzyme sensitive to small changes in the AMP concentration. Once the AMPK enzyme is activated, it turns on catabolic pathways and turns off several processes that consume ATP (Hardie and Hawley, 2001).

Some view AMPK as a "master metabolic switch" because it also plays a key role in other metabolic processes (Fujii et al., 2006; Hardie, 2004; Winder and Hardie, 1999). Specifically, AMPK increases glucose uptake by skeletal muscle during contractions by mobilizing the GLUT-4 receptors to the muscle membrane (see Figure 2). It also potentially

phosphorylates several key proteins to increase the rates of fat oxidation and glucose metabolism to buffer the ATP concentration in response to a low cellular energy charge (Hardie, 2004). It phosphorylates glycogen synthase and hormone sensitive lipase (Kemp et al., 1999). The actual target for phosphorylation by AMPK that triggers translocation of GLUT-4 to the plasma membrane is unknown (Hardie, 2004). It also inhibits enzymes involved in glycogen, fatty acid and cholesterol synthesis (Hardie, 2004). AMPK seems to be a fuel gauge that recognizes ATP depletion and limits further ATP utilization by anabolic pathways (Kemp et al., 1999). Kemp et al. (1999) reported that AMPK initiates a series of compensatory changes that maintain cellular ATP levels, and that a decreased PCr levels stimulate AMPK activity. It is important to note that the AMPK system responds to the AMP to ATP ratio, not simply the AMP level.

Mu et al. (2003) found that transgenic mice not expressing AMPK enzyme suffered several metabolic effects. These mice could not maintain adequate glycogen stores. The mice became lazy most likely because muscles were more easily fatigued. The investigators also showed that stimulatory effects of contraction on muscle glucose uptake were partially eliminated in the AMPK deficient mice and the effects of hypoxia effect were completely eliminated. The authors concluded that AMPK is critical for both long term glycogen storage and acute changes in glucose uptake that occur during periods of exercise and hypoxia. Thus, AMPK may also be responsible for many long-term adaptations of muscle, such as endurance training, and especially an increase in mitochondrial content and oxygen capacity (Hardie, 2004).

AICAR EFFECTS

The compound, AICAR, when injected in animal models, causes alterations in carbohydrate metabolism. These adaptations are similar to those caused by exercise and training, such as increased glucose transport (Aschenbach et al., 2002; Wright et al., 2004; Hayashi et al., 1998; Musi et al., 2001; Sakoda et al., 2002; Musi and Goodyear, 2003). AICAR is taken up by cells and phosphorylated by adenosine kinase to ZMP, a monophosphorylated nucleotide (Hardie and Hawley, 2001; Wright et al., 2004). ZMP mimics the effects of AMP on the AMPK cascade of events (Hardie and Hawley, 2001; Wright et al., 2004). AMP can exist in high concentrations when the cell has been incubated with AICAR. AICAR represents a way to activate AMPK without disturbing the ATP, ADP or AMP cellular levels (Aschenbach et al., 2002; Hardie and Hawley, 2001). Winder and Hardie (1999) found that, in addition, AICAR activates the AMPK enzyme by increasing the ZMP concentration. This produces kinetic changes in the muscle like those seen after exercise, including increased fatty acid oxidation and glucose uptake, and a decrease in maximal velocity and malonyl-CoA. In several studies AICAR has been shown to stimulate glucose transport in skeletal muscle. These findings led to the concept that AMPK activation mediates activation of glucose uptake by contractile activity (Wright et al., 2004).

The role of AMPK has been clarified with results from AICAR studies, specifically those using the rat model (Hardie and Hawley, 2001). Ideally, these results could be translated to AMPK metabolic disorders including diabetes (Hardie and Hawley, 2001). Diabetes creates an increase in glucose levels and, specifically in Type 2, a reduced insulin sensitivity of glucose metabolizing tissues (Hardie and Hawley, 2001). AMPK could potentially reverse many Type 2 diabetes abnormalities.

Hardie (2004) discussed the beneficial effects that exercise may have on development of Type 2 diabetes. This particular syndrome may be mediated by AMPK. As demonstrated in various animal models, such as obese rats, AICAR improves insulin resistance. This may lead to drug developments that activate AMPK en route to improving the Type 2 diabetes condition.

SUMMARY

Given the link between muscle energetics and SR function, some have suggested that increased energy expenditure and depressed SR function during exercise might be linked. Indeed, Batts (1997, 2002) showed that metabolic stress, using epinephrine administration, resulted in reduced SR Ca^{2+} uptake and ATPase activity. However, epinephrine evokes other alterations such as increased heart rate, respiratory rate and body temperature. It is possible that these changes confounded the direct effects of epinephrine on carbohydrate metabolism. This investigation will focus on the potential role of AMPK activation and SR function during exercise. It is proposed that imposing energy stress, using artificial activation of AMPK, will evoke metabolic stress resulting in changes in glycogen metabolism. If the hypothesis that metabolic stress contributes to altered SR Ca^{2+} transport during exercise is true, the AICAR treatment should evoke changes in SR function that are qualitatively similar to those caused by fatigue.

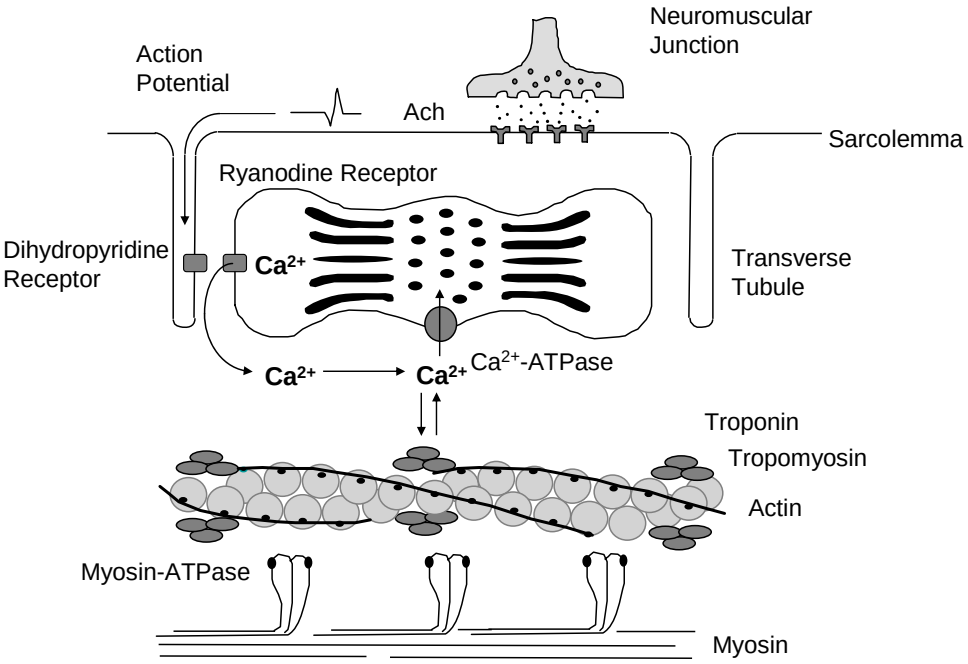

Figure 1. The steps involved in skeletal muscle contraction. Modified from Williams (1994).

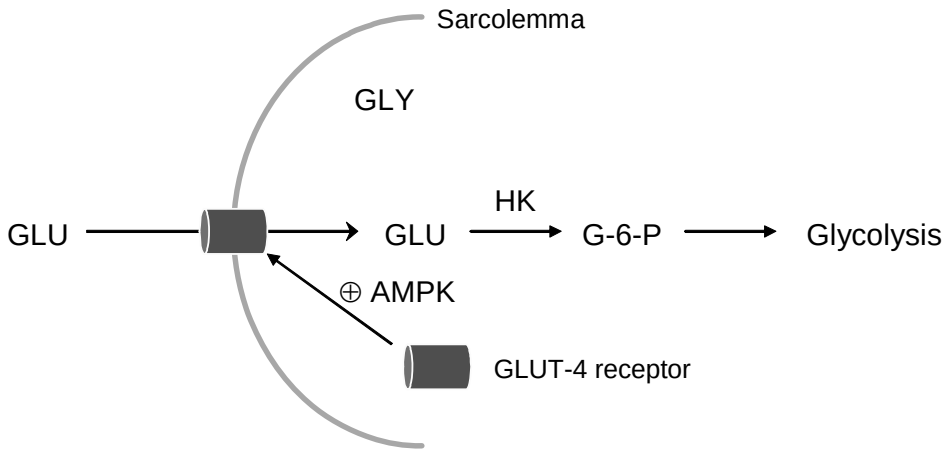

Figure 2. Schematic diagram showing the effects of AMPK activation on skeletal muscle glucose uptake.

CHAPTER THREE:

METHODOLOGY

RESEARCH DESIGN

Two studies were conducted. Study 1 examined the effects of in vivo AICAR administration on muscle metabolites and SR function. Study 2 focused on in vitro AICAR administration and fatigue on muscle metabolites and SR function.

