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The Effects of Aging on Skeletal Muscle AMPK Activation and an Analysis of Chronic AICAR Treatment on the Aging Phenotype

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ABSTRACT

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AMP-activated protein kinase (AMPK), a metabolic regulator, acts in opposition to many of the effects of aging and may provide insights into the development of sarcopenia. However, the effect of aging on AMPK activation is unclear. The purpose of this dissertation was to: 1) clarify the controversy concerning the activation of AMPK in response to endurance-like exercise in aged skeletal muscle; 2) address mechanisms for the age-associated alterations in AMPK activation; and 3) address the known benefits of chronic AICAR treatment in aged skeletal muscle.

First, to clarify the effect of age on AMPK activation, young adult (YA) (8 mo.) and old (O) (30 mo.) male Fischer$_{344}$ x Brown Norway F1 hybrid rats received an *in situ* bout of endurance-type contractions produced via electrical stimulation of the sciatic nerve (STIM). AMPK activation was attenuated in aging muscle as demonstrated by decreased AMPKα phosphorylation and AMPKα2 protein content and activity in O vs. YA muscle after STIM. In contrast, AMPKα1 content was greater in O vs. YA muscle, and α1 activity increased with STIM in O but not YA muscles.

Second, the effect of age on the AMPK heterotrimer composition and nuclear localization was assessed as mechanisms for the altered AMPK activation. The AMPK heterotrimer composition was altered in aging skeletal muscle with lower AMPKγ2 and γ3 content and decreased association of AMPKγ3 with AMPKα1 and α2. Furthermore, activation of AMPK is known to increase translocation of AMPK to the nucleus in YA muscle; however, translocation of phosphorylated AMPK, AMPKα2, and AMPKγ3 were impaired in the aging rat muscle after STIM.

Finally, chronic activation of AMPK with 5'-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is known to increase mitochondrial content, activate autophagy, and repress protein synthesis; pathways that are altered with aging. The known benefits of chronic AICAR treatment were assessed in YA (5 mo.) and O (23 mo.) male C57Bl/6 mice. Mice were treadmill tested prior to and after one month of AICAR treatment. *In vitro* muscle contractions were performed following AICAR treatment. AICAR treatment improved the O mice treadmill endurance and the YA mice rate of fatigue and recovery. Additionally, AICAR increased citrate synthase activity, decreased SQSTM1/p62 protein content , and decreased Myf6 protein content in both the YA and O mice suggesting increased mitochondrial activity, autophagy, and decreased muscle regeneration. Therefore, chronic AICAR treatment may alter metabolic pathways to improve the exercise response in both YA and O mice.

Keywords: AMPK, aging, skeletal muscle, heterotrimer, AICAR, metabolism

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CHAPTER 1: Review of the Literature

Metabolic Pathways are Altered with Sarcopenia

Sarcopenia, or age-related skeletal muscle atrophy, affects 20-50% of adults over the age of 60 years (Berger & Doherty, 2010b) and contributes to the decline of muscle mass and strength that leads to frailty with aging (Thompson, 1994; Verdijk *et al.*, 2010a). Age-related alterations in muscle fibers lead to decreased maximal isometric force during contraction and result in muscle weakness and frailty (Thompson, 1994). Sarcopenia and frailty significantly impact the quality of life of elderly individuals. Furthermore, frailty in the elderly is an indicator of an increased risk for disease, functional dependency, and/or death (Evans *et al.*, 2010). The increased risks associated with sarcopenia may be attributed to alterations in skeletal muscle metabolism and function as a result of aging.

Impaired skeletal muscle metabolism associated with aging has been linked to an increased number of dysfunctional mitochondria. As a result of aging, mitochondria become enlarged with structural deterioration of the inner membrane along with an increased lack of polarity (Terman *et al.*, 2006a). These alterations in the mitochondrial structure result in decreased functionality of the mitochondria. Additionally, the mitochondrial electron transport chain complexes show decreased activity with age, limiting the amount of adenosine triphosphate (ATP) generation (Kumaran *et al.*, 2004b). Generation of ATP is further diminished through an overall decrease in the mRNA transcripts that encode mitochondrial proteins, further suppressing the oxidative capacity of the mitochondria (Short *et al.*, 2005; Menshikova *et al.*, 2006). The decreased oxidative capacity of the mitochondria decreases skeletal muscle metabolism and function, contributing to the muscle wasting associated with sarcopenia.

The mitochondrial impairment in aged tissue leads to decreased rates of fatty acid oxidation. Elderly individuals show a 25-35% decrease in fatty acid oxidation following exercise in comparison to young adults (Coggan *et al.*, 1992b; Sial *et al.*, 1996). Inversely correlated to the decrease in fatty acid oxidation, elderly individuals have increased triglyceride levels (Park *et al.*, 2006). As a result, the elderly show increased deposition of fatty acids in the liver, skeletal muscle, and pancreas (Slawik & Vidal-Puig, 2006) which often coincides with a change of body composition leading to increased body fat mass or obesity (Calles-Escandón & Poehlman, 1997).

The increased number of dysfunctional mitochondria may further be amplified by the decreased rate of autophagy associated with aging. Autophagic vacuoles regulate the turnover rate of mitochondria and other organelles thereby slowing the accumulation of damaged cellular components (Pfeifer, 1978). Increasing age is associated with fewer and smaller autophagic vacuoles with decreased lysosome interaction (Del Roso *et al.*, 2003). The inefficiency of autophagic vacuoles with age results in an increased accumulation of damaged organelles. Furthermore, the age-related decrease and delay in autophagic vacuoles results in decreased binding of substrate proteins along with decreased lysosomal degradation rates (Cuervo & Dice, 2000). Therefore, old age results in an accumulation of damaged organelles and oxidative stress along with alterations in protein turnover, further contributing to the effects of sarcopenia (Combaret *et al.*, 2009).

Age further results in deregulation of protein homeostasis by not only altering protein degradation but also protein synthesis. Total body protein decreases in elderly individuals by 14% with the majority of the protein loss being attributed to a decrease in muscle protein content (Cohn *et al.*, 1980). The decrease in muscle protein content is correlated to a 45% decrease in skeletal muscle mass with sarcopenia (Cohn *et al.*, 1980). Skeletal muscle contraction and amino

acid supplementation, both known to increase protein synthesis, have a reduced effect in the elderly compared to younger individuals (Dardevet *et al.*, 2000; Fry *et al.*, 2011). Therefore, elderly individuals have an impaired response to stimuli known to increase the rate of protein synthesis, which may contribute to the symptoms of sarcopenia.

AMPK is a Metabolic Regulator

Adenosine monophosphate (AMP) -activated protein kinase (AMPK), a metabolic regulator, acts in opposition to many of the effects of aging and may provide insight into the effects of aging. AMPK is a heterotrimeric protein composed of a catalytic α subunit along with regulatory β and γ subunits (Davies *et al.*, 1994; Woods *et al.*, 1996a; Cheung *et al.*, 2000). The α and β subunits each have two distinct isoforms referred to as α1 and α2 or β1 and β2 respectively (Salt *et al.*, 1998b). The γ subunit has three distinct isoforms referred to as γ1, γ2, and γ3 (Thornton *et al.*, 1998). In rat skeletal muscle, the α2 and β2 isoforms are most commonly expressed; however, both the β1 and β2 isoforms play a role in AMPK α2 activation (Thornton *et al.*, 1998). Liver kinase B1 (LKB1), a serine /threonine kinase, phosphorylates the α subunit on the threonine 172 residue of AMPK to activate AMPK in skeletal muscle in response to endurance exercise (Hawley *et al.*, 1996; Winder & Thomson, 2007). In addition, endurance exercise creates an energy-challenged state, which increases the amount of AMP in comparison to ATP (Sakamoto *et al.*, 2005; Winder *et al.*, 2006). AMP binds to the gamma subunit of AMPK to make AMPK a better substrate for LKB1 (Hawley *et al.*, 1995; Hawley *et al.*, 1996; Scott *et al.*, 2007) and a worse substrate for Protein phosphatase 2C (PP2C), an AMPK phosphatase (Davies *et al.*, 1995a; Marley *et al.*, 1996). AMP additionally induces a conformational change in AMPK to further induce allosteric activation of AMPK (Hawley *et al.*, 1995; Scott *et al.*, 2004; Witczak *et al.*, 2008b). The γ subunits regulate the AMP dependence of

the AMPK heterotrimer. γ2 complexes have a higher AMP dependence than γ1 subunits, which have a higher AMP dependence than γ3 subunits (Cheung *et al.*, 2000).

Increased activation of AMPK is associated with increased translocation of AMPK to the nucleus. AMP-regulated allosteric activation of AMPK increases nuclear localization of the AMPKα2 isoform (Salt *et al.*, 1998b; McGee *et al.*, 2003). Translocation of AMPKα2 from the cytosol to the nucleus allows AMPK to regulate gene expression (Witczak *et al.*, 2008b). The β1 isoform is also localized to the nucleus through myristoylation and phosphorylation (Warden *et al.*, 2001a). The γ1 isoform further demonstrates preferential nuclear localization compared to the other two isoforms (Turnley *et al.*, 1999b). Thus, when localized to the nucleus, AMPK may regulate cell function by altering gene expression, but when localized to the cytoplasm it may have direct effects on metabolism (e.g. by directly promoting fatty acid oxidation (Foretz *et al.*, 1998b), increasing glucose transport (Foretz *et al.*, 1998b), autophagy (Meley *et al.*, 2006) and inhibiting processes that consume ATP such as lipogenesis (Foretz *et al.*, 1998b), protein synthesis (Bolster *et al.*, 2002; Reiter *et al.*, 2005) and cholesterol synthesis (Henin *et al.*, 1995)).

AICAR is a Pharmacological Activator of AMPK

AICAR, or 5'-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, is a pharmacological activator of AMPK. AICAR increases the accumulation of 5-amino-4-imidazole-carboxamide ribotide (ZMP) in the cell in a dose-dependent manner (Sabina *et al.*, 1985; Sullivan *et al.*, 1994). ZMP mimics 5'-AMP to increase AMPK activation through both allosteric activation and promotion of phosphorylation of AMPK (Sullivan *et al.*, 1994; Corton *et al.*, 1995).

AICAR is sufficient to activate AMPK in skeletal muscle similar to the activation response of exercise and muscle contraction (Merrill *et al.*, 1997). Acute AICAR treatment in

skeletal muscle increases fatty acid oxidation (Merrill *et al.*, 1997), glucose uptake (Merrill *et al.*, 1997) autophagy (Sanchez *et al.*, 2012) and protein synthesis(Bolster *et al.*, 2002). Furthermore, chronic AICAR treatment increases mitochondrial gene expression and enzyme activity (Holmes *et al.*, 1999; Winder *et al.*, 2000; Zhou *et al.*, 2000; Narkar *et al.*, 2008) and increases running endurance (Narkar *et al.*, 2008). Therefore, it has been proposed that AICAR treatment may be sufficient to both enhance and/or compensate for the exercise training response (Narkar *et al.*, 2008).

The Effect of Age on AMPK Activation is Controversial

The effect of aging on AMPK activation and composition is not well understood. Some studies have supported the hypothesis that AMPK is hyperactivated in aging skeletal muscle. Aging is associated with decreased mitochondrial function, which will limit the availability of ATP and increase the ratio of AMP to ATP under cell stress conditions such as exercise. An increased ratio of AMP to ATP suggests that aging would result in increased activation levels of AMPK in response to exercise. Muscle wasting is increased in old age, which may be correlated to a decrease in muscle fibers and protein synthesis (Thompson, 1994; D'Antona & Nisoli, 2010). Activation of AMPK down regulates protein synthesis by inhibiting activation of the mTOR pathway (Bolster *et al.*, 2002). Additionally, activation of the mTOR pathway after in situ muscle contraction is attenuated more in aged rats compared to young rats (Parkington *et al.*, 2004), resulting in a greater inhibition of protein synthesis. Since AMPK inhibits protein synthesis through inhibiting the mTOR pathway, the muscle wasting that occurs in response to aging further supports a possible increase in the activation levels of AMPK.

The hypothesis that AMPK activation levels increase in correlation with the aging process has been supported in studies that analyzed AMPK levels in response to resistance-type

training. Thomson *et al.* (Thomson & Gordon, 2005) showed that in response to overload-induced hypertrophy, aged skeletal muscle show a decreased percent hypertrophy in fast-twitch muscle but higher activation levels of AMPK than young skeletal muscle. Increased levels of AMPK activation in aged skeletal muscle was further shown by Thomson *et al.* (Thomson *et al.*, 2009) using high-frequency electrical stimulation (HFES) of the sciatic nerve to mimic resistance-type muscle contractions. HFES in aged skeletal muscle resulted in hyperactivation of AMPK in aged fast-twitch muscle along with increased AMPKα2 activity. Thomson *et al.* further showed that pharmacological activation of AMPK using AICAR increased AMPK activation but showed no significant difference in activation with age. The increased levels of AMPK activation in response to resistance exercise as a result of aging in rats observed by Thomson *et al.* are similar to results observed in humans by Drummond *et al.* (Drummond *et al.*, 2008). In response to resistance exercise, elderly individuals showed increased AMPKα phosphorylation of Thr172 in contrast to younger individuals. Therefore, resistance exercise appears to result in a greater increase in AMPK activation in aged skeletal muscle in comparison to young skeletal muscle.

The effects of aging on metabolic pathways acts in opposition to AMPK activation; therefore, it may alternatively be hypothesized that decreased AMPK activity might contribute to age-related muscle metabolic dysfunction. In skeletal muscle, AMPK promotes the formation of oxidative fibers by increasing the transformation of type IIb to type IIa/x muscle fibers (Röckl *et al.*, 2007). In contrast, older individuals show increased amounts of type I slow-twitch muscle fibers as opposed to type IIa fast twitch muscle fibers (Thompson, 1994). AMPK also promotes mitochondrial biogenesis to increase muscle mitochondria density (Bergeron *et al.*, 2001b) and therefore ATP production. AMPK activation results in an increased expression of peroxisome

proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) (Garcia-Roves *et al.*, 2008) which regulates the expression of mitochondrial proteins through peroxisome proliferator-activated receptor alpha (PPARα) (Vega *et al.*, 2000), increasing mitochondrial biogenesis. AMPK activation further increases mitochondrial turnover rate by allowing the induction of autophagy (Pfeifer, 1978; Sanchez *et al.*, 2012). Age alters expression of proteins that regulate autophagy and proteosomal degradation, namely a decrease in autophagy-related gene 7 (Atg7) expression and an increase in p62/SQSTM1 expression (Cui *et al.*, 2012). AMPK acts in opposition to aging by increasing autophagy. AMPK binds to ULK1 (UNC-51-like kinase) (the mammalian homologue of yeast Atg1 that works concertedly with Atg7) to block the inhibition of ULK1 by mTOR (Lee *et al.*, 2010; Sanchez *et al.*, 2012). Binding of AMPK to ULK1 allows the induction of autophagy in skeletal muscle. The role AMPK plays in opposition to the effects of aging suggests that there is a decrease in AMPK activity as a result of aging.

In support of this hypothesis, Reznick *et al.* (Reznick *et al.*, 2007b) demonstrated that at rest, phosphorylated AMPK was not different between young adult and old rats. However, rats given AICAR, a pharmacologic AMPK activator, or rats exercise trained for 5 days on a treadmill showed a 55% increase in phosphorylation of Thr172 of AMPKα in the young adult rats (3 mo.) in comparison to the old rats (28 mo.). These results were further supported by Qiang *et al.* (Qiang *et al.*, 2007). At rest, phosphorylated AMPK was actually lower in the old rats compared to young rats. AICAR was administered subcutaneously for one week to young and old rats. The old rats showed a 63% impairment in phosphorylated AMPK levels with AICAR, meaning there was a drastic impairment in AMPK activation in old rats compared to young rats. More recently it was supported that in both male, and more significantly in female, aged rats show a marked attenuation in the levels of phosphorylated AMPK in contrast to young

rats at rest (Paturi *et al.*, 2010). However, no form of exercise was implemented to activate AMPK and may need to be accounted for. Therefore, endurance exercise or pharmacological activation of AMPK may result in impaired levels of phosphorylated active AMPK in older rats in comparison to younger rats, contradictory to the results seen in response to resistance and overload exercise discussed earlier.

Addressing the Conflict of AMPK Activation in Aging Muscle

The research performed prior to this study shows conflicting results in AMPK activity levels in aged rats versus young adult rats depending on the experimental procedure performed. The current literature suggests that resistance exercise results in increased AMPK activation levels in aged rats whereas endurance exercise results in decreased AMPK activation levels in aged rats. A possible explanation for the discrepancy may be differences in the testing procedures used to test AMPK activation levels or comparison between resistance versus endurance exercise. One such example may be seen in the study performed by Reznick *et al.* (Reznick *et al.*, 2007b). Older rats run on the treadmill were run at a much lower speed for the 5 days in comparison to the young rats. This decreased intensity of endurance bout in the aged rats may account for decreased AMPK activation levels. Therefore, a more standardized testing procedure was needed to validate the results in response to endurance exercise.

In addition, AMPK activation is regulated by the localization and heterotrimeric composition of the subunits. Prior to this study little research has been performed in analyzing the differences in the localization and heterotrimeric composition of AMPK in skeletal muscle in aged versus young adult rats. Current research has indicated that aged muscle exhibits a decrease in AMPKα2 content but an increase in AMPKα1 content (Thomson *et al.*, 2009; Rivas *et al.*, 2011). However, no current research has addressed the overall heterotrimer composition of

AMPK and localization of AMPK in aging muscle. Localization and heterotrimeric composition were therefore evaluated to further understand the differences reported in AMPK activation in young adult versus old rats in response to an endurance-type exercise electrical stimulation.

Furthermore, current research on the response to AICAR treatment in aged skeletal muscle did not address the effects of chronic AICAR treatment on endurance exercise performance and age-related signaling pathways. We proposed that the decreased mitochondrial biogenesis, autophagy, and fatty acid oxidation seen with aging is linked to impaired activation of AMPK. This study addresses the effects of chronic AICAR treatment in old and young mice following treadmill running and *in vitro* muscle contractions. Treadmill endurance, rate of muscle fatigue, AMPK activation, mitochondria function, autophagy, and protein synthesis were compared between old and young mice that receive AICAR versus a saline control treatment. Increasing understanding of the effects of AMPK activation on the aging phenotype will further aid in the understanding and treatment of sarcopenia to increase the quality of life of elderly individuals.

Objectives

The purpose of this dissertation was to clarify the controversy in the current literature concerning the activation of AMPK in response to endurance-like exercise in aged skeletal muscle. In addition, the following study is the first to address the nuclear localization of AMPK after endurance-like exercise and the heterotrimer composition of AMPK in aged skeletal muscle to understand how age-associated alterations in AMPK activation may contribute to the misregulation of metabolic pathways seen with sarcopenia. Additionally, this dissertation addresses the known beneficial effects that have been observed with chronic AICAR treatment in aged skeletal muscle and how activation of AMPK may improve the aging phenotype.

The specific purpose in Chapter 2 was to determine the effect of aging on the level of AMPK activation and heterotrimer composition in skeletal muscle after an endurance-like muscle contraction using electrical stimulation in rats. We hypothesized:

1) AMPK activation levels would be attenuated in the aged skeletal muscle in contrast to the young adult skeletal muscle in response to endurance-like muscle contractions
2) Aged skeletal muscle would have decreased levels of the α2, β1, and γ1 isoforms and increased levels of γ2 and γ3 compared to young skeletal muscle.

In Chapter 3 the purpose was to determine the effect of aging on AMPK localization in skeletal muscle after an endurance-like muscle contraction using electrical stimulation in rats. We hypothesized that nuclear localization of AMPK would decrease in aged skeletal muscle after endurance-like muscle contractions.

The specific purpose in Chapter 4 was to determine the effect of chronic AICAR treatment on contractile performance and age-related signaling pathways. This was examined in

young adult and old mice given four weeks of AICAR treatment followed by treadmill testing and *in vitro* muscle contractions. We hypothesized:

1) AMPK activation, treadmill endurance, and muscle rate of recovery would improve and muscle fatigue would decrease with chronic AICAR treatment in aged mice in comparison to saline treated mice.
2) Chronic AICAR treatment would increase mitochondrial gene expression and enzyme activity, increase the expression of autophagic markers, and decrease mTOR activation in aged mice.

Impact

This dissertation is the first to address the nuclear localization and heterotrimeric composition of AMPK in aged skeletal muscle to understand age-related alterations of AMPK activation in response to endurance-type muscle contractions. Furthermore, this dissertation is the first to address the effect of chronic AICAR treatment in aged skeletal muscle. Understanding age-associated alterations in AMPK activation and how chronic AMPK activation through AICAR affects the aging phenotype will open new doors to understanding sarcopenia and how to improve the metabolic misregulations associated with sarcopenia.

CHAPTER 2: The Effects of Age and Muscle Contraction on AMPK Activity and Heterotrimer Composition

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Abstract

Sarcopenia is characterized by increased skeletal muscle atrophy due in part to alterations in muscle metabolism. AMP-activated protein kinase (AMPK) is a master regulator of skeletal muscle metabolic pathways. Functional AMPK is a heterotrimer composed of alpha, beta and gamma subunits. Each subunit can be represented by one of two (α1/α2, β1/β2) or three (γ1/γ2/γ3) isoforms. Previous work has shown that overall AMPK activation with endurance-type exercise is blunted in old vs. young skeletal muscle. However, details regarding the activation of the specific isoforms of AMPK, as well as the heterotrimeric composition of AMPK in old skeletal muscle are unknown. Our purpose here, therefore, was to determine the effect of old-age on 1) the activation of the α1 and α2 catalytic subunits of AMPK in skeletal muscle by a continuous contraction bout, and 2) the heterotrimeric composition of skeletal muscle AMPK. We studied gastrocnemius (GAST) and tibialis anterior (TA) muscles from young adult (YA; 8 mo old) and old (O; 30 mo old) male $Fischer_{344}$ x Brown Norway F1 hybrid rats after an *in situ* bout of endurance-type contractions produced via electrical stimulation of the sciatic nerve (STIM). AMPKα phosphorylation and AMPKα1 and α2 activities were unaffected by age at rest. However, AMPKα phosphorylation and AMPKα2 protein content and activity were lower in O vs. YA after STIM. Conversely, AMPKα1 content was greater in O vs. YA muscle, and α1 activity increased with STIM in O but not YA muscles. AMPKγ3 concentration and its association with AMPKα1 and α2 was lower in O vs. YA GAST. We conclude that

activation of AMPKα1 is enhanced, while activation of α2 is suppressed immediately after repeated skeletal muscle contractions in O vs. YA skeletal muscle. These changes are associated with changes in the AMPK heterotrimer composition. Given the known roles of AMPK α1, α2 and γ3, this likely contributes to sarcopenia and associated muscle metabolic dysfunction.

Introduction

Sarcopenia, or age-related skeletal muscle atrophy, affects 20-50% of adults over the age of 60 years (Berger & Doherty, 2010a) and is defined by a decline of muscle mass and strength that leads to frailty with aging (Thompson, 1994; Janssen *et al.*, 2002; Verdijk *et al.*, 2010b). Sarcopenia is associated with many alterations in skeletal muscle metabolism such as decreased insulin sensitivity (Goodman & Ruderman, 1979), decreased fatty acid oxidation (Sial *et al.*, 1996; Calles-Escandón & Poehlman, 1997; Park *et al.*, 2006), decreased muscle protein content (Dardevet *et al.*, 2000; Fry *et al.*, 2011), and an increase in dysfunctional mitochondria (Kumaran *et al.*, 2004b; Terman *et al.*, 2010). These metabolic disruptions may contribute to the decreased fiber size and contractility seen with aging muscle (Lexell *et al.*, 1988; Evans & Campbell, 1993; Larsson *et al.*, 1997).

AMP-activated protein kinase (AMPK) is a cellular energy sensor that acts as a master regulator of skeletal muscle metabolic pathways including many that are affected by aging. AMPK is activated as it becomes phosphorylated at Thr172 by the upstream kinase LKB1. AMPK phosphorylation and activation is dependent upon an increased cellular AMP/ATP ratio. Both nucleotides, as well as ADP, compete for binding to AMPK. AMP binding leads to AMPK phosphorylation through a conformational shift that makes AMPK a better substrate for LKB1 (Hawley *et al.*, 1995; Hawley *et al.*, 1996; Scott *et al.*, 2007; Gowans *et al.*, 2013) and a worse substrate for dephosphorylation by protein phosphatase 2C (Davies *et al.*, 1995b; Marley *et al.*,

1996). Binding of AMP also allosterically activates AMPK in addition to its effect on phosphorylation (Hawley *et al.*, 1995; Scott *et al.*, 2004; Witczak *et al.*, 2008a; Gowans *et al.*, 2013).

AMPK is a heterotrimeric protein composed of α, β, and γ subunits (Stapleton *et al.*, 1996; Woods *et al.*, 1996a). The subunits of AMPK all have multiple isoforms and differences in isoform composition affects AMPK localization and function. The α subunit contains the catalytic domain and the site of Thr172 phosphorylation. The α subunit has two different isoforms, α1 or α2. The β subunit serves as a scaffolding or regulatory subunit and also has two isoforms, β1 and β2. The γ subunit contains nucleotide binding sites and determines the degree of AMP dependence of the AMPK heterotrimer. γ2 complexes have a higher AMP sensitivity than γ1 subunits, which have a higher AMP sensitivity than γ3 subunits (Cheung *et al.*, 2000).

During exercise, the AMP to ATP ratio increases by way of the adenylate kinase reaction, thereby potently activating AMPK (Hawley *et al.*, 1995; Winder & Hardie, 1996; Hawley *et al.*, 2003; Dreyer *et al.*, 2006; Jensen *et al.*, 2007). In both mice and humans, the α2/β2/γ1 AMPK complex is predominant. However, exercise and muscle contraction appear to primarily activate complexes containing α2 and γ3 isoforms, although γ1 can be activated as well (Birk & Wojtaszewski, 2006; Treebak *et al.*, 2007). The two catalytic α isoforms have different substrate specificity leading to regulation of different metabolic pathways (Stapleton *et al.*, 1996). While some redundancy between isoforms certainly exists, the α1 subunit is particularly important in negatively regulating muscle cell size through regulation of the mTOR pathway (Mounier *et al.*, 2009), while the α2 subunit appears more able to localize to the nucleus than α1, presumably to control gene transcription (Salt *et al.*, 1998b).

Activation of AMPK in response to endurance exercise appears to be blunted in old skeletal muscle (Reznick *et al.*, 2007b; Ljubicic & Hood, 2009). However, the effect of old-age on isoform-specific activation of the catalytic α subunit (i.e. α1 vs. α2) after exercise is unknown. Likewise, it is not known how old-age affects the heterotrimer composition of AMPK, and how this might relate to the effect of aging on AMPK activation. Since the effects of both exercise and AMPK activation (e.g. increased fatty acid oxidation, glucose transport, and mitochondrial capacity) are in opposition to many effects of aging (Foretz *et al.*, 1998b; Bergeron *et al.*, 2001b; Zong *et al.*, 2002), understanding how the AMPK system is altered in aged muscle may lead to improved strategies for combatting age-related dysfunction.

