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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 which regulate many cellular processes that are disrupted in old-age. Functional AMPK is a heterotrimer composed of alpha, beta and gamma subunits, and each subunit can be represented in the heterotrimer by one of two ($\alpha 1/\alpha 2$, $\beta 1/\beta 2$) or three ($\gamma 1/\gamma 2/\gamma 3$) isoforms. Altered isoform composition affects AMPK localization and function. 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 $\alpha 1$ and $\alpha 2$ 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 Fischer₃₄₄ x Brown Norway F1 hybrid rats after an *in situ* bout of endurance-type contractions produced via electrical stimulation of the sciatic nerve (STIM). AMPK α phosphorylation and AMPK $\alpha 1$ and $\alpha 2$ activities were unaffected by age at rest. However, AMPK α phosphorylation and AMPK $\alpha 2$ protein content and activity were lower in O vs. YA after STIM. Conversely, AMPK $\alpha 1$ content was greater in O vs. YA muscle, and $\alpha 1$ activity increased with STIM in O but not YA muscles. AMPK $\gamma 3$ overall concentration and its association with AMPK $\alpha 1$ and $\alpha 2$ was lower in O vs. YA GAST. We conclude that activation of AMPK $\alpha 1$ is enhanced, while activation

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.E.H. designed and performed experiments, collected and analyzed data, prepared figures, drafted and reviewed the manuscript. D.E.H. designed and performed experiments, analyzed data, and reviewed manuscript. A.J.C. performed experiments, analyzed data, and reviewed the manuscript. C.R.H. supervised experiments, analyzed data, and reviewed the manuscript. D.M.T. designed, supervised and performed experiments, collected and analyzed data, prepared figures, drafted, edited and revised the manuscript.

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of $\alpha 2$ 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 $\alpha 1$, $\alpha 2$ and $\gamma 3$, this may contribute to sarcopenia and associated muscle metabolic dysfunction.

Keywords

AMPK; skeletal muscle; aging

1. INTRODUCTION

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

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 (Gowans et al. 2013; Hawley et al. 1996; Hawley et al. 1995; Scott et al. 2007) and a worse substrate for dephosphorylation by protein phosphatase 2C (Davies et al. 1995; Marley et al. 1996). Binding of AMP also allosterically activates AMPK in addition to its effect on phosphorylation (Gowans et al. 2013; Hawley et al. 1995; Scott et al. 2004; Witczak et al. 2008).

AMPK is a heterotrimeric protein composed of α , β , and γ subunits (Stapleton et al. 1996; Woods et al. 1996). 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, $\alpha 1$ or $\alpha 2$. The β subunit serves as a scaffolding or regulatory subunit and also has two isoforms, $\beta 1$ and $\beta 2$. The γ subunit contains nucleotide binding sites and determines the degree of AMP dependence of the AMPK heterotrimer. $\gamma 2$ complexes have a higher AMP sensitivity than $\gamma 1$ subunits, which have a higher AMP sensitivity than $\gamma 3$ subunits (Cheung et al. 2000).

During exercise, the AMP to ATP ratio increases by way of the adenylate kinase reaction, thereby potentially activating AMPK (Dreyer et al. 2006; Hawley et al. 2003; Hawley et al.

1995; Jensen et al. 2007; Winder and Hardie 1996). In both mice and humans, the $\alpha 2/\beta 2/\gamma 1$ AMPK complex is predominant. However, exercise and muscle contraction appear to primarily activate complexes containing $\alpha 2$ and $\gamma 3$ isoforms, although $\gamma 1$ can be activated as well (Birk and 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 $\alpha 1$ subunit is particularly important in negatively regulating muscle cell size through regulation of the mTOR pathway (Mounier et al. 2009), while the $\alpha 2$ subunit appears more able to localize to the nucleus than $\alpha 1$, presumably to control gene transcription (Salt et al. 1998).