STUDY 1 – IN VIVO ADMINISTRATION

Animals: All procedures used were approved by the Virginia Tech Animal Care Committee. Subjects used for this investigation were Swiss Webster mice (35-50g) housed 2 to 4 animals per cage. Animals were allowed standard rodent chow ad libitum. Subjects were assigned to either a control group or an experimental group each containing eight mice. Mice in the experimental group were injected with AICAR (0.85 mg/kg *i.p.*) from a 47.6 mg/ml stock saline solution. Control animals were injected with an equal volume of saline. All injections were given at approximately 9:00am each morning. Animals were then allowed to rest quietly for 60 minutes and then were then anesthetized by injecting 0.1ml of a 1.0ml solution containing 0.2ml ketamine, 0.1ml xylazine, and 0.7ml saline.

Tissue Processing: Once animals reached a surgical plane of anesthesia, both gastrocnemius muscles were removed from all 16 mice. One gastrocnemius muscle was placed in a 1.5ml ice-cold homogenizing buffer (20mM HEPES, 0.2% sodium azide, 0.2mM PMSF, and 1mM EDTA). It was minced with scissors and homogenized (3x15sec bursts) using a VirTishear homogenizer. Prior to homogenizing, the probe was chilled and the samples remained on ice throughout this procedure. The homogenate was centrifuged (1600xg) and the supernatant removed, frozen (-80ºC), and was stored for later analysis of SR function (Ca^{2+} uptake and release rates and ATPase activity). The contralateral muscle

was placed in labeled foil, immediately frozen in liquid N_2, and then freeze-dried for analysis of ATP, ADP, PCr, glycogen, glucose and G-6-P.

STUDY 2 – IN VITRO INCUBATION

Animals: Swiss-Webster mice (described above) were also used for Study 2. Mice were anesthetized as described above and both EDL muscles were surgically removed.

Tissue Processing: During the surgical procedure, EDL length was measured, in situ, with the ankle in "neutral" position (halfway between full plantar and dorsi flexion). Muscles were then removed and the tendons were tied, at resting muscle length, to glass capillary tubes (8-0 surgical silk). They were then incubated for 60 minutes in physiological saline solution which contained 120.5mM NaCl, 4.8mM KCl, 1.2mM $MgSO_4$, 20.4mM $NaHCO_3$, 1.6mM $CaCl_2$, 1.2mM NaH_2PO_4, 1.0mM pyruvate and 5.0mM dextrose (95% O_2 / 5% CO_2, 37°C). For one muscle of each pair, the above solution contained AICAR (4mM). After a 60 minute incubation period, muscles were removed, placed in ice-cold homogenizing buffer and homogenized as described above. The homogenate was then centrifuged at 1600xg for 15 minutes. The supernatant was then stored at -80°C for later analysis of SR Ca^{2+} uptake and ATPase activity. In a separate group of animals, EDL muscles were incubated then freeze dried for analysis of ATP, ADP, PCr, glycogen, glucose and G-6-P.

For comparison, EDL muscles were removed from the animals and mounted between a fixed post and an isometric force transducer in a temperature controlled incubation chamber (37°C) containing the incubation solution described above. One muscle of the pair was stimulated to contract using 100ms trains of pulses (80Hz) delivered at one per second for 5

minutes. The contralateral muscle served as the rested control. Immediately after stimulation, each muscle was removed, placed in ice-cold homogenizing buffer and homogenized as described above. The homogenate was then centrifuged at 1600xg for 15 minutes. The supernatant was then stored at -80°C for later analysis of SR Ca^{2+} uptake and ATPase activity. In a separate group of animals, muscles were incubated then freeze dried for analysis of ATP, ADP, PCr, glycogen, glucose and G-6-P.

SPECIFIC METHODS

Metabolite Measurements: ATP, ADP and PCr measurements were made using high-performance liquid chromatography (HPLC). A portion of each freeze-dried muscle was weighed, minced, and then homogenized in 5 volumes of ice-cold perchloric acid (PCA). After incubating the samples on ice for 20 minutes, the pH was neutralized with KOH. Samples were then centrifuged at 1600xg to sediment proteins and precipitated KCl. HPLC analysis was carried out using a modification of the approach described by Bernocchi et al. (1994) using a Waters HPLC system equipped with a diode array detector. Separations were done on a Suplechem C18 3µm reversed phased column (0.46x15cm) with an injection volume of 20µl. The mobile phase consisted of a gradient using two buffers. The first buffer (buffer A) contained 0.1M KH_2PO_4, 5mM tetrabutylammonium hydrogen sulfate (TBAHS) and 2.5% (v/v) acetonitrile (pH, 6.0). The second buffer (buffer B) contained 0.1M KH_2PO_4, 5mM tetrabutylammonium hydrogen sulfate (TBAHS) and 25% (v/v) acetonitrile (pH, 5.5). The column was eluted with buffer A for 3 minutes, for 2 minutes with buffer A and buffer B (steadily increasing to 11%), for 25 minutes with a steady increase to 100% buffer B. The column was re-equilibrated for 20 minutes with 100% buffer A. Flow was

maintained at 0.8ml•min^{-1} and separations were performed at room temperature. Detections of ADP and ATP were done at 260nm and PCr at 205nm. Peaks were identified using coelution with standards (see Figure 3) and quantified against injected standards solutions. A typical chromatogram recorded at 260nm using ATP and ADP standards is shown in 3. Typical sets of calibration data are shown in Figures 4 and 5.

Muscle glycogen, glucose and G-6-P levels were determined by the glucoamylase method described by Keppler and Decker (1984). SR glycogen was determined using an approach modified from the total glycogen measurement methods as described by Lees et al. (2001). In both cases, glycogen was hydrolyzed by glucoamylase (EC 3.2.1.3). Glucose was then measured spectrophotometrically using an NADPH absorbance wavelength of 340nm.

Sarcoplasmic Reticulum Function: Sarcoplasmic reticulum function was determined by examining SR calcium uptake and release rates. To determine the calcium uptake and release rates, 250µg of sample protein was added to 1.5ml of buffer (100mM KCl, 20mM HEPES, 5mM $MgCl_2$, 5mM KH_2PO_4, 2mM ATP and antiparylazo III, pH. 7.0, 37°C). Uptake was initiated by adding 1.2µmol·mg^{-1} $CaCl_2$ and was allowed to continue until free [Ca^{2+}] in the cuvette declined to a plateau. At this point, 250µM $AgNO_3$ or 500µM suramin was added to stimulate release. APIII absorbance was determined by subtracting the absorbance at 790nm from that at 710nm. Values were converted to free Ca^{2+} concentrations against a standard curve using known amounts of free Ca^{2+}. Peak rates of uptake and release were determined as the steepest positive and negative slopes of the free [Ca^{2+}] time curves. A typical recording of an uptake and release experiment is shown in Figure 6 and APIII calibration in Figure 7.

Ca^{2+} ATPase activities were determined using a coupled-enzyme assay (Luckin et al., 1991). Samples (250µg protein) were incubated in buffer containing 25mM HEPES, 100mM KCl, 10mM $MgCl_2$, 1mM EGTA, 0.2% NaN_2, 2µM A23187, 5U·ml^{-1} lactate dehydrogenase, 7.5U·ml^{-1} pyruvate kinase, 3.0mM phophoenolpyruvate and 0.6mM NADH (pH 7.0, 37°C). Samples were then placed in a diode array spectrophotometer (Aligent) and the temperature set to 37°C. The disappearance of NADH was monitored continuously at 340nm and converted to ATPase activity. The reaction was started with ATP and basal activity recorded for 3 minutes. Ca^{2+} was then added (2.0µM free Ca^{2+}) and total activity was recorded for an additional 3 minutes. Ca^{2+}-stimulated activity was computed by subtracting basal activity from total activity.

Protein Measurements: Sample protein was determined by the Bradford protocol as described by BioRad. Bovine serum albumin (BSA, 1mg·ml^{-1}) served as the standard (10-50 µg). The assay solution was a dilution of the BioRad stock of 1 part stock to 4 parts water. Standards and samples (total volume of 40µl) was added to each cuvette then allowed to incubate for 20 minutes. Absorbance values (460nm) were read and protein amounts calculated against the standard curve.

SDS-PAGE: The content of the Ca^{2+} ATPase was quantified using sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed following the method of Laemmli (1970). Each lane was loaded with 10 µg of protein and run on a mini-Protean II cell from Bio-Rad with a 5% separating and a 4% stacking gel. The running conditions were set at 45mA (constant) until the tracking dye ran off the gel. Gels were stained overnight in a solution containing: 0.1% Coomassie blue R-250, 40% methanol, and 10% acetic acid. After staining, the gels were destained for ~1 h in a solution containing

50% H_2O, 40% methanol, and 10% acetic acid. The bands corresponding to the molecular weight of the SR Ca^{2+}-ATPase were scanned using MultiImage Light Cabinet and analyzed using an AlphaImager 2000 Documentation and Analysis System (Alpha Innotech). From these scanned images, optical densities of these bands were determined.