Accordingly, this study has two purposes: 1) to evaluate catalytic isoform-specific AMPK activation in young adult and old skeletal muscle after an electrically stimulated *in situ* endurance-type contraction bout, and 2) to determine whether differences in AMPK activation could be accounted for by alterations in AMPK subunit isoform composition.

Materials and Methods

Animal Care

Experimental procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. All animals were housed in a temperature controlled (20-21°C) environment with a 12hr: 12hr light-dark cycle and fed standard chow and water *ad libitum*. Young adult (YA) (8 mo.; n=8) and old (O) (30 mo.; n=8) male $Fischer_{344}$ x Brown Norway (FBN) hybrid rats were used as recommended by the National Institute of Aging for age-related research based on studies indicating that this strain has fewer detrimental pathologies than inbred strains and 50% mortality at about 32 months of age (Lipman *et al.*, 1996; Lushaj *et al.*, 2008).

Electrical Stimulation of the Sciatic Nerve

Rats were anesthetized using vaporized isoflurane (2–3%) in supplemental oxygen sufficient to achieve surgical anesthetic depth. Twenty minutes after anesthetization, the sciatic nerve of the left hindlimb was isolated just proximal to the point of trifurcation. Contraction of the hindlimb musculature was elicited by stimulating the sciatic nerve at 100 Hz for 10 min at one 10 msec pulse per sec and 15 volts (Grass Model S48 Stimulator, Quincy, MA). During the contraction bout, the foot was held at approximately 90°. Sciatic nerve stimulation activates both the chronically weight-bearing plantarflexors as well as the dorsiflexors of the hindlimb, including the gastrocnemius (GAST) and tibialis anterior (TA) muscles. GAST and TA were removed immediately after contraction and frozen at the temperature of liquid nitrogen. Both muscles were analyzed to allow assessment of the AMPK system in two distinct muscles with different ambulatory functions, with the gastrocnemius being a weight-bearing muscle, while the TA only stabilizes and dorsiflexes the ankle during ambulation. The right hindlimb was not subjected to electrical stimulation and was removed prior to stimulation of the left hindlimb and served as a resting control. All tissue samples were frozen between metal tongs cooled to the temperature of liquid nitrogen and then frozen at -95°C until further analysis.

Homogenization

Muscles were pulverized on liquid nitrogen then glass-ground homogenized in 19-volumes of homogenization buffer (50 mM Tris-HCl, pH 7.4; 250 mM mannitol; 50 mM NaF; 5 mM Sodium Pyrophosphate; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 50 mM B-glycerophosphate; 1 mM sodium orthovanadate; 1 mM DTT; 1 mM benzamidine; 0.1 mM phenylmethane sulfonyl fluoride; 5 ug/ml soybean trypsin inhibitor). The raw homogenate was freeze- thawed three times to ensure disruption of intracellular membranes and then centrifuged

at 10,000 x g for 10 min. Supernatants were analyzed for protein content using the DC Protein Assay (Biorad Laboratories, Hercules, CA, USA). Supernatants were stored in microcentrifuge tubes at -95°C until further analysis.

Western Blot and Immunodetection

Homogenates were diluted in sample loading buffer (125 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue) and then loaded on 5% [phosphorylated ACC (pACC), total ACC (tACC)], 7.5% [phosphorylated AKT (pAKT), phosphorylated AMPK (pAMPK), total AMPK (tAMPK), LKB1, AMPKγ3], and 10% [AMPKα1, AMPKα2, AMPKβ1, AMPKβ2, AMPKγ1, AMPKγ2, phosphorylated ERK (pERK), and phosphorylated p38 MAPK (p-p38)] Tris·HCl gels (Bio-Rad Criterion System, Hercules, CA). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were stained with Ponceau S and visually inspected for equal protein loading. Membranes were then washed with Tris-buffered saline plus 0.1% Tween-20 (TBST), blocked with 5% non-fat dry milk in TBST for 1 hour, and probed overnight at 4°C with primary antibody diluted in 1% bovine serum albumin (BSA) dissolved in TBST, pH 7.6.

Primary antibody manufacturers and dilutions were as follows: pAMPK (Thr172) (#4188/#2535, 1:5000), tAMPK (#2532, 1:2000), pACC (Ser79) (#3661, 1:5000), AMPKβ1 (#4182, 1: 2000), AMPKβ2 (#4148, 1:2000), AMPKγ1 (#4187, 1:4000), pERK (Thr202/Tyr204) (#4370, 1:2000), p-p38 (Thr180/Tyr182) (#4511, 1:2000), and pAKT (Ser473) (#4060, 1:2000) from Cell Signaling Technology (Beverly, MA, USA); AMPKα1 (A300-507A, 1:4000), AMPKα2 (A300-508A, 1:20000) from Bethyl Laboratories, Inc. (Montgomery, TX, USA); AMPKγ2 (sc-20165, 1:2000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); AMPKγ3 (custom made as described previously (Durante *et al.*, 2002), 1:2000) from Affinity Bioreagents

(Golden, CO, USA); tACC (streptavidin-horseradish peroxidase, RPN1231V, 1:2000) from GE Healthcare Biosciences (Pittsburgh, PA, USA); and LKB1 (#07-694, 1:5000) from Upstate (Lake Placid, NY, USA).

Membranes were probed with the HRP-conjugated mouse anti-rabbit secondary antibody (#211-032-171) from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, USA) or HRP-conjugated goat anti-mouse secondary antibody (sc-2314) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for 1 hour at room temperature. Membranes were washed 4 x 5 min with TBST. ECL Plus Western Blotting Detecting Solution (GE Healthcare Bio-Sciences, Piscataway, NJ) was applied for 2 min. Chemiluminescent signals were detected with autoradiography film and quantified using Gel-Pro Analyzer 6.0 (Media Cybernetics, Inc. Bethesda, MD) or AlphaEase FC software (Alpha Innotech Corp., San Leandro, CA).

AMPK Activity Assay

AMPK activity of α1 and α2 subunits immunoprecipitated from GAST and TA homogenates was measured by the incorporation of radiolabeled phosphate from ATP into SAMS (HHMRSAMSGLHLVKRR-OH) peptide. Activity was assessed and expressed as picomoles per gram tissue per minute, as described previously (Park *et al.*, 2002).

Co-Immunoprecipitation

To determine the effect aging has on the heterotrimer subunit composition, 15 µg of α1 or α2 antibody and 1ml ice cold TBS were added to about 80 µl (packed volume) of Exactacruz IP matrix (Santa Cruz no. sc-45039) and rotated for 1 hour at 4°C. The IP matrix was pelleted by centrifuging for 60 sec. at 13,000 x g, then washed three times with 1 ml ice cold TBS and resuspended in 420 µl ice cold TBS. 50 µl aliquots of the resuspended matrix were transferred to centrifuge tubes and centrifuged. After removal of the supernatant, 400 µg of GAST homogenate

was added to the matrix-antibody pellet which then was brought up to 500 µl total volume with ice cold homogenization buffer. Samples were rotated end-over-end overnight at 4°C. After rotation, samples were centrifuged for 30 sec. at 10,000 x g. The supernatant was transferred to a fresh tube and saved. The pellet was washed three times with ice cold homogenization buffer and then resuspended in 80 µl of loading buffer and boiled for 3 min at 100°C. Samples were then loaded onto 10% gels for western blotting and immunodetection as described previously.

Glycogen Concentration Assay

GAST tissue samples were ground into powder under liquid nitrogen. 0.5 mL cold 30% KOH was added to 50 mg of sample and placed in a boiling water bath for 30 min. Samples were then neutralized with 10.7 M acetic acid. 0.1 ml of sample was transferred to a 12 x 75 mm tube containing 0.9 ml amyloglucosidase buffer (amyloglucosidase, 50 mM acetate buffer (50 mM acetic acid, 50 mM sodium acetate), pH 4.7). Samples were incubated at 55°C for 1 hour, vortexed, and centrifuged for 10 min. 0.1 ml of sample was transferred to a cuvette containing 0.88 ml reaction buffer (25.4 mg $MgCl_2$, 0.4 mg dithiothreitol (DTT), 6.9 mg ATP, 7.7 mg NADP, 25 ml 100 mM Tris buffer, pH 8.8). Initial read was set at 340 nm. G6PDH/ HK enzyme mixture (100 units GSPDH, Sigma G-6378, 50 mM Tris, 80 units/mg solid Hexokinase, Sigma H5375, pH 7.6) was then added to each cuvette, mixed and then read for final O.D. after 15 min.

High Performance Liquid Chromatography (HPLC)

High-energy phosphate concentrations were determined by HPLC. Muscles were pulverized at the temperature of liquid nitrogen and then homogenized in 6% perchloric acid. Homogenates were then centrifuged at 800 rpm for 5 min to remove protein. This was followed by neutralization of the supernatant with neutralization solution (2 N potassium hydroxide, 0.4 M

potassium chloride, 0.4 M imidazole) to pH 7.0. Homogenates were then vortexed and centrifuged at 800 rpm for 5 min. 120 µl of the supernatant was placed in a fresh tube, dehydrated, and stored at −80°C. Just prior to HPLC, samples were rehydrated in 120 µl ddH_2O. Adenine nucleotides (ATP, ADP, AMP) and IMP were quantified by reverse-phase HPLC, as described by Tullson et al. (1990) (Tullson *et al.*, 1990). Phosphocreatine (PCr) concentrations were measured by ion exchange HPLC as described by Wiseman et al. (1992)(Wiseman *et al.*, 1992). Metabolites were expressed as micromoles per gram wet weight and corrected to the total adenine nucleotide content plus IMP of rested muscle (6.7 µmol/g wet wt) to account for fluid shifts that occur in response to muscle contractions as previously described (Hancock *et al.*, 2005; Thomson *et al.*, 2010).

Statistics

Statistical comparisons using Microsoft Excel or GraphPad Prism statistical analysis software (GraphPad Software Inc., La Jolla, CA) were made using a student *t* test or repeated measures ANOVA to determine statistical significance ($p \leq 0.05$) with Fisher's LSD post-hoc analysis employed where appropriate. Values are reported as means ± SE.

Results

Effects of Age on AMPK Activation and Phosphorylation

The energetic response to the contraction bout was assessed by measuring the concentration of glycogen and high-energy phosphate metabolites in the gastrocnemius (Table 2.1). IMP and AMP were elevated at rest in O vs. YA muscles. Following STIM, there was a significant reduction in glycogen, PCr, and ATP for both the YA and O rats. This corresponded to an accumulation of IMP in both the YA and O rats. (O rats had a greater drop in ATP as well

as a greater increase in IMP than YA rats.) Response to the contraction bout was further confirmed by measuring phosphorylation of energetic stress-related mitogenic and metabolic proteins. STIM increased phosphorylation of ERK, AKT, and p38 similarly in both the YA and O rats (Fig. 2.1), further verifying the effectiveness of the stimulus to elicit a similar energetic response in both age groups by the contraction bout.

AMPK activity was next assessed by determining pAMPK protein content and AMPKα1 and α2 activity. pAMPK content increased with STIM in both O and YA rats; however, the increase in pAMPK was significantly attenuated by 63% and 75% respectively in the GAST and TA after STIM in O rats compared to YA suggesting impaired overall activation of AMPK in O rats in response to STIM (Figure 2.2A). The overall protein content level of total AMPK was decreased in O vs. YA muscle (Figure 2.2B). AMPKα2 activity followed the same trend as seen with pAMPK with increased activity after STIM in both O and YA rats; however, that increase was attenuated by 19% and 23% respectively in the GAST and TA in O versus YA rats (Figure 2.2D). In contrast, AMPKα1 activity increased by 30% and 38% in the GAST and TA respectively after STIM in O rats while α1 activity was unaffected by STIM in YA rats (Figure 2.2C).

Effects of Age on LKB1 and ACC

Protein content of LKB1 was unaffected by age (Figure 2.3A). Total protein content of Acetyl CoA Carboxylase (ACC), a known downstream target of AMPK, was greater in aged fast twitch muscle in comparison to YA rats (Figure 2.3C) but pACC significantly increased with STIM in both O and YA rats (Figure 2.3B).

Effects of Age on AMPK Subunit Isoform Protein Content

The effect of age on the AMPK system was further addressed by measuring the protein content levels of the AMPK isoforms. AMPKα1 protein content in O versus YA muscle was 45% and 59% higher in the GAST and TA respectively (Figure 2.4A). In contrast, AMPKα2 content was attenuated by 18% in the GAST in O versus YA rats (Figure 2.4B), but not significantly different for the TA. Protein content levels of AMPKβ1, β2, and γ1 were not significantly different between age groups (Figure 2.5A, 2.5B, 2.6A). AMPKγ2 content in O versus YA rats was 75% and 49% lower in the GAST and TA respectively (Figure 2.6B). AMPKγ3 subunit isoform content in O versus YA rats was also 85% and 78% lower in the GAST and TA respectively (Figure 2.6C).

Effects of Age on AMPK Subunit Heterotrimer Composition

Immunoprecipitation of the catalytic isoforms AMPKα1 and α2 was followed by western blotting to detect content of the β1, β2, γ1, γ2, or γ3 subunits associated with the two alpha subunits in the GAST homogenates. After immunoprecipitation using the AMPKα1 and α2 antibodies, the entire western blot signal for the respective alpha subunits was contained in the pellets and not in the supernatants, indicating that the respective alpha subunits were pulled down completely from the homogenates (data not shown). The immunoprecipitated AMPKα1 content increased by 128% in O versus YA rats, and immunoprecipitated α2 content decreased by 33% in O vs. YA rats (Figure 2.7), consistent with AMPK content in the whole homogenates shown in Figure 2.4.

The amount of γ1 that co-immunoprecipitated with α1 increased similarly (130%) in O muscle, while the amount of coimmunoprecipitated γ2 was unchanged and the amount of coimmunoprecipitated γ3 was decreased by 82% (Figure 2.7A). Decreased association of

AMPKα1 with γ3 was verified by immunoprecipitating γ3 and then western blotting for α2 (data not shown). This suggests a shift from α1: γ3 complexes to α1: γ1 complexes in old vs. young muscle. The amount of β1 and β2 subunits coimmunoprecipitated with α1 did not change significantly (Figure 2.7A). Since α1 content increased without a change in associated β subunit content, this suggests that at least part of the increase in α1 content is not associated in the typical heterotrimeric complex with AMPKβ subunits, which may limit the effect this has on overall AMPKα1 activity.

The 33% drop in immunoprecipitated α2 isoform in O muscle was associated with a similar but non-significant drop in coimmunoprecipitated γ1 content, an even greater and significant 83% drop in coimmunoprecipitated γ3 content, and a non-significant increase in coimmunoprecipitated γ2 content (Figure 2.7B). Taken together the disproportionate drop in γ3 vs. α2 in this case suggests a shift away from α2: γ3 complexes, likely toward α2: γ2complexes with old age. The decline in β1 and β2 content that coimmunoprecipitated with α2 in O muscle was similar to the decline in immunoprecipitated α2, suggesting that interaction between α2 and the β subunits is not altered with aging (Figure 2.7B).

Discussion

The purposes of this study were to: 1) evaluate catalytic isoform-specific AMPK activation in young adult and old skeletal muscle after an electrically stimulated *in situ* endurance-type contraction bout, and 2) determine whether differences in AMPK activation could be accounted for by alterations in AMPK subunit isoform composition. Our findings support the results by Reznick et al. (Reznick *et al.*, 2007a) indicating that overall AMPKα phosphorylation and *in vitro* activity of AMPKα2 is attenuated with age in response to endurance-type muscle contraction. However, we also found that AMPKα1 protein content and

contraction-induced activity increases with age, and aging results in decreased association of the AMPKα1 and α2 subunits with the γ3 isoform, and increased association of α1 with γ1 and α2 with γ2.

The endurance-type *in situ* muscle stimulation procedure for contraction of the hindlimb musculature was identical for both the YA and O rats. The contraction bout used here was selected because it strongly activates AMPK in healthy young muscle (Winder & Hardie, 1996). Similar type stimulation has been shown to lead to endurance-type adaptations when performed chronically in rabbits (Patel *et al.*, 1998). The energetic and signaling stress elicited by the contraction protocol used here (Table 1 and Figure 1) suggest that it is a suitable endurance exercise model. Although we did not measure muscle force production during the stimulation bout, ERK, Akt, and p38 phosphorylation were similar between ages, glycogen and PCr depletion was not significantly altered by aging, and ATP depletion and IMP accumulation were increased in the aged muscle. This strongly indicates that the impaired AMPK activation cannot be accounted for by a decreased energetic or overall signaling stimulus in the aged muscle.

Although AMPK phosphorylation and AMPKα2 activity increased with STIM in both YA and O rats, the increase in AMPK phosphorylation and AMPKα2 activity was attenuated in aged compared to YA rats in response to the endurance-type muscle contraction, consistent with findings observed after treadmill running (Reznick *et al.*, 2007b; Ljubicic & Hood, 2009) and *in situ* contractions. This hypo-activation of AMPK was associated with lower AMPKα2 and total AMPKα protein expression. AMPKα2 is the predominant isoform expressed in skeletal muscle (Stapleton *et al.*, 1996) and should therefore closely reflect the total AMPK protein content.

Altered isoform expression of the regulatory γ subunit likely contributed as well to the impaired activation of AMPKα2 with contraction. While protein expression of the regulatory β

subunit isoforms was not altered with age, the γ2 and γ3 isoform protein content was decreased in O muscles, consistent with findings in aged human muscle (Mortensen *et al.*, 2009). Furthermore, the association of AMPKα2 with AMPKγ2 increased while its association with AMPKγ3 decreased. Since AMPKγ3 is the predominant isoform activated in response to exercise, particularly as a α2β2γ3 heterotrimer (Yu *et al.*, 2004; Birk & Wojtaszewski, 2006), this decreased α2/γ3 association in aged muscle likely contributed to the impaired activation of AMPK with contraction. The AMPKα2 and γ3 isoforms are predominantly expressed in fast-twitch glycolytic muscle (Mahlapuu *et al.*, 2004; Yu *et al.*, 2004). Previous research has reported that aging muscle atrophy is primarily accounted for by a reduction in fast-twitch muscle (Lexell *et al.*, 1983; Holloszy *et al.*, 1991; Lexell, 1995). It is not surprising therefore that a reduction in fast-twitch muscle in aged muscle would result in decreased content of the AMPKγ3 and α2 isoforms (Chen *et al.*, 1999; Putman *et al.*, 2007).

Given AMPK's well-defined roles in muscle, the decreased content and activity of AMPKα2 may contribute to many of the dysfunctional characteristics of aging skeletal muscle. Age-related muscle dysfunction is thought to result from many factors including impaired mitochondrial biogenesis and turnover, decreased autophagy, excessive inflammation and enhanced ROS production. AMPK, on the other hand, stimulates mitochondrial biogenesis (Winder *et al.*, 2000; Bergeron *et al.*, 2001a; Zong *et al.*, 2002) and autophagy (Sanchez *et al.*, 2012), while it reduces pro-inflammatory signaling (Green *et al.*, 2011) and ROS production (Irrcher *et al.*, 2009).

In contrast to the α2 isoform, we observed increased activation of AMPKα1 in O muscle after the contraction bout. Likewise, AMPKα1 content was higher in O vs. YA muscles, which is consistent with previous reports from our (Thomson *et al.*, 2009) and other's laboratories (Rivas

et al., 2011). Its hyperactivation by contraction was also likely mediated by a decrease in its association with the γ3 subunit, and an increase in its association with the γ1 subunit since α1 heterotrimers containing γ2 or γ3 are not activated by muscle contraction (Treebak *et al.*, 2014). We had not expected any association of the α1 isoform with γ3 based on previous data from mice showing that immunoprecipitated AMPKγ3 only associates with the α2 isoform (Mahlapuu *et al.*, 2004; Treebak *et al.*, 2009). We verified our results by immunoprecipitating AMPKγ3 and then western blotting for AMPKα1, which confirmed an association between those subunits in skeletal muscle. This discrepancy in results is likely due to species differences, since our experiment was performed in rat muscle, while the conflicting data was in mouse muscle. This is supported by the findings of Cheung et al (Cheung *et al.*, 2000) who demonstrated that AMPKγ isolated from rat liver can associate with either α subunit.

As was the case for the impaired activation of AMPKα2, the elevated AMPKα1 activity in old muscle after contraction may have important clinical significance. AMPKα1 has been shown to play an isoform-specific role in inhibiting the mTOR pathway and protein synthesis in skeletal muscle (Mounier *et al.*, 2009; Mounier *et al.*, 2011). Thus, this could contribute to the deficit in contraction-induced activation of mTOR and protein synthesis observed with old age (Parkington *et al.*, 2004; Thomson & Gordon, 2006), and, over time, result in an imbalance in protein turnover that may contribute to sarcopenia.

Although our findings clearly indicate that AMPK composition and activity are altered in aged sarcopenic muscle, the functional consequence of these alterations in the AMPK system remain unclear. The phosphorylation of ACC by AMPK is considered to be a reliable indicator of overall *in vivo* AMPK activity (Gowans *et al.*, 2013) and is important because the phosphorylation of AMPK as measured by western blot and the AMPK activity assay do not

reflect the *in vivo* allosteric activation of AMPK by an increased ratio of AMP/ATP. ACC phosphorylation after contractions was not subdued in aged muscle, as would be expected given the decreased AMPK phosphorylation and α2 activity, and in fact was slightly elevated in O vs. YA TA muscles. One possibility suggested by this finding is that the functional *in vivo* activity of AMPK toward ACC was not impaired in the aged muscle. This might be due, at least in part, to the fact that ATP content dropped more and IMP content (reflective of free AMP content) increased more in O vs. YA muscles after contractions, indicating that the AMP/ATP ratio was greater in the old muscles after contraction. This could result in greater allosteric activation of the phosphorylated AMPK in the old muscles, offsetting the decreased level of total phosphorylated AMPK. Alternatively, there is evidence that alternative exercise-inducible ACC kinases other than AMPK exist (Dzamko *et al.*, 2008), which could likewise compensate for decreased AMPK activity. Furthermore, the increased amount of total ACC protein content in the O muscles may also have contributed as well by providing an increased mass of substrate for AMPK to act upon. Our results in this regard conflict with those of Reznick et al. who observed decreased phosphorylation of ACC in old muscles after treadmill exercise along with decreased AMPK phosphorylation. This difference is likely due to the differing contraction models used in the studies. Clearly, further work will need to be done to clarify the control of ACC phosphorylation by AMPK and perhaps other kinases during exercise.

Despite the differences in AMPK subunit expression and heterotrimer composition in the aged muscle, no differences were found in AMPK activity between YA and O resting, unstimulated muscles. This is consistent with previous findings in rodent (Ljubicic & Hood, 2009; Thomson *et al.*, 2009)and human (Drummond *et al.*, 2008) muscle, and makes sense given the broad view of AMPK as a stress-sensing system. The differences in AMPK activation that

we observed occurred after relatively intense contractions. This begs the question, then, of how altered AMPK signaling might play a role in age-related muscle dysfunction in individuals who spend little to no time engaged in intense physical activity. We propose that the altered AMPK signaling that we observe in aged muscle may contribute to sarcopenia and its associated metabolic disruption in the following manner. As an individual engages in activities of daily living, an energetic stress is applied to the activated muscle fibers. In old fibers, AMPKα1 is activated earlier and to a greater degree than in young muscle, leading to impaired anabolic signaling consistent with the findings of Mounier et al. (Mounier *et al.*, 2009) described above. At the same time, AMPKα2 activation is suppressed, contributing to the many metabolic disruptions associated with sarcopenia. Validation of this hypothesis will require continued research.

In conclusion, we found that 1) activation of AMPKα2 immediately after a continuous bout of muscle contractions is attenuated but AMPKα1 activation is enhanced in aged skeletal muscle, and 2) AMPK γ subunit isoform expression and association with the α subunits is altered in a manner consistent with both the decreased activation of AMPKα2 and α1 with stimulation. Based on current understanding of AMPK actions in skeletal muscle, these alterations in AMPK activity in old muscle may contribute to muscle dysfunction in sarcopenia, and therapies designed to reverse these changes would be expected to improve the aging skeletal muscle phenotype.

Acknowledgements

We would like to acknowledge Kyler Black and Richard Compton for their assistance in collecting data contributing to this manuscript, as well as William W. Winder for his intellectual contributions to the project.

Table 2.1: High Energy Phosphate Concentrations in Gastrocnemius Muscles

	YA		O	
	REST	STIM	REST	STIM
Glycogen	8.87 ± 0.41	4.28 ± 0.64[a]	8.80 ± 0.54	6.54 ± 0.66[a]
ATP	5.37 ± 0.03	4.22 ± 0.17[a]	4.63 ± 0.33[b]	3.69 ± 0.25[ab]
ADP	0.57 ± 0.01	0.57 ± 0.09	0.55 ± 0.01	0.44 ± 0.02
AMP	0.023 ± 0.002	0.029 ± 0.005	0.048 ± 0.009[b]	0.030 ± 0.003
IMP	0.72 ± 0.03	2.02 ± 0.22[a]	1.45 ± 0.33[b]	2.51 ± 0.27[ab]
PCr	25.04 ± 1.10	13.32 ± 2.48[a]	22.46 ± 0.82	12.27 ± 1.52[a]

High-energy phosphates (μ mol/g wet wt) n=5-8. Glycogen (mg glycogen/ g tissue wt) n=8. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

Table 2.2: Summary of AMPK Heterotrimer Modifications in Aging Skeletal Muscle

Isoform	Protein Content	Association with AMPKα1	Association with AMPKα2
α1	↑	N/A	N/A
α2	↓	N/A	N/A
β1	-	-	↓
β2	-	-	-
γ1	-	↑	-
γ2	↓	-	-
γ3	↓	↓	↓

Symbols indicate the change in content in comparison to the YA rats. (↑) indicates an increase; (↓) indicates a decrease; (-) indicates no change; (N/A) not applicable.

