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AMPK Activation Stimulates Myofibrillar Protein Degradation and Expression of Atrophy-Related Ubiquitin Ligases by Increasing FOXO Transcription Factors in C2C12 Myotubes

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In skeletal muscle, AMP-activated protein kinase (AMPK) is a metabolic master switch regulating glucose and lipid metabolism. Recently, AMPK has been implicated in the control of protein synthesis in skeletal muscle, but the effect of AMPK activation on myofibrillar protein degradation has yet to be elucidated. The present study was designed to examine the effect of 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR)-induced AMPK signaling on effector mechanisms of myofibrillar protein degradation and the expression of atrophy-related genes (atrogin-1/MAFbx, MuRF1, proteasome C2 subunit, calpains, cathepsin B, and caspase-3) in C2C12 myotubes. AICAR stimulated myofibrillar protein degradation (as measured by N^{τ}-methylhistidine release), while also increasing the levels of atrogin-1/MAFbx and MuRF1 mRNA, but the expression of other atrophy-related genes was not enhanced by AICAR treatment in C2C12 myotubes. AICAR also stimulated the level of FOXO transcription factors mRNA and protein in C2C12 myotubes. These results indicate that activation of AMPK stimulates myofibrillar protein degradation through the expression of atrogin-1/MAFbx and MuRF1 by increasing FOXO transcription factors in skeletal muscles.

Key words: AMP-activated protein kinase (AMPK); myofibrillar protein degradation; atrogin-1/MAFbx; MuRF1; FOXO

AMP-activated protein kinase (AMPK) acts as an energy sensor that responds to changes in the intracellular ratio of AMP:ATP.[1] Activation of AMPK results in the stimulation of a variety of cellular processes involved in the production of ATP, *e.g.*, glucose uptake[2,3] and fatty acid oxidation,[4,5] and repression of energy-consuming processes, *e.g.*, fatty acid[4] and protein synthesis.[6–8] The repression of protein synthesis by AMPK is associated with decreased signaling to the protein kinase, referred to as the mammalian target of rapamycin (mTOR).[6]

Research using *in vitro* systems has shown that AMPK can be activated under artificial conditions, *e.g.*, by treatment with high fructose or 2-deoxyglucose, heat shock or an inhibitor of oxidative phosphorylation.[9] Pharmacological use of 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) to activate AMPK directly without altering cellular concentrations of ATP, ADP, and AMP is common.[10] Activation of AMPK in skeletal muscle, whether through endurance exercise or administration of AICAR, results in repressed protein synthesis and decreased signaling through mTOR,[6,11,12] but, the effect of AMPK activation on myofibrillar protein degradation has yet to be elucidated at the molecular level.

Like other tissues, skeletal muscle contains multiple proteolytic systems, each of which could play an important role in muscle atrophy. Intracellular proteolysis is carried out by lysosomal and non-lysosomal pathways in which intracellular proteases are directly responsible for the degradation of proteins. Calpains, cysteine proteases in the cytosol, are thought to be the main agents of non-lysosomal Ca^{2+}-dependent proteolysis that occurs within the myofibril, and have been found to be capable of carrying out the initial step in myofibrillar proteolysis.[13,14] Cathepsins, the main agents of lysosomal degradation, are known to contribute to muscle protein breakdown.[15] Lysosomal proteases degrade sarcoplasmic proteins and released myofibrillar proteins.[16] Recently, caspase-3, an apoptotic protease, has been found to be an initial trigger of accelerated muscle proteolysis in catabolic conditions.[17] The major proteolytic pathway involves an ubiquitin-proteasome system, which is ubiquitous throughout the body, is dependent on ATP, and degrades ubiquitin-conjugated proteins *via* the 26S proteasome.[18] In skeletal muscle, the ubiquitin-proteasome system is also the main proteolytic pathway for overall proteolysis.[19] Studies with animals have consistently demonstrated that protein degradation by the ubiquitin-proteasome system increases in muscle undergoing atrophy.[20–23] Evidence suggests that atrogin-1/MAFbx and MuRF1, E3 ubiquitin ligases, play a pivotal role in muscle atrophy.[24,25] Atrogin-1/MAFbx and

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MuRF1 are expressed only in skeletal muscle. Their expression is increased under catabolic conditions that result in muscle atrophy.[21,22,24,25]

Recent evidence strongly suggests that FOXO transcription factors (FOXO1, FOXO3a, and FOXO4; a subfamily of the forkhead type transcription factors) play a central role in the regulation of gene expression during skeletal muscle atrophy.[26,27] Both FOXO1 and FOXO3a appear to upregulate expression of muscle-specific ubiquitin ligases and to decrease muscle size,[28,29] but the regulation and function of forkhead transcription factors in skeletal muscle are poorly understood.