Activation of AMPK in response to endurance exercise appears to be blunted in old skeletal muscle (Ljubicic and Hood 2009; Reznick et al. 2007b). However, the effect of old-age on isoform-specific activation of the catalytic α subunit (i.e. $\alpha 1$ vs. $\alpha 2$) 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 (Bergeron et al. 2001a; Foretz et al. 1998; 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.

2. MATERIALS AND METHODS

2.1. 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 months; n=8) and old (O) (30 months; n=8) male Fischer₃₄₄ 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).

2.2. 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 minutes at one 10 millisecond pulse per second 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.

2.3. 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 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor). The raw homogenate was freeze-thawed three times to ensure disruption of intracellular membranes and then centrifuged at $10,000 \times g$ for 10 minutes. Supernatants were analyzed for protein content using the DC Protein Assay (Bio Rad Laboratories, Hercules, CA, USA). Supernatants were stored in microcentrifuge tubes at -95°C until further analysis.

2.4. 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, AMPK γ 3], and 10% [AMPK α 1, AMPK α 2, AMPK β 1, AMPK β 2, AMPK γ 1, AMPK γ 2, 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 to ensure equal protein loading and transfer to the PVDF. 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 catalog numbers, dilutions and manufacturers were as follows: pAMPK, Thr172 (#4188/#2535, 1:5000), tAMPK (#2532, 1:2000), pACC, Ser221 (#3661, 1:5000), AMPK β 1 (#4182, 1:2000), AMPK β 2 (#4148, 1:2000), AMPK γ 1 (#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); AMPK α 1 (A300–507A, 1:4000), AMPK α 2 (A300–508A, 1:20000) from Bethyl Laboratories, Inc. (Montgomery, TX, USA); AMPK γ 2 (sc-20165, 1:2000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); AMPK γ 3 (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).

2.5 AMPK Activity Assay

AMPK activity of $\alpha 1$ and $\alpha 2$ subunits immunoprecipitated from GAST and TA homogenates was measured by the incorporation of radiolabeled phosphate from ATP into SAMS (HHMRSAMSGHLHVKRR-OH) peptide. Activity was assessed and expressed as picomoles per gram tissue per minute, as described previously (Park et al. 2002).

2.6 Co-Immunoprecipitation

To determine the effect aging has on the heterotrimer subunit composition, 15 μ g of $\alpha 1$ or $\alpha 2$ antibody and 1ml ice cold TBS were added to about 80 μ l (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 seconds at 13,000 x g, then washed three times with 1 ml ice cold TBS and resuspended in 420 μ l ice cold TBS. 50 μ l aliquots of the resuspended matrix were transferred to centrifuge tubes and centrifuged. After removal of the supernatant, 400 μ g of GAST homogenate was added to the matrix-antibody pellet which then was brought up to 500 μ l total volume with ice cold homogenization buffer. Samples were rotated end-over-end overnight at 4°C. After rotation, samples were centrifuged for 30 seconds 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 μ l of loading buffer and boiled for 3 minutes at 100°C. Samples were then loaded onto 10% gels for western blotting and immunodetection as described previously.

2.7. 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 minutes. 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 minutes. 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 minutes.

2.8. 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 minutes 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 μ l of the supernatant was placed in a fresh tube, dehydrated, and stored at 80°C. Just prior to HPLC, samples were rehydrated in 120 μ l ddH₂O. 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 μ mol/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).

2.9. Statistics

Statistical comparisons using Microsoft Excel or NCSS statistical analysis software (NCSS, Kaysville, UT) were made using a student *t* test or repeated measures ANOVA to determine statistical significance (*p* < 0.05) with Fisher's LSD post-hoc analysis employed where appropriate. Values are reported as means $\pm$ SE.

3. RESULTS

3.1. Effects of age on AMPK phosphorylation and activation

The energetic response to the contraction bout was assessed by measuring the concentration of glycogen and high-energy phosphate metabolites in the gastrocnemius (Table 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 mitogenic and metabolic proteins. STIM increased phosphorylation of AKT, ERK, and p38 similarly in both the YA and O rats (Fig. 1), further verifying the effectiveness of the stimulus in both age groups by the contraction bout.