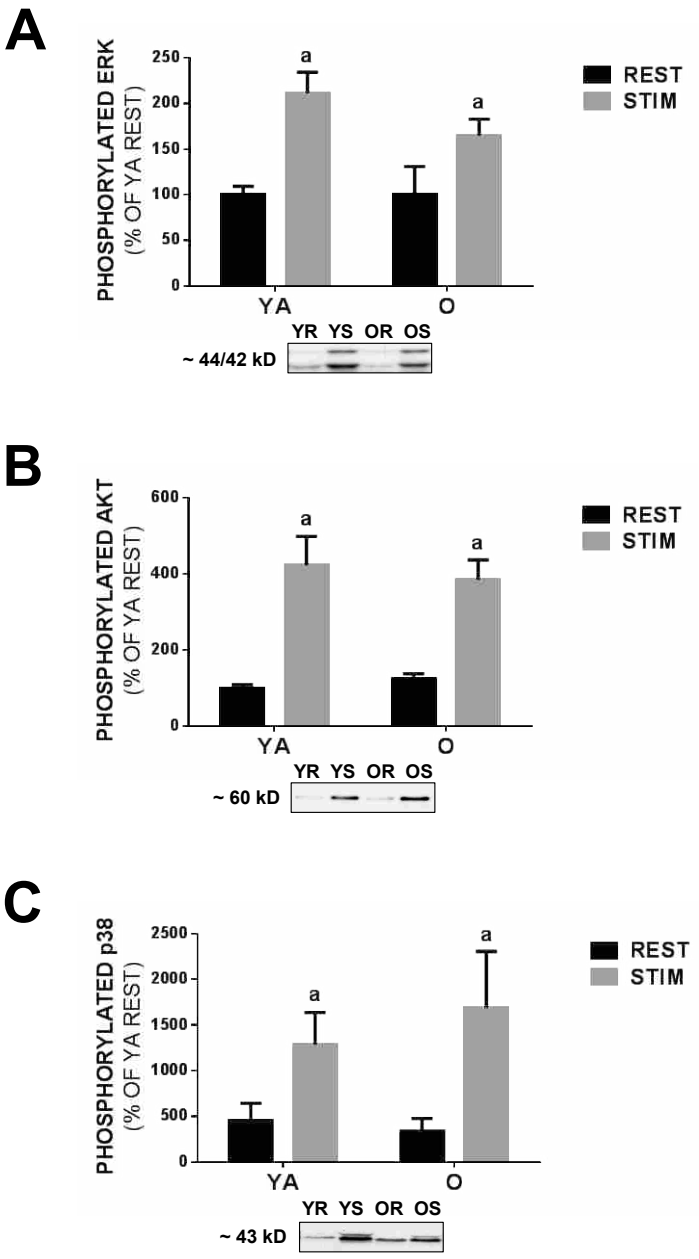

Figure 2.1: Phosphorylation of Mitogen and Metabolic Proteins Increases after an Electrically Stimulated Endurance-Type *In Situ* Contraction Bout (STIM) in YA and O Rats. Gastrocnemius (GAST) muscles from young adult (YA) (8 mo.) and old (O) (30 mo.) Fisher Brown Norway male rats were stimulated for 10 min. (1 pulse per second, 15V, 10 msec. duration) and removed immediately after the contraction bout. Western blot analysis of GAST for (A) phosphorylated ERK, (B) phosphorylated AKT, and (C) phosphorylated p38. N=7-8 / group. YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means ± SEM. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

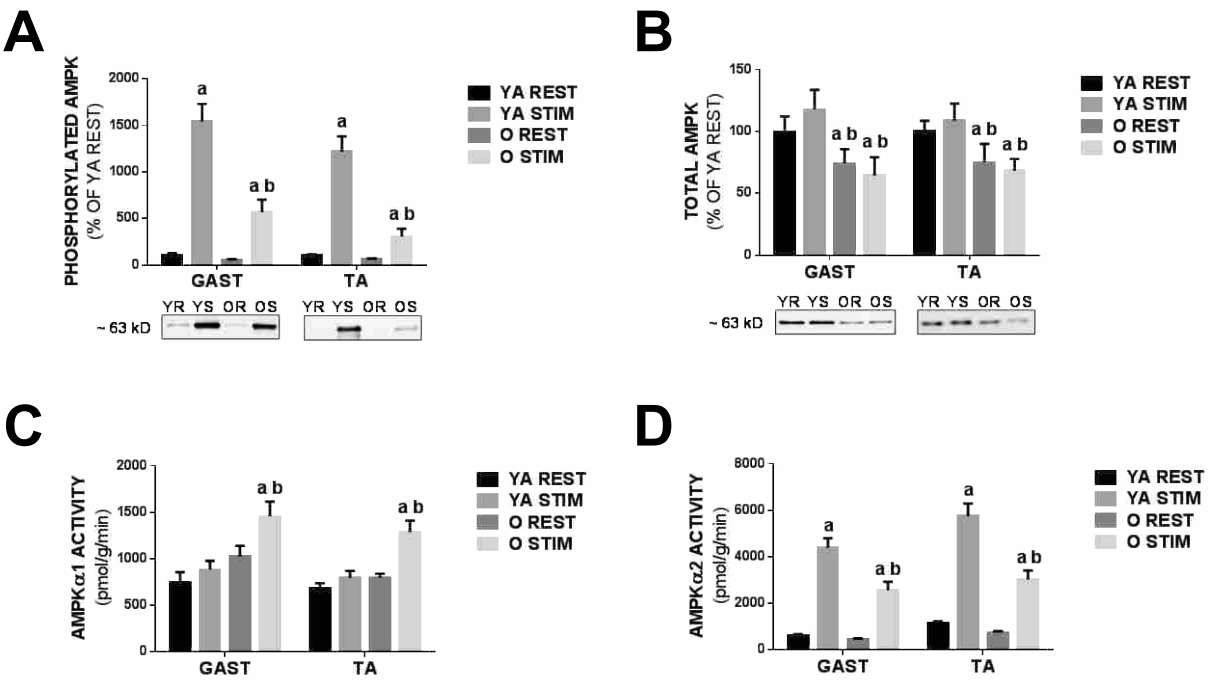

Figure 2.2: AMPK Phosphorylation and AMPKα2 Activity Are Attenuated While AMPKα1 Activity is Increased in O vs. YA Fast-Twitched Muscles. Western blotting analysis of GAST and tibialis anterior (TA) for (A) phosphorylated AMPK and (B) total AMPK. Activity assay of (C) AMPKα1 and (D) AMPKα2. N=8 / group. YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means ± SEM. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

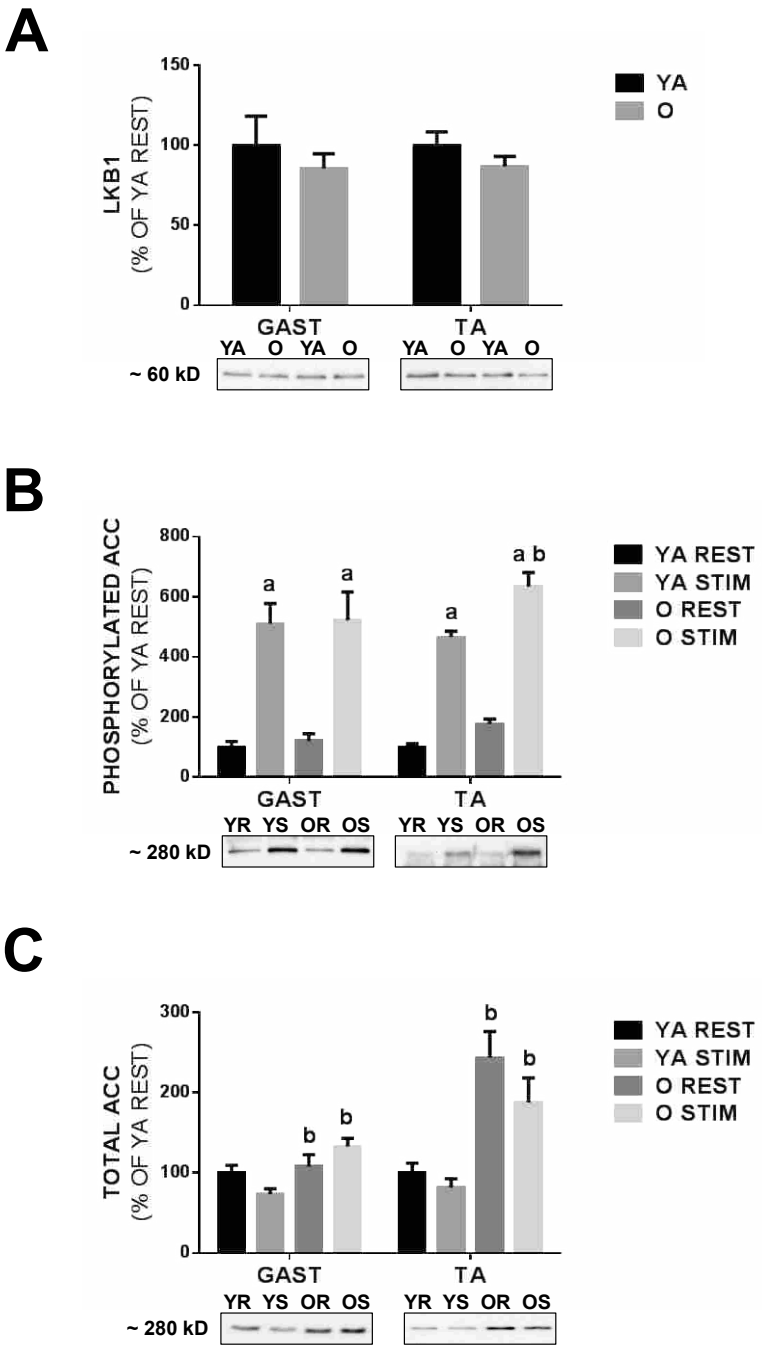

Figure 2.3: LKB1 Content and ACC Response to STIM are Unaffected by Age. Western blotting analysis of GAST and TA for (A) LKB1, (B) phosphorylated ACC, (C) total ACC. N=7-8 / group. YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means ± SEM. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

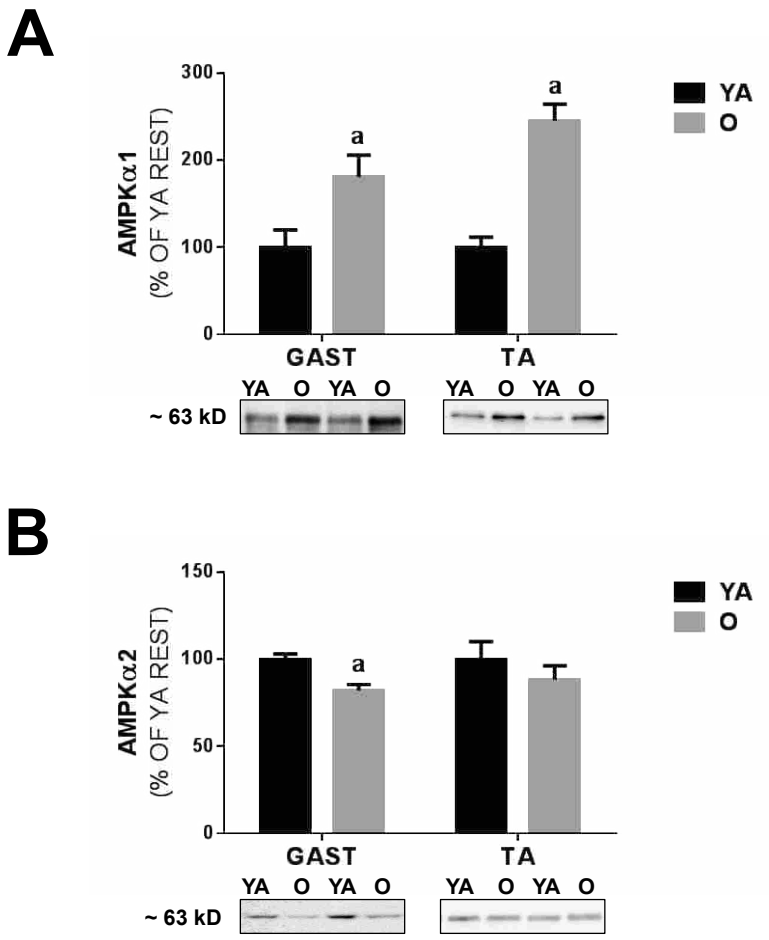

Figure 2.4: AMPKα1 Protein Content is Increased in O vs. YA Fast Twitch Muscle while AMPKα2 Content is Decreased. Western blotting analysis of GAST and TA for (A) AMPKα1 and (B) AMPKα2. N=8 / group. YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means ± SEM. a = significant difference from YA ($p < 0.05$).

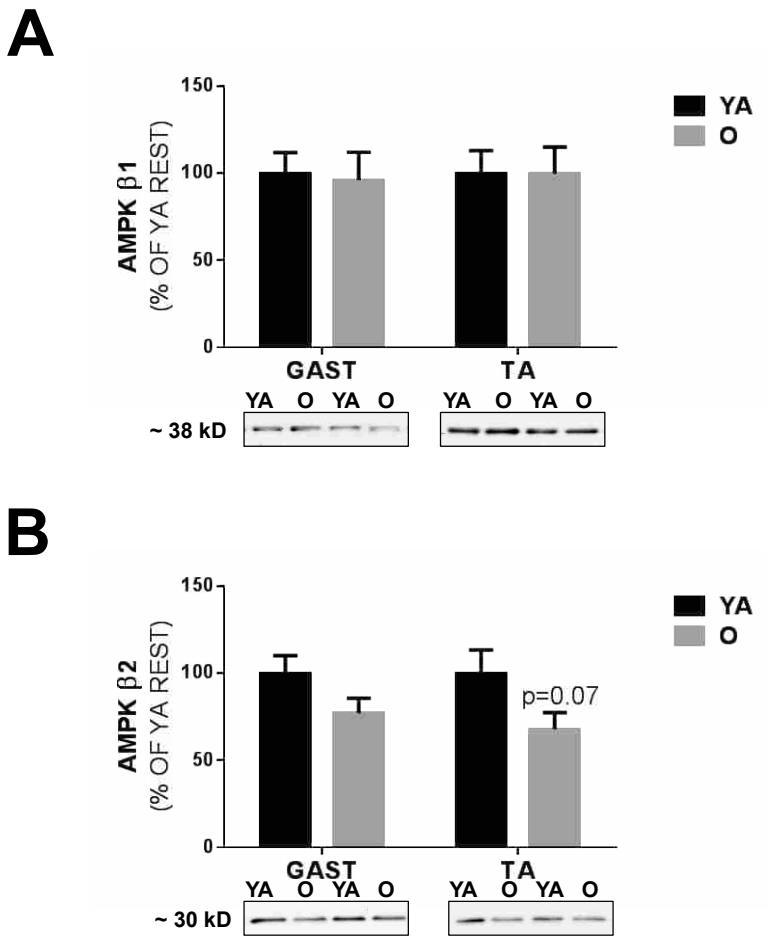

Figure 2.5: AMPKβ1 and β2 Protein Content are Unchanged in O vs. YA Fast Twitch Muscle. Western blotting analysis of GAST and TA for (A) AMPKβ1 and (B) AMPKβ2. N=8 / group. YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means ± SEM.

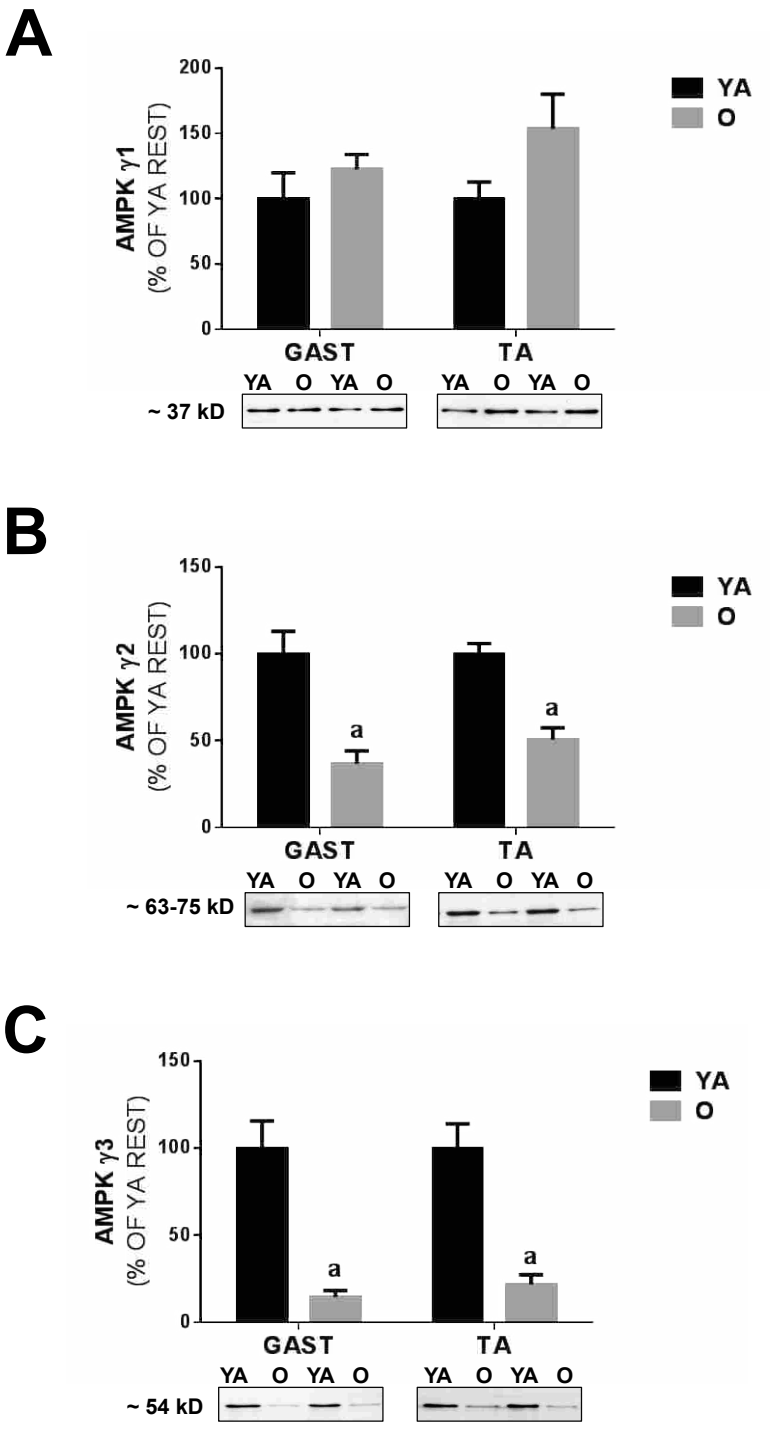

Figure 2.6: AMPKγ2 and γ3 Protein Content are Decreased in O vs. YA Rats. Western blotting analysis of GAST and TA for (A) AMPKγ1, (B) AMPKγ2 and (C) AMPKγ3. N=8 / group. YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means ± SEM. a = significant difference from YA ($p < 0.05$).

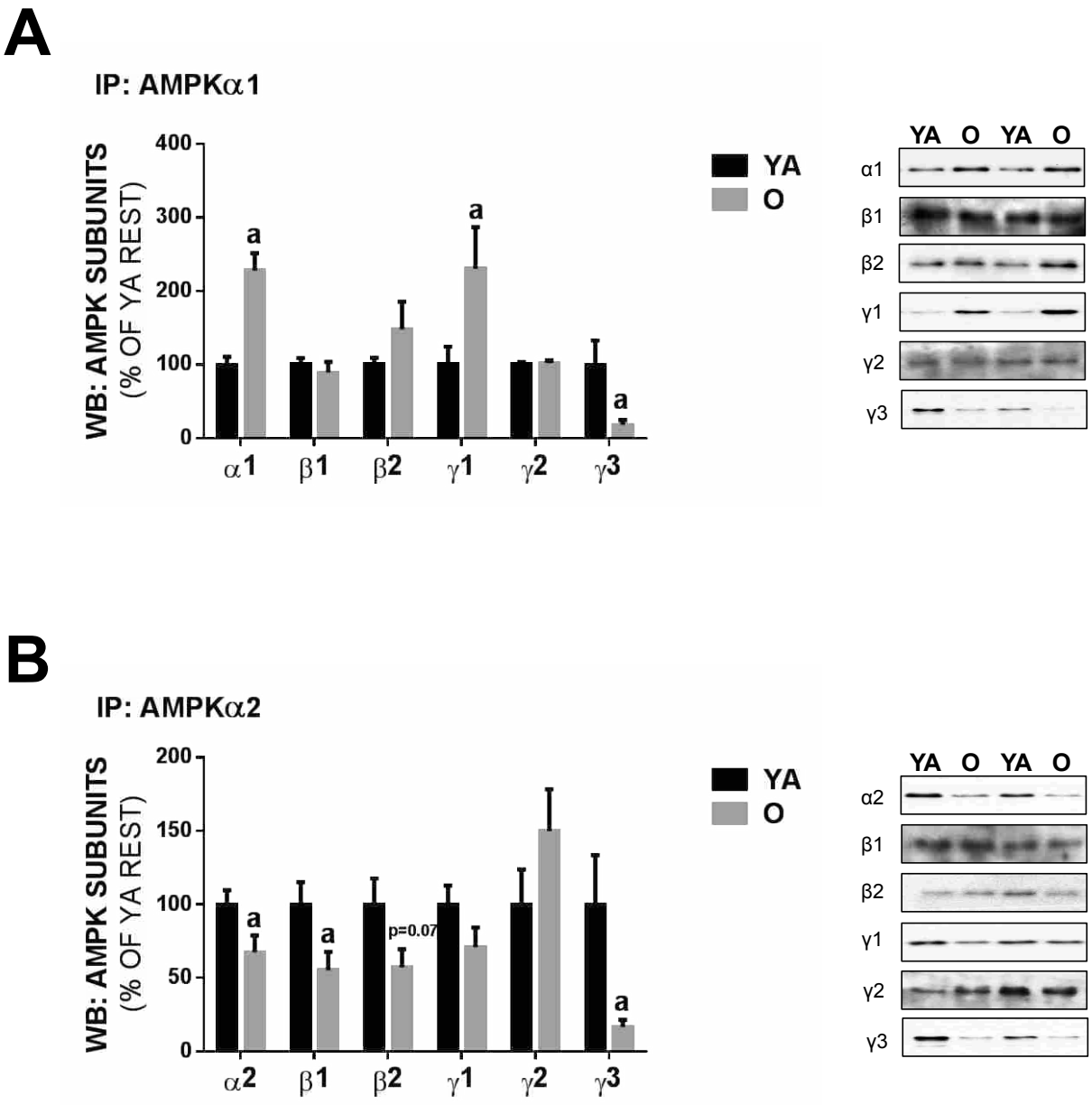

Figure 2.7: Association of the AMPKα1 and α2 Isoform with the Regulatory Isoforms is Altered in Aged Rats. Immunoprecipitation (IP) of the catalytic AMPKα1 or α2 isoform from GAST was followed by western blotting of the non-catalytic subunits to determine content. (A) IP of AMPKα1 followed by western blotting for AMPKα1, AMPKβ1, AMPKβ2, AMPKγ1, AMPKγ2, and AMPKγ3. (B) IP of AMPKα2 followed by western blotting for AMPKα2, AMPKβ1, AMPKβ2, AMPKγ1, AMPKγ2, and AMPKγ3. N=7-8/group. Values are means ± SEM. a = significant difference from YA ($p < 0.05$).

CHAPTER 3: The Effects of Age and Muscle Contraction on AMPK Nuclear Localization

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Abstract

Sarcopenia is the gradual degeneration of skeletal muscle mass due to alterations in skeletal muscle metabolism. AMP-activated protein kinase (AMPK) is a master regulator of skeletal muscle metabolic pathways and regulates many metabolic pathways that are disrupted with aging. AMPK regulates different metabolic pathways depending on its localization in the cytosol or nucleus. Exercise activates AMPK and increases translocation of AMPK to the nucleus. However, the effect that aging has on AMPK translocation is unknown. To study the effect of age on AMPK cellular localization, we studied gastrocnemius (GAST) and tibialis anterior (TA) muscles from YA (8 mo old) and O (30 mo old) male Fischer$_{344}$ x Brown Norway F1 hybrid rats after an *in situ* bout of endurance-type contractions produced via electrical stimulation of the sciatic nerve (STIM). Muscles from the resting contralateral leg served as controls (REST). Nuclear localization of phosphorylated AMPK was impaired in aging muscle after STIM. Additionally, nuclear localization of AMPKα2 and AMPKγ3 were impaired in aging muscle in response to STIM. Furthermore, AMPK and the heterotrimer isoform protein contents were also altered in aging muscle, independent of cellular localization, with an overall decrease in LKB1, total AMPK, phosphorylated AMPK, AMPKα2, AMPKβ2, and AMPKγ2 and an increase in AMPKα1 and AMPKγ1. The impaired translocation of activated AMPK to the nucleus, along with the altered content of the AMPK isoforms, may provide a mechanism for altered regulation of AMPK-regulated metabolic pathways in aging skeletal muscle.

Introduction

AMP-activated protein kinase (AMPK), a metabolic regulator protein, acts in opposition to many of the effects of aging and may provide insight into the effects of aging. AMPK is a heterotrimeric protein composed of a catalytic α subunit along with regulatory β and γ subunits (Davies *et al.*, 1994; Woods *et al.*, 1996a; Cheung *et al.*, 2000). The α and β subunits have two distinct isoforms referred to as α1 and α2 or β1 and β2 respectively (Salt *et al.*, 1998b). The γ subunit has three isoforms referred to as γ1, γ2, and γ3 (Thornton *et al.*, 1998).

AMPK is activated in response to cellular stressors, such as exercise, which increase the ratio of AMP (adenosine monophosphate) to ATP (adenosine triphosphate) (Winder & Hardie, 1996; Sakamoto *et al.*, 2005; Winder *et al.*, 2006). Increased AMP-regulated allosteric activation of AMPK increases the nuclear localization of the AMPKα2 isoform (Salt *et al.*, 1998a; McGee *et al.*, 2003). Translocation of AMPKα2 from the cytosol to the nucleus allows AMPK to regulate gene expression (Witczak *et al.*, 2008a). The β1 isoform is also localized to the nucleus through myristoylation and phosphorylation (Warden *et al.*, 2001b). The γ1 isoform further demonstrates preferential nuclear localization compared to the other two γ isoforms (Turnley *et al.*, 1999a).

The exact mechanism that drives AMPK into the nucleus remains unknown. However, the nuclear localization of Snf1, the yeast analog of AMPK, has been shown to be glucose-regulated (Vincent *et al.*, 2001) while in mammals, AMPK localization is regulated by the MEK-signaling pathway in response to cellular stress (Kodiha *et al.*, 2007). Additionally, the catalytic subunit of AMPK contains highly conserved carboxy-terminal hydrophobic amino acids that function as a nuclear export signal back into the cytoplasm (Kazgan *et al.*, 2010). Thus, when localized to the nucleus, AMPK may regulate cell function by altering gene expression through

phosphorylation of transcriptional regulators such as PGC1α (Jäger *et al.*, 2007) and PPARα/γ/δ (Leff, 2003; Bronner *et al.*, 2004; Narkar *et al.*, 2008). Alternatively, when AMPK is localized to the cytoplasm it may have direct effects on metabolism (e.g. by directly promoting fatty acid oxidation (Foretz *et al.*, 1998a), increasing glucose transport (Foretz *et al.*, 1998a), autophagy (Meley *et al.*, 2006) and inhibiting processes that consume ATP such as lipogenesis (Foretz *et al.*, 1998a), protein synthesis (Bolster *et al.*, 2002; Reiter *et al.*, 2005) and cholesterol synthesis (Henin *et al.*, 1995)).

