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AMP-Activated Protein Kinase Enhances the Expression of Muscle-Specific Ubiquitin Ligases Despite Its Activation of IGF-1/Akt Signaling in C2C12 Myotubes

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ABSTRACT

Two muscle-specific ubiquitin ligases (UL), muscle atrophy F box (MAFbx) and muscle RING finger 1 (MuRF1), are crucial for myofibrillar protein breakdown. The insulin like growth factor-1 (IGF-1) pathway inhibits muscle UL expression through Akt-mediated inhibition of FoxO transcription factors, while AMP-activated protein kinase (AMPK) promotes UL expression. The underlying cellular mechanism, however, remains obscure. In this study, the effect of AMPK and its interaction with IGF-1 on ubiquitin ligases expression was investigated. C2C12 myotubes were treated with 0, 0.1, 0.3, and 1.0 mM 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) in the presence or absence of 50 ng/ml IGF-1. IGF-1 activated Akt, which enhanced phosphorlytion of FoxO3a at Thr 318/321 and reduced the expression of UL. Intriguingly, though activation of AMPK by 0.3 and 1.0 mM AICAR synergized IGF-1-induced Akt activation, the expression of UL was not attenuated, but strengthened by AMPK activation. AICAR treatment decreased FoxO3a phosphorylation at 318/321 in the cytoplasm and induced FoxO3 nuclear relocation. mTOR inhibition increased basal MAFbx expression and reversed the inhibitory effect of IGF-1 on UL expression. In conclusion, our data show that AMPK activation by AICAR stimulates UL expression despite the activation of Akt signaling, which may be due to the possible antagonistic effect of FoxO phosphorylation by AMPK on phosphorylation by Akt. In addition, AMPK inhibition of mTOR may provide an additional explanation for the enhancement of UL expression by AMPK. J. Cell. Biochem. 108: 458–468, 2009.

KEY WORDS: AMP-ACTIVATED PROTEIN KINASE; UBIQUITIN LIGASE; C2C12; FOXO; INSULIN-LIKE GROWTH FACTOR-1; PROTEIN DEGRADATION; SKELETAL MUSCLE

Maintenance of skeletal muscle (SM) depends upon the dynamic balance of anabolic and catabolic reactions which determine the level of muscle proteins [McKinnell and Rudnicki, 2004]. Several key molecular mediators of hypertrophy and atrophy have been identified [Stitt et al., 2004]. Insulin and insulin-like growth factor 1 (IGF-1) stimulate SM growth mainly mediated by the phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt) signaling pathway [Alessi et al., 1996; Latres et al., 2005; Engelman et al., 2006]. IGFs, major downstream targets of growth hormone (GH), are essential for the regulation of growth and body size both prenatally and postnatally [Oldham and Hafen, 2003]. When released, IGF-1 binds to the IGF receptor and activates Akt, which integrates anabolic and catabolic responses by altering the phosphorylation of its numerous substrates, such as phosphorylation of mammalian target of rapamycin (mTOR) and glycogen synthase kinase-3 (GSK-3) to increase protein synthesis [Glass, 2003].

Muscle atrophy is induced by a variety of conditions including cancer, cachexia, sepsis, and starvation [Mitch and Goldberg, 1996]. SM atrophy is not simply the reverse of SM hypertrophy. Atrophy is associated with an increased rate of protein breakdown which is correlated with the activation of cellular proteases, most notably the ATP-dependent ubiquitin proteasome system (UPS) [Jagoe and Goldberg, 2001; Stitt et al., 2004]. The UPS involves two successive steps, namely polyubiquitination and proteasome-mediated degradation. Protein ubiquitination is regulated by multiple enzymes, including the ubiquitin activating enzyme E1, the ubiquitin-conjugating enzyme E2, and E3 ubiquitin ligases [Wray et al., 2003]. E3 plays a major role in the selection of proteins for conjugation and degradation because the specific binding of protein substrate to E3 happens prior to the reaction with ubiquitin [Hershko and Ciechanover, 1998]. Accumulating evidences show that two muscle specific E3 ubiquitin ligases, muscle atrophy F box (MAFbx, also

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known as Atrogin-1) and muscle RING finger 1 (MuRF1), play a critical role in mediating the loss of muscle proteins [Glass, 2003; Lecker et al., 2006; Krawiec et al., 2007]. Their expression increases dramatically in catabolic states, such as muscle immobilization, denervation, and fasting [Bodine et al., 2001; Stitt et al., 2004].