AMPK activity was next assessed by determining pAMPK protein content and AMPK α 1 and α 2 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 (Fig. 2A). The overall protein content level of total AMPK was decreased in O vs. YA muscle (Fig. 2B). AMPK α 2 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 (Fig. 2D). In contrast, AMPK α 1 activity increased by 30% and 38% in the GAST and TA respectively after STIM in O rats while α 1 activity was unaffected by STIM in YA rats (Fig. 2C).

3.2. Effects of age on LKB1 and ACC

Protein content of LKB1 was unaffected by age (Fig. 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 (Fig. 3C) but pACC significantly increased with STIM in both O and YA rats (Fig. 3B).

3.3. 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. AMPK α 1 protein content in O versus YA muscle was 45% and 59% higher in the GAST and TA respectively (Fig. 4A). In contrast, AMPK α 2 content was attenuated by 18% in the GAST in O versus YA rats (Fig. 4B), but not significantly different for the TA. Protein content levels of AMPK β 1, β 2, and γ 1 were not significantly different between age groups (Fig. 5A, 5B, 6A). AMPK γ 2 content in O versus YA rats was 75% and 49% lower in the GAST and TA respectively (Fig. 6B). AMPK γ 3 subunit isoform content in O versus YA rats was also 85% and 78% lower in the GAST and TA respectively (Fig. 6C). These differences are summarized in Table 2.

3.4 Effects of age on AMPK subunit heterotrimer composition

Immunoprecipitation of the catalytic isoforms AMPK α 1 and α 2 was followed by western blotting to detect content of the β 1, β 2, γ 1, γ 2, or γ 3 subunits associated with the two alpha subunits in the GAST homogenates. After immunoprecipitation using the AMPK α 1 and α 2 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 AMPK α 1 content increased by 128% in O versus YA rats, and immunoprecipitated α 2 content decreased by 33% in O vs. YA rats (Fig. 7), consistent with AMPK content in the whole homogenates shown in Fig. 4.

The amount of γ 1 that co-immunoprecipitated with α 1 increased similarly (130%) in O muscle, while the amount of coimmunoprecipitated γ 2 was unchanged and the amount of coimmunoprecipitated γ 3 was decreased by 82% (Fig 7A). Decreased association of AMPK α 1 with γ 3 was verified by immunoprecipitating γ 3 and then western blotting for α 2 (data not shown). This suggests a shift from α 1: γ 3 complexes to α 1: γ 1 complexes in old vs. young muscle. The amount of β 1 and β 2 subunits coimmunoprecipitated with α 1 did not change significantly (Fig 7A). Since α 1 content increased without a change in associated β subunit content, this suggests that at least part of the increase in α 1 content is not associated in the typical heterotrimeric complex with AMPK β subunits, which may limit the effect this has on overall AMPK α 1 activity.

The 33% drop in immunoprecipitated $\alpha 2$ isoform in O muscle was associated with a similar but non-significant drop in coimmunoprecipitated $\gamma 1$ content, an even greater and significant 83% drop in coimmunoprecipitated $\gamma 3$ content, and a non-significant increase in coimmunoprecipitated $\gamma 2$ content (Fig 7B). Taken together the disproportionate drop in $\gamma 3$ vs. $\alpha 2$ in this case suggests a shift away from $\alpha 2$: $\gamma 3$ complexes, likely toward $\alpha 2$: $\gamma 2$ complexes with old age. The decline in $\beta 1$ and $\beta 2$ content that coimmunoprecipitated with $\alpha 2$ in O muscle was similar to the decline in immunoprecipitated $\alpha 2$, suggesting that interaction between $\alpha 2$ and the β subunits is not altered with aging (Fig 7B).