Many AMPK regulated metabolic pathways, particularly fatty acid oxidation (Coggan *et al.*, 1992b; Sial *et al.*, 1996), lipogenesis (Park *et al.*, 2006), protein synthesis (Cohn *et al.*, 1980; Dardevet *et al.*, 2000), autophagy (Cuervo & Dice, 2000; Del Roso *et al.*, 2003), and Glut4 expression (Houmard *et al.*, 1995) (Lin *et al.*, 1991), are altered in aged skeletal muscle. Changes in the cellular localization of AMPK may contribute to the alterations in AMPK activation and regulation of metabolic pathways seen in aging skeletal muscle. Accordingly, the purpose of this study was to evaluate the cellular localization of AMPK in young adult and old skeletal muscle after an electrically stimulated endurance-type contraction bout.

Materials and Methods

Animal Care

Experimental procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. All animals were housed in a temperature controlled (20-21°C) environment with a 12h: 12h light-dark cycle and fed standard chow and water *ad libitum*. Young adult (YA) (8 mo.; n=8) and old (O) (30 mo.; n=8) male $Fischer_{344}$ x Brown Norway (FBN) hybrid rats were used as recommended by the National Institute of Aging for age-related research based on studies indicating that this strain has fewer detrimental pathologies

than inbred strains and 50% mortality at about 32 months of age (Lipman *et al.*, 1996; Lushaj *et al.*, 2008).

Electrical Stimulation of the Sciatic Nerve

Rats were anesthetized using vaporized isoflurane (2–3%) in supplemental oxygen sufficient to achieve surgical anesthetic depth. Twenty minutes after anesthetization, the sciatic nerve of the left hindlimb was isolated just proximal to the point of trifurcation. Contraction of the hindlimb musculature was elicited by stimulating the sciatic nerve using HFES (100 Hz; Grass Model S48 Stimulator, Quincy, MA) for 10 min at one 10 msec pulse per second at 15 volts (STIM). During the contraction bout, the left foot was held at approximately 90° to the tibia. The right hindlimb was not subjected to electrical stimulation and was removed prior to stimulation of the left hindlimb and served as a resting control (REST).

The gastrocnemius (GAST), tibialis anterior (TA), and extensor digitorum longus (EDL) were removed immediately after contraction. The GAST was frozen between metal tongs cooled to the temperature of liquid nitrogen and then frozen at -95ºC until further analysis. The EDL was frozen in isopentane at the temperature of liquid nitrogen and then frozen at -95ºC until further analysis.

Nuclear Isolation

To measure nuclear localization of AMPK, the nuclear fraction (NUC) of the right and left GAST and TA tissue samples from FBN rats were separated from the cytosolic fraction (CYT). Tissue samples were homogenized on a glass-on-glass homogenizer in 10 µl of lysis buffer (10 mM NaCl, pH 7.4; 1.5 mM MgCl; 20 mM HEPES; 20% glycerol; 0.1% triton X-100; 1 mM sodium orthovanadate; 1 mM DTT; 1 mM benzamidine; 200 mM phenylmethane sulfonyl fluoride; 5 ug/µl soybean trypsin inhibitor) per mg of tissue and placed in microcentrifuge tubes.

Samples were then centrifuged at 5000 x g for 5 min. The supernatant was collected in a separate tube and cleared by centrifuging at 6000 x g for 3 x 5 min and then stored at -95°C for further testing. The pellet was washed and suspended 3 times in 500 µl lysis buffer and centrifuged at 5000 x g between each wash. The pellet was then resuspended in 3µl of lysis buffer plus NaCl (360µl stock lysis buffer plus 49.8µl of 5 M NaCl) per mg of tissue. The pellet samples were rotated for 2 hours at 4°C to lyse the nuclei and then centrifuged at 15,000 x g for 15 min at 4°C. The supernatant was collected and stored at -95°C for further testing.

Western Blot and Immunodetection

Homogenates were diluted in sample loading buffer (125 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue) and then loaded on 7.5% [phosphorylated AMPK (pAMPK), total AMPK (tAMPK), LKB1, AMPKγ3], and 10% [Lamin B, GAPDH, AMPKα1, AMPKα2, AMPKβ1, AMPKβ2, AMPKγ1, AMPKγ2] Tris·HCl gels (Bio-Rad Criterion System, Hercules, CA). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were stained with Ponceau S and visually inspected for equal protein loading. Membranes were then washed with Tris-buffered saline plus 0.1% Tween-20 (TBST), blocked with 5% non-fat dry milk in TBST for 1 hour, and probed overnight at 4°C with primary antibody diluted in 1% bovine serum albumin (BSA) dissolved in TBST, pH 7.6.

Primary antibody manufacturers and dilutions were as follows: pAMPK (Thr172) (#4188/#2535, 1:5000), tAMPK (#2532, 1:2000), AMPKβ1 (#4182, 1: 2000), AMPKβ2 (#4148, 1:2000), and AMPKγ1 (#4187, 1:4000) from Cell Signaling Technology (Beverly, MA, USA); AMPKα1 (A300-507A, 1:4000), AMPKα2 (A300-508A, 1:20000) from Bethyl Laboratories, Inc. (Montgomery, TX, USA); AMPKγ2 (sc-20165, 1:2000), and Lamin B (M-20) (sc-6217,

1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); AMPKγ3 (custom made as described previously (Durante *et al.*, 2002), 1:2000) from Affinity Bioreagents (Golden, CO, USA); LKB1 (#07-694, 1:5000) from Upstate (Lake Placid, NY, USA); and GAPDH (MAB374, 1:200,000) from EMD Millipore (Billerica, MA, USA).

Membranes were probed with the appropriate secondary antibody [HRP-conjugated mouse anti-rabbit (#211-032-171) from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, USA); HRP-conjugated donkey anti-goat (sc-2020) or HRP-conjugated donkey anti-mouse (sc-2314) from Santa Cruz Technology] for 1 hour at room temperature. Membranes were washed 4 x 5 min with TBST. ECL Plus Western Blotting Detecting Solution (GE Healthcare Bio-Sciences, Piscataway, NJ) was applied for 2 min. Chemiluminescent signals were detected with autoradiography film and quantified using Gel-Pro Analyzer 6.0 (Media Cybernetics, Inc. Bethesda, MD) or AlphaEase FC software (Alpha Innotech Corp., San Leandro, CA).

Tissue Sectioning/Immunohistochemistry

EDL muscle samples from FBN rats frozen in isopentane following electrical stimulation extracted during tissue harvesting were placed in a mold containing Tissue Tek® O.C.T. compound (Ted Pella Inc., Redding, CA), covered, and then flash frozen in isopentane for 30 sec. Tissue samples were placed in aluminum foil and stored at -95°C until ready to be sectioned. Tissues were prepared for sectioning by placing in the cryostat microtome at -21°C for 30 min prior to cutting to allow the sample to equilibrate. The tissue sample was then mounted using O.C.T. compound and cut into 10 μm muscle sections at -21°C in cryostat microtome, placed on glass slides, and allowed to air dry. Glass slides with samples were fixed using acetone.

Cellular Localization

5% Normal Goat Serum (NGS) blocking solution was applied to the FBN EDL muscle slide samples for 30 min at room temperature and then removed. The primary antibodies pAMPKα (Cell Signaling Technology no. 4188) and caveolin (Santa Cruz Technology no. sc-5310) were diluted 1:250 (0.4 µl antibody in 100 µl 5% NGS-PBS) and then applied to the respective slide for 60 min at room temperature and washed 3 x 5 min in 1% PBS. The secondary antibodies Alexa Fluor® 546 goat anti-rabbit IgG (Invitrogen) and goat anti-mouse IgA-FITC (Santa Cruz Technology no. sc-3692) were diluted 1:500 (0.2 µl antibody in 100 µl 5% NGS-PBS) and applied for 30 min in the dark at room temperature and then washed 3 x 5 min in 1% PBS. The nuclei were stained with the DAPI stain for 2 min and washed 5-10 min with PBS. A coverslip was then mounted with Fluoromount-G to capture the images using a fluorescent microscope filter set.

Statistics

Statistical comparisons using Microsoft Excel or GraphPad Prism statistical analysis software (GraphPad Software Inc., La Jolla, CA) were made using a student *t* test or repeated measures ANOVA to determine statistical significance ($p \leq 0.05$) with Fisher's LSD post-hoc analysis employed where appropriate. Values are reported as means ± SE.

Results

Effect of Age on AMPK Cellular Localization

Cellular localization (CYT vs. NUC) was determined by comparing the protein content between age (YA vs. O) and condition (REST vs. STIM). GAPDH and Lamin B were detected to verify the enrichment of the CYT and NUC fractions (Figure 3.1). Overall, the total protein

content of AMPK in both the CYT and NUC fractions was significantly lower in O rats compared to YA rats (Figure 3.2B). LKB1 protein content also tended to be lower in the O versus YA rats but results were not significant (Figure 3.2A). Additionally, for both AMPK and LKB1, protein content was not significantly different between the CYT and NUC fractions or with STIM in both the YA and O rats (Figure 3.2A,B).

On the other hand, phosphorylated AMPK (pAMPK) protein content was not overall significantly different between age or condition in the GAST CYT fraction and the TA CYT and NUC fractions. However, in the GAST NUC fraction, STIM significantly increased pAMPK protein content in the YA and O but the increase was significantly attenuated in the aging muscle (Figure 3.2C).

Immunohistochemistry of the EDL indicated an overall decrease in colocalization of pAMPK with both the nuclei and muscle fibers in the O versus YA rats, indicating an overall decrease in pAMPK in aging rats. Additionally, we were unable to detect any significant difference in pAMPK with STIM in either the YA or O rats (Figure 3.3).

Effect of Age on the Cellular Localization of the AMPKα Catalytic Isoforms

AMPKα1 protein content in the GAST was greater in the O rats than the YA rats. However, cellular localization was not affected by age or STIM. There was no significant difference of AMPKα1 protein content in the TA (Figure 3.4A).

In contrast, AMPKα2 protein content in both the GAST and TA tended to be lower in the O versus YA rats. Additionally, AMPKα2 protein content was not affected by STIM in the O rats. However, in the TA NUC fraction of the YA rats, AMPKα2 increased significantly with STIM (Figure 3.4B).

Effect of Age on the Cellular Localization of the Regulatory AMPKβ Isoforms

Age and condition did not alter the CYT or NUC content of AMPKβ1 (Figure 3.5A). However, AMPKβ2 content was significantly lower in the O versus YA rats in the GAST CYT fraction and tended to be lower in the TA. However, in the NUC fractions of the GAST and TA, AMPKβ2 protein content was not different between age or condition (Figure 3.5B).

Effect of Age on the Cellular Localization of the Regulatory AMPKγ Isoforms

AMPKγ1 protein content in the O rats was greater than the YA rats for the GAST CYT fraction. Additionally, with STIM, AMPKγ1 significantly increased in the YA GAST CYT fraction to match levels found in the O rats. However, in the CYT and NUC fractions of the TA and the NUC fraction of the GAST, AMPKγ1 was not affected by age or condition (Figure 3.6A).

On the other hand, AMPKγ2 was significantly lower in the CYT fraction of the O rats compared to the YA rats in both the GAST and TA but was not affected by STIM. In the NUC fractions, age and condition did not significantly change the content of AMPKγ2 (Figure 3.6B).

Lastly, AMPKγ3 was not affected by age or condition in the CYT fraction of the GAST or in the CYT and NUC fraction of the TA. However, in the GAST NUC fraction, AMPKγ3 significantly increased with STIM in the YA rats but not the O rats (Figure 3.6C).

Discussion

This study found that the nuclear localization of phosphorylated AMPK is attenuated in aging rats after an electrically stimulated endurance-type contraction bout. More specifically, the nuclear localization of AMPKα2 and AMPKγ3 are attenuated in aging muscle in response to STIM. Furthermore, this study supports previous findings from Chapter 2 that AMPK and the heterotrimer isoform protein content are altered in aging muscle with a decrease in protein

content of LKB1, total AMPK, pAMPK, AMPKα2, AMPKβ2, and AMPKγ2 and an increase in AMPKα1 and AMPKγ1.

The nuclear isolation procedure was sufficient to isolate the cytosolic and nuclear fractions as verified by the presence of Lamin B restricted mainly to the NUC fraction and GAPDH restricted to the CYT fraction. The predominant AMPK heterotrimeric subunit composition activated during exercise in murine and human skeletal muscle is α2β2γ3 (Cheung *et al.*, 2000; Durante *et al.*, 2002; Mahlapuu *et al.*, 2004; Yu *et al.*, 2004; Birk & Wojtaszewski, 2006; Steinberg *et al.*, 2010). Activation of AMPK was previously shown to result in increased nuclear localization of the AMPKα2 isoform (Salt *et al.*, 1998a; McGee *et al.*, 2003). Furthermore, AMPKα2 is the predominant isoform expressed in skeletal muscle (Stapleton *et al.*, 1996) and should therefore closely reflect the phosphorylated AMPK protein content. Consistently, we found that the nuclear protein content of pAMPK and AMPKα2 in the TA increased in response to STIM compared to REST in the YA rats. Additionally, nuclear protein content of AMPKγ3 in the GAST increased in response to STIM in the YA rats. The disparate response seen between the GAST and the TA may be due to the anatomical positioning of the two muscles along with the load naturally placed on the muscle throughout the lifespan of the rat.

Conversely, in the O rats the nuclear protein content of pAMPK also increased in response to STIM but was significantly attenuated in the aging muscle. However, AMPKα2 and AMPKγ3 did not increase in response to STIM in the NUC fraction of the aging muscle. The decreased nuclear localization of activated AMPK may contribute to many of the dysfunctional characteristics of aging skeletal muscle. One example is through the regulation of GLUT4 by AMPK. Activation of AMPK and translocation to the nucleus regulates the expression of

GLUT4 (glucose transporter type 4) (Holmes *et al.*, 1999; Holmes *et al.*, 2005), which in turn regulates glucose uptake. Age is associated with decreased GLUT4 expression and basal glucose uptake (dos Santos *et al.*, 2012). Therefore, it is likely that the decreased nuclear localization of AMPK in aging muscle may affect the gene expression of other genes to contribute to the aging phenotype.

We additionally found that aging skeletal muscle was associated with an altered protein content of LKB1, AMPK, and the AMPK heterotrimer isoforms. We found an overall decrease in the protein content of LKB1, total AMPK, pAMPK, AMPKα2, AMPKβ2, and AMPKγ2. As stated previously, AMPKα2 is the predominant isoform expressed in skeletal muscle (Stapleton *et al.*, 1996). Additionally, AMPKγ2 is the predominant isoform expressed in all tissues (Mahlapuu *et al.*, 2004). The decrease protein content of AMPKα2 and AMPKγ2 in aging muscle further supports the overall decrease in total AMPK and pAMPK content. This overall decrease in content of AMPK in aging muscle may further contribute to the aging phenotype by limiting the availability of AMPK in aging muscle to regulate metabolic pathways.

Interestingly, we also saw an increase in the cytosolic concentration of AMPKα1 and AMPKγ1. AMPKα1 has been shown to play an isoform-specific role in inhibiting the mTOR pathway and therefore protein synthesis in skeletal muscle (Mounier *et al.*, 2009; Mounier *et al.*, 2011). The increase in content of AMPKα1 in the cytosol could contribute to the deficit in contraction-induced activation of mTOR and protein synthesis observed with old age (Parkington *et al.*, 2004; Thomson & Gordon, 2006). Previously in Chapter 2 we found that in aging muscle, AMPKα1 has an increased association with AMPKγ1. Therefore, the increased availability and interaction between AMPKα1 and AMPKγ1 in aging muscle may compensate for the decrease in the predominant α2β2γ3 heterotrimer composition. This change in the AMPK

heterotrimer composition along with decreased nuclear localization may contribute to the aging phenotype by increasing the inhibition of protein synthesis.

In conclusion, the nuclear localization of phosphorylated AMPK after an electrically stimulated endurance-type contraction bout is attenuated in aging rat skeletal muscle. This is correlated with an overall decrease in the protein content of LKB1, total AMPK, pAMPK, AMPKα2, AMPKβ2, and AMPKγ2 in aging muscle. The decreases in the AMPK heterotrimer isoforms are accompanied by an increase in the protein content of AMPKα1 and AMPKγ1 in the aging rat muscle. These alterations in the composition and localization of AMPK in aging muscle in response to muscle contraction may contribute to the aging phenotype by changing the localization and therefore regulation of downstream metabolic pathways.

Acknowledgements

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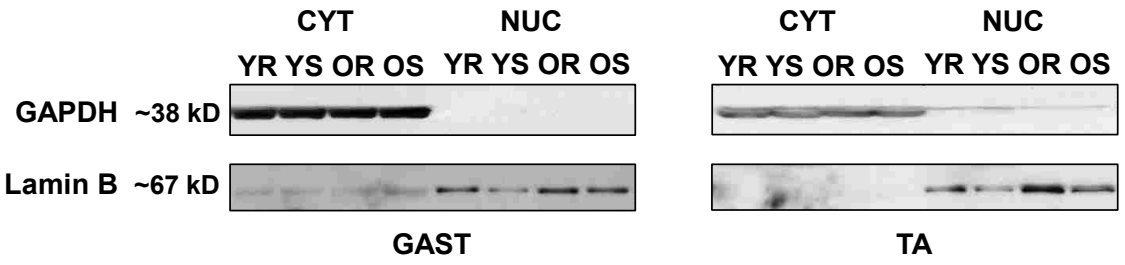

Figure 3.1: Purification of the Cytosolic and Nuclear Fractions from YA and O Rat Skeletal Muscle. Gastrocnemius (GAST) and tibialis anterior (TA) muscles from YA (8 mo.) and O (30 mo.) Fisher Brown Norway male rats were stimulated for 10 min (1 pulse per second, 15V, 10 msec duration) and removed immediately after the contraction bout. Nuclear isolation was performed to separate the cytosolic (CYT) and nuclear (NUC) fractions. Western blot images for GAPDH and Lamin B. N=8 / group. YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM.

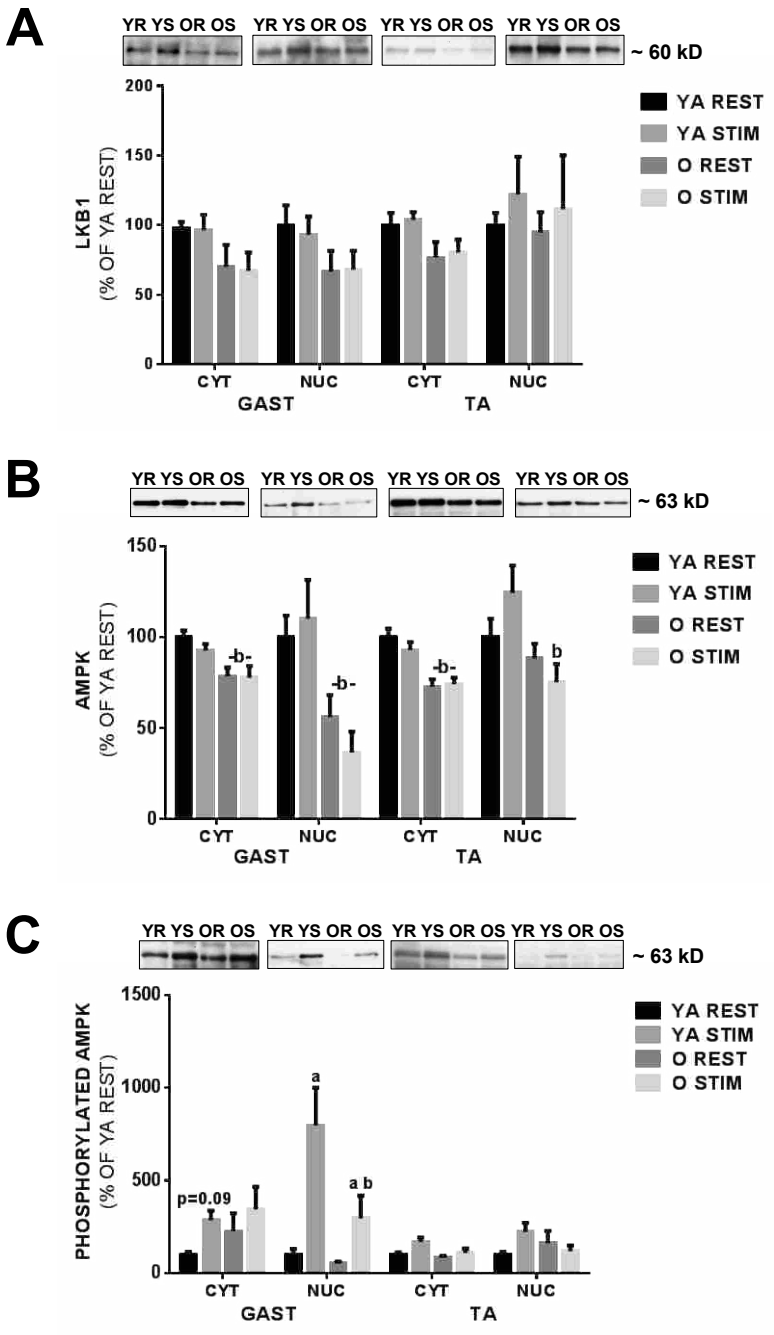

Figure 3.2: Nuclear Localization of pAMPK in Response to STIM in the GAST is Attenuated in Aging Rat Skeletal Muscle. Western blot analysis of GAST and TA for (A) LKB1, (B) total AMPK (C) pAMPK. N=8 / group. CYT = cytosolic fraction; NUC = nuclear fraction; YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means ± SEM. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

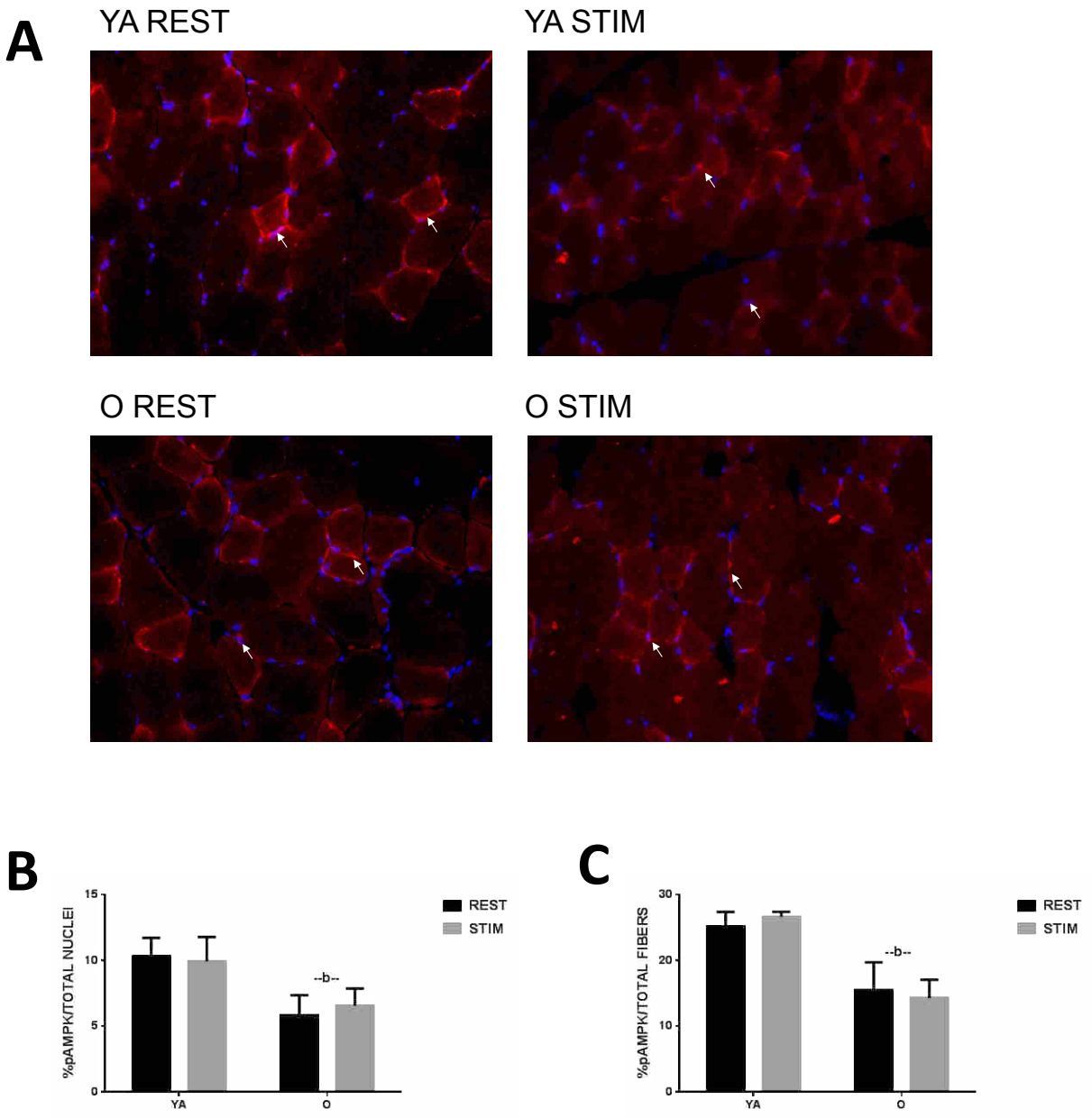

Figure 3.3: Colocalization of pAMPK in the Muscle Nuclei and Fibers is Decreased in Aging Rat Skeletal Muscle. (A) Immunohistochemistry of the extensor digitorum longus (EDL) muscle for pAMPK, (B) Quantitative analysis of the percent of muscle nuclei colocalized with pAMPK, (C) Quantitative analysis of the percent of muscle fibers colocalized with pAMPK,. N=7-8 / group. CYT = cytosolic fraction; NUC = nuclear fraction; YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means ± SEM. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