STATISTICS

Differences in each parameter between the two conditions (control vs. AICAR treated) were determined using both non-paired (Study 1) and paired (Study 2) t-tests. The level of significance was set at $p<0.05$.

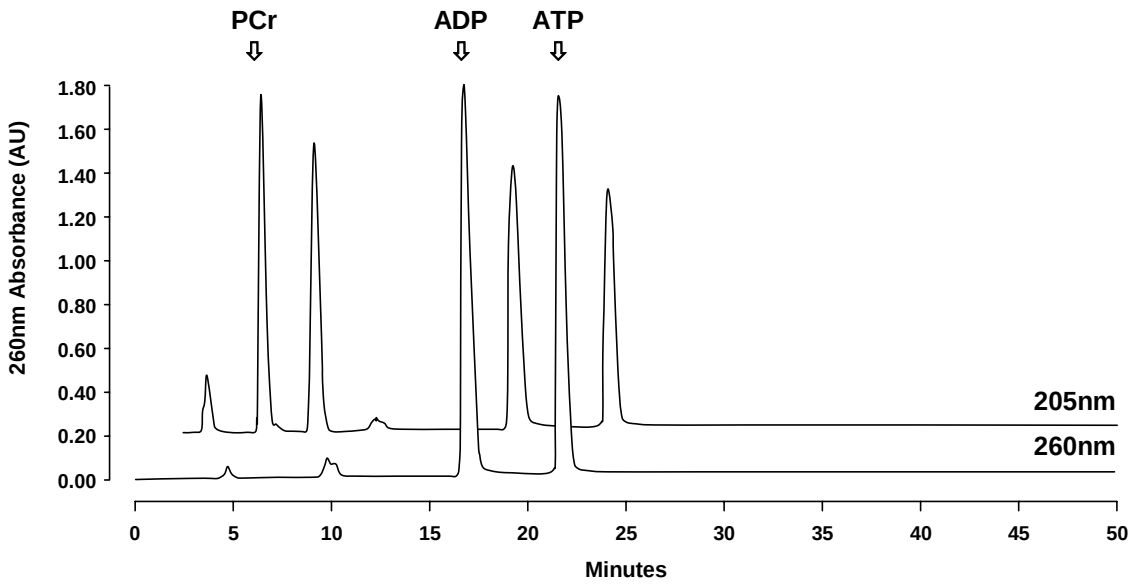

Figure 3. Separation of ATP, ADP and PCr by HPLC. This example shows 2.5 µmol ADP and ATP standards. For clarity, the 205nm chromatogram is shifted up and 2.5 minutes to the right.

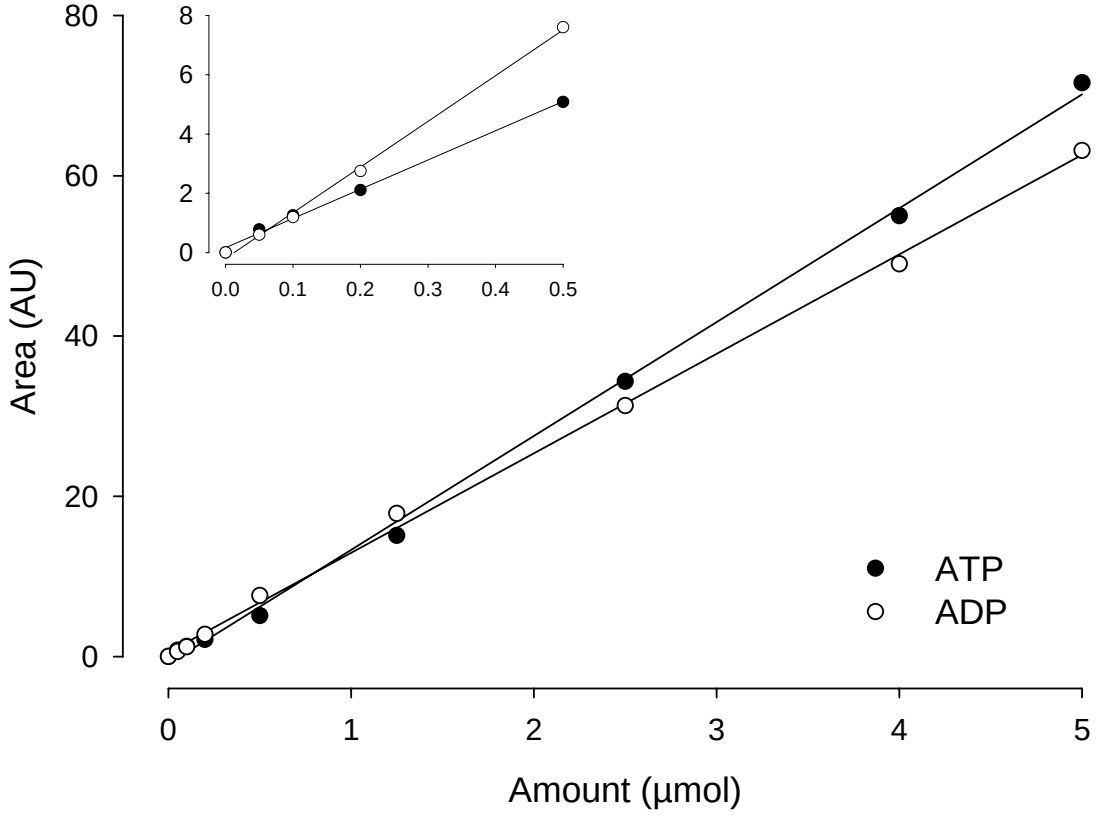

Figure 4. A typical set of calibration data. The relationships between chromatogram area and the amount of ATP and ADP added during calibration. *Inset:* Enlargement showing calibration using ≤ 0.5 µmol.

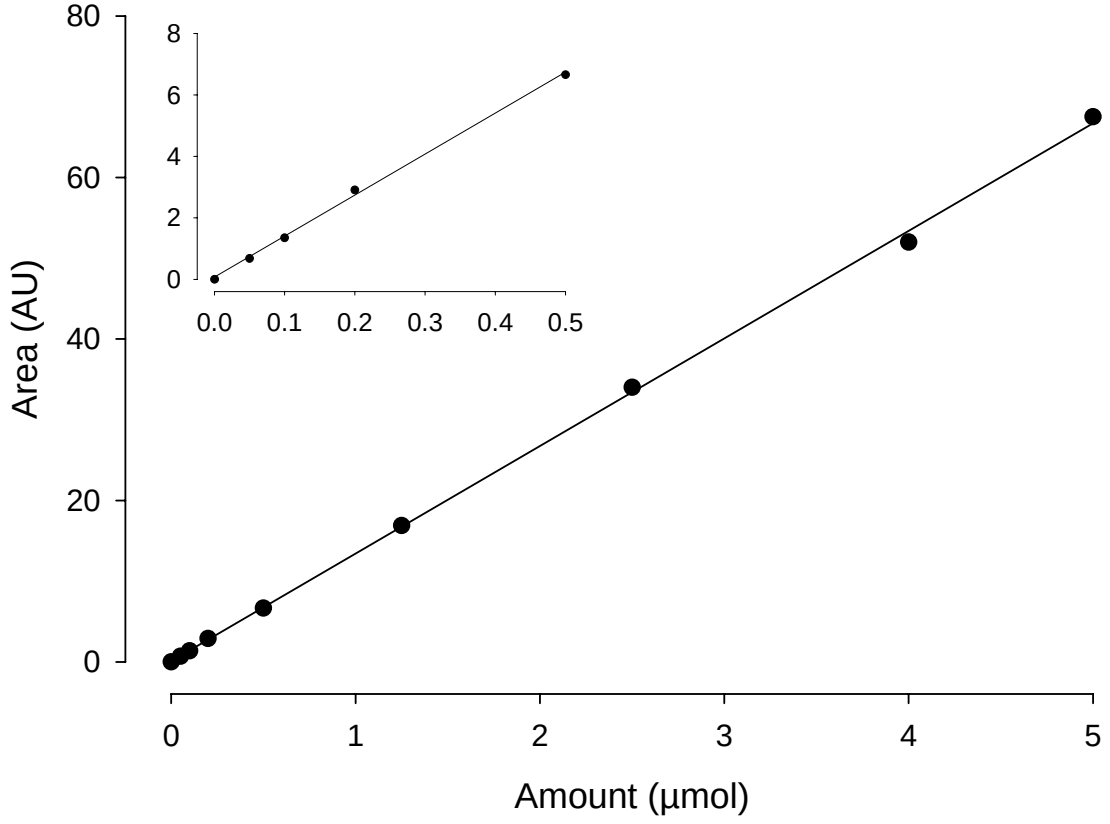

Figure 5. A typical set of calibration data. The relationships between chromatogram area and the amount of PCr added during calibration. *Inset:* Enlargement showing calibration using ≤ 0.5 µmol.