4. 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 AMPK α phosphorylation and *in vitro* activity of AMPK $\alpha 2$ is attenuated with age in response to endurance-type muscle contraction. However, we also found that AMPK $\alpha 1$ protein content and contraction-induced activity increases with age, and aging results in decreased association of the AMPK $\alpha 1$ and $\alpha 2$ subunits with the $\gamma 3$ isoform, and increased association of $\alpha 1$ with $\gamma 1$.

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 (Ljubcic and Hood 2009; Thomson et al. 2009) and human (Drummond et al. 2008) muscle, and makes sense given the broad view of AMPK as part of a stress-sensing system.

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 and 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 (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, Akt, ERK, 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 AMPK $\alpha 2$ activity increased with STIM in both YA and O rats, the increase in AMPK phosphorylation and AMPK $\alpha 2$ activity was attenuated in aged compared to YA rats in response to the endurance-type muscle contraction, consistent with findings observed after treadmill running (Ljubcic and Hood 2009; Reznick et al. 2007b) and *in situ* contractions. This hypo-activation of AMPK was associated with lower

AMPK α 2 and total AMPK α protein expression. AMPK α 2 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 AMPK α 2 with contraction. While protein expression of the regulatory β subunit isoforms was not altered with age, the γ 2 and γ 3 isoform protein content was decreased in O muscles, consistent with findings in aged human muscle (Mortensen et al. 2009). Furthermore, the association of AMPK α 2 with AMPK γ 3 decreased. Since AMPK γ 3 is the predominant isoform activated in response to exercise, particularly as a α 2 β 2 γ 3 heterotrimer (Birk and Wojtaszewski 2006; Yu et al. 2004), this decreased α 2/ γ 3 association in aged muscle likely contributed to the impaired overall activation of AMPK with contraction. The AMPK α 2 and γ 3 isoforms are predominantly expressed in fast-twitch glycolytic muscle (Mahlpuu 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 (Holloszy et al. 1991; Lexell 1995; Lexell et al. 1983). It is not surprising therefore that a reduction in fast-twitch muscle with old-age would result in decreased content of the AMPK γ 3 and α 2 isoforms (Chen et al. 1999; Putman et al. 2007).

Given AMPK's well-defined roles in muscle, the decreased content and activity of AMPK α 2 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 (Bergeron et al. 2001b; Winder et al. 2000; 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 α 2 isoform, we observed increased activation of AMPK α 1 in O muscle after the contraction bout. Likewise, AMPK α 1 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 γ 3 subunit, and an increase in its association with the γ 1 subunit since α 1 heterotrimers containing γ 2 or γ 3 are not activated by muscle contraction (Treebak et al. 2014). We had not expected any association of the α 1 isoform with γ 3 based on previous data from mice showing that immunoprecipitated AMPK γ 3 only associates with the α 2 isoform (Mahlpuu et al. 2004; Treebak et al. 2009). We verified our results by immunoprecipitating AMPK γ 3 and then western blotting for AMPK α 1, 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 *in vitro* that all AMPK γ isoforms isolated from rat liver can associate with either α subunit isoform.

As was the case for the impaired activation of AMPK α 2, the elevated AMPK α 1 activity in old muscle after contraction may have important clinical significance. AMPK α 1 has been

shown to play an isoform-specific role in inhibiting the mTOR pathway and protein synthesis in skeletal muscle (Mounier et al. 2011; Mounier et al. 2009). 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 and 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 $\alpha 2$ 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 the 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.

In conclusion, we found that 1) activation of AMPK $\alpha 2$ immediately after a continuous bout of muscle contractions is attenuated but AMPK $\alpha 1$ 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 AMPK $\alpha 2$ and $\alpha 1$ 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.