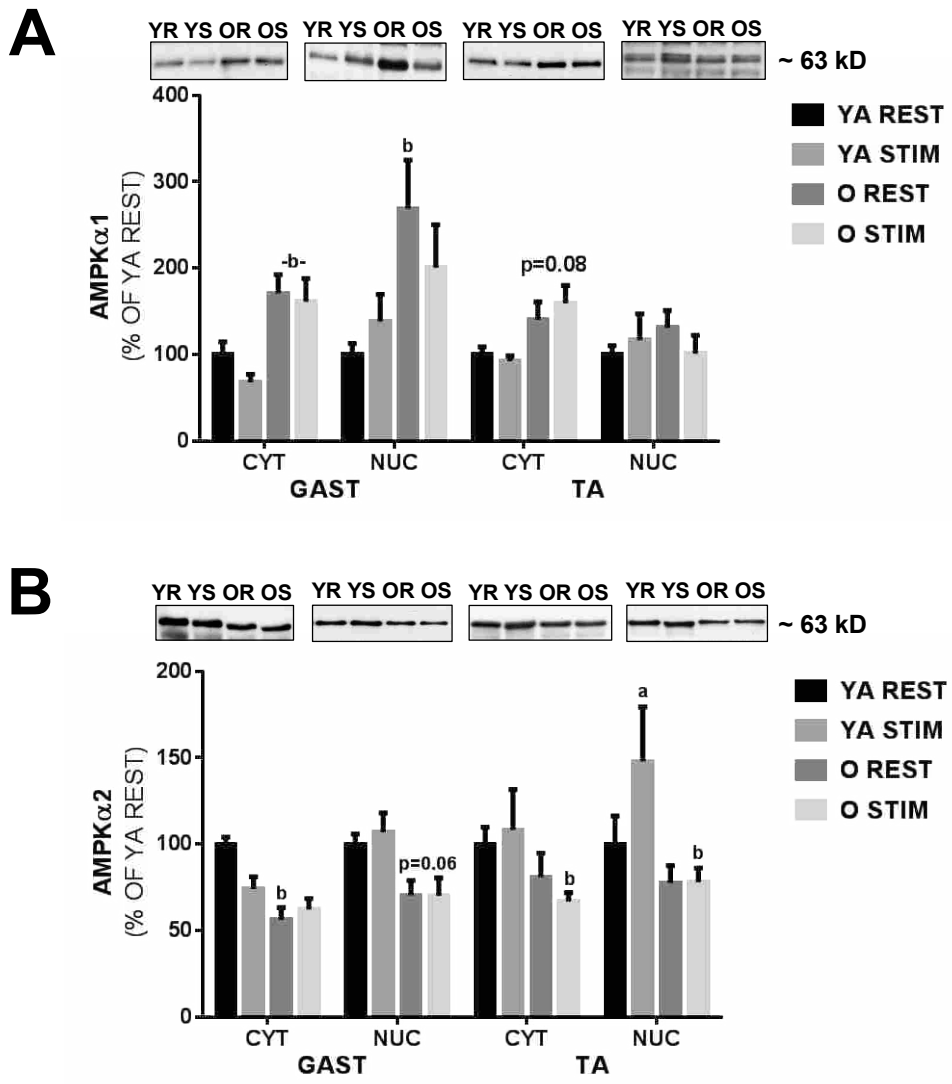

Figure 3.4: Nuclear Localization of AMPKα2 in Response to STIM in the TA is Attenuated in Aging Rat Skeletal Muscle. Western blotting analysis of GAST and TA for (A) AMPKα1 and (B) AMPKα2. N=8 / group. CYT = cytosolic fraction; NUC = nuclear fraction; YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means ± SEM. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

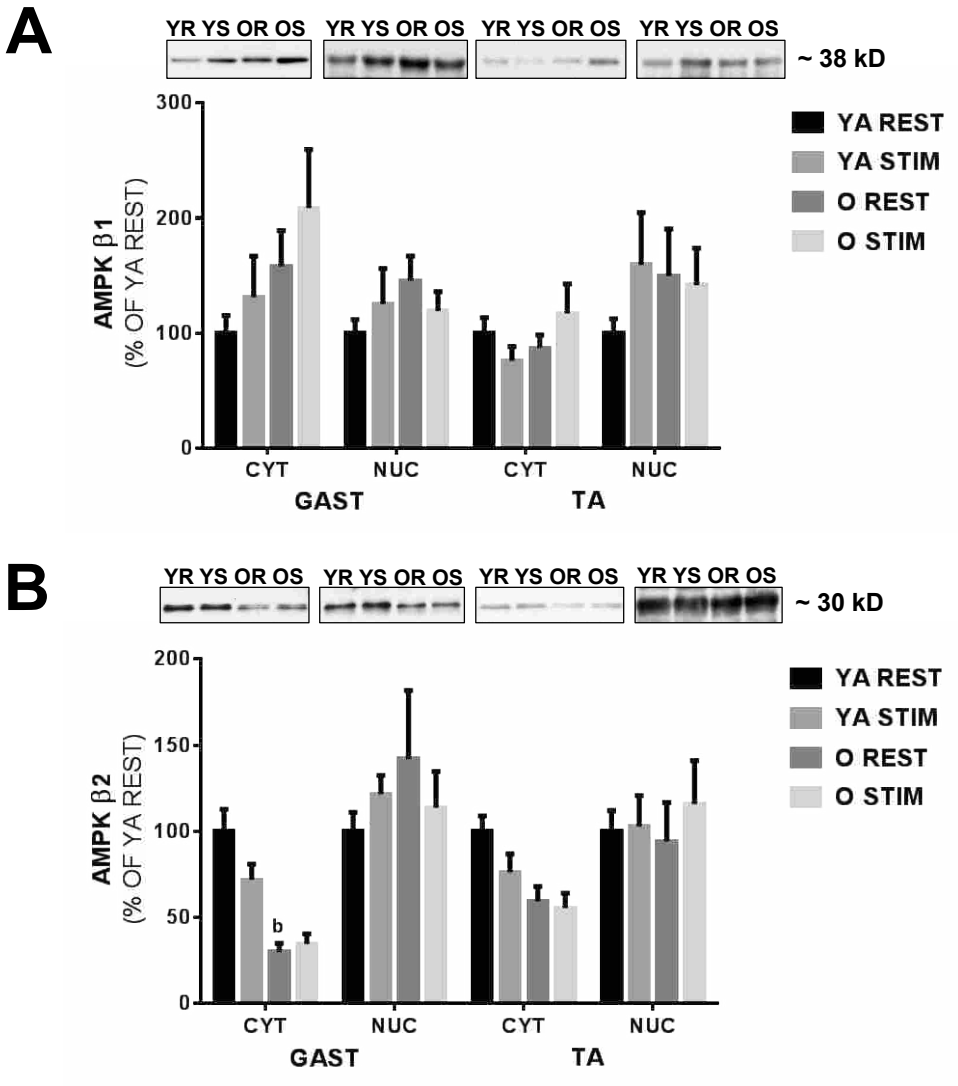

Figure 3.5: Nuclear Localization of AMPKβ1 and β2 are Unaffected in Aging Rat Skeletal Muscle. Western blotting analysis of GAST and TA for (A) AMPKβ1 and (B) AMPKβ2. N=8 / group. CYT = cytosolic fraction; NUC = nuclear fraction; YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means ± SEM. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

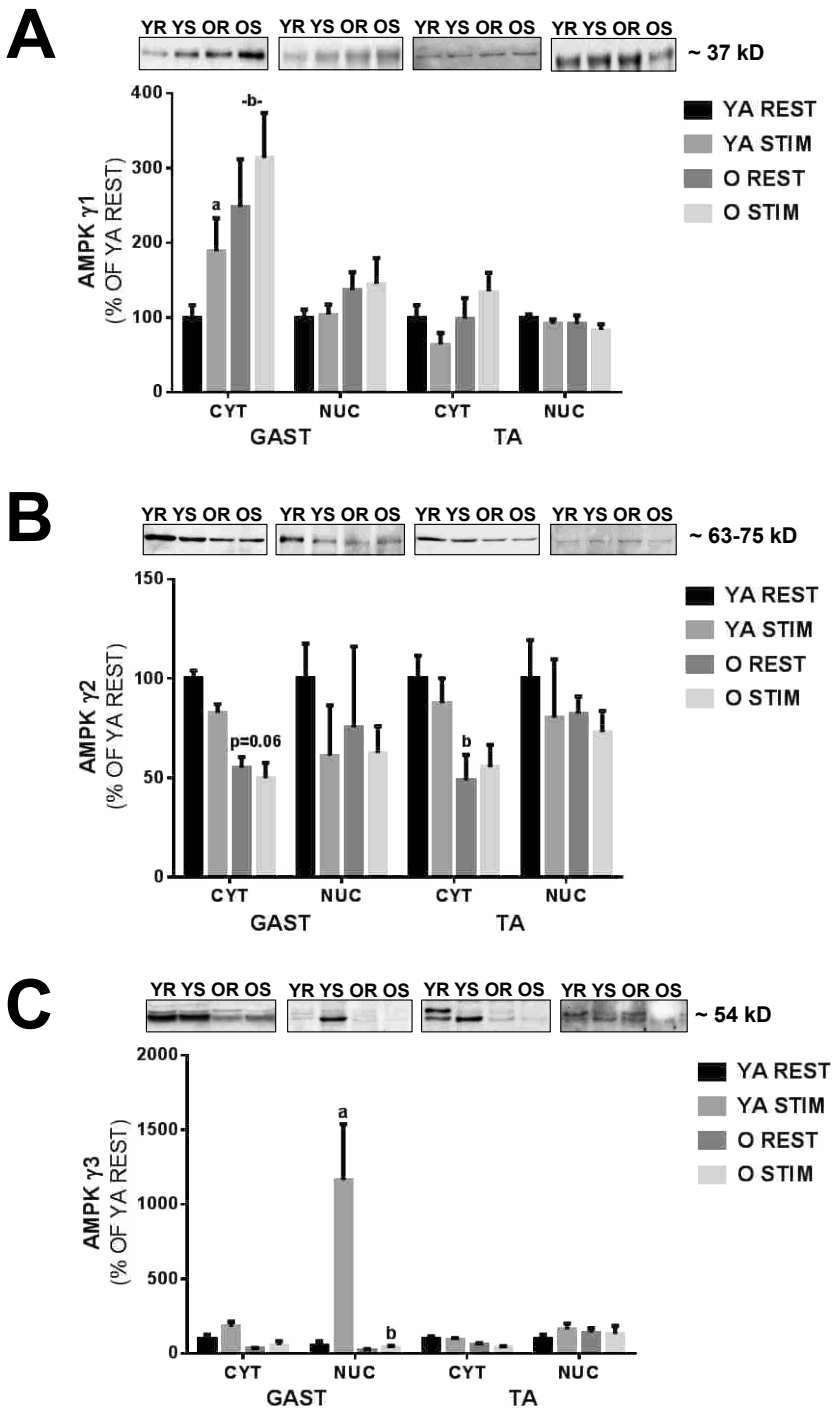

Figure 3.6: Nuclear Localization of AMPKγ3 in Response to STIM in the GAST is Attenuated in Aging Rat Skeletal Muscle. Western blotting analysis of GAST and TA for (A) AMPKγ1, (B) AMPKγ2 and (C) AMPKγ3. N=8 / group. CYT = cytosolic fraction; NUC = nuclear fraction; YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means ± SEM. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

CHAPTER 4: The Effect of Chronic AICAR Treatment on the Aging Phenotype

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Abstract

Sarcopenia is characterized by alterations in many metabolic pathways. AMP-activated protein kinase (AMPK), a metabolic regulator, acts in opposition to many of the effects of aging and may serve as a potential therapeutic target. Acute activation of AMPK has been shown to increase mitochondrial content, activate autophagy, and repress protein synthesis in normal skeletal muscle; pathways that are altered with aging. However, the effect of chronic activation of AMPK on metabolic pathways in aging skeletal muscle has yet to be addressed. Therefore, the purpose of this study was to assess the effect of chronic activation of AMPK in aging skeletal muscle by administering 5'-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) injections for one month in YA (5 mo old) and O (23 mo old) male C57Bl/6 mice. Mice were treadmill tested prior to and after one month of AICAR treatment. Following treatment, *in vitro* muscle contractions were performed on the right extensor digitorum longus (EDL) muscle. Chronic AICAR treatment improved the treadmill endurance in O mice and the rate of fatigue and recovery in response to *in vitro* muscle contractions in YA mice. Additionally, chronic AICAR increased citrate synthase activity, decreased SQSTM1/p62 protein content, and decreased Myf6 protein content in both the YA and O mice. Therefore, chronic AICAR treatment alters downstream metabolic pathways in skeletal muscle to improve the exercise response in both YA and O mice.

Introduction

The increased risks associated with sarcopenia, or age-related skeletal muscle atrophy, may be attributed to alterations in skeletal muscle metabolism and function. Age-associated impairments in skeletal muscle metabolism include an increased number of dysfunctional mitochondria (Kumaran *et al.*, 2004a; Terman *et al.*, 2006b), increased fat deposition due to decreased fat oxidation (Coggan *et al.*, 1992a; Sial *et al.*, 1996; Calles-Escandon & Poehlman, 1997; Park *et al.*, 2006; Slawik & Vidal-Puig, 2006), accumulation of damaged organelles through decreased autophagy (Pfeifer, 1978; Cuervo & Dice, 2000; Del Roso *et al.*, 2003; Combaret *et al.*, 2009), and a decrease in muscle protein content (Cohn *et al.*, 1980; Dardevet *et al.*, 2000; Fry *et al.*, 2011).

Adenosine monophosphate (AMP) -activated protein kinase (AMPK), a metabolic regulator, acts in opposition to many of the age-associated impairments in skeletal muscle metabolism. AMPK is a heterotrimeric protein composed of a catalytic α subunit along with regulatory β and γ subunits (Davies *et al.*, 1994; Woods *et al.*, 1996b; Cheung *et al.*, 2000). Liver kinase B1 (LKB1), a serine /threonine kinase, phosphorylates the α subunit on the threonine (Thr) 172 residue of AMPK to activate AMPK in skeletal muscle in response to endurance exercise (Hawley *et al.*, 1996; Winder & Thomson, 2007). In addition, endurance exercise creates an energy deficient state, which increases the amount of AMP in comparison to adenosine triphosphate (ATP) (Sakamoto *et al.*, 2005; Winder *et al.*, 2006). AMP binds to the γ subunit of AMPK to make AMPK a better substrate for LKB1 (Hawley *et al.*, 1995; Hawley *et al.*, 1996; Scott *et al.*, 2007) and a worse substrate for Protein phosphatase 2C (PP2C), an AMPK phosphatase (Davies *et al.*, 1995b; Marley *et al.*, 1996). AMP additionally induces a conformational change in AMPK to further induce allosteric activation of AMPK (Hawley *et al.*,

1995; Scott *et al.*, 2004; Witczak *et al.*, 2008a). Activation of AMPK regulates metabolic pathways to stimulate processes that produce ATP such as fatty acid oxidation (Foretz *et al.*, 1998a), glucose uptake (Foretz *et al.*, 1998a), and autophagy (Meley *et al.*, 2006) and inhibits processes that consume ATP such as lipogenesis (Foretz *et al.*, 1998a), protein synthesis (Bolster *et al.*, 2002; Reiter *et al.*, 2005) and cholesterol synthesis (Henin *et al.*, 1995).

AICAR, or 5'-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, is a pharmacological activator of AMPK. AICAR increases the accumulation of 5-amino-4-imidazole-carboxamide ribotide (ZMP) in the cell in a dose-dependent manner (Sabina *et al.*, 1985; Sullivan *et al.*, 1994). ZMP mimics AMP to increase AMPK activation through allosteric activation and promotion of phosphorylation (Sullivan *et al.*, 1994; Corton *et al.*, 1995). AICAR is sufficient to activate AMPK in skeletal muscle similar to activation by exercise and muscle contraction (Merrill *et al.*, 1997). Acute AICAR treatment in skeletal muscle increases fatty acid oxidation (Merrill *et al.*, 1997), glucose uptake (Merrill *et al.*, 1997) autophagy (Sanchez *et al.*, 2012) and protein synthesis (Bolster *et al.*, 2002). Furthermore, chronic AICAR treatment increases mitochondrial gene expression and enzyme activity (Holmes *et al.*, 1999; Winder *et al.*, 2000; Zhou *et al.*, 2000; Narkar *et al.*, 2008) and increases running endurance in young, healthy animals (Narkar *et al.*, 2008). Therefore, chronic AICAR treatment may be sufficient to enhance and/or compensate for the age-related changes in metabolic regulation.

Our lab has previously addressed the effect of acute AICAR treatment on AMPK activation in aged muscle. Acute AICAR treatment increased phosphorylation of Thr172 of AMPK and activity of AMPKα2 one hour post-treatment in both young adult and old rat muscle tissue (Thomson *et al.*, 2009). However, although results were not significant, there appeared to be a blunted response to the AICAR treatment in the old rats compared to the young adult rats.

Reznick *et al.* also demonstrated that rats infused with AICAR for one hour showed an increase in phosphorylation of AMPK and increased activity of AMPKα2 in the young adult rats (3 mo.); whereas AMPK activity in the old rats (28 mo.) was blunted (Reznick *et al.*, 2007b). These findings were further supported by Qiang *et* al (Qiang *et al.*, 2007). Qiang *et* al administered AICAR subcutaneously for one week to young and old rats. The old rats showed a 63% impairment in phosphorylated AMPK levels with AICAR, signifying a drastic impairment in AMPK activation in old rats compared to young rats. Therefore, although the response is blunted in aged muscle, AICAR treatment is sufficient to activate AMPK. However, the known effects of chronic AICAR treatment on metabolic pathways have yet to be addressed in aging muscle. We hypothesized that one month chronic AICAR treatment would sufficiently activate AMPK to improve the regulation of mitochondrial biogenesis, autophagy, and protein synthesis in aged skeletal muscle.

Materials and Methods

Animal Care

Experimental procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. All animals were housed in a temperature controlled (20-21°C) environment with a 12h: 12h light-dark cycle and fed standard chow and water *ad libitum*. YA (5 mo.; n=24) and O (23 mo.; n=27) male C57Bl/6 mice were used as recommended by the National Institute of Aging for age-related research.

AICAR injections

Acute AICAR mice were subcutaneously injected with AICAR dissolved in saline (0.5 mg/g body weight, 50 mg AICAR/ml saline) or with an equivalent volume of saline without AICAR. Mice were anesthetized by isoflurane inhalation (2-4% with nitrous oxide in supplemental oxygen) sufficient to achieve surgical anesthetic depth forty minutes after the AICAR injection. Muscle tissue samples were removed 1 hour post-injection and frozen to the temperature of liquid nitrogen and stored at -95°C until further analysis.

Chronic AICAR mice were subcutaneously injected with AICAR dissolved in saline (up to 0.5 mg/g body weight, 50 mg AICAR/ml saline) or with an equivalent volume of saline without AICAR daily for 31 days. (Mice were gradually brought up to the full dosage by injecting 0.3 mg/g body weight, 50 mg AICAR/ml saline for one week; 0.4 mg/g body weight, 50 mg AICAR/ml saline for one week; and 0.5 mg/g body weight, 50 mg AICAR/ml saline for the remaining two weeks.)

Treadmill Test

Mice were run on a rodent treadmill prior to the start of chronic injections and one week prior to harvest. Mice were run at a temperature of 60°F on a 7% grade at for 12 m/min for 3 min, followed by 16 m/min for 3 min, and then 20m/min until exhaustion (defined as remaining unresponsive to prodding with a brush at the back of the treadmill for 5 consecutive seconds). Results were compared between pre and post-injections treadmill testing.

Measurement of *In Vitro* Contractile Properties of the EDL

After chronic AICAR injections, mice were anesthetized by isoflurane inhalation (2-4% with nitrous oxide in supplemental oxygen) sufficient to achieve surgical anesthetic depth. The right EDL was removed and attached to a servomotor (300B Dual-Mode Lever System; Aurora

Scientific, Aurora, Canada) using a 4-0 silk suture and clamp-secured to a stationary platform in a jacketed tissue bath filled with Ringer's solution (137 mM NaCl, 24 mM $NaHCO_3$, 11 mM D-glucose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $NaH_2PO_4.H_2O$, 1 mM $MgSO_4$, pH 7.4; aerated with 95% O_2/CO_2 at 37°C). The muscle was allowed to equilibrate to the bath for 10 min. The optimal length was determined from a resting tension of 0.2-0.5 mN at 100 volts. Force frequency relationship was determined using a train duration of 500 msec at 10, 20, 40, 80, 100, 150, 200, and 250 Hz (S88X Grass Stimulator; Astro-Med, Inc., West Warwick, RI, USA) after which the muscle was stimulated for 5 min at 150 Hz with a train frequency of 0.2/sec with a train duration of 150 msec to determine the rate of fatigue. Acute muscle recovery was determined by five additional stimulations after resting 5 min. Following stimulation, the EDL was frozen to the temperature of liquid nitrogen and stored at -95°C until further analysis. The white quadricep tissues samples were removed and frozen to the temperature of liquid nitrogen and stored at -95°C until further analysis.

Homogenization

White quadricep muscles were pulverized on liquid nitrogen then glass-ground homogenized in 19-volumes of homogenization buffer (50 mM Tris-HCl, pH 7.4; 250 mM mannitol; 50 mM NaF; 5 mM Sodium Pyrophosphate; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 50 mM B-glycerophosphate; 1 mM sodium orthovanadate; 1 mM DTT; 1 mM benzamidine; 0.1 mM phenylmethane sulfonyl fluoride; 5 ug/ml soybean trypsin inhibitor). The raw homogenate was freeze- thawed three times to ensure disruption of intracellular membranes and then centrifuged at 10,000 x g for 10 min. Supernatants were analyzed for protein content using the DC Protein Assay (Biorad Laboratories, Hercules, CA, USA). Supernatants were stored in microcentrifuge tubes at -95°C until further analysis.

AMPK Activity Assay

AMPK activity of the α1 and α2 subunits immunoprecipitated from acute AICAR white quadricep homogenates was measured by the incorporation of radiolabeled phosphate from ATP into the SAMS peptide using a scintillation counter. Immunoprecipitates were prepared by adding rabbit anti-AMPKα1 or α2 antibody to a microcentrifuge tubes containing protein G sepharose. 40µl of spun homogenate samples were added to the rabbit antibody and protein G sepharose pellet, mixed overnight at 4°C and washed with ice-cold IP buffer (50 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1mM EGTA, 1mM DTT, 1 mM benzamidine, 0.1 nN PMSF, 5 µg/ml soybean trypsin inhibitor, pH 7.4) and 1 M NaCl. The pellet was then washed with lysate assay buffer (62.5 mM Na HEPES, 62.5 mM NaCl, 62.5 mM NaF, 6.25 mM sodium pyrophosphate, 1.25 mM EDTA, 1.25 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 µg/ml soybean trypsin inhibitor, pH 7.4), centrifuged, and the supernatant aspirated. The pellet was resuspended in HEPES-Brij (HB) buffer (25 mM HEPES, 0.02% Brij, 1 mM DTT, pH 7.4) and transferred to microcentrifuge tubes. The reaction was started by adding 15 µl of the working assay cocktail (40 mM HEPES, 0.2 mM SAMS peptide (HHMRSAMSGLHLVKRR-OH), 0.2 mM AMP, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM $MgCl_2$, 0.2 mM ATP, pH 7.0) at timed intervals of 30 sec. Samples were incubated at 150 rpm in a thermomixer at 30°C for 10 min. 15 µl of reaction mix was transferred to a quarter circle of P81 filter paper, washed five times in 1% phosphoric acid, and then washed with ddH_20 and acetone and allowed to dry. The filter paper was then added to a vial and placed in the scintillation counter along with a blank control vial and a positive control hot assay cocktail vial to calculate the specific activity. Activity was

assessed and expressed as picomoles phosphate incorporated into SAMS peptide per gram tissue per minute.

Western Blot and Immunodetection

White quadricep homogenates were diluted in sample loading buffer (125 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue) to load onto Tris·HCl gels (Bio-Rad Criterion System, Hercules, CA). Electrophoresis was applied for 45-55 min at 200 volts. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which were then probed for specific proteins via immunodetection. The antibodies used were as follows: pACC (Ser79) (#3661), Akt (#9272), pAkt (Ser473) (#4060), AMPKα (#2532), pAMPKα (Thr172) (#4188), Atg5 (#8540), Atg7 (#8558), Atg12 (#4180), Beclin1 (#3738), 4EB-P1 (#9644), p4EB-P1 (Thr37/46) (#2855), eEF2 (#2332), peEF2 (Thr56) (#2331), eIF2α (#9722), peIF2α (Ser51) (#9721), LC3A (#4599), LC3B (#2775), mTOR (#2983), pRaptor (Ser792) (#2083), S6 (#2217), pS6 (Ser235/236) (#4858), p70 S6K (#2708), p-p70 S6K (Thr389) (#9234), STAT3 (#9139), pSTAT3 (Tyr705) (#9145), SQSTM1/p62 (#5114), Ulk1 (#4773), pUlk1 (ser555) (#5869), and pUlk1 (ser757) (#6888) from Cell Signaling Technology (Beverly, MA, USA); LKB1 (#07-694) from Upstate (Lake Placid, NY, USA); PGC1α (#AB3242), UCP-3 (#PA1-055) from Affinity Bioreagents (Rockford, IL, USA); Cytochrome C (#13156), Hexokinase II (#6521), MAFbx (#33782), Myf5 (#302), Myf6 (#301), MyoD (#304) from Santa Cruz Biotechnology (Dallas, TX, USA); Cox4 (#A6403) and Oxphos (#457999) from Invitrogen Life Technology (Grand Island, NY, USA); and ACC (streptavidin-horseradish peroxidase, RPN1231V) from GE Healthcare Biosciences (Pittsburgh, PA, USA). Chemiluminescent signals from blots were analyzed using AlphaEase FC software (Alpha Innotech Corp., San Leandro, CA) to determine relative protein levels.

Tissue Sectioning/Immunohistochemistry

Tibialis anterior (TA) muscle samples from the chronically treated mice were extracted during tissue harvesting and placed in a mold containing Tissue Tek® O.C.T. compound (Ted Pella Inc., Redding, CA), covered, and then flash frozen in isopentane for 30 sec. Tissue samples were placed in aluminum foil and stored at -95°C until ready to be sectioned. Prior to sectioning, the samples were placed in the cryostat microtome at -21°C for 30 min to allow the samples to equilibrate. The tissue samples were mounted using O.C.T. compound and then cut into 8 μm muscle sections at -21°C, placed on glass slides, and allowed to air dry.

CD31 Immunofluorescence

Skeletal muscle capillarity was assessed by determining the capillary to fiber ratio on chronically-treated muscle samples. Sectioned samples were fixed in acetone and washed in PBS, permeabilized in 0.3% Triton X-100 in PBS for 10 min at 4°C, and blocked in 5% Normal Goat Serum (NGS) in PBS for 30 min at room temperature (RT). After blocking, samples were incubated for at least 1 hour at RT (or overnight at 4°C) in 1:50 dilution of CD31 primary antibody (AbD Serotec, no. MCA2388, Raleigh, NC, USA) in 5% NGS in PBS and then washed 3 X 5 min with PBS. Samples were incubated for 30 min at RT in 1:100 dilution of secondary antibody (cy3-conjugated goat anti-rat IgG) in the dark or an opaque plastic jar. Samples were then washed 3 X 5 min in PBS in an opaque plastic jar. Coverslips were applied using Fluormount and images were captured using fluorescent microscopy with the TRITC filter set and the 20X objective. To analyze, the total number of capillaries per field were counted and expressed as a ratio to the total number of muscle fibers per field.

Succinate Dehydrogenase Staining

Sectioned samples from the chronically-treated mice were placed in a pre-warmed substrate solution [sodium succinate (Sigma S2378) and nitrotetrazolium blue (Sigma N6639) in 0.2 M phosphate buffer] in a 37°C water bath. Slides were washed in distilled water 3 X 1 min, dried and mounted with a coverslip using mounting medium. Images were captured using light microscopy. Purple formazan precipitate is deposited at sites of mitochondria. Oxidative fibers are darker than glycolytic to allow for the percentage of cells that are SDH-positive to be determined.