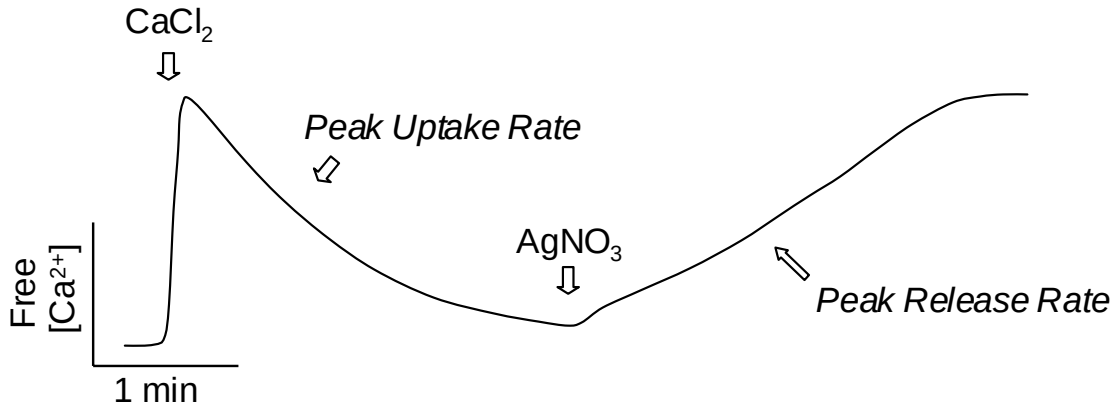

Figure 6. Typical recording during the measurement of SR Ca^{2+} uptake and release using the mouse gastrocnemius homogenate fraction.

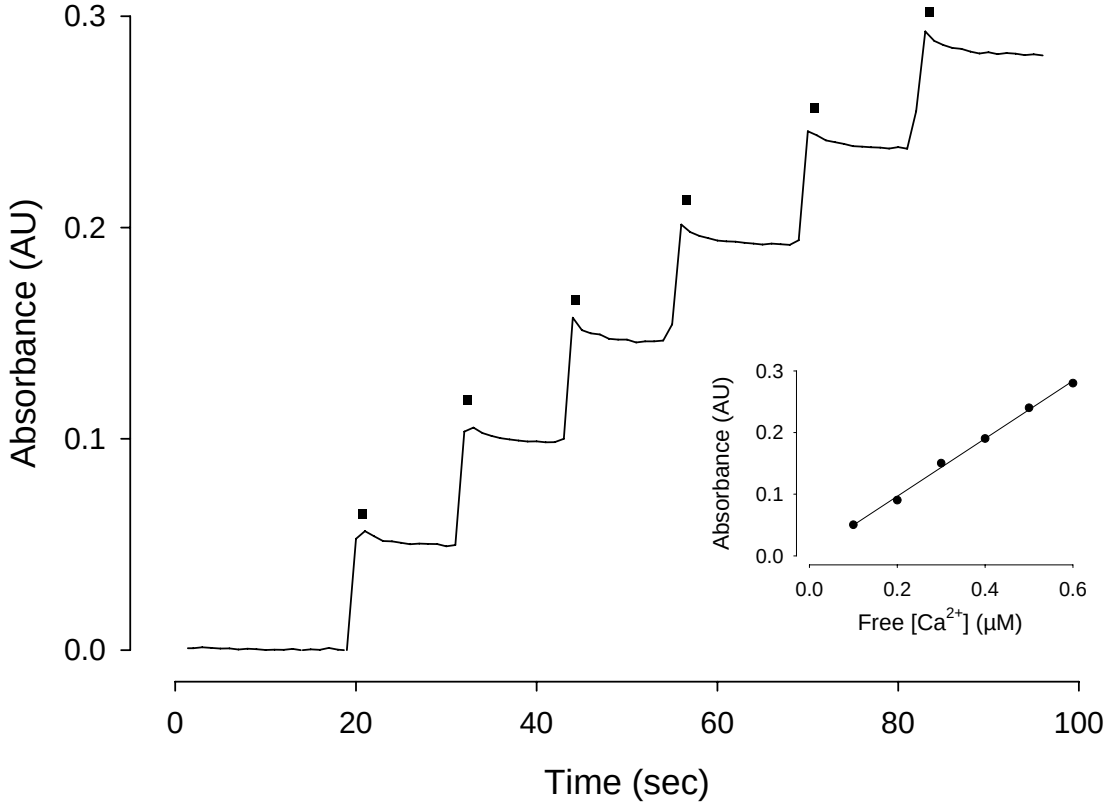

Figure 7. A typical set of APIII calibration data. Pulses of $CaCl_2$ were added at the indicated times (·). *Inset:* Calibration curve generated from the raw data.

CHAPTER FOUR:

RESULTS

STUDY 1 – IN VIVO ADMINISTRATION

Tissue Masses: AICAR treatment did not affect muscle masses. Wet mass for control and AICAR animals were 3.53±0.24 and 3.82±0.23 mg·g body mass $^{-1}$ ($p>0.05$), respectively. Dry masses were 0.88±0.06 and 0.95±0.04 mg·g body mass $^{-1}$ ($p>0.05$), respectively.

Metabolite Levels: In animals injected with AICAR, neither muscle glucose nor glycogen contents were significantly altered by AICAR treatment but G-6-P content was increased by 137% (Table 1). Contents of high-energy phosphate compounds (ATP, ADP and PCr) were not altered by AICAR treatment. From the HPLC analyses, a peak corresponding to AMP was noticeably elevated in the AICAR treated samples. It is possible that this peak represents the AICAR-induced accumulation of ZMP. However, the exact nature of this peak is not known.

SR Ca^{2+} Uptake and Release Rates: SR Ca^{2+} handling was modified by AICAR treatment. Peak SR Ca^{2+} uptake rate was reduced by 36% in the experimental group (Figure 8). SR Ca^{2+} release rates induced by $AgNO_3$ and suramin were both depressed by approximately 13-15%. In addition, Ca^{2+}-stimulated ATPase activity was reduced in the AICAR treated animals by 42% (Figure 9). Basal activity was not affected.

Changes in Ca^{2+} handling rates could not be explained by changes in the amount of Ca^{2+} pump or release channel proteins. SDS-PAGE showed that SERCA content was not different between control and AICAR treated animals (0.96±0.08 vs 0.98±0.13 AU, $p>0.05$).

SDS-PAGE also showed that glycogen phosphorylase (GP) content remained consistent with AICAR treatment (0.96±0.08 vs 0.98±0.04 AU, $p>0.05$) (Figure 10).

STUDY 2 – IN VITRO INCUBATION

Effects of AICAR Treatment: The effects of AICAR administered to EDL muscles in vitro on metabolite levels are shown in Table 2. The changes in all six metabolites were qualitatively similar to those changes observed after in vivo AICAR administration. There were no changes in muscle glycogen or glucose contents but there was a significant elevation of G-6-P levels (200%). Glucose, ATP, ADP and PCr were not significantly altered.

In vitro AICAR treatment also altered the rates of SR Ca^{2+} handling (Figure 11). The peak rate of uptake was depressed by 28% and the peak rate of Ag-induced release was lowered by 16%. These reductions were similar in magnitude and direction to those changes found following in vivo AICAR treatment. Due to the small size of the EDL muscle, not enough sample was available to measure suramin-induced release. Likewise, Ca^{2+} ATPase activities could not be measured due to limited sample availability.

Effects of Fatiguing Stimulation: Electrical stimulation caused large changes in muscle metabolite levels (Table 3). As can be seen glycogen and PCr were significantly reduced while ADP, glucose and G-6-P levels were elevated ($p<0.05$). ATP content was not significantly altered. By comparison, the increase in G-6-P was much larger in the stimulated muscles that in the AICAR treated muscles. This was the case when comparing the stimulated muscles with either the AICAR treated EDL or gastrocnemius muscles.

Fatigue also induced large changes in the rates of SR Ca^{2+} uptake and release. The peak rate of uptake was reduced by 31% and the peak rate of release was depressed by 41%. The change in uptake rate following stimulation was comparable to either the EDL or gastrocnemius muscles treated with AICAR. The rate of Ca^{2+} release was noticeably greater in the stimulated muscles than in either of the AICAR treated muscles (Figure 12).

Table 4 and Figure 13 show the relative chances in glycogen, G-6-P as well as SR Ca^{2+} uptake and release rates due to AICAR treatment and stimulation. As can be seen, stimulation caused more pronounced changes in glycogen, G-6-P and Ca^{2+} release rate but similar changes in the rate of Ca^{2+} uptake.

Metabolite	Control	AICAR
Glycogen	176.01 ± 7.79	170.08 ± 7.53
Glucose	22.28 ± 1.11	24.89 ± 2.93
G-6-P	0.92 ± 0.05	2.18 ± 0.07 *
ATP	22.03 ± 1.49	21.33 ± 2.42
ADP	5.09 ± 0.29	6.88 ± 0.78
PCr	51.71 ± 3.63	52.39 ± 2.84

Values are expressed in μmol/mg dry mass. *p<0.05 versus control.

Table 1. Metabolite levels in gastrocnemius muscles of control and AICAR treated animals in vivo (Study 1).