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GRANTS

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ABBREVIATIONS

ACC	acetyl CoA carboxylase
ADP	adenosine diphosphate
AKT	protein kinase B
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AMPK	AMP-activated protein kinase
ERK	extracellular signal-regulated kinase
GAST	gastrocnemius
HPLC	high performance liquid chromatography
IMP	inosine monophosphate
IP	immunoprecipitation
O	old
OR	old at rest
OS	old with STIM
PCr	phosphocreatine
STIM	<i>in situ</i> electrical stimulation
TA	tibialis anterior
YA	young adult
YR	young adult at rest
YS	young adult with STIM

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HIGHLIGHTS

- Aging is associated with decreased muscle AMPK γ 2 and AMPK γ 3 content.
- Association of AMPK α 1 and AMPK α 2 isoforms with the AMPK γ 3 isoform decreases with age.
- *In vitro* AMPK α 2 activation is impaired after contractions in old vs. young adult muscle.
- *In vitro* AMPK α 1 activation is increased after contractions in old vs. young adult muscle.

Figure 1. Phosphorylation of exercise-induced signaling proteins increases after an electrically stimulated endurance-type *in situ* contraction bout (STIM) in young adult (YA) and old (O) rats Gastrocnemius (GAST) muscles from YA (8 months) and O (30 months) Fisher Brown Norway male rats were stimulated for 10 min (1 pulse per second, 15V, 10 ms duration) and removed immediately after the contraction bout. Western blot analysis of GAST for (A) phosphorylated AKT (Ser473), (B) phosphorylated ERK (Thr202/Tyr204), and (C) phosphorylated p38 (Thr180/Tyr182). N=7–8 / group. YR= YA at REST; YS= YA with STIM; OR= O at REST; OS= O with STIM. Values are means $\pm$ SEM. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

Figure 2. AMPK phosphorylation and AMPK α 2 activity are attenuated while AMPK α 1 activity is increased in O vs. YA fast-twitch muscles

Western blotting analysis of GAST and tibialis anterior (TA) for (A) phosphorylated AMPK (Thr172) and (B) total AMPK. Activity assay of (C) AMPK α 1 and (D) AMPK α 2. N=8 / group. Values are means $\pm$ SEM. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

Figure 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. Values are means $\pm$ SEM. a = significant difference from corresponding REST muscle; b = significant difference from corresponding condition in the YA muscle ($p < 0.05$).

Figure 4. AMPK $\alpha 1$ protein content is increased in O vs. YA fast twitch muscle while AMPK $\alpha 2$ content is decreased

Western blotting analysis of GAST and TA for (A) AMPK $\alpha 1$ and (B) AMPK $\alpha 2$. N=8 / group. Values are means $\pm$ SEM. a = significant difference from YA ($p < 0.05$).

Figure 5. AMPK β 1 and β 2 protein content are unchanged in O vs. YA fast twitch muscle
Western blotting analysis of GAST and TA for (A) AMPK β 1 and (B) AMPK β 2. N=8 / group. Values are means $\pm$ SEM.

Figure 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. Values are means ± SEM. a = significant difference from YA (p < 0.05).

Figure 7. Association of the AMPK α 1 and α 2 isoform with the regulatory isoforms is altered in aged rats

Immunoprecipitation (IP) of the catalytic AMPK α 1 or α 2 isoform from GAST was followed by western blotting of the non-catalytic subunits to determine content. (A) IP of AMPK α 1 followed by western blotting for AMPK α 1, AMPK β 1, AMPK β 2, AMPK γ 1, AMPK γ 2, and AMPK γ 3. (B) IP of AMPK α 2 followed by western blotting for AMPK α 2, AMPK β 1, AMPK β 2, AMPK γ 1, AMPK γ 2, and AMPK γ 3. N=7–8/group. Values are means $\pm$ SEM. a = significant difference from YA ($p < 0.05$).

Table 1

High-energy phosphate concentrations in gastrocnemius muscle

	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 (μmol/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

Summary of AMPK heterotrimer isoform modifications in aging skeletal muscle

Isoform	Protein Content	Association with AMPK α 1	Association with AMPK α 2
α 1	↑	N/A	N/A
α 2	↓	N/A	N/A
β 1	-	-	↓
β 2	-	-	-
γ 1	-	↑	-
γ 2	↓	-	-
γ 3	↓	↓	↓

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.