N^{τ}-methylhistidine is an amino acid formed by the post-translational methylation of specific histidine residues in the myofibrillar proteins actin and myosin. Since N^{τ}-methylhistidine cannot charge tRNA, it cannot be reutilized for protein synthesis. Because of this, and also because it does not undergo catabolism, the output of N^{τ}-methylhistidine has been used as an index of myofibrillar protein degradation.[30,31] but, it is not known whether this reflects protein degradation caused by AICAR, and the mechanism of the effects of AMPK activation in skeletal muscles is not understood.

The present study using C2C12 myotubes is first to demonstrate that AICAR-induced activation of AMPK stimulates myofibrillar protein degradation through the expression of atrophy-related ubiquitin ligases by increasing FOXO transcription factors in skeletal muscles.

Materials and Methods

Materials. AICAR was purchased from Sigma Aldrich (St. Louis, MO). The other chemicals were the best grade commercially available.

Cell culture. C2C12 myoblasts were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum. At a confluence, myoblasts were induced to fuse by changing the medium to DMEM containing 2% horse serum. Cells were maintained in the 2% horse serum (differentiation medium) for 3 d. The myotubes were treated 3 d later, when myoblast fusion was complete.

Protein degradation. C2C12 myotubes in 6-well plates were incubated with AICAR (0, 0.1, 0.5 and 1.0 mM) for 24 h in DMEM. The medium was then collected, and the N^{τ}-methylhistidine concentration was measured by HPLC after derivatization of fluorescamine by perchloric acid and heating.[32] The cell monolayer was washed three times with ice-cold PBS, and the cells were dissolved in 1 N NaOH. Protein concentrations were measured by the Lowry method using bovine serum albumin as the standard.[33]

RNA isolation and real-time PCR. Total RNA was extracted from C2C12 myotubes by the guanidine thiocyanate/phenol–chloroform extraction method with TRIzol Reagent (Invitrogen Life Technologies) according to the manufacturer's directions. cDNA was synthesized from 1–1,000 ng of total RNA using random hexamer (TaKaRa, Tokyo) and ReverTra Ace (TOYOBO, Tokyo). Real-time PCR primers were purchased from Qiagen (QuantiTect Primer Assays) for mouse atrogin-1/MAFbx (QT00158543), mouse MuRF1 (QT 00119917), mouse proteasome C2 subunit (QT 0013-7347), mouse μ-calpain (QT00112168), mouse m-calpain (QT00106876), mouse calpain-3/p94 (QT0011-3652), mouse cathepsin B (QT00145719), mouse caspase-3 (QT00260169), mouse FOXO1 (QT00116186), mouse FOXO3a (QT00168623), and mouse glyceraldehyde-3-phosphate dehydrogenase (QT00199388, GAPDH). Gene expression was measured by real-time PCR using a LightCycler (Roche Diagnostics, Mannheim, Germany) instrument with the QuantiTect SYBR Geen PCR system (Qiagen). GAPDH expression was used as an internal control.

Western blot analysis. The cells were washed twice with PBS and lysed in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 2 mM Na_3VO_4, 0.1 mM okadaic acid, 25 mM β-glycerophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 pg/ml leupeptin). Soluble proteins were recovered after a 10-min centrifugation ($10{,}000 \times g$), and their concentrations determined according to the method established by Lowry using a protein assay kit (Bio-Rad). Equal amounts of protein were boiled for 5 min and analyzed by electrophoresis in a 10% SDS–polyacrylamide gel, and then transferred to a nitrocellulose membrane. Subsequently, the membranes were treated with blocking buffer (5% nonfat dry milk in PBS) for 1 h at 4 °C. The blocked membranes were probed with primary antibodies and further incubated with a secondary antibody conjugated with horseradish peroxidase. Bound IgG was detected using an enhanced chemiluminescence system (Amersham Biosciences). The primary antibodies used in this study were anti-AMPKα (rabbit polyclonal IgG, Cell Signaling, #2532) 1:1,000; anti-phospho-AMPKα (Thr 172) (rabbit polyclonal IgG, Cell Signaling, #2531) 1:1,000; anti-FOXO1 (rabbit polyclonal IgG, Cell Signaling, #9462) 1:1,000; anti-FOXO3a (rabbit polyclonal IgG, UPSTATE, #07-702) 1:1,000.