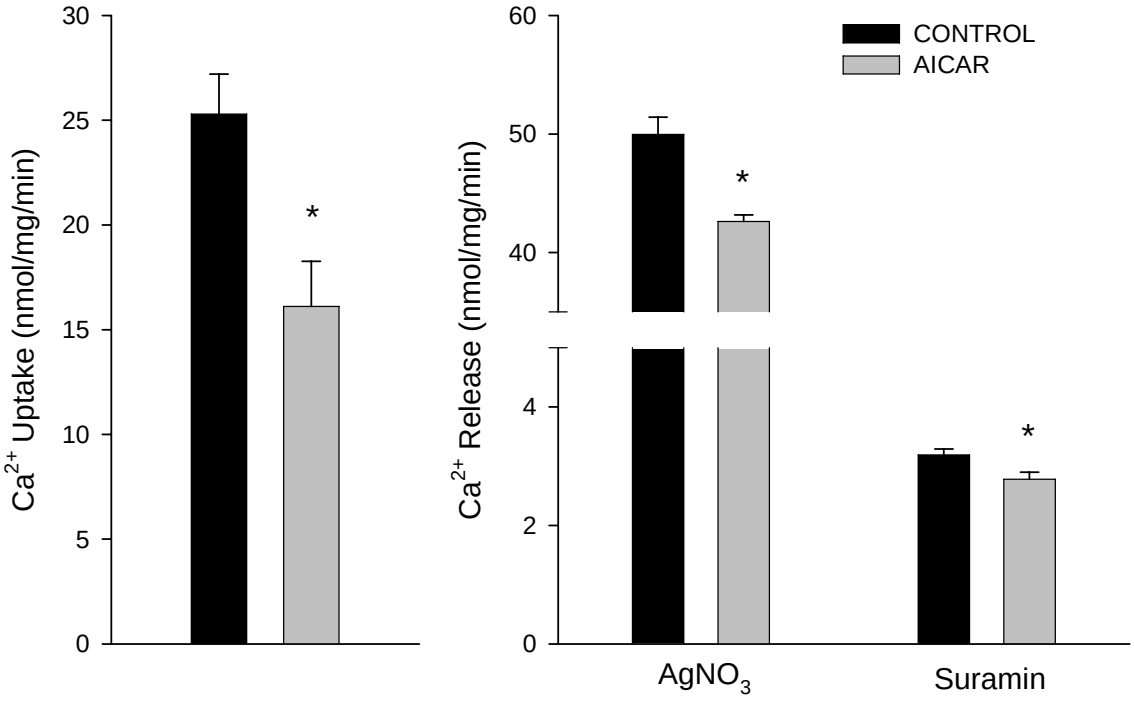

Figure 8. Peak rates of in vivo gastrocnemius Ca^{2+} uptake as well as peak $AgNO_3$- and suramin-stimulated Ca^{2+} release rates ($p<0.05$ between conditions).

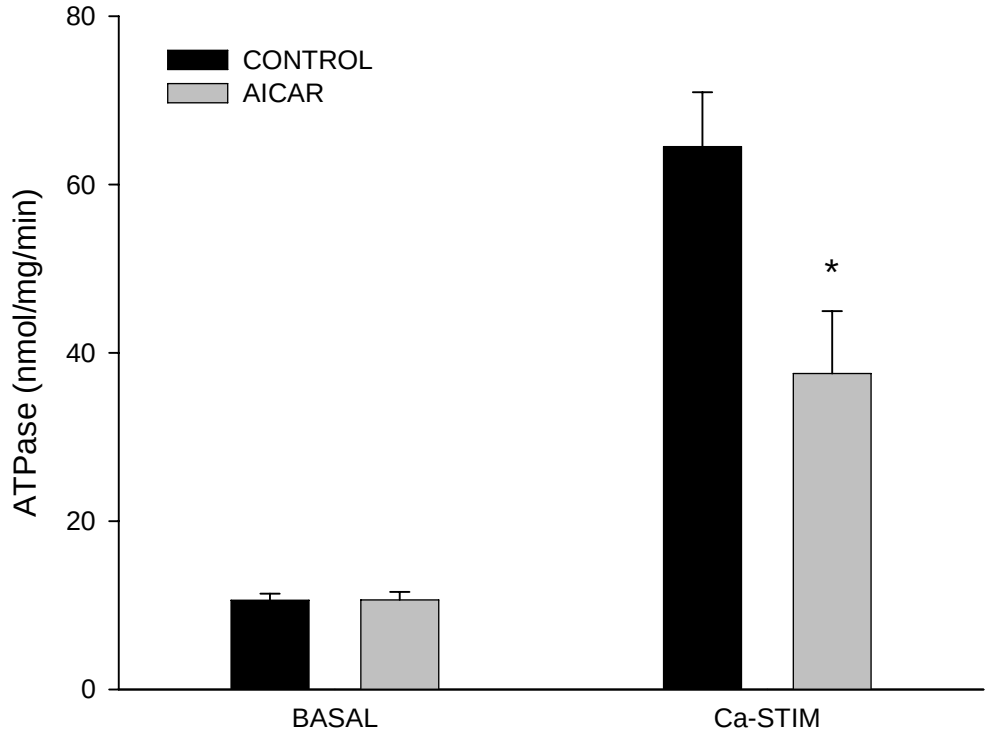

Figure 9. Basal and Ca^{2+}-stimulated (Ca-STIM) Ca^{2+} ATPase activities in control and AICAR treated animals in vivo (*$p<0.05$ between conditions).

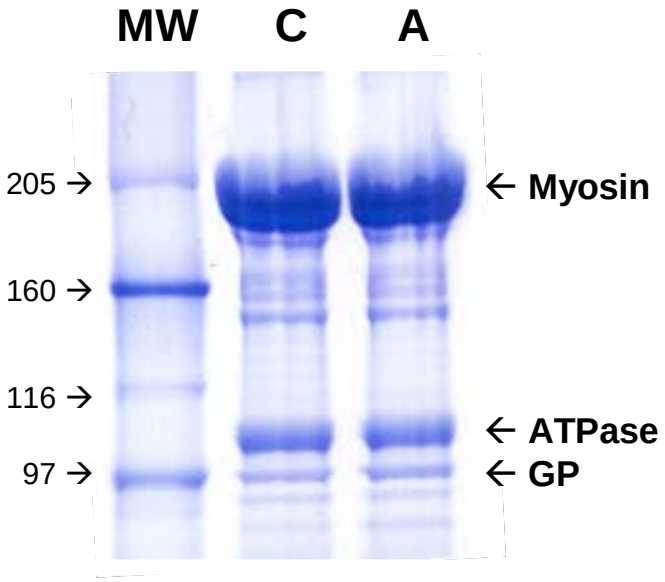

Figure 10. SDS-PAGE showing the relative amounts of the Ca^{2+} ATPase and glycogen phosphorylase (GP) in the gastrocnemius homogenate fractions in vivo (Study 1) (Lees et al., 2001). MW, molecular weight markers; C, control; A, AICAR treated.

Metabolite	Control	AICAR
Glycogen	129.06 ± 4.63	123.53 ± 6.69
Glucose	21.66 ± 3.79	23.07 ± 3.09
G-6-P	0.92 ± 0.19	2.79 ± 0.19 *
ATP	24.19 ± 1.49	23.82 ± 2.70
ADP	4.28 ± 0.29	3.90 ± 0.61
PCr	57.29 ± 2.72	60.96 ± 1.70

Values are expressed in μmol/mg dry mass. *p<0.05 versus control.

Table 2. Metabolite levels in EDL muscles following incubation in 4mM AICAR in vitro (Study 2).

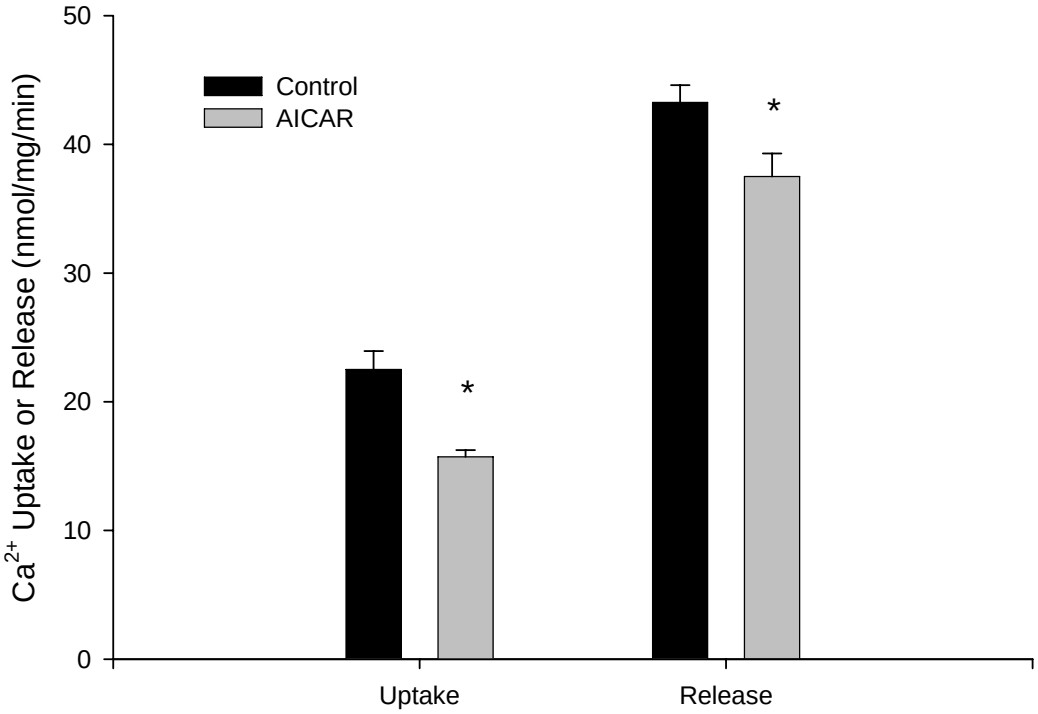

Figure 11. The effect of in vitro EDL incubation on SR Ca^{2+} uptake and $AgNO_3$-induced Ca^{2+} release rates.