Forkhead box-containing protein, O-subfamily (FoxO) transcription factors (FoxO1, FoxO3a, FoxO4, and FoxO6 in mammals) are active players in longevity and tumor suppression by up-regulating target genes involved in stress resistance, metabolism, cell cycle arrest and apoptosis in response to a variety of environmental stimuli [Calnan and Brunet, 2008]. FoxOs induce the E3 ubiquitin ligase expression and cause SM atrophy [Sandri et al., 2004]. Activation of IGF-1/PI3K/Akt pathway results in the blockade of FoxOs [Brunet et al., 1999]. In myotubes, Akt phosphorylates FoxOs which excludes FoxOs from nuclei, preventing their functions as transcription factors to induce UL expression [Latres et al., 2005].

AMP-activated protein kinase (AMPK) is a serine–threonine heterotrimeric kinase which acts as a sensor of cellular energy status. AMPK is switched on by an increase in the AMP/ATP ratio, which leads to the phosphorylation of AMPK at Thr 172 by AMPK kinases [Woods et al., 2005; Hardie, 2008]. Hormones and other extracellular signals can modulate the AMPK system independent of cellular energy level [Towler and Hardie, 2007]. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) as a AMP mimic, activates AMPK directly without altering cellular concentrations of ATP and AMP [Sakoda et al., 2002]. Pharmacological activation of AMPK results in inhibition of protein synthesis through its suppression of mTOR signaling and translational elongation [Chan and Dyck, 2005; Reiter et al., 2005; Du et al., 2007], but its role in myofibrillar protein degradation has only been studied recently. AMPK activation after exposure to its agonists AICAR or metformin enhanced the mRNA expression of MAFbx/Atrogin1 and MuRF1 both in C2C12 myotubes and in murine skeletal muscle [Krawiec et al., 2007]. In another study also using C2C12 myotubes, activation of AMPK by AICAR increased ULs thereby enhancing proteolysis, which was associated with increase of FoxO1 and FoxO3 mRNA and protein levels [Nakashima and Yakabe, 2007]. However, other studies showed that AMPK activation by AICAR in C2C12 myotubes decreased FoxO mRNA dose-dependently [Nystrom and Lang, 2008]. In H4-hepatoma cell lines, AMPK activation leads to an almost complete disappearance of FoxO1 proteins [Barthel et al., 2002]. These contradictory observations indicate that the exact cellular mechanisms by which AMPK promotes UL are not understood. Since IGF/Akt pathway and AMPK have different roles in regulating muscle protein degradation, the objective of the present study was to examine the effect of AMPK and its interaction with IGF-1 on the muscle specific UL expression and the associated signaling pathways in C2C12 myotubes.

MATERIALS AND METHODS

CHEMICALS AND ANTIBODIES

Chemicals for cell culture were purchased from Sigma–Aldrich (St. Louis, MO). Insulin like growth factor-1 (IGF-1) was purchased from BD Biosciences (San Jose, CA). 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was purchased from Toronto Research Chemicals (Ontario, Canada). Rapamycin and Compound C were purchased from EMD Chemicals Inc. (Gibbstown, NJ). Antibodies against AMPK α subunit, phospho-AMPKα at Thr 172, ACC, phospho-ACC at Ser 79, Akt, phospho-Akt at Ser 473, GSK-3β, phospho-GSK-3β at Ser 9, mTOR, phospho-mTOR at Ser 2448, phospho-4E-binding protein (4E-BP1) at Thr 37/46, FoxO1, phospho-FoxO1 at Thr 24, FoxO3a, phospho-FoxO3a at Ser 318/321, histone H3, and horseradish peroxidase linked secondary antibody were purchased from Cell Signaling (Danvers, MA). Antibodies against FoxO3a at Ser 413 and Ser 588 were kind gifts from Dr. Anne Brunet (Stanford University). Antibodies against MAFbx and MuRF1 were purchased from Santa Cruz Biotechnology (Delaware, CA). Anti-β-actin antibody was obtained from Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA 52242).