Statistical analysis. Data were analyzed by Student's *t*-test. A p value of <0.05 was considered statistically significant. Each result was expressed as the mean ± SD.

Results

AICAR induced phosphorylation of AMPK in C2C12 myotubes

C2C12 cells were treated with various concentrations of AICAR (0.1, 0.5, and 1.0 mM) for 6 or 24 h. Western

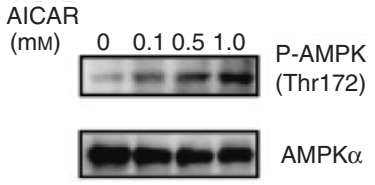

Fig. 1. Effect of AICAR Treatment on Phosphorylation of AMPK at Thr172 in C2C12 Myotubes.

Cells were incubated in DMEM for 6 h with the AMPK activator AICAR (0, 0.1, 0.5, and 1.0 mM). Treated and control cells were harvested with RIPA buffer, and lysate was assessed for the phosphorylation status of AMPK at the Thr172 site by immunoblot analysis (see "Materials and Methods" for further details).

blot analysis of extracts from cells treated with AICAR for 6 h revealed dose-dependent phosphorylation of the enzyme at Thr-172 (Fig. 1), providing evidence of increasing AMPK activity in response to AICAR.

AICAR increased myofibrillar protein degradation in C2C12 myotubes

We examined the effects of AICAR (0.1, 0.5, and 1.0 mM) on protein content and the degradation of myofibrillar protein using N^{τ}-methylhistidine released into the medium in C2C12 myotubes. AICAR (0.5 and 1.0 mM) deceased protein content in C2C12 myotubes (Fig. 2A). On the other hand, the amount of N^{τ}-methylhistidine released into the medium was increased by AICAR (0.5 and 1.0 mM, Fig. 2B). These results indicate that AMPK activation stimulates myofibrillar protein degradation in C2C12 myotubes. Activation of AMPK by AICAR also inhibits protein synthesis in C2C12 myotubes.[6] The present study provides clear evidence that AMPK, in addition to regulating protein synthesis, also regulates myofibrillar protein degradation in C2C12 myotubes.

AICAR induced expression of atrogin-1MAFbx and MuRF1 but not of other atrophy-related genes in C2C12 myotubes

The effects of AICAR (0.1, 0.5, and 1.0 mM) on the expression of atrophy-related genes (atrogin-1/MAFbx, MuRF1, proteasome C2 subunit, calpains, cathepsin B, and caspase-3) in C2C12 myotubes were studied. AICAR increased atrogin-1 mRNA expression (0.5 and 1.0 mM Fig. 3A). The expression of MuRF1 mRNA was also increased by AICAR (1.0 mM, Fig. 3B). However, the expression of proteasome C2 subunit, μ-calpain, m-calpain, calpain-3/p94, cathepsin B, and caspase-3 mRNA was not increased by AICAR (Fig. 3C–H). This result indicates that AMPK activation stimulates the expression of atrogin-1/MAFbx and MuRF1 in C2C12 myotubes.

AICAR increased the mRNA and protein levels of FOXO transcription factors in C2C12 myotubes

The effects of AICAR (0.1, 0.5, and 1.0 mM) on the mRNA and protein levels of FOXO transcription factors in C2C12 myotubes were studied. AICAR increased the

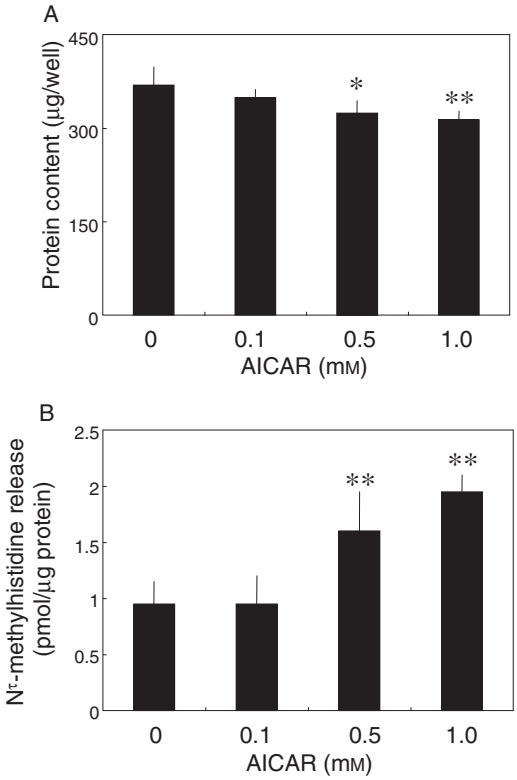

Fig. 2. Effect of AICAR on Myofibrillar Protein Degradation in C2C12 Myotubes.