Metabolite	Rested	Fatigued
Glycogen	115.77 ± 9.14	22.67 ± 2.67 *
Glucose	14.67 ± 1.02	27.28 ± 1.57 *
G-6-P	0.63 ± 0.12	11.12 ± 1.00 *
ATP	25.95 ± 1.07	23.88 ± 1.09
ADP	2.49 ± 0.13	4.00 ± 0.32 *
PCr	65.71 ± 4.13	7.11 ± 1.36 *

Values are expressed in μmol/mg dry mass. *p<0.05 versus control.

Table 3. Metabolite levels in EDL muscles following in vitro stimulation to fatigue (Study 2).

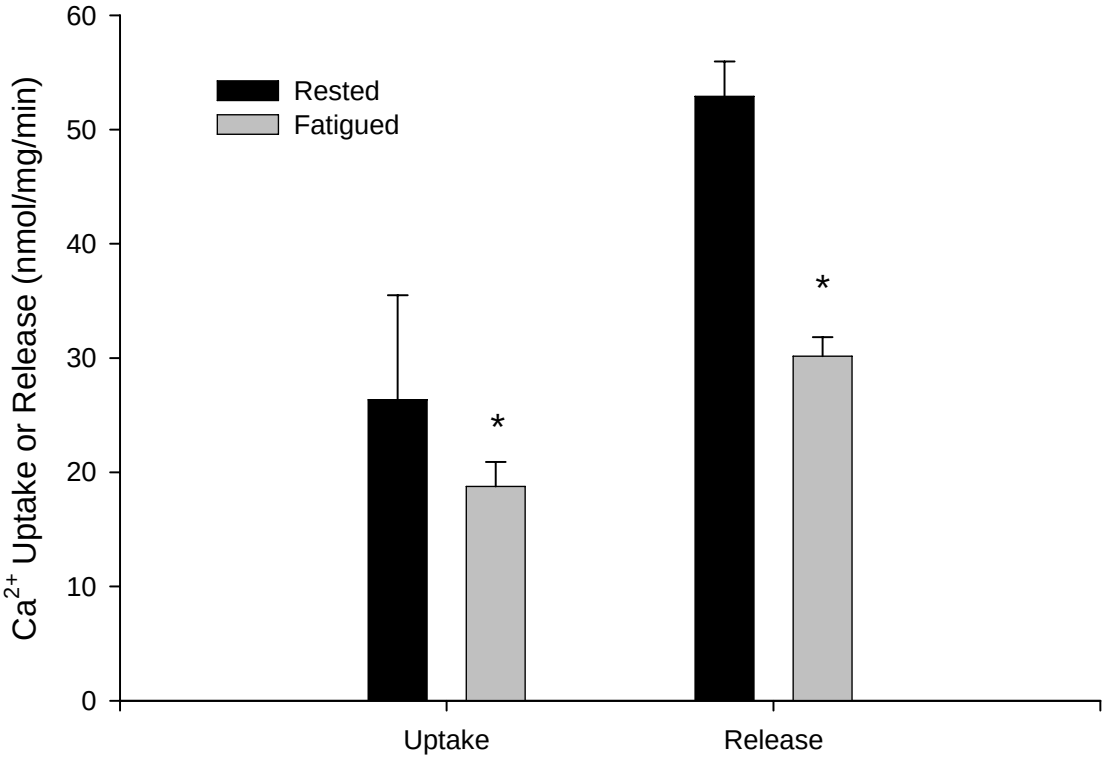

Figure 12. Effects of stimulation on SR Ca^{2+} uptake and $AgNO_3$-stimulated Ca^{2+} release rates in vitro (Study 2).

	In Vitro	In Vivo	Stimulation
Glycogen	ns	ns	-80.4
G-6-P	+137.0	+203.3	+1665.1

Table 4. Comparison of the effects of AICAR and stimulation on muscle glycogen and G-6-P concentrations. Values are expressed as the percent change compared to control.

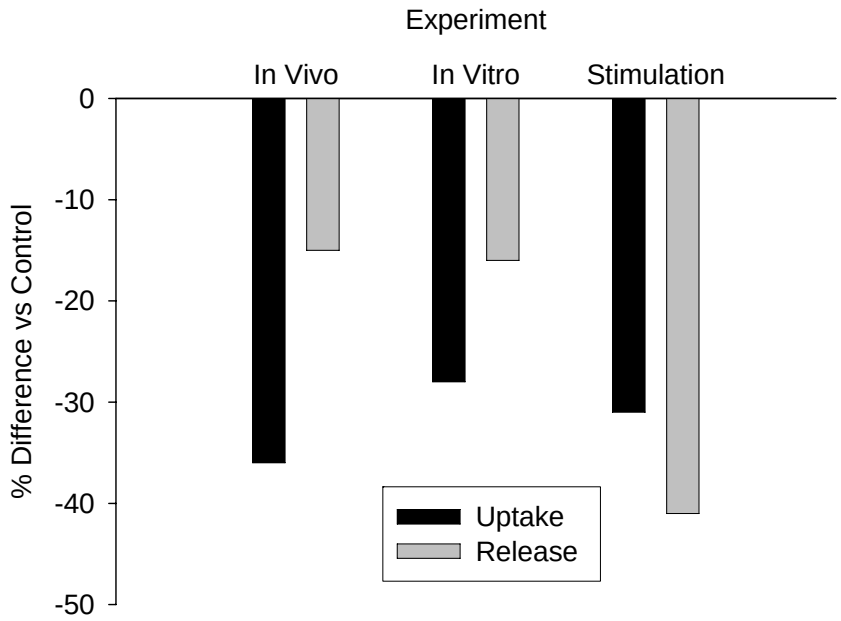

Figure 13. Comparison of the effects of AICAR treatment and stimulation on SR Ca^{2+} uptake and release rates.

CHAPTER FIVE:

DISCUSSION

MAJOR FINDINGS

The major finding of this investigation was that changes in SR function, as a result of the AICAR treatment, were qualitatively similar to those caused by fatiguing exercise. That is, the directions of change were similar but the magnitudes were somewhat different. AICAR is a compound that when injected into animal models is taken up by cells and phosphorylated by adenosine kinase to ZMP which activates AMPK. Thus, AICAR represents a way to activate AMPK without disturbing the ATP, ADP or AMP cellular levels (Aschenbach et al., 2002; Hardie and Hawley, 2001). According to Winder and Hardie (1999), AICAR produces metabolic changes in the muscle qualitatively similar to those resulting from exercise. Thus, the present results are consistent with the notion that metabolic stress during exercise affects SR function.

EFFECTS OF AICAR ON METABOLISM

Following both in vivo and in vitro administration of AICAR, G-6-P levels were increased without changes in other metabolites (i.e. ATP, ADP). Metabolite measurements are similar to other reports which have shown the effects of using acute AICAR administration. Aschenbach et al. (2002) showed large increases in G-6-P following AICAR injection. This was accompanied by no change in glycogen content 60 minutes post-injection (the time used in the present study). Only after 180 minutes following injection did glycogen content significantly increase. Several recent studies have shown similar results using in vivo and in vitro treatment (Miyamoto et al., 2007; Fediuc et al., 2006; Christopher et al., 2006).

AICAR has been shown to stimulate glucose uptake into skeletal muscle (Hayashi et al., 1998; Musi et al., 2001; Sakoda et al., 2002; Musi and Goodyear, 2003; Wright et al., 2004). The present finding of increased G-6-P content in the absence of reduced glycogen suggest that glucose uptake was increased in treated muscles in vivo and in vitro. It is likely that glucose entering the muscle is quickly converted into G-6-P via the hexokinase reaction. Aschenbach et al. (2002) also argued that the observed increase in blood lactate after AICAR treatment probably results from increased glucose uptake by muscle contributing to glycolytic flux as opposed to glycogen breakdown. Thus it appears that the major effect of AMPK activation by AICAR on carbohydrate metabolism is stimulation of glucose uptake rather than glycogen breakdown.