Myosin Heavy Chain (MHC) Expression

Sectioned slides were blocked in 10% normal goat serum (NGS) (Jackson Immunoresearch, no. 005-000-121, West Grove, PA, USA) for 60 min. A primary antibody cocktail [MHC I (Dev. Studies Hybridoma Bank, no. BA-F8, Iowa City, Iowa, USA), MHC IIa (Dev. Studies Hybridoma Bank, no. SC-71), and MHC IIb (Dev. Studies Hybridoma Bank, no. BF-F3) in 10% NGS] was applied to each section for 120 min. Slides were washed in PBS 3 x 5 min. A secondary antibody cocktail [AlexaFluor 350 IgG_2b (Invitrogen, no. A21140, Grand Island, New York, USA), AlexaFluor 488 IgG_1 (Invitrogen, no. A21121), and AlexaFluor 555 IgM (Invitrogen, no. A21426) in 10% NGS] was applied to each section for 60 min. Slides were washed in PBS 3 x 5 min and visualized using a fluorescent microscope.

Citrate Synthase Activity Assay

Mitochondrial function was further assessed by performing a citrate synthase activity assay. 0.025 ml of chronic AICAR homogenates were diluted into 1.225 ml of 100 mM Tris, pH 8.0 and vortexed gently. The following reagents were added to a 1 ml quartz cuvette: 0.60 ml of 100 mM Tris, pH 8.0; 0.10 ml of 3.0 mM Acetyl-CoA; 0.10 ml of 1.0 mM DTNB, and 0.10 ml

of the diluted homogenate. The cuvette was mixed by inversion and placed in a spectrophotometer at 30°C for 7 min. The change in optical density (O.D.) for 3 min at 1 min intervals (4 readings) at 412 nm was recorded. The reaction was started by adding 0.10 ml of 5 mM oxaloacetate and mixed by inversion several times. The change in O.D. for 3 min at 1 min intervals (4 readings) was recorded. Calculations were determined by the change in O.D. per minute and the amount of tissue in the reaction cuvette.

Statistics

Statistical comparisons using Microsoft Excel or GraphPad Prism statistical analysis software (GraphPad Software Inc., La Jolla, CA) were made using a student *t* test or repeated measures ANOVA to determine statistical significance ($p \leq 0.05$) with Fisher's LSD post-hoc analysis employed where appropriate. Values are reported as means ± SE.

Results

Acute AICAR Treatment Increases AMPK Activity in YA and O Mice

Acute AICAR treatment was administered to verify the activation of AMPK with the specified AICAR dosage. Phosphorylation of AMPK (pAMPK) was increased in both the YA and O mice compared to saline treated mice one-hour post AICAR injection (Figure 4.1A). However, pAMPK increased by 467% in the O mice whereas the YA mice only increased by 159%. Interestingly, acute AICAR treatment increased phosphorylated Acetyl Co-A Carboxylase (pACC) in both the YA and O mice to the same extent (Figure 4.1C). Acute AICAR did not alter total AMPK or ACC protein content (Figure 4.1B, D).

Activation of AMPK in response to acute AICAR treatment was further assessed by determining the activity of the catalytic subunits. AMPKα1 activity did not increase with AICAR

treatment but AMPKα2 activity increased to a similar extent in both the YA and O mice with AICAR compared to saline treated mice (Figure 4.2).

Chronic AICAR Treatment Alters the Body Weight of YA and O Mice, Improves the Rate of Fatigue in YA mice, and Improves Treadmill Endurance in O Mice

Prior to AICAR treatment, YA and O mice had similar body weights (Figure 4.3A). However, the muscle weight per body weight of the O mice was significantly less than the YA mice (Figure 4.3B). One month chronic AICAR treatment in the YA mice increased the average body weights compared to the saline treated YA mice by about 5% (Figure 4.3A). AICAR treatment did not affect the O mice body weight. Therefore, the O AICAR mice weighed about 4.5% less than the YA AICAR treated mice after one month (Figure 4.3A).

Mice were treadmill tested pre- and post-treatment with AICAR or saline. YA mice treadmill endurance time was unaffected by AICAR treatment. However, in the O mice, running performance decreased with saline treatment but AICAR prevented the drop in performance between pre and post treadmill running (Figure 4.3C). *In vitro* muscle contractions were additionally performed to assess the rate of fatigue and recovery with AICAR treatment. AICAR treatment increased the initial force production in both the YA and O mice (Figure 4.3D). However, AICAR treatment did not improve the rate of fatigue or recovery of the O mice. On the other hand, YA mice fatigued at a slower rate within 2-3 min of contraction and recovered quicker with AICAR than their saline treated counterparts (Figure 4.3E).

Chronic AICAR Treatment does not Change AMPK Protein Content in YA and O Mice

AMPK and pAMPK protein content were assessed to determine the effect of chronic activation of AMPK by AICAR. pAMPK and total AMPK content were not significantly different between saline and AICAR treated YA and O mice (Figure 4.4A, B). pACC and LKB1 also were unaffected by AICAR in YA and O mice (Figure 4.4C, E). However, the protein

content of pACC was on average about 49% greater in O mice versus YA mice regardless of treatment (Figure 4.4C). Alternatively, AICAR treated O mice had greater total ACC protein content than both the O saline treated mice and the YA AICAR and saline treated mice (Figure 4.4D).

Chronic AICAR Treatment Increases Citrate Synthase Activity in YA and O Mice

Mitochondrial enzyme activity and gene content was measured to assess the downstream effects of chronic AICAR treatment in aged muscle. Citrate synthase activity significantly increased with AICAR in both the YA and O mice (Figure 4.5A) but succinate dehydrogenase expression was unaffected by AICAR in YA and O mice (Figure 4.5B, C). PGC1α and UCP3 protein content were also unaffected by AICAR treatment in both the YA and O mice (Figure 4.6A, D). Cytochrome C, Cox4, and oxphos complexes I, II, III, and IV all tended to increase with AICAR treatment in YA mice, but were not significant (Figure 4.6B, C, E). Mitochondrial protein content was not affected by AICAR in the O mice.

Chronic AICAR Treatment does not Overall Inhibit the mTOR Pathway

The effect of chronic AICAR treatment on protein synthesis in YA and O mice was determined by assessing the regulation of the mTOR pathway. Akt, pAkt, S6K, pS6K, pS6, 4EB-P1, p4EB-P1, eIF2α, eEF2, and peEF2 were all unaffected by AICAR treatment in both the YA and O mice (Figure 4.7A-D, F-I, K-L). However, the O mice had greater protein contents of pAkt, pS6, p4EB-P1, and eEF2 than the YA mice (Figure 4.7B, F, H, K). On the other hand, S6 content was significantly greater with AICAR treatment than saline in both the YA and O mice (Figure 4.7E). The protein content of S6 was also significantly greater in O mice than YA mice for both the saline and AICAR treatments. Additionally, peIF2α content was significantly lower in both the YA and O AICAR treated mice (Figure 4.7J).

Chronic AICAR Treatment Decreases SQSTM1/p62 Content as an Indicator of Autophagic Flux

Autophagic proteins were measured to determine the effect chronic AICAR treatment has on the recycling of cellular components to maintain cell survival. AICAR treatment did not significantly alter the protein content of Beclin1, Atg5, Atg12, Atg7, MAFbx, LC3A, ULK1, pULK1 (ser555 or ser757) (Figure 4.8A-D, F, G, J-L). However, both YA and O mice had lower SQSTM1/p62 content with AICAR treatment than saline treated mice. Additionally, SQSTM1/p62 protein content was lower in O versus YA saline-treated mice (Figure 4.8E). LC3B I and II content were greater in O versus YA saline treated mice (Figure 4.8H, I). AICAR treatment in the O mice decreased the protein content of LC3B I and II but had no effect in the YA mice (Figure 4.8H, I). However, there was no significant difference in the ratio between LC3 II and I with AICAR versus saline in the YA and O mice (data not shown).

Chronic AICAR Treatment Decreases Myf6 but does not Alter Skeletal Muscle Fiber Type Composition or Capillarity

The effect of chronic AICAR treatment on myogenic regulating factors was assessed by western blotting. AICAR treatment did not alter Myf5 and MyoD protein contents, but both were significantly greater in aged muscle than YA muscle (Figure 4.9A, C). Myf6 was also greater in the O saline versus YA saline treated mice; however, with AICAR treatment, both the YA and O mice had significantly less Myf6 protein content (Figure 4.9B).

Fiber type composition was determined by MHC staining of the TA muscles in both YA and O mice. There was no significant difference in fiber type composition between YA and O mice treated with saline or AICAR; however, AICAR treatment did tend to increase type I fiber expression in both YA and O mice, but results were not significant (Figure 4.10).

An increase in capillarity was assessed as a potential mechanism for the improvement in run time and rate of fatigue. However, chronic AICAR treatment did not increase the number of capillaries found associated with each fiber in either the YA or O muscle (Figure 4.11).

Discussion

This study supports previous results by Pagala et al. (1998) that saline treated O mice have reduced treadmill endurance but greater resistance to fatigue with *in vitro* muscle contractions than YA saline treated mice (Pagala *et al.*, 1998). Furthermore, we found that chronic AICAR treatment prevented a reduction in run time in aged mice to match the YA endurance levels. Chronic AICAR treatment also improved the rate of fatigue and recovery in YA mice to match that of the O mice. These differences may be attributed to differences between an isolated muscle and whole body interactions (i.e. an increase in the heart size or blood flow to increase oxygen supply to the muscle).

Verification that the AICAR dosage was effective in both the YA and O mice was demonstrated by assessing the activation of AMPK one-hour post injection. Similar to previous research, the acute AICAR dosage was sufficient to activate AMPK in the YA and O skeletal muscle (Merrill *et al.*, 1997). Additionally, AICAR treatment preferentially activated the AMPKα2 isoform in comparison to the AMPKα1 isoform (Qiang *et al.*, 2007; Reznick *et al.*, 2007b; Thomson *et al.*, 2009). However, in contrast to previous research performed in rats (Qiang *et al.*, 2007; Reznick *et al.*, 2007b; Thomson *et al.*, 2009), we found that acute AICAR treatment increased pAMPK content to a greater extent in the O mice compared to the YA mice. The use of mice instead of rats in this study may account for the difference seen with AICAR as has been reported in vasculature from mice by Lesniewski *et al.* (Lesniewski *et al.*, 2012).

Alternatively, chronic AICAR injections did not alter the pAMPK or total AMPK protein content in either the YA or O mice. AICAR injections were stopped one day prior to post-treadmill testing; therefore, pAMPK were expected to return to baseline levels. In contrast to previous studies performed where pACC was lower in O rats (Qiang *et al.*, 2007), we found that pACC protein content was higher in O saline treated mice compared to YA saline treated mice. Previous research has also indicated that chronic AICAR treatment decreases ACC activity (Winder *et al.*, 2000) but in the current study western blotting indicated no difference in the protein content of pACC between saline and AICAR treated YA and O mice. Similar to previous research, total ACC content was not different between YA and O saline treated mice (Qiang *et al.*, 2007). Unexpectedly, chronic AICAR treatment significantly increased the protein content of total ACC in aged mice but not YA mice. Acute AICAR promotes fatty acid oxidation (Merrill *et al.*, 1997) but the increase in total ACC without the increase in phosphorylated ACC with chronic AICAR treatment may actually suggest an increase in fatty acid biosynthesis in the aged mice (Davis *et al.*, 2000). The increase in total ACC with chronic AICAR further suggests that AICAR regulates the transcription of ACC in skeletal muscle, similar to that seen in cardiac muscle (Adam *et al.*, 2010).

As noted previously, chronic AICAR treatment prevented a reduction in overall run time in aged mice compared to their saline-treated counterparts to match the YA endurance levels. Additionally, AICAR treatment improved the maximum force per muscle mass generated with *in vitro* muscle contractions in both the YA and O mice. However, AICAR treatment only improved the rate of fatigue and recovery with *in vitro* contractions in the YA mice not the O mice. We hypothesized that the improvements seen between the YA and O mice may be due to alterations in metabolic pathways regulated by AMPK.

It has previously been shown that chronic AICAR treatment increases mitochondrial enzyme content and activity (Winder *et al.*, 2000; Bergeron *et al.*, 2001b; Suwa *et al.*, 2003). In contrast, aging is associated with impaired mitochondrial function (Kumaran *et al.*, 2004a; Terman *et al.*, 2010). Therefore, it was hypothesized that chronic AICAR treatment would improve mitochondrial activity and therefore contribute to the improved treadmill endurance and rate of fatigue in the YA and O mice. As expected, chronic AICAR treatment increased citrate synthase activity in both the YA and O mice, suggesting improved mitochondrial activity with AICAR treatment. Conversely, succinate dehydrogenase expression was unaffected by AICAR treatment or age. Additionally, the protein content of PGC1α, cytochrome C, Cox4, UCP3, and the oxidative phosphorylation proteins were not significantly increased with AICAR treatment. However, there was an increasing trend in cytochrome C, Cox4, and complex II, III, and IV of the oxidative phosphorylation proteins with AICAR treatment in the YA mice but not the O mice. Therefore, chronic AICAR increased citrate synthase activity in both the YA and O mice and may tend to increase other mitochondrial enzymes in the YA mice but not the O mice. These differences in the mitochondria of YA mice but not the O mice may contribute to the improved rate of fatigue and recovery of the isolated YA EDL muscle that was not seen in the O mice. However, these results do not explain the overall improvement in treadmill endurance seen with the O mice.

One proposed mechanism for the differences was that while AICAR may not increase mitochondrial content and activity in the aging skeletal muscle, it may increase the rate of turnover of dysfunctional mitochondria to improve treadmill endurance. The impaired mitochondrial function seen with aging may be attributed to a reduction in autophagy and therefore a reduction in the removal of damaged organelles (Pfeifer, 1978; Cuervo & Dice, 2000;

Del Roso *et al.*, 2003; Combaret *et al.*, 2009). Alternatively, activation of AMPK is known to increase the rate of autophagy in skeletal muscle cells (Meley *et al.*, 2006; Sanchez *et al.*, 2012). Therefore, we hypothesized that chronic AICAR activation would increase the rate of autophagy and improve endurance by removing dysfunctional mitochondria. Contrary to our hypothesis, average autophagy levels in the O mice were not significantly lower in the YA mice. In fact, LC3BI and II, two of the primary markers of autophagosome formation (Karim *et al.*, 2007; Tanida *et al.*, 2008), were actually greater in the aged mice than the YA mice with saline treatment. Chronic AICAR treatment in the aged mice instead lowered the levels of both LC3BI and II to match those found in the YA mice. A decrease in LC3BI should indicate an increase in autophagy but a decrease in LC3BII should be indicative of decreased autophagy (Kabeya *et al.*, 2000; Tanida *et al.*, 2008; Barth *et al.*, 2010). These results are therefore conflicting concerning the rate of autophagy with chronic AICAR treatment in the aged rats. However, LC3BII is also degraded by the lysosome, making LC3BII a difficult marker to interpret and not always the best indicator of autophagy (Mizushima & Yoshimori, 2007). Therefore, LC3BII and LC3BI may not be the best indicators of autophagy.

Another common marker used to measure autophagy is SQSTM1/p62. Protein content of SQSTM1/p62 decreased with chronic AICAR treatment in both the YA and O rats. SQSTM1/p62 interacts with ubiquitin to tag the autophagic vesicle for degradation. Increased rates of autophagy result in a decrease in content of SQSTM1/p62 due to degradation of the autophagic vesicle (Mizushima & Yoshimori, 2007). Therefore, although the other measured proteins involved in autophagy were not affected, there does appear to be an increase in autophagic vesicle degradation in YA and O mice which would indicate an increase in autophagic flux with chronic AICAR treatment. Therefore, the effect of chronic AICAR

treatment on autophagic flux and the removal of damaged organelles to improve cellular metabolism and efficiency are inconclusive, but suggests a possible increase in autophagy.

Chronic activation of AMPK with AICAR may further contribute to the changes seen in YA and O mice through the regulation of protein synthesis and muscle hypertrophy by the mTOR pathway. Previous research has indicated that the mTOR pathway is upregulated in aged skeletal muscle as an attempt to maintain the loss of muscle mass associated with sarcopenia (Kimball *et al.*, 2004). Inhibition of mTOR in aging muscle has been shown to improve the age-related decline in spontaneous activity levels and the aging phenotype (Harrison *et al.*, 2009; Wilkinson *et al.*, 2012). AICAR is a known inhibitor of the mTOR pathway in skeletal muscle (Bolster *et al.*, 2002). Therefore, we hypothesized that chronic AICAR treatment may improve the aged mice endurance activity levels through inhibition of the mTOR pathway. In support of this, the current study supports previous findings that some proteins involved in the mTOR pathway are upregulated in the O saline mice compared to the YA saline mice (Kimball *et al.*, 2004; Parkington *et al.*, 2004). Namely, pAKT, S6, pS6, p4EB-P1, and eEF2, had a higher protein content in the O versus YA mice. This increase in the protein content of some proteins involved in the mTOR pathway may serve as a compensatory mechanism to counterbalance the skeletal muscle atrophy associated with aging. In addition to the mTOR pathway proteins and in support of previous research (Musarò *et al.*, 1995), we further found that the myogenic factors myf5, myf6, and myoD were elevated in the aged muscle compared to the YA muscle, again suggesting a compensatory mechanism to counteract the increasing cell death in aging muscle.

However, contrary to our hypothesis, there was no overall inhibition of the mTOR pathway as would have been expected with chronic AICAR treatment in either the YA or the O mice. In contrast, chronic AICAR treatment increased the protein content of S6 in the YA and O

mice, which would suggest greater regulation of protein translation and cell growth; however, AICAR did not change the levels of pS6. Additionally, chronic AICAR treatment decreased the protein content of peIF2α, suggesting a potential increase in translation initiation in both the YA and O mice. The small effect that chronic AICAR treatment has on the mTOR pathway may be due to the short-term effect of AMPK activation on mTOR. Many studies have shown that AICAR inhibits the mTOR pathway for about 20-30 min but within 40-60 min mTOR signaling is no longer affected (Thomson & Gordon, 2006; Williamson *et al.*, 2006). Therefore, chronic AICAR may not inhibit the mTOR pathway but may instead lead to activation of mTOR-related proteins and protein synthesis. Alternatively, chronic AICAR decreased the protein content of Myf6, which may suggest a decrease in muscle regeneration with AICAR treatment (Braun *et al.*, 1990).

In addition to muscle growth and differentiation, we addressed the effect of chronic AICAR treatment on fiber-type composition. Previous research has indicated that chronic AICAR treatment has no effect on muscle fiber-type composition (Bamford *et al.*, 2003; Putman *et al.*, 2007). Likewise, we found that chronic AICAR treatment did not alter the muscle fiber type composition or capillarity. However previous studies have also indicated that in mouse EDL muscles, aged mice have a preferential reduction in type II fibers (Lexell *et al.*, 1983) and therefore an increased percentage of slow oxidative fibers (Alnaqeeb & Goldspink, 1987). Suwa *et al.* (1985) also found that chronic AICAR treatment decreased the amount of type IIB glycolytic fibers and therefore increased the percentage of IIX oxidative fibers in rat EDL muscles (Suwa *et al.*, 2003). Likewise, we found that there did appear to be an increasing trend in the oxidative type I fibers with AICAR treatment compared to saline treatment, although the results were not significant. An increase in slow oxidative fibers in the aged mice may improve

the response to AICAR. Slow oxidative fibers have a higher percentage of the AMPKα2 subunit than the AMPKα1 subunit (Putman *et al.*, 2007). As discussed previously, AICAR preferentially activates the AMPKα2 subunit. The increase in the AMPKα2 with AICAR in aged muscle with the change in fiber type composition may contribute to the increased endurance with treadmill running in the aged mice but cannot completely account for the overall improvements seen in the aging muscle.

Future Directions

Although chronic AICAR slightly changed the protein content of a few metabolic proteins, we cannot conclusively say that chronic AICAR treatment significantly impacted the age-related signaling pathways of mitochondrial biogenesis, autophagy, or protein synthesis. However, the YA mice did have a decrease in the rate of fatigue and improved recovery with AICAR treatment and the O mice had improved treadmill endurance. Therefore, other metabolic pathways not assessed in this study may be influencing the improved endurance. In addition, differences in the YA and O mice with AICAR treatment may be due to the difference between evaluating the endurance of a single muscle versus whole body interactions. Chronic AICAR treatments had a more robust improvement in mitochondrial activity and gene expression in the YA mice that may have improved the rate of fatigue and recovery of a single muscle in isolation. However, the improvement in treadmill endurance in the O mice may be regulated by interactions between many factors. Although improvements in mitochondrial biogenesis, autophagy, and protein synthesis were not overall significantly affected by chronic AICAR treatment, there may be other tissues or factors affected besides skeletal muscle (i.e. an increase in the heart size or blood flow to increase oxygen supply to the muscle) to improve the treadmill endurance in the aged muscle.

Additionally, other signaling pathways may be affected by the chronic AICAR treatment. One potential target is hexokinase II to increase the glycolytic potential of the YA and O mice muscles. Previous research has indicated that AICAR treatment increases the protein content of Hexokinase II (Holmes *et al.*, 1999; Ojuka *et al.*, 2000; Winder *et al.*, 2000). Similarly, we found that Hexokinase II protein content increased with AICAR treatment in both the YA and O mice (See Supplemental Figure 4.1A). The combination of increased glycolytic potential and mitochondrial activity may decrease the rate of fatigue and improve endurance capabilities.

Another potential pathway that may contribute to the improved endurance to *in vitro* muscle contractions and treadmill running may be through inflammatory pathways. Aged muscle has an increase in low-grade inflammation (Salminen *et al.*, 2008; de Magalhães *et al.*, 2009). Alternatively, we found that chronic AICAR treatment significantly decreased phosphorylated STAT3 protein content in both the YA and O mice without changing the total STAT3 protein content (See Supplemental Figure 4.1B, C). This may indicate a decrease in inflammation with chronic AICAR treatment but will require further investigation.

In conclusion, chronic AICAR treatment improved the treadmill endurance in O mice and the rate of fatigue and recovery in response to *in vitro* muscle contractions in YA mice. However, the exact mechanism for the improvements remain unknown. Chronic AICAR increased citrate synthase activity in both the YA and O mice and tended to increase mitochondrial gene content in the YA mice. Additionally, chronic AICAR treatment decreased SQSTM1/p62 and Myf6 protein content in both the YA and O mice, suggesting increased autophagy and decreased muscle regeneration with treatment. However, the differences seen with AICAR treatment on treadmill endurance and rate of fatigue cannot conclusively be attributed to changes in protein synthesis, autophagy, fiber type distribution, or muscle

differentiation. Therefore, the improvements in endurance may be due to improvements in factors other than skeletal muscle energetics (e.g. cardiac function, blood supply, etc.). Aging muscle may have been impacted more by the chronic AICAR treatment than YA muscle due to greater deterioration in whole body energetics allowing for a greater range of improvement with treadmill endurance. Alternatively, improvements in isolated skeletal muscle energetics improved the rate of fatigue and recovery in the YA skeletal muscle but not aged muscle. This may primarily be due to the improvements in mitochondrial protein expression and activity that was seen in the YA muscle versus the O muscle with chronic AICAR treatment. Therefore, chronic AICAR treatment can regulate metabolic pathways that are altered as a result of aging to improve the exercise capacity in response to treadmill running and *in vitro* muscle contractions.