A, Changes in protein content in C2C12 myotubes incubated at 0, 0.1, 0.5, and 1.0 mM AICAR in DMEM. Stimulation with AICAR lasted for 24 h. Data are expressed as means ± SD (n = 6). B, Changes in the release of N^{τ}-methylhistidine in C2C12 myotubes incubated at 0, 0.1, 0.5, and 1.0 mM AICAR in DMEM. Stimulation with AICAR lasted for 24 h. Data are expressed as means ± SD (n = 6). Student's *t*-test was performed. Significantly different from that of the untreated control ($^{**}p < 0.01$, $^{*}p < 0.05$).

expression of FOXO1 mRNA (0.5 and 1.0 mM, Fig. 4A). FOXO3a mRNA expression was also increased by AICAR (1.0 mM, Fig. 4B). The protein level of FOXO1 and FOXO3a was also increased by AICAR in a dose-dependent manner (Fig. 4C). These results indicate that AMPK activation stimulates the mRNA and protein levels of FOXO transcription factors in C2C12 myotubes.

Discussion

In the present study, we investigated the direct effects of AICAR, as an AMPK activator, on myofibrillar protein degradation, and attemped to characterize the cellular responses involved in this process. AMPK is recognized as having a well-established role in the regulation of energy production and nutrient flux in skeletal muscle during periods of energetic stress. The concept of AMPK acting as an energy sensor suggests that cellular processes that utilize ATP and are not vital to short term survival are potential control points for regulation by the protein kinase.[34] An association between AICAR-induced reduction in protein synthesis