EFFECTS OF AICAR ON SR FUNCTION

Acute treatment with AICAR resulted in SR function changes that are qualitatively similar to those observed after fatiguing activity. Under both conditions of AICAR treatment and short-term stimulation, SR Ca^{2+} uptake and release rates as well as Ca^{2+} ATPase activities were reduced by approximately 15-35%. The reductions in SR function observed following stimulation compare well with what is reported to occur following stimulation or prolonged exercise (for review see Williams and Klug, 1995). Thus, it is evident that, under the conditions used in this study, acute AICAR treatment evokes changes in SR function that are qualitatively similar to those observed following fatiguing activity.

Batts (1997; 2002) showed similar results using an epinephrine treatment. Epinephrine evoked a metabolic stress by stimulating glycogen breakdown. This, in turn, affected SR function depressing both Ca^{2+} transport and Ca^{2+}-stimulated ATPase activity. In

these studies, epinephrine treatment resulted in significant decrease in muscle glycogen content which was accompanied by a large increase in G-6-P concentration. Thus is it likely that the metabolic effects of epinephrine differed somewhat from those of AICAR administration. Despite this, when viewed collectively, the present study and those of Batts (1997, 2002) suggest that a portion of the change in SR function that accompanies fatigue is due to the metabolic stress induced by contractile activity.

Terada et al. (2003) showed that incubation of rat epitrochlearis muscles in 2mM AICAR did not affect resting myoplsamic Ca^{2+} levels. They suggested that activation of AMPK did not affect net Ca^{2+} movement across either the sarcoplasmic or sarcolemmal membranes. However, it is important to point out that Terada et al. (2003) did not electrically stimulate the muscles. So, firm conclusions regarding SR Ca^{2+} release and uptake during activity cannot be made. Evans et al. (2005) showed that O_2 sensing cells (pulmonary arterial myocytes) responded to AICAR administration by increasing SR Ca^{2+} release. They suggest that activation of cyclic ADP ribose (cADPR) by AMPK mediated the release and that this mechanism is, in part, responsible for hypoxic vasoconstriction. However it should be noted that a key role for cADPR in skeletal muscle SR Ca^{2+} release remains questionable in that the actions of cADPR on Ca release in skeletal muscle is different from that described in other cells (Morrissette et al. 1993).

To date, no one has proposed a pathway linking AMPK activity and SR function. Unfortunately, the present results do not establish such a link. It is unlikely that the AICAR-induced decrease in SR function was the result of increased metabolite levels. The present results show that the high energy phosphate compounds ATP, ADP, and PCr were not altered by the AICAR treatment. Muscle glycogen levels remained constant and only G-6-P was

increased. G-6-P has been shown to directly influence SR function (Williams et al. 1998). In isolated SR vesicles, the addition of G-6-P (5mM) caused reduced SR Ca^{2+} release and uptake rates as well as induced a small Ca^{2+} release. Kermode et al. (1998) also showed that G-6-P caused a slight, but significant, stimulation of SR Ca^{2+} release channel activity. However, in the present study, the SR was removed from an environment where metabolic levels were altered and placed in one that more closely simulated a resting cell. Thus it is unlikely that the SR function change seen here resulted from the accumulation of G-6-P or other metabolites.

It is possible that one of the downstream targets of AMPK affects SR Ca^{2+} release and or uptake. Hardie et al. (2001; 2003; 2006) showed that AMPK activation has multiple downstream targets that have both metabolic and non-metabolic roles (Figure 14). It is possible that one or more of these targets influences SR function. For example, Fryer et al. (2000) show that AMKP stimulation by AICAR activates nitric oxide synthase (NOS) in H-2Kb cells (skeletal muscle). It is known that the production of nitric oxide directly inhibits both ryanodine receptor – mediated SR Ca^{2+} release and ATPase – stimulated Ca^{2+} uptake by the SR (Heunks et al., 2001; Viner et al., 2000). Also, several groups suggest that activation of AMPK in both skeletal and cardiac muscle inhibits creatine kinase (Neumann et al., 2002; Ponticos et al., 1998). AMPK is thought to phosphorylate CK which, in turn, limits its maximal activity. Several groups argue that CK function is critically important for proper function of the SR Ca^{2+} pump and Ca^{2+} sequestration (Korge and Campbell, 1994; Rossi et al., 1990; Dahlstedt et al., 2003; Kindig et al., 2005).

It is important to point out that the present data do not suggest that either of the mechanisms described above is responsible for the observed effects of AICAR on SR

function. However, the present study does suggest that there might be a link between AMPK activation and SR function. In support of this idea, Woollhead et al. (2005) have shown that AICAR depresses the Na/K ATPase in lung cells. Further, from a teleological standpoint, this seems plausible. SR function is directly responsible for nearly all of the energy consumed by exercising muscle. SR Ca^{2+} release activates the contractile machinery which accounts for nearly 2/3 of total energy consumption while the SR Ca^{2+}pump is responsible for the remaining 1/3 (Rall, 1982; Lou et al., 1997). Several have suggested that AMPK acts as a "metabolic thermostat" (Fujii et al., 2006; Hardie 2004; Winder and Hardie, 1999). Under times of metabolic stress, activation of AMPK stimulates the ATP synthesis pathways while inhibiting ATP utilization (Hardie 2004). It seems reasonable to suggest that one target of AMPK would be the SR. By inhibiting SR Ca^{2+} release and uptake during activity, the utilization of ATP can be curtailed and ATP stores maintained. Unfortunately, confirmation of such a mechanism is lacking.

CONCLUSIONS

Both in vivo and in vitro treatment with AICAR induced metabolic stress in skeletal muscle. This was associated with depressed SR function. The rates of Ca^{2+} uptake, release and ATPase activity were all reduced compared to untreated muscles. These changes were qualitatively similar to those evoked by fatiguing activity. AICAR evokes metabolic changes via activation of AMPK. While the present results do not identify a specific pathway linking AMPK activity and SR function, they do support that idea that changes in SR function during activity are due, in part, to metabolic stress. During maximal exercise the rate of ATP utilization exceeds replenishment. It appears that muscle cells are able to sense

this compromised energy state, transmit a signal to activate alternate pathways of ATP production while inhibiting ATP consumption. Ultimately the cell is able to compensate for this condition of metabolic stress and avoid irreversible skeletal muscle damage. It remains to be seen what role, if any, the SR AMPK link plays in this process.

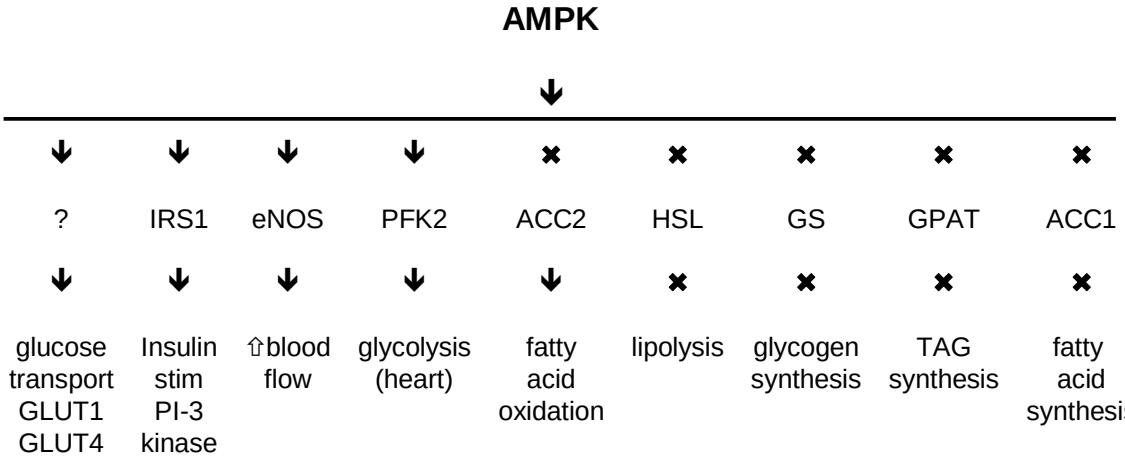

Figure 14. Potential downstream targets of AMPK activation. Modified from Hardie (2003).