C2C12 CELL CULTURE

C2C12 myoblasts were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. At confluence, myoblasts were induced to differentiation in 2% horse serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin for 6 days. Differentiation medium was replaced every 48 h. Then, C2C12 myotubes in 6-well plates were incubated with AICAR (0, 0.1, 0.3, and 1.0 mM) with or without 50 ng/ml IGF-1 or 10 μg/ml Compound C for 4 and 24 h in DMEM. Also, myotubes were treated with 50 nM okadaic acid or 100 nM rapamycin with or without 50 ng/ml IGF-1 for 4 and 24 h in DMEM. Cells were then collected for analyses.

WESTERN BLOT ANALYSIS

C2C12 myotubes were washed with PBS and lysed in a buffer containing 50 mM HEPES (pH 7.4), 2% SDS, 1% NP-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na_3VO_4, and 100 mM NaF. Soluble proteins were recovered after a 10-min centrifugation (10,000*g*), and their concentrations were determined according to the Bradford method (Bio-Rad Laboratories, Hercules, CA) [Zhu et al., 2006]. For the subcellular fractionation, nuclear and cytoplasmic extracts from C2C12 myotubes were prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) and followed the instruction by the manufacturer. Proteins in cell lysates were separated by SDS–PAGE and transferred to a nitrocellulose membrane. Subsequently, the membranes were treated with blocking buffer (5% nonfat dry milk in TBS/T buffer containing 150 mM NaCl, 10 mM Tris pH8.0 and 0.1% Tween 20) for 1 h. The blocked membranes were probed with primary antibodies and further incubated with a secondary antibody conjugated with horseradish peroxidase. Membranes were visualized using Enhanced Chemiluminescence (ECL) Western blotting reagents (Amersham Bioscience, Piscataway, NJ) and exposure to film (MR, Kodak, Rochester, NY). Density of bands was quantified by using Imager Scanner II and ImageQuant TL software (Amersham Bioscience). Band density was normalized according to the β-actin

content except nuclear extract where histone H3 was used for normalization.

IMMUNOPRECIPITATION

After washing with cold PBS, C2C12 myotubes were lysed with ice-cold lysis buffer containing 20 mM Tris (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1 mM PMSF, 2.5 mM sodium pyrophosphate, 1 μg/ml leupeptin, 1 mM Na_3VO_4 and 100 mM NaF. Myotubes were then collected and microcentrifuged for 10 min at 14,000*g* at 4°C, and 200 μl supernatant was pre-cleared with 20 μl Protein A Sepharose bead slurry (50%) (Rockland Inc., Gilbertsville, PA) and incubated at 4°C for 1 h. The supernatant (150 μl) was mixed with anti-foxo3a antibody (1:50 dilution) and incubated with rocking overnight at 4°C. Protein A Sepharose bead slurry (50%, 20 μl) was added and continued to incubate with rocking for 2 h at 4°C. Immunoprecipitated samples were collected and washed with 100 μl lysis buffer three times; then it was re-suspended with 20 μl SDS sample buffer containing 4% β-mercaptoethanol. After heating to 95°C for 5 min, the samples were analyzed by SDS–PAGE and Western blotting using antibodies against phospho-FoxO3a at Ser 413 and Ser 588.

PHASE-CONTRAST MICROSCOPY

C2C12 Cells were seeded on coverslips, cultured and treated as described above. Myotube morphology was photographed under a phase contrast microscope (Nikon mirophot, Nikon Inc., Melville, NY) at 40× magnification after 24 h treatment.