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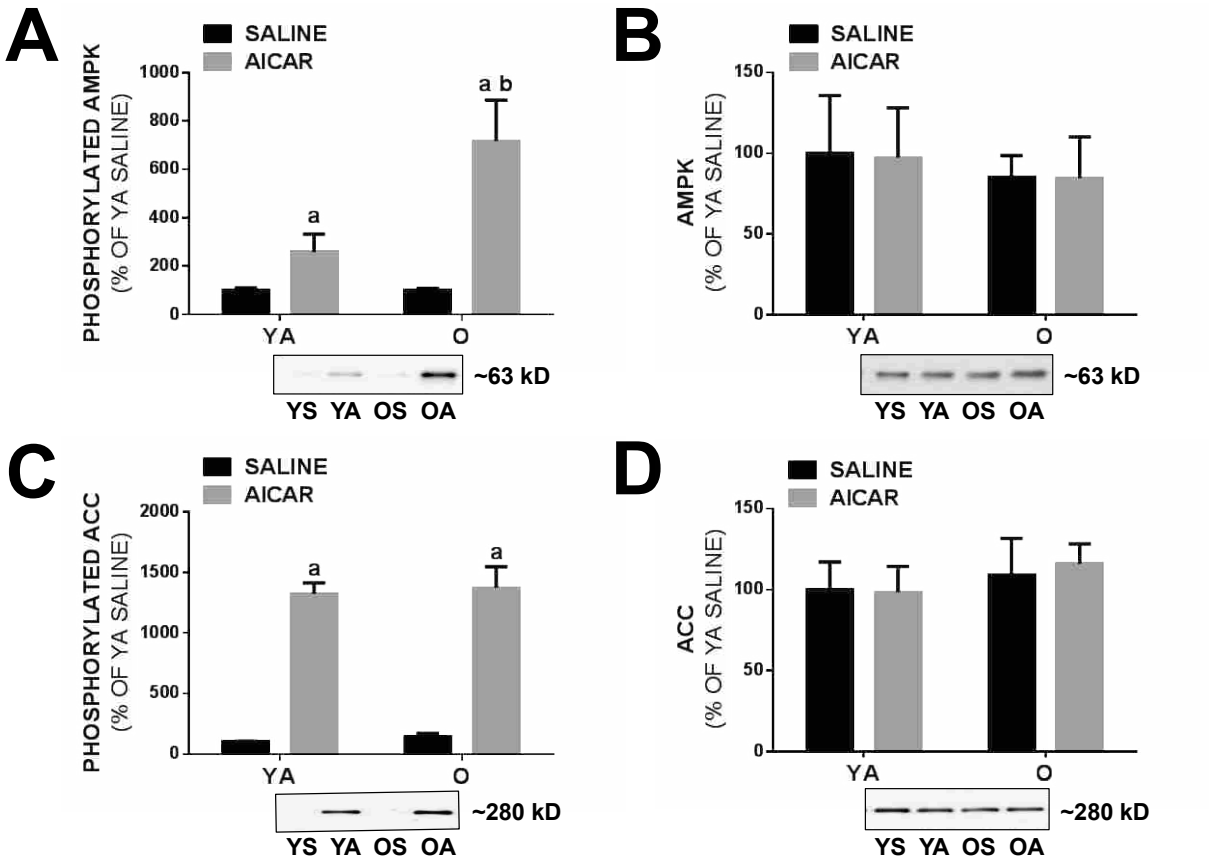


Figure 4.1: Acute AICAR Injections Increase Phosphorylation of AMPK and ACC. White quadricep (WQ) muscles from YA (5 mo.) and O (23 mo.) male C57Bl/6 mice were subcutaneously injected with AICAR dissolved in saline (0.5 mg/g body weight, 50 mg AICAR/ml saline) or with an equivalent volume of saline without AICAR. Western blot analysis of WQ for (A) pAMPK, (B) total AMPK, (C) pACC, and (D) total ACC. N=4 / group. YS= YA saline treated; YA= YA AICAR treated; OS= O saline treated; OA= O AICAR treated. Values are means ± SEM. a = significant difference from corresponding saline treated muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

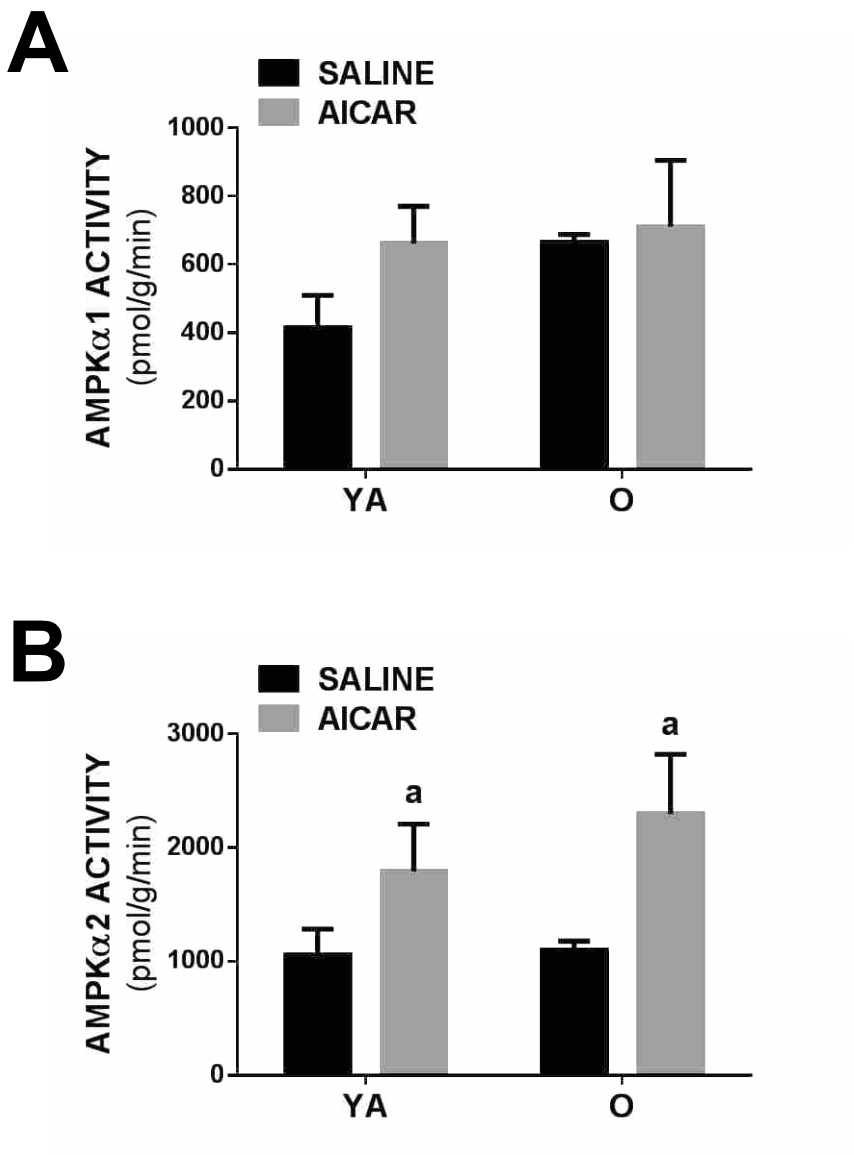


Figure 4.2: Acute AICAR Injections Increase AMPKα2 Activity but not AMPKα1. Activity assay of the WQ for (A) AMPKα1 and (B) AMPKα2. N=4 / group. Values are means ± SEM. a = significant difference from corresponding saline treated muscle ($p < 0.05$).

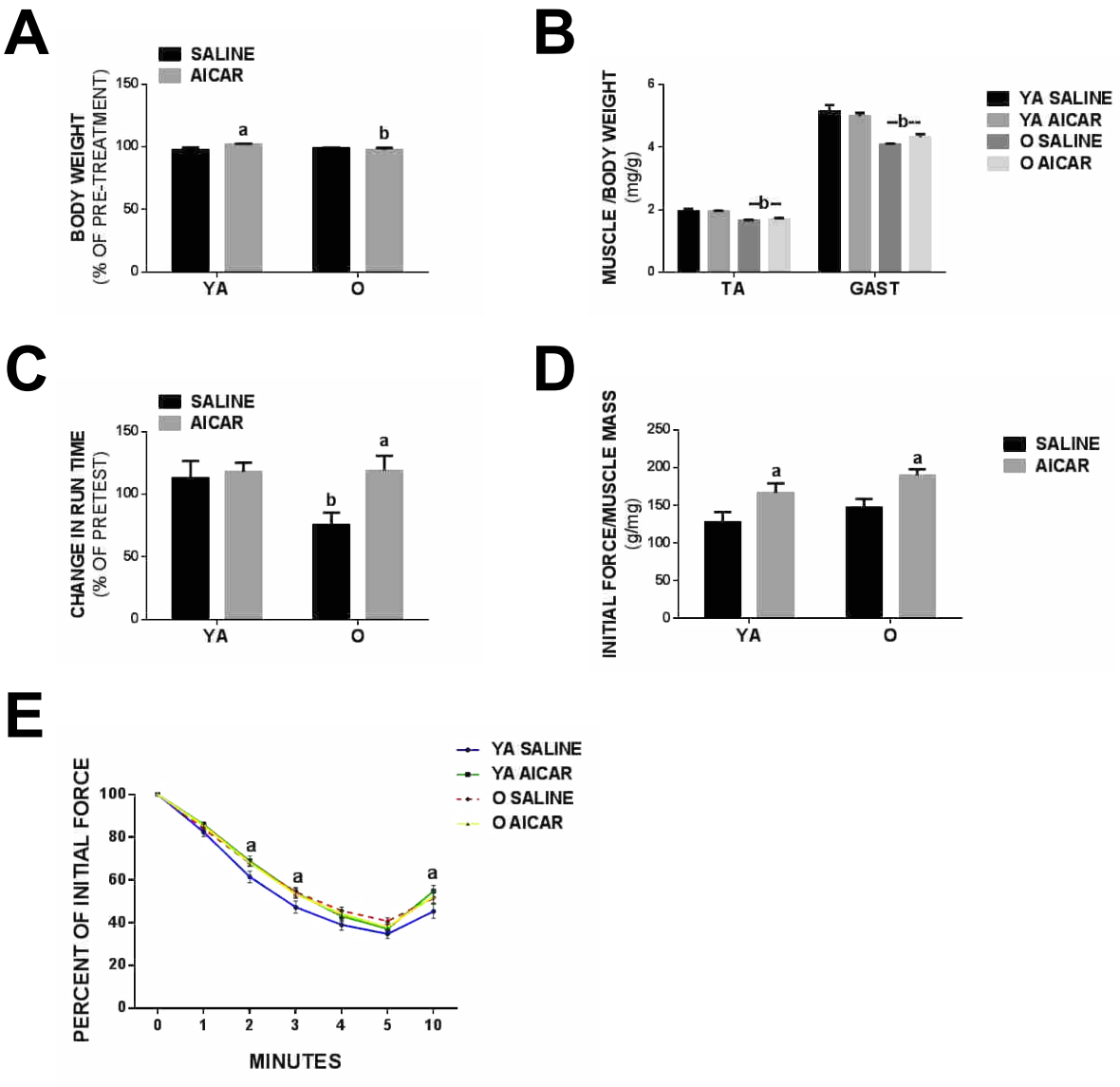


Figure 4.3: Chronic AICAR Injections Increase Body Weight in YA and O Mice, Improve Treadmill Endurance in O Mice, and Improve the Rate of Fatigue and Recovery in YA Mice. YA and O mice were subcutaneously injected with AICAR dissolved in saline (0.5 mg/g body weight, 50 mg AICAR/ml saline) or with an equivalent volume of saline without AICAR for 31 days. Mice were weighed and treadmill tested prior to and following injections. (A) Total body weights, (B) muscle weight per body weight for tibialis anterior (TA) and gastrocnemius (GAST), (C) change in run time. After injections the right extensor digitorum longus (EDL) muscle was removed and stimulated for 5 min at 150 Hz with a train frequency of 0.2/sec with a train duration of 150 msec to determine the rate of fatigue. (D) initial force per EDL muscle weight. Acute muscle recovery was then determined by 5 additional stimulations after 5 min. (E) rate of fatigue and recovery. N=9-13 / group. Values are means ± SEM. a = significant difference from corresponding saline treated muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

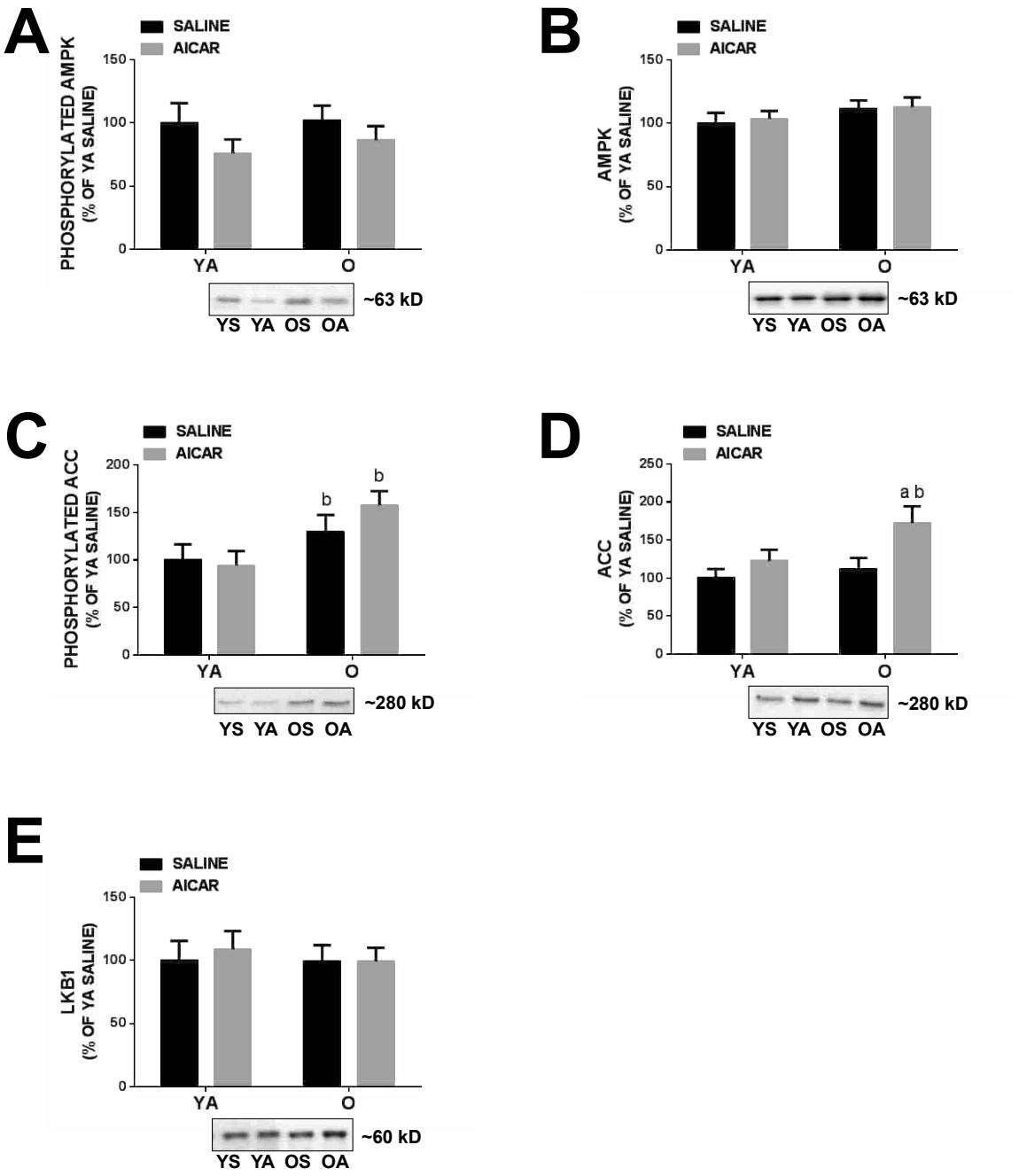


Figure 4.4: Chronic AICAR Injections Increase the Protein Content of Total ACC in O Mice but do not Alter pAMPK, Total AMPK, pACC, and LKB1 Content. Western blotting analysis of WQ for (A) pAMPK, (B) total AMPK, (C) pACC, (D) total ACC, and (E) LKB1. N=9-13 / group. YS= YA saline treated; YA= YA AICAR treated; OS= O saline treated; OA= O AICAR treated. Values are means ± SEM. a = significant difference from corresponding saline treated muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

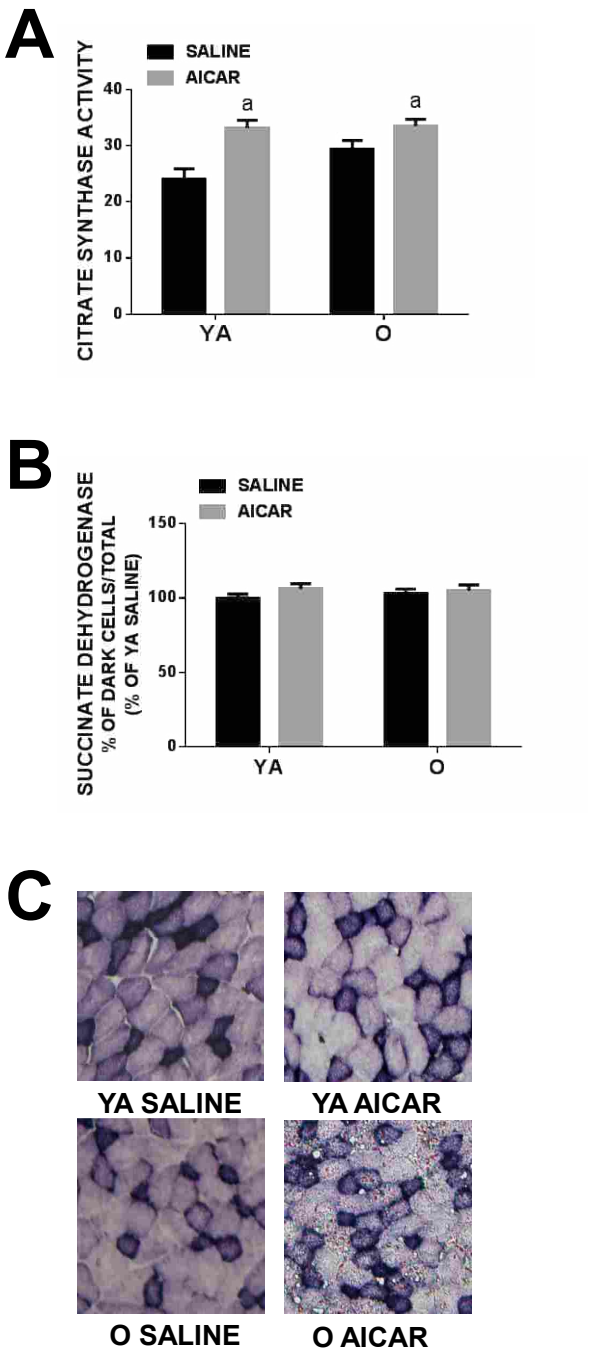


Figure 4.5: Chronic AICAR Injections Increase Citrate Synthase Activity but not SDH Expression. Activity assay of the WQ for (A) Citrate synthase. Immunohistochemistry of the tibialis anterior (TA) for (B) Succinate dehydrogenase (SDH) quantification and (C) SDH images. N=9-13 / group. Values are means ± SEM. a = significant difference from corresponding saline treated muscle ($p < 0.05$).

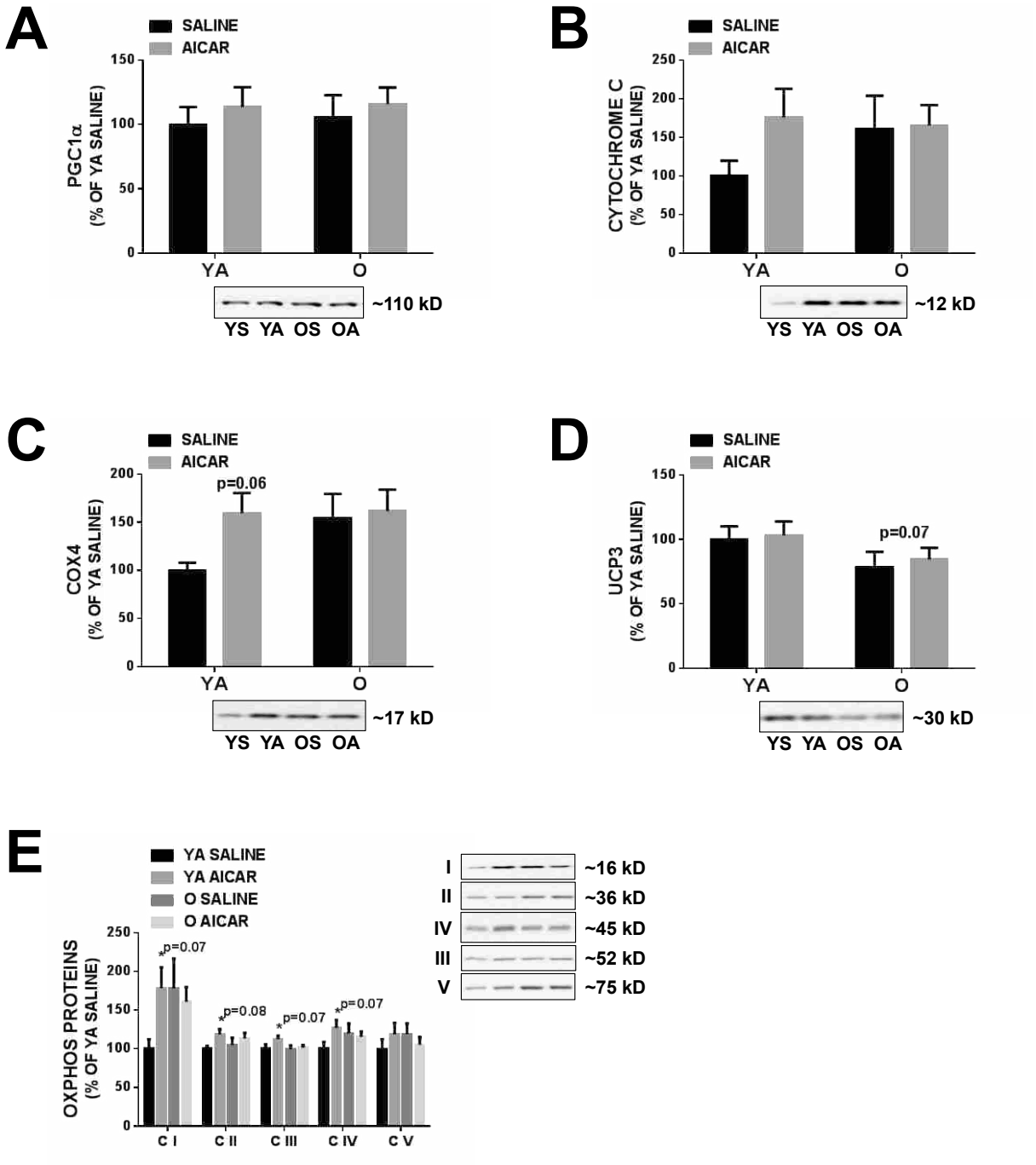


Figure 4.6: Chronic AICAR Injections Tend to Increase Mitochondrial Protein Content in YA but not O Mice. Western blotting analysis of WQ for (A) PGC1α, (B) Cytochrome C, (C) Cox4, (D) UCP3, and (E) Oxphos proteins. N=9-13/ group. YS= YA saline treated; YA= YA AICAR treated; OS= O saline treated; OA= O AICAR treated. Values are means ± SEM. ($p < 0.05$).

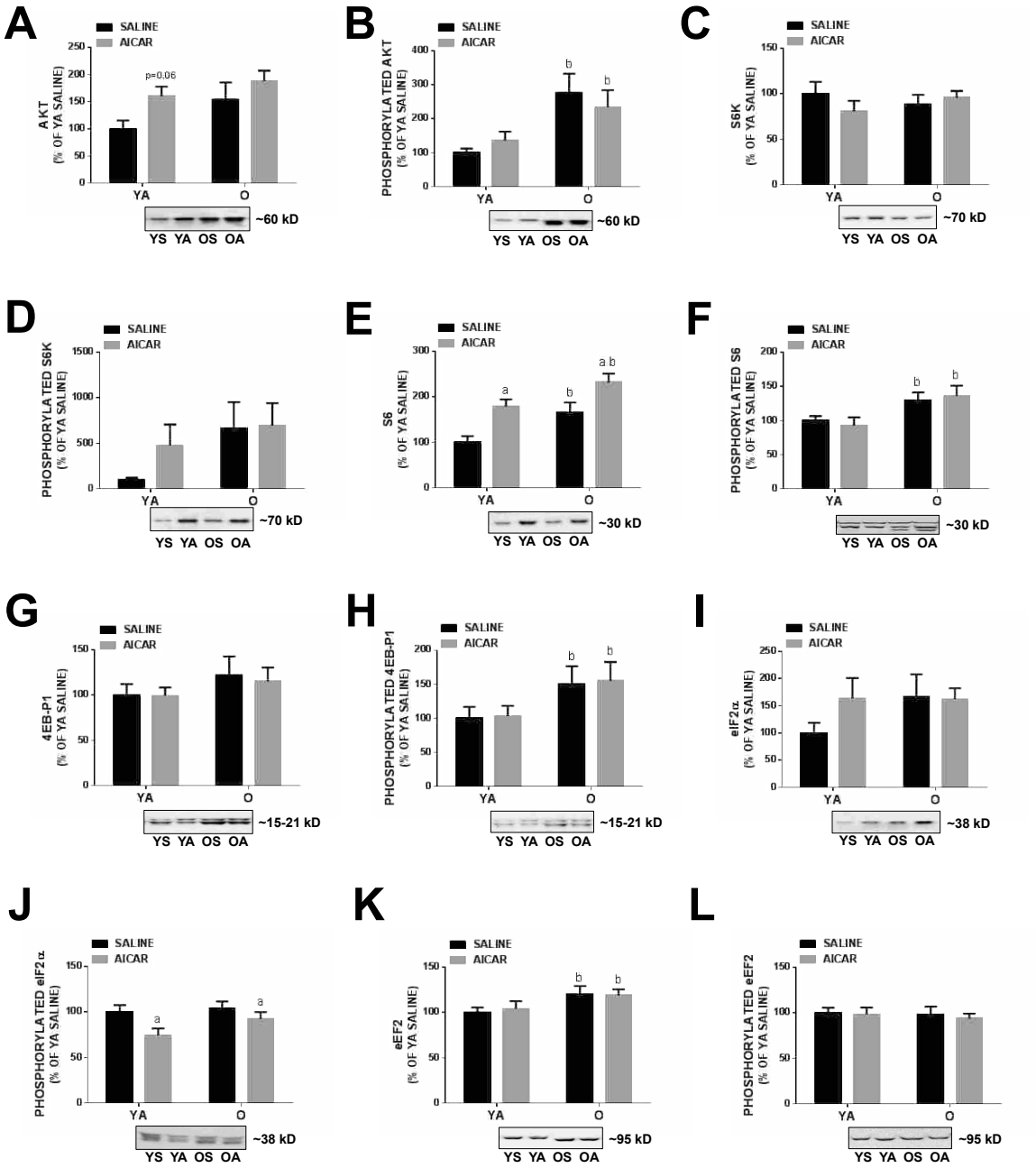


Figure 4.7: Chronic AICAR Injections Increase S6 but Decrease peIF2α Protein Content. Western blotting analysis of WQ for (A) Akt, (B) pAkt, (C) S6K, (D) pS6K, (E) S6, (F) pS6, (G) 4EB-P1, (H) p4EB-P1, (I) eIF2α, (J) peIF2α, (K) eEF2, and (L) peEF2. N=9-13/group. YS= YA saline treated; YA= YA AICAR treated; OS= O saline treated; OA= O AICAR treated. Values are means ± SEM. a = significant difference from corresponding saline treated muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

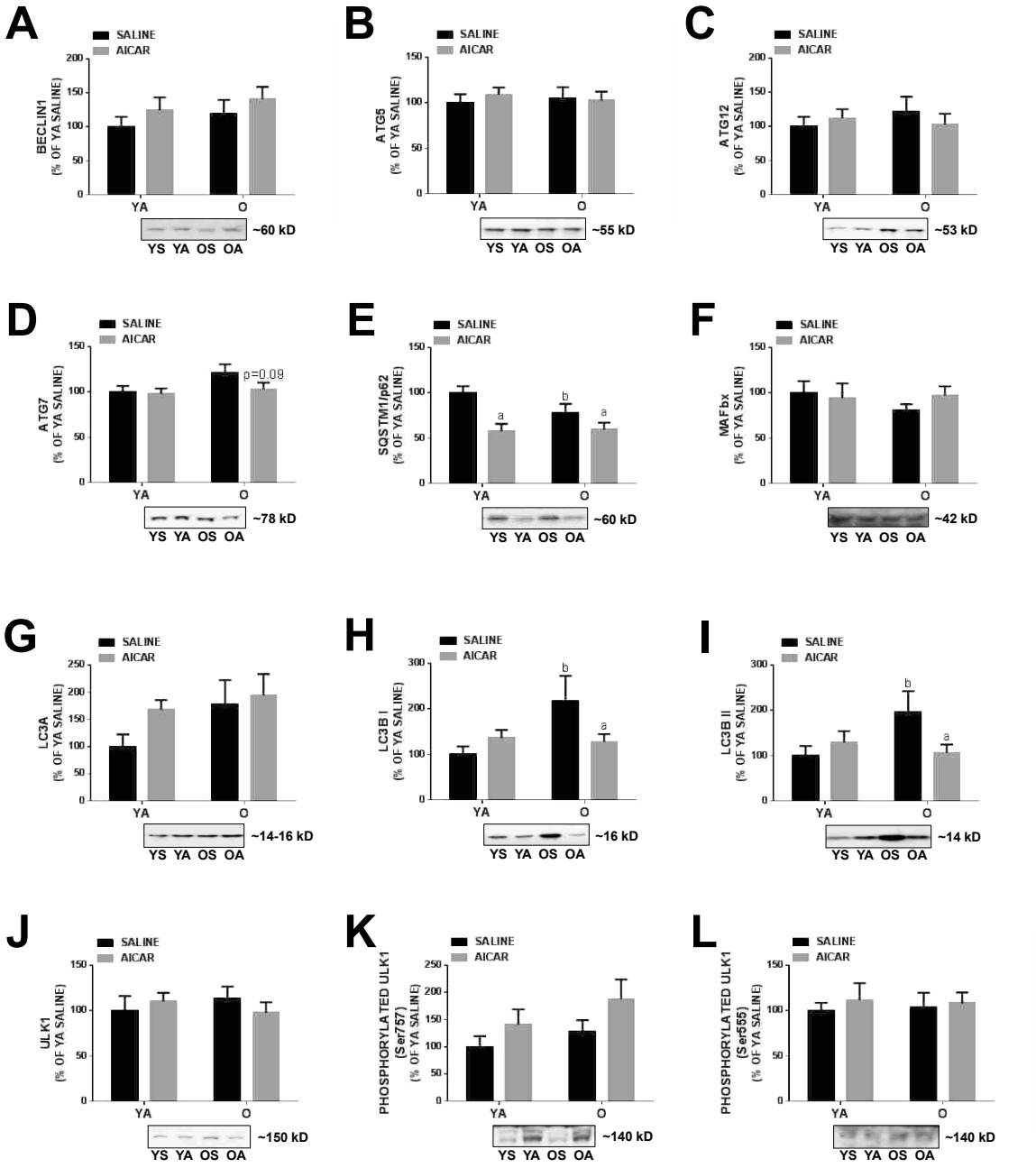


Figure 4.8: Chronic AICAR Injections Decrease SQSTM1/p62 Protein Content. Western blotting analysis of WQ for (A) Beclin1 (B) Atg5, (C) Atg12, (D) Atg7, (E) SQSTM1/p62, (F) MAFbx, (G) LC3A, (H) LC3BI, (I) LC3BII, (J) Ulk1, (K) pUlk1 (ser555), and (L) pUlk1 (ser757). N=9-13/group. YS= YA saline treated; YA= YA AICAR treated; OS= O saline treated; OA= O AICAR treated. Values are means ± SEM. a = significant difference from corresponding saline treated muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