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CHAPTER SEVEN:

APPENDICES

APPENDIX A: Raw Data for in vivo gastrocnemius treatment (Study 1) – Morphology

	Animal Mass (g)	Wet Mass (mg)	Dry Mass (mg)	Wet Mass (mg/g)	Dry Mass (mg/g)
CONTROL					
1	48.6	139.60	34.90	4.28	0.72
2	47.6	189.20	47.30	2.93	0.99
3	44.4	190.00	47.50	3.32	1.07
4	50.2	147.20	36.80	2.87	0.73
5	49.4	188.80	47.20	3.98	0.96
6	51.3	170.50	42.70	3.82	0.83
Mean	**48.57**	**170.88**	**42.73**	**3.53**	**0.88**
SEM	**0.98**	**9.24**	**2.31**	**0.24**	**0.06**
AICAR					
1	37.64	123.6	30.90	3.356	0.82
2	44.22	173.2	43.30	4.002	0.98
3	50.45	212.0	53.00	3.412	1.05
4	43.28	148.4	37.10	3.284	0.86
5	49.04	203.6	50.90	4.152	1.04
6	44.84	172.1	43.00	4.727	0.96
Mean	**44.91**	**172.15**	**43.03**	**3.82**	**0.95**
SEM	**1.86**	**13.54**	**3.38**	**0.23**	**0.04**
p	**0.113**	**0.940**	**0.943**	**0.408**	**0.366**

APPENDNIX B: Raw Data for in vivo gastocnemius treatment (Study 1) – Metabolites

	Glycogen (µmol/mg)	Glucose (µmol/mg)	G-6-P (µmol/mg)	ATP (µmol/mg)	ADP (µmol/mg)	PCr (µmol/mg)
CONTROL						
1	162.31	16.5	0.97	26.04	5.81	51.7
2	195.03	21.59	1.03	22.79	3.73	67.87
3	184.72	20.53	1.06	19.29	5.15	44.33
4	144.11	23.94	0.73	16.51	5.33	54.65
5	181.96	23.86	0.85	25.51	5.42	44.55
6	187.90	21.3	0.86	22.01	5.08	47.16
Mean	**176.01**	**21.29**	**0.92**	**22.03**	**5.09**	**51.71**
SEM	**7.79**	**1.11**	**0.05**	**1.49**	**0.29**	**3.63**
AICAR						
1	183.56	32.44	2.19	21.33	6.00	54.31
2	174.39	19.02	1.91	19.71	6.88	53.93
3	148.22	18.73	2.03	31.82	6.85	52.30
4	164.60	34.81	2.23	22.57	5.24	42.60
5	198.23	19.44	2.39	18.58	5.79	47.89
6	155.80	24.9	2.32	13.95	10.54	63.34
Mean	**170.80**	**24.89**	**2.18**	**21.33**	**6.88**	**52.39**
SEM	**7.53**	**2.93**	**0.07**	**2.42**	**0.78**	**2.84**
p	**0.641**	**0.277**	**0.000**	**0.811**	**0.055**	**0.885**

APPENDIX C: Raw Data for in vivo gastrocnemius treatment (Study 1) – SR Function

	Uptake (mol/mg/min)	Ag Release (mol/mg/min)	Sur Release (mol/mg/min)	B-ATPase (mol/mg/min)	Ca-ATPase (mol/mg/min)	Ca-ATPase (AU)
CONTROL						
1	19.97	49.96	2.96	9.50	41.62	0.89
2	23.84	55.73	3.28	10.95	58.16	0.65
3	24.99	44.97	3.08	7.24	73.86	1.02
4	23.63	51.25	3.60	12.41	60.21	1.17
5	34.04	49.55	2.98	11.69	88.66	1.15
6	25.30	48.32	3.22	11.74	64.50	0.89
Mean	**25.30**	**49.96**	**3.19**	**10.59**	**64.50**	**0.96**
SEM	**1.91**	**1.45**	**0.10**	**0.78**	**6.46**	**0.08**
AICAR						
1	16.12	42.35	2.90	10.27	12.13	1.17
2	14.79	42.00	3.19	8.43	36.81	0.99
3	16.53	45.23	2.83	10.63	62.20	0.96
4	8.15	42.62	2.38	8.14	52.19	0.99
5	16.36	42.38	2.88	14.32	37.55	0.86
6	24.71	41.16	2.47	12.02	24.44	0.89
Mean	**16.11**	**42.62**	**2.78**	**10.63**	**37.55**	**0.98**
SEM	**2.15**	**0.56**	**0.12**	**0.94**	**7.39**	**0.04**
p	**0.010**	**0.001**	**0.026**	**0.970**	**0.021**	**0.876**

APPENDIX D: Raw Data for in vitro EDL incubation (Study 2) – Metabolites

	Glycogen (µmol/mg)	Glucose (µmol/mg)	G-6-P (µmol/mg)	ATP (µmol/mg)	ADP (µmol/mg)	PCr (µmol/mg)
CONTROL						
1	144.18	26.16	0.74	27.68	3.77	49.60
2	136.11	22.72	1.17	21.06	5.63	63.48
3	127.77	5.21	0.90	22.88	4.22	67.04
4	120.02	29.66	1.58	26.62	3.91	55.48
5	112.83	17.19	0.95	19.20	4.36	52.41
6	133.43	29.00	0.15	27.72	3.79	55.71
Mean	**129.06**	**21.66**	**0.92**	**24.19**	**4.28**	**57.29**
SEM	**4.63**	**3.79**	**0.19**	**1.49**	**0.29**	**2.72**
AICAR						
1	149.19	24.36	2.87	12.75	5.35	55.17
2	115.76	29.00	1.93	30.98	4.80	63.08
3	116.57	29.84	2.76	29.92	3.38	60.88
4	111.06	10.25	3.30	23.98	5.32	66.78
5	138.77	18.09	3.01	24.15	2.94	62.40
6	109.82	26.89	2.87	21.13	1.61	57.44
Mean	**123.53**	**23.07**	**2.79**	**23.82**	**3.90**	**60.96**
SEM	**6.69**	**3.09**	**0.19**	**2.70**	**0.61**	**1.70**
p	**0.512**	**0.778**	**0.000**	**0.906**	**0.590**	**0.279**

APPENDIX E: Raw Data for in vitro EDL incubation (Study 2) – SR Function

	Uptake (mol/mg/min)	Ag Release (mol/mg/min)
CONTROL		
1	23.92	42.23
2	24.40	44.15
3	24.55	46.12
4	25.23	38.20
5	20.81	41.57
6	16.15	47.21
Mean	**22.51**	**43.25**
SEM	**1.42**	**1.34**
AICAR		
1	14.36	40.93
2	16.03	43.04
3	15.54	39.99
4	17.54	35.21
5	16.50	33.91
6	14.37	31.95
Mean	**15.72**	**37.50**
SEM	**0.51**	**1.80**
p	**0.001**	**0.029**

APPENDIX F: Raw Data for Study 2 – Fatigue Metabolites

	Glycogen (µmol/mg)	Glucose (µmol/mg)	G-6-P (µmol/mg)	ATP (µmol/mg)	ADP (µmol/mg)	PCr (µmol/mg)
CONTROL						
1	114.93	15.09	0.69	27.85	2.27	69.38
2	135.22	16.27	0.67	26.73	3.12	74.32
3	137.84	14.22	0.51	20.76	2.47	60.33
4	121.70	14.58	1.05	25.98	2.30	79.04
5	108.60	10.24	0.73	27.15	2.49	53.00
6	76.34	17.60	0.13	27.20	2.28	58.19
Mean	**115.77**	**14.67**	**0.63**	**25.95**	**2.49**	**65.71**
SEM	**9.14**	**1.02**	**0.12**	**1.07**	**0.13**	**4.13**
STIMULATED						
1	25.84	27.67	11.07	22.93	3.13	3.40
2	29.53	26.85	9.08	20.61	3.75	8.81
3	14.85	33.63	11.72	28.12	5.02	8.98
4	23.80	28.68	8.90	25.09	3.29	10.74
5	28.19	24.19	10.37	24.63	4.02	2.55
6	13.81	22.67	15.56	21.88	4.82	8.19
Mean	**22.67**	**27.28**	**11.12**	**23.88**	**4.00**	**7.11**
SEM	**2.76**	**1.57**	**1.00**	**1.09**	**0.32**	**1.36**
p	**0.000**	**0.000**	**0.000**	**0.205**	**0.001**	**0.000**

APPENDIX G: Raw Data for Study 2 – Fatigue SR Function

	Uptake (mol/mg/min)	Ag Release (mol/mg/min)
CONTROL		
1	27.45	59.72
2	25.71	62.61
3	28.31	54.38
4	35.30	46.27
5	35.60	50.98
6	23.80	43.57
Mean	**29.36**	**52.92**
SEM	**2.03**	**3.04**
STIMULATED		
1	23.61	32.64
2	15.21	27.89
3	20.37	29.02
4	23.73	30.75
5	10.27	36.25
6	19.40	24.48
Mean	**18.77**	**30.17**
SEM	**2.13**	**1.66**
p	**0.005**	**0.000**

CHAPTER EIGHT:

VITA

VITA

May 21, 2007

Stacey Elizabeth Vidt was born July 24, 1980 to Dr. and Mrs. Louis G. Vidt. She grew up just outside of Pittsburgh, Pennsylvania and graduated from Hampton High School. Following a successful high school academic and athletic career, she arrived at Virginia Tech to pursue an undergraduate degree and further her running career. In May 2003, she graduated with a Bachelor of Science degree in Human Nutrition, Foods and Exercise and a minor in Chemistry.

The decision to remain at Virginia Tech and pursue a graduate degree was not a hard decision. Working with Dr. Jay Williams, she continued her studies working toward a Master of Science degree in Muscle Physiology. Nearing the end of her studies, she was hired as the Assistant Cross Country Coach at Virginia Tech, thus adding a full-time job in addition to her thesis writing. Although she had little time to spare, she continued to train for marathons and has successfully completed two. Following her thesis defense, she will research other career options hoping to find a job in or related to the field of muscle physiology, and of course, continue her training.