REVERSE TRANSCRIPTION AND REAL-TIME QUANTITATIVE PCR

Total RNA was isolated from C2C12 myotubes using TRI reagent (Sigma, St. Louis, MO) which was reverse transcribed to synthesize first-strand cDNA using a kit (Bio-Rad Laboratories). The cDNAs were stored at −80°C for subsequent analysis. Real-time (RT)-PCR was performed using an iQ5 RT-PCR detection system (Bio-Rad Laboratories) using SYBR Green RT-PCR kit from Bio-Rad Laboratories and the following primers: mouse atrogin-1/MAFbx, forward, 5′-GCAGAGAGTCGGCAAGTC-3′, and reverse, 5′-CAGGTCGGTGATCGTGAG-3′; mouse MuRF-1, forward, 5′-CAACCTGTGCCGC-AAGTG-3′, and reverse, 5′-CAACCTCGTGCC-TACAAGATG-3′; and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward, 5′-ACCCCCAATGTATCCGTTGT-3′, and reverse, 5′-TACTCCTTGGAGGCCATGTA-3′ [Wang et al., 2006]. PCR was performed in duplicate using the following cycle parameters: 94°C for 2 min and 40 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 10 min. Melting point dissociation curves and agarose gel electrophoresis were performed to confirm that only a single product of the expected size was amplified. Results were expressed using the comparative cycle threshold (Ct) method. The ΔCt values were calculated as following: Ct of the detecting gene − Ct of the reporter gene GAPDH.

STATISTICAL ANALYSIS

For cell culture studies, at least three independent experiments were conducted. All data were expressed as mean ± standard errors (SE). Data from each time point (4 or 24 h) were analyzed independently using the GLM procedure of SAS (SAS Institute, Inc., Cary, NC). Data were analyzed by ANOVA (SAS Institute, Inc.), and Tukey's