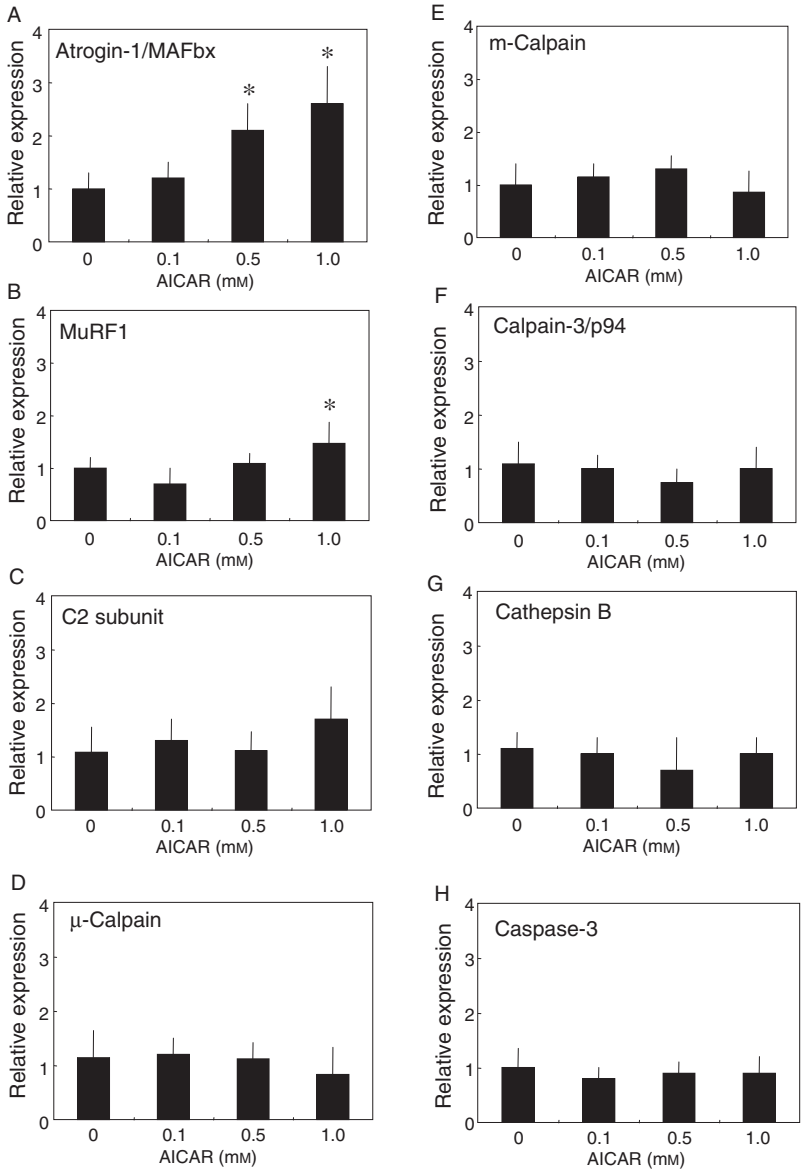

Fig. 3. Effect of AICAR on mRNA Expression of Atrophy-Related Genes in C2C12 Myotubes.

C2C12 myotubes were incubated with AICAR (0, 0.1, 0.5, and 1.0 mM) for 6 h. Results are expressed as the relative expression and are means ± SD, n = 6. RNA quantification was performed as indicated in "Materials and Methods." Student's *t*-test was performed. Significantly different from that of the untreated control ($^{**}p < 0.01$, $^{*}p < 0.05$).

and repression of mTOR-related signaling has been demonstrated,[6,35] but, the effect of AMPK activation on myofibrillar protein degradation has yet to be elucidated at the molecular level. In muscle cell cultures, the amount of N^{τ}-methylhistidine released into the medium, as an index of myofibrillar protein degradation, was increased by serum deprivation[36] and glucocorticoid treatment.[37] In this study, AICAR induced the release of N^{τ}-methylhistidine into the medium from C2C12 myotubes (Fig. 2B). This result indicates that AMPK activation stimulates myofibrillar protein degradation in skeletal muscle. The effects of AICAR as an AMPK activator on myofibrillar protein degradation in skeletal muscle have not previously been reported.

Multiple proteolytic systems play a major role in protein loss and muscle wasting. The intracellular proteolytic processes found in skeletal muscle involve various proteases such as lysosomal acidic cathepsins,[15] Ca^{2+}-dependent calpains,[13,14] and apoptotic caspases.[17] Protein can also be degraded by the ATP-dependent ubiquitin-proteasome system.[38] Ubiquitin-proteasome has been found to constitute an essential pathway of accelerated proteolysis in various animal models of muscle wasting.[19,20] However, the precise roles of all these degradation systems in the breakdown of skeletal muscle proteins are yet to be determined. In this study, AICAR stimulated mRNA expression of atrogin-1/MAFbx and MuRF1 in C2C12 myotubes (Fig. 3A and B), but the expression of other atrophy-related genes (proteasome subunits, calpains, cathepsin B, and cas-