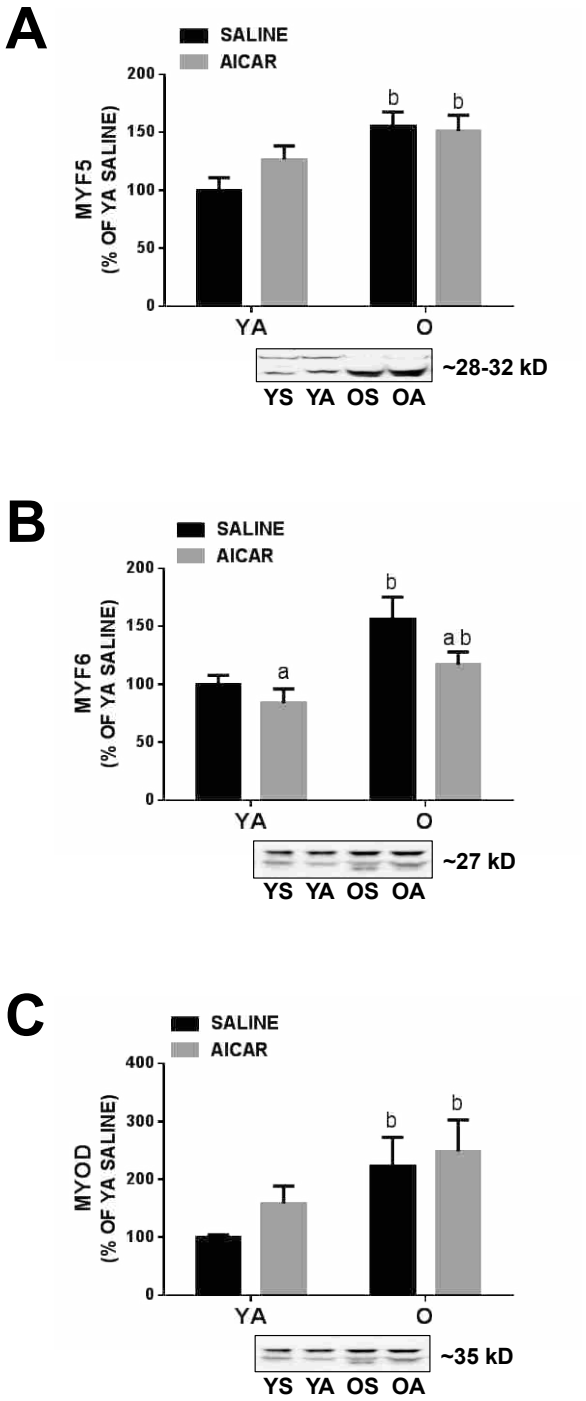


Figure 4.9: Chronic AICAR Injections Decrease Myf6 Protein Content. Western blotting analysis of WQ for (A) Myf5, (B) Myf6, and (C) MyoD. N=9-13/group. YS= YA saline treated; YA= YA AICAR treated; OS= O saline treated; OA= O AICAR treated. Values are means ± SEM. a = significant difference from corresponding saline treated muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

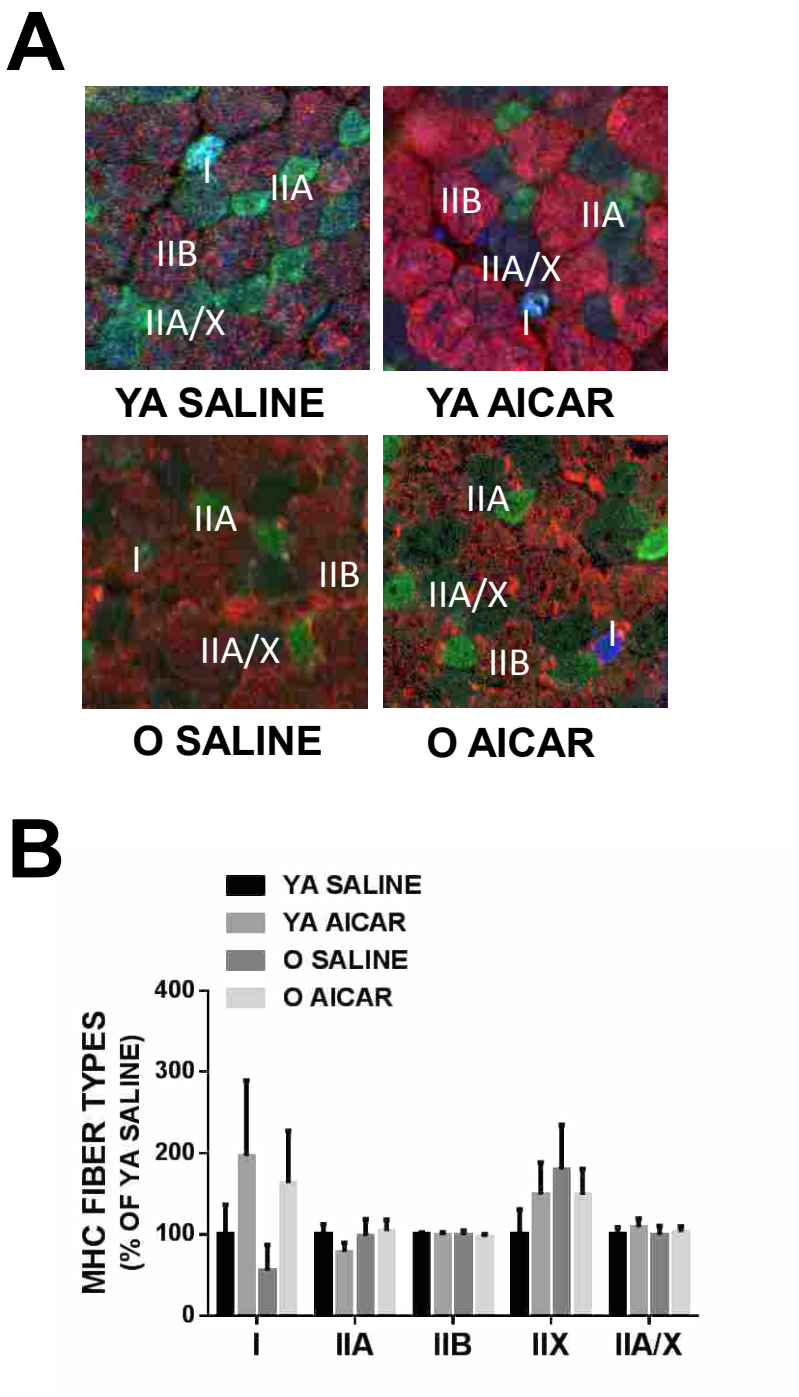


Figure 4.10: Chronic AICAR Injections do not Alter Myosin Heavy Chain Content. Immunohistochemistry for myosin heavy chain (MHC) fiber type composition. N=9-13/group. Values are means ± SEM.

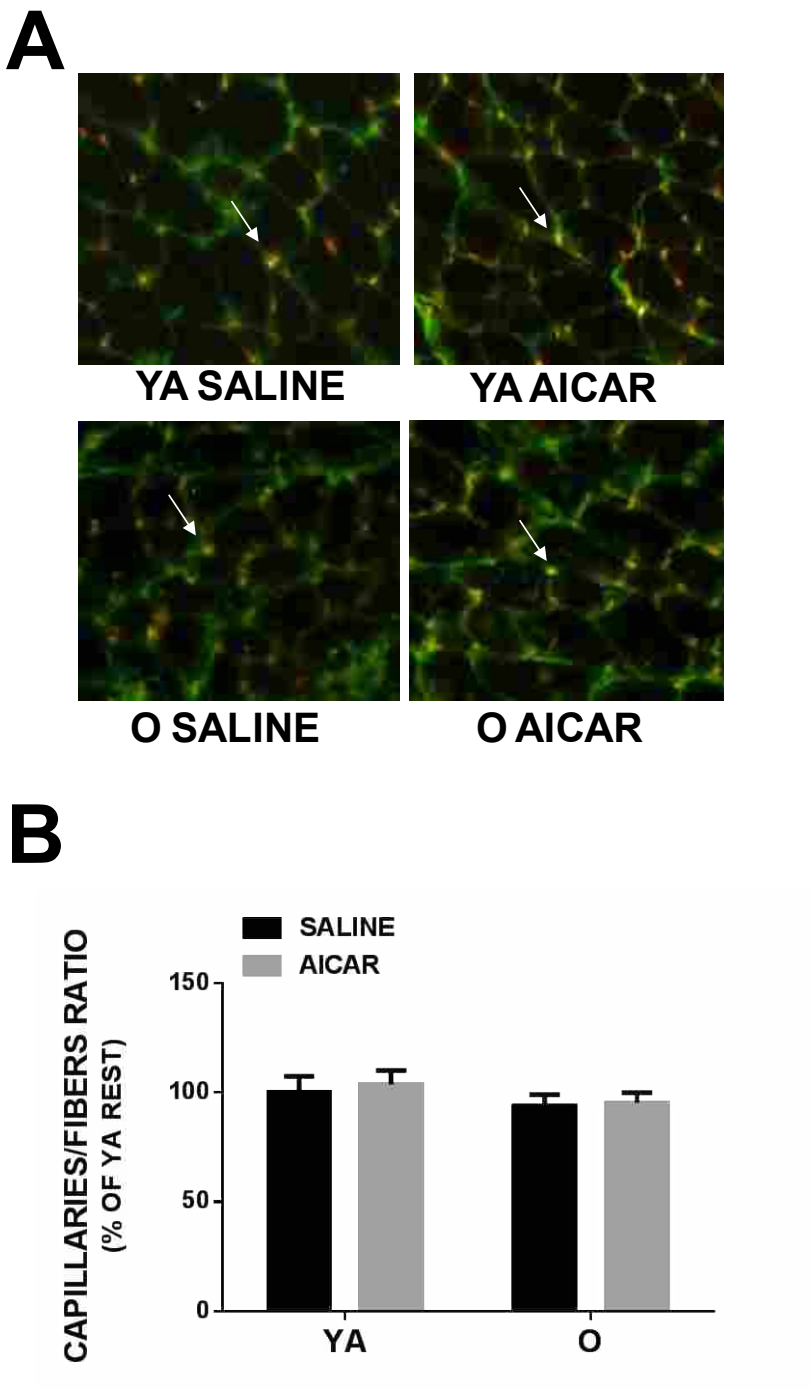
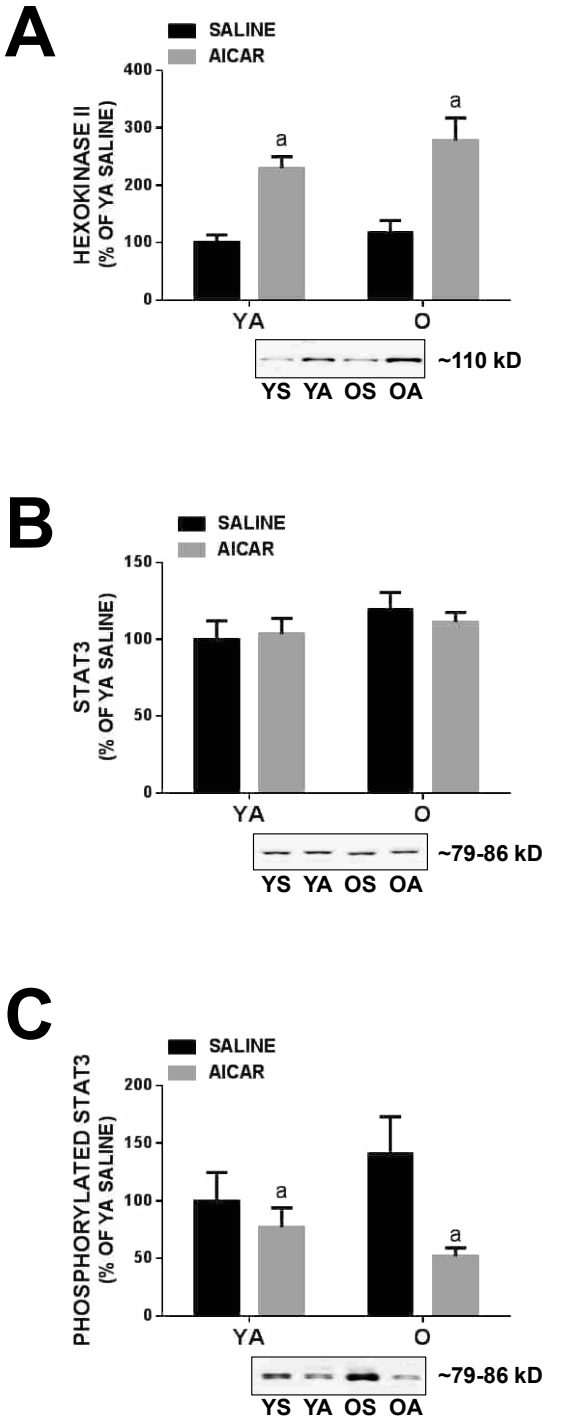


Figure 4.11: Chronic AICAR Injections do not Increase Capillarity. Immunohistochemistry for CD31 as a marker of capillaries. N=9-13/group. Values are means ± SEM.



Supplemental Figure 4.1: Chronic AICAR Injections Increase Hexokinase II but Decrease pSTAT3 Protein Content. Western blot analysis in WQ for (A) Hexokinase II (B) STAT3, and (C) pSTAT3. N=9-13/group. YS= YA saline treated; YA= YA AICAR treated; OS= O saline treated; OA= O AICAR treated. Values are means ± SEM. a = significant difference from corresponding saline treated muscle ($p < 0.05$).

CHAPTER 5: Summary

The overall purpose of the studies described in chapters 2-4 was to better understand the age-associated alterations in AMPK in skeletal muscle and chronic activation of AMPK as it relates to the development of sarcopenia. Specifically, the following questions were addressed:

1) Is AMPK activation in response to endurance-like exercise attenuated in aged skeletal muscle?
2) Does the AMPK heterotrimer composition change in aged skeletal muscle?
3) Does the nuclear localization of AMPK after endurance-like exercise change in aging skeletal muscle?
4) Does chronic activation of AMPK improve the aging phenotype in response to endurance-type exercise?
5) Are the known beneficial effects that have been observed with chronic AICAR treatment on metabolic pathways found in aged skeletal muscle?

We had hypothesized that AMPK activation after endurance-like muscle contractions would be impaired in aged skeletal muscle as a result of altered changes in the AMPK heterotrimer composition and localization. Therefore, we further hypothesized that chronic activation of AMPK with AICAR treatment would improve the aging phenotype. This study found the following answers to the above-mentioned questions:

AMPK Activation is Attenuated with Endurance-Like Exercise in Aged Skeletal Muscle.

In support of our hypothesis, we found that AMPKα phosphorylation was lower in O vs. YA rat muscles after STIM. This suggests an attenuated activation of AMPK in response to electrical stimulation. More specifically, AMPKα2, the predominant catalytic subunit activated

in response to endurance-type exercise, was also lower in O vs. YA muscles with STIM. However, AMPKα1 activity was greater in O vs. YA muscle with STIM.

Furthermore, ACC, ERK, AKT, and p38 phosphorylation increased with STIM. However, phosphorylation, particularly ACC phosphorylation, was not lower after contractions in O vs. YA muscles. These results suggest that while *in situ* AMPK activity is suppressed, *in vivo* AMPK activity may not be suppressed by old-age in rat skeletal muscle.

The AMPK Heterotrimer Composition is Altered in Aging Skeletal Muscle

In support of the change in catalytic subunit activity, AMPKα2 protein content was lower and AMPKα1 protein content was greater in O vs. YA rats after STIM. Additionally, AMPKγ2 and γ3 protein contents were lower in O vs. YA muscle. However, AMPKβ1, β2, and γ1 protein contents were unaffected by age.

The changes in the individual isoform protein contents suggest a change in the availability of the AMPK isoforms and therefore alterations in the AMPK heterotrimer composition in aging skeletal muscle. In support of this, we found that association of AMPKα2 with AMPKγ2 increased while association with AMPKγ3 decreased. We also observed decreased association of AMPKα1 with the γ3 isoform in O vs. YA muscles. However, there was not a comparable increase in coimmunoprecipitated β1 or β2 subunits with the increase in AMPKα1 in aging rat skeletal muscle.

AMPK Nuclear Localization after Endurance-Like Exercise is Impaired in Aging Skeletal Muscle

In support of our hypothesis, nuclear localization of phosphorylated AMPK was impaired in aging muscle after STIM. Additionally, nuclear localization of AMPKα2 and AMPKγ3 were also impaired in aging muscle in response to STIM. Results also reconfirmed the alteration of the

AMPK heterotrimer isoform protein content. Specifically, LKB1, total AMPK, phosphorylated AMPK, AMPKα2, AMPKβ2, and AMPKγ2 protein content decreased and AMPKα1 and AMPKγ1 protein content increased. Consequently, aging not only alters the composition and activity of AMPK, but also the availability of AMPK to either regulate gene transcription in the nucleus or directly interact with cytosolic targets to regulate metabolic pathways.

Chronic Activation of AMPK Improved Treadmill Endurance in Aging Skeletal Muscle and In Vitro Muscle Contraction Endurance in Young Adult Skeletal Muscle

As we hypothesized, one month of chronic activation of AMPK with AICAR treatment improved the treadmill endurance of O mice. Interestingly, chronic AICAR treatment did not improve the treadmill endurance of YA mice. Conversely, chronic AICAR treatment improved the rate of fatigue and recovery with *in vitro* muscle contractions in YA mice but not O mice.

Chronic AICAR Treatment does not Conclusively Alter Mitochondrial Biogenesis, Protein Synthesis, or Autophagy in Aged Skeletal Muscle

The improvements in endurance-type exercise with chronic AICAR treatment suggest changes in metabolic pathways. However, the improvements we saw in both the YA and O mice cannot conclusively be attributed to changes in protein synthesis, autophagy, fiber type distribution, or muscle differentiation. Therefore, the improvements in endurance may be due to improvements in factors other than skeletal muscle energetics (e.g. cardiac function, blood supply, etc.). Aging muscle may have been impacted more by the chronic AICAR treatment than YA muscle due to greater deterioration in whole body energetics allowing for a greater range of improvement. Alternatively, improvements in isolated skeletal muscle energetics improved the rate of fatigue and recovery in the YA skeletal muscle but not aged muscle. This may primarily be due to the improvements in mitochondrial protein expression and activity that was seen in the YA muscle versus the O muscle with chronic AICAR treatment.

Conclusion

In conclusion, the AMPK heterotrimer composition and activity is altered in aging muscle. Additionally, chronic activation of AMPK by AICAR treatment can improve the treadmill endurance of aging mice. These improvements with chronic AICAR suggest that the alteration in AMPK heterotrimer composition and activation in aging muscle may contribute to the aging phenotype by altering AMPK signaling. However, chronic AICAR treatment does not overall significantly impact mitochondrial, mTOR, or autophagy signaling proteins in aging muscle to account for the improvements in exercise endurance.

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Yu H, Fujii N, Hirshman MF, Pomerleau JM & Goodyear LJ. (2004). Cloning and characterization of mouse 5'-AMP-activated protein kinase gamma3 subunit. *Am J Physiol Cell Physiol* 286, C283-292.

Zhou M, Lin BZ, Coughlin S, Vallega G & Pilch PF. (2000). UCP-3 expression in skeletal muscle: effects of exercise, hypoxia, and AMP-activated protein kinase. *Am J Physiol Endocrinol Metab* 279, E622-629.

Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ & Shulman GI. (2002). AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proceedings of the National Academy of Sciences* 99, 15983-15987.

CURRICULUM VITAE

SHALENE HARDMAN
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EDUCATION

Brigham Young University, Provo, UT
Ph.D. in Physiology and Developmental Biology 2014
Dissertation: The effects of aging on skeletal muscle AMPK activation and an analysis of chronic AICAR treatment on the aging phenotype

Brigham Young University, Provo, UT
B. S. in Physiology and Developmental Biology 2002-2006

TEACHING EXPERIENCE

Salt Lake Community College, Taylorsville, UT
Adjunct Faculty- BIO 2425: Human Physiology 2014
Taught lab course material, set up and managed lab, administered exams and grades

Brigham Young University, Provo, UT
Teaching Assistant- PDBIO 482: Developmental Biology 2010-2014
Taught weekly review sessions, assisted in writing exams and grading

Teaching Practicum- PDBIO 305: Human Physiology 2013
Taught lecture and administered exams under the direction of a faculty mentor

Laboratory Instructor- PDBIO 305: Human Physiology 2010, 2013
Taught lab lecture, set up and managed lab, administered grades.

Teaching Assistant- BIO 100: Principles of Biology 2004-2006, 2009
Taught weekly discussion groups, lab practicums, assisted in grading

Thanksgiving Point, Lehi, UT
Education Assistant 2006
Developed and carried out summer day camps and K-6 grade school field trips.

Girl Scouts of Utah, Murray, UT
Unit Counselor 2003
Developed and led summer camps to teach leadership and outdoor skills.

Jordan School District, Sandy, UT
Teaching Assistant 2001-2002
Assisted Occupational Therapists in teaching children with disabilities.

RESEARCH EXPERIENCE

Brigham Young University, Provo, UT (Advisor: Dr. David Thomson)
Graduate Research-Exercise Metabolism & Sarcopenia 2010-present
Lab skills: Radiation certified, western blots, immunohistochemistry, immunoprecipitation, tissue sectioning, HPLC, PCR, cell culture, microscopy, rodent handling and surgeries, and undergraduate mentoring.

Nelson Laboratories, Inc., Salt Lake City, UT
Lab analyst/Study Director I/Supervisor 2006-2009
Responsibilities: Protocol generation, validations, quality control, training and evaluation of personnel, instructor for National Registry of Microbiology preparation course on microbial identification, Bacteriostasis/Fungistasis (B/F) testing, and Environmental Monitoring.
Lab skills: B/F testing; sterility procedures; environmental monitoring; microbial identification through MIDI, Vitek, API systems; gram stains; cell culture; BSL-3 training, and GLP/ GMP procedures.

Brigham Young University, Provo, UT (Advisor: Dr. Robert Seegmiller)
Undergraduate Research- Osteoarthritis 2005-2006
Lab skills: Mouse colony maintenance, microscopy, animal dissections, tissue sectioning, data analysis, and immunohistochemistry.

LEADERSHIP AND COMMUNITY SERVICE

Anatomy Academy Mentor- Greenwood Elementary 2013-2014

BYU Graduate Student Society PDBIO department representative 2013-2014

CUSEF Science Fair Judge- Provo, UT 2013-2014

Provo Youth Mentor- Provo, UT 2005-2006, 2011-2012

Tutor- Physiology, Biology, Chemistry- Orem/Provo, UT 2005-2006, 2012

AWARDS

Research Assistantship- PDBIO Department, BYU, Provo, UT 2010-2014

Teaching Assistantship- PDBIO Department, BYU, Provo, UT 2010-2014

Teaching Assistantship- Biology Department, BYU, Provo, UT 2009

Full Tuition Scholarship- BYU, Provo, UT 2003-2006

MEMBERSHIPS

American Association for the Advancement of Science

PUBLICATIONS

Tanner CB, Madsen SR, Hallowell DM, Goring DM, Moore TM, Hardman SE, Heninger MR, Atwood DR, and Thomson DM. Mitochondrial and performance adaptations to exercise training in mice lacking skeletal muscle LKB1. Am J Physiol Endocrinol Metab 2013.

Merrill JF, Thomson DM, Hardman SE, Hepworth SD, Willie S, and Hancock CR. Iron deficiency causes a shift in AMP-activated protein kinase (AMPK) subunit composition in rat skeletal muscle. Nutr Metab (Lond) 9: 104, 2012.

ORAL AND POSTER PRESENTATIONS

Hardman SE, Merrill JF, Thomson DM, Hancock CR. The effect of iron deficiency on AMPK subunit isoform composition. Poster session presented at: Experimental Biology Conference; 2013 Apr 20-24; Boston, MA.

Jacobs MB, Hardman SE, Moore TM, Lew JJ, Thomson DM. RAGE and STAT3 signaling in chronic AICAR-treated young adult and old skeletal muscle. Poster session presented at: American Physiological Society Conference; 2012 Oct 10-13; Westiminster, CO.

Anderson SK, Hardman SE, Nelson BJ, Oleskey ZP, Rindlisbach CA, Thomson DM. Role of LKB1 in skeletal muscle regeneration after injury. Poster session presented at: American Physiological Society Conference; 2012 Oct 10-13; Westiminster, CO.

Hardman SE, Hall DE, Mitchell AJ, Black KM, Compton RA, Thomson DM. The Effects of Age and Muscle Contraction on AMPK Activity. Oral and poster session presented at: Experimental Biology Conference; 2012 Apr 21-25; San Diego, CA.

Hall DE, Hardman SE, Jacobs MB, Mitchell AJ, Thomson DM. The effects of aging on AMPK subunit expression and complex formation. Poster session presented at: Experimental Biology Conference; 2012 Apr 21-25; San Diego, CA.

Davis J, Hardman SE, Hansen M. Substrate stiffness alters cell-cell detachment during HGF-induced epithelial-mesenchymal transition. Poster session presented at: 50th annual meeting of the American Society of Cell Biologists; 2010 Dec 11-15; Philadelphia, PA.