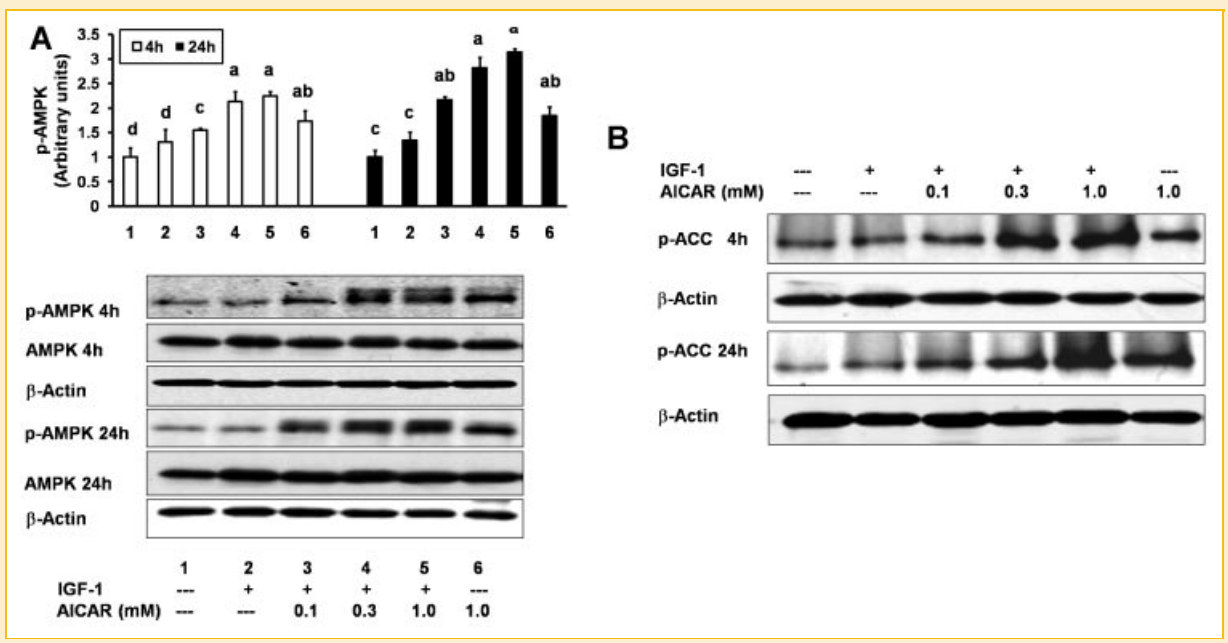

Fig. 1. AICAR induced phosphorylation of AMPK and ACC in C2C12 Myotubes. C2C12 myotubes were incubated with various concentrations of AICAR (0.1, 0.3, and 1.0 mM) in the presence and absence of 50 ng/ml IGF-1 for indicated durations. A: Total and phosphorylated AMPK at Thr 172; (B) phosphorylated ACC at Ser 79. Bars depict means ± SE of three independent experiments. Data from each time point (4 or 24 h) were analyzed independently. Different letters indicate significant differences between treatments at $P < 0.05$.

Studentized Range test was used for multi-comparison to determine significant difference among means ($P < 0.05$).

RESULTS

AICAR STIMULATED AMPK ACTIVITY IN C2C12 MYOTUBES

AICAR is an adenosine analog taken up by cells and phosphorylated to form 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl-5monophosphate (ZMP), which stimulates AMPK activity [Sakoda et al., 2002]. As shown in Figure 1A, AICAR treatment increased the phosphorylation of AMPK at Thr 172, which is required for AMPK activation [Stein et al., 2000], while IGF-1 treatment alone had no effect on AMPK activity. However, when combining IGF-1 with AICAR, the AMPK phosphorylation was enhanced. No difference in the expression of AMPKα subunit was observed. Since AICAR is a specific activator of AMPK [Lopez et al., 2003], to confirm the activation AMPK by AICAR, we further analyzed its direct substrate Acetyl-CoA Carboxylase (ACC) phosphorylation at Ser 79. As illustrated in Figure 1B, ACC phosphorylation was correlated with AMPK activation. The trends of AMPK activation by AICAR were similar for 4 and 24 h time points.

AMPK AND IGF-1 ACTIVATED AKT SYNERGISTICALLY

As shown in Figure 2A, Akt phosphorylation at Ser 473 was increased in response to IGF-1 treatment. Strikingly, IGF-1 in combination with 0.3 or 1.0 mM AICAR treatment increased the phosphorylation level of Akt compared to IGF-1 treatment only. While 1.0 mM AICAR treatment alone did not induce Akt phosphorylation compared with control, showing that AMPK only enhances Akt activity when exogenous IGF-1 is available. No difference in the expression of total Akt was observed. Since GSK-3β is a direct substrate of Akt, we further examined GSK-3β phosphorylation at Ser 9. As shown in Figure 2B, the increase of GSK-3β phosphorylation level was in parallel with the phosphorylation of Akt, consistent with the potentiation of Akt by AMPK. We further used Compound C, a specific inhibitor of AMPK activity, to verify the effect of AMPK on Akt phosphorylation. Inhibition of AMPK activity with Compound C reduced Akt phosphorylation in