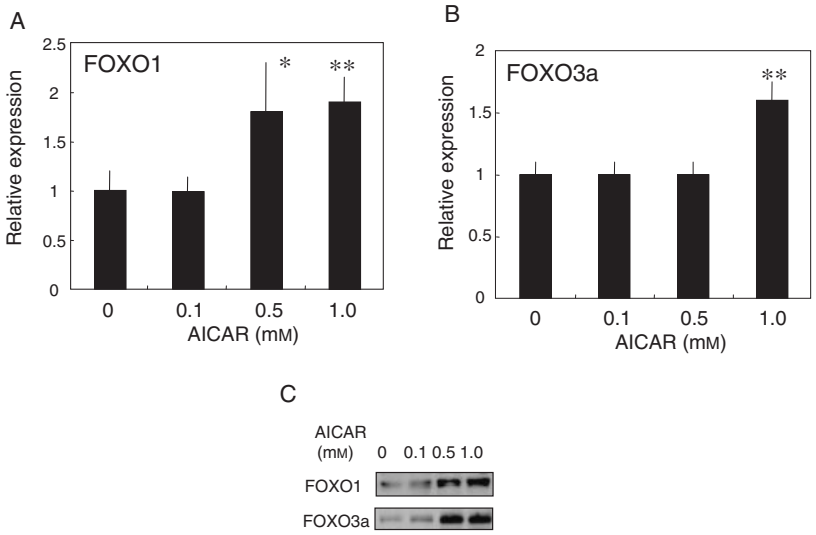

Fig. 4. Effect of AICAR on the Expression of FOXO Transcription Factor mRNA (A and B) and Protein (C) in C2C12 Myotubes.
C2C12 myotubes were incubated with AICAR (0, 0.1, 0.5, and 1.0 mM) for 6 h. RNA quantification was performed as indicated in "Materials and Methods." Results are expressed as the relative expression and are means ± SD, n = 6. Student's *t*-test was performed. Significantly different from that of the untreated control ($^{**}p < 0.01$, $^{*}p < 0.05$). Treated and control cells were harvested with RIPA buffer, and lysate was assessed for the protein of FOXO1 and FOXO3a by immunoblot analysis (see "Materials and Methods" for further details).

pase) was not increased by AICAR treatment (Fig. 3C–H). These results show that AMPK activation stimulates myofibrillar protein degradation through the expression of atrogin-1/MAFbx and MuRF1 in skeletal muscles. Recently, Krawiec *et al.* reported that AMPK activation stimulates the transcription level of atrogin-1/MAFbx and MuRF1 mRNA as muscle atrophy-realted genes in C2C12 myotubes.[39] Our results support theirs, but they did not examine the effect of myofibrillar protein degradation by AICAR in skeletal muscles. The present study is the first to demonstrate that induction of the expression of atrogin-1/MAFbx and MuRF1 suggests an increase in myofibrillar protein degradation with AICAR treatment.

Recently, the FOXO family of transcription factors was found to regulate skeletal muscle atrophy both *in vitro* and *in vivo*.[26,27] Both FOXO1 and FOXO3a transcription factors upregulate expression of the ubiquitin ligase, atrogin-1/MAFbx and MuRF1, and are necessary for increased protein degradation during muscle atrophy.[28,29] In the present study, AICAR increased the mRNA and protein levels of FOXO1 and FOXO3a (Fig. 4A, B, and C). The present study is the first to show changes in the expression of atrogin-1/MAFbx and MuRF1, and FOXO transcription factors in skeletal muscles. We found that AICAR stimulated myofibrillar protein degradation at least in part through the expression of atrogin-1/MAFbx and MuRF1 by increasing FOXO transcription factors in C2C12 myotubes.

Our data indicate that activation of AMPK by AICAR results in significant stimulation of the degradation of muscle protein. Various metabolic states involving acute and chronic energetic imbalances, such as fasting, exercise, and cachexia, have been shown to increase myofibrillar protein degradation.[40–43] Identifying a molecular signal that initiates changes in protein degradation under these conditions remains a challenge. The concept that AMPK acts as an energy sensor is reinforced by the present finding that increased AMPK activity is a regulator of skeletal muscle protein degradation under conditions of energetic stress. The stimulation of myofibrillar protein degradation observed in the present study was mediated by alterations in the expression of the atrophy-related genes atrogin-1/MAFbx and MuRF-1. AMPK has been found to be a sensor of cellular energy status, regulating reciprocal changes in the levels of AMP and/or ATP.[34] An increase in the ratio of AMP to ATP such as that observed in response to starvation, exercise, muscle contraction, and other conditions involving skeletal muscle catabolism lead to activation of AMPK.[44] The FOXO family members FOXO1, FOXO3a, and FOXO4 are all expressed in skeletal muscle, and their expression is increased during caloric restriction.[45] FOXO transcription factor expression is also increased in skeletal muscle in energy-deprived states, as in fasting, diabetes, and after treadmill running.[46,47] In this study, AMPK was involved in the up-regulation of FOXO transcription factors in skeletal muscles. The present study shows that AMPK activation directly stimulates myofibrillar protein degradation and expression of atrogin-1/MAFbx and MuRF1 by increasing the level of FOXO transcription factors (FOXO1 and FOXO3a) in C2C12 myotubes. The results also further establish AMPK as a unique energy sensor that not only modulates glucose and fatty acid metabolism but also appears to regulate, in part, the catabolic functions of myofibrillar protein degradation.

A limitation of this study is that findings were obtained only from cultured cell lines and did not directly provide information on conditions in the skeletal muscle and *in vivo*. Further investigation with administration of AICAR *in vivo* will be necessary. In conclusion, this study shows that AICAR stimulates myofibrillar protein degradation and up-regulates the expression of atrogin-1/MAFbx and MuRF1 at the transcriptional level by increasing FOXO transcription factors in C2C12 myotubes, possibly through activation of AMPK.

Acknowledgments

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