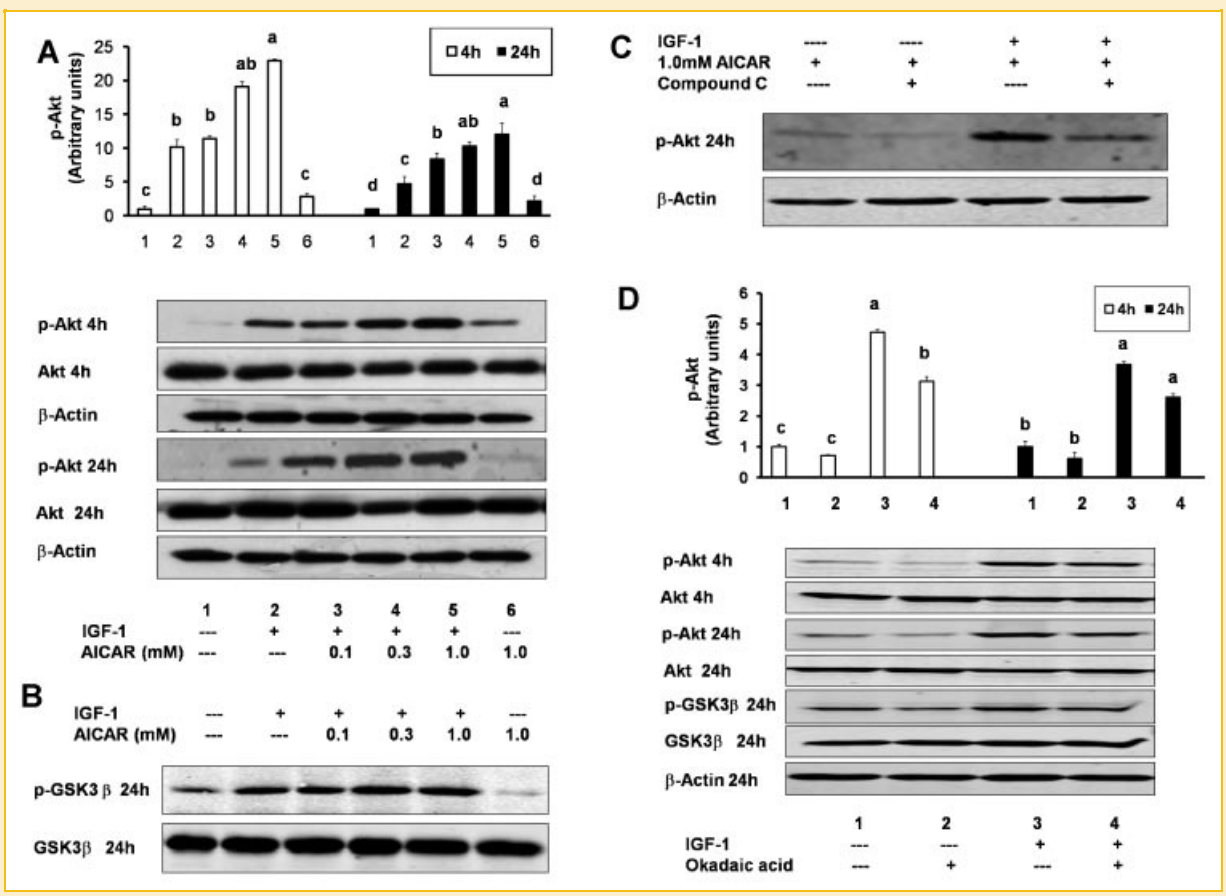

Fig. 2. AICAR synergized Akt activity induced by IGF-1 in C2C12 Myotubes. C2C12 myotubes were incubated with various concentrations of AICAR (0.1, 0.3, and 1.0 mM) in the presence and absence of 50 ng/ml IGF-1 for indicated durations. A: Total and phosphorylated Akt at Ser 473; (B) total and phosphorylated SK-3β at Ser 9; (C) Compound C, an inhibitor of AMPK, and Akt phosphorylation. C2C12 myotubes were incubated with 10 μg/ml Compound C for 24 h; (D) Akt phosphorylation at Ser 473. C2C12 myotubes were incubated with 50 ng/ml IGF-1 and 50 nM okadaic acid. Bars depict means ± SE of three independent experiments. Data from each time point (4 or 24 h) were analyzed independently. Different letters indicate significant differences between treatments at $P < 0.05$.

the presence of IGF-1 (Fig. 2C), clearly supporting the notion that AMPK activates Akt. Taken together, these findings demonstrated that AMPK has a synergistic effect with IGF-1 on the activation of Akt.

INHIBITION OF PROTEIN PHOSPHATASE 2A BY OKADAIC ACID DID NOT INCREASE PHOSPHORYLATION OF AKT

We observed that IGF-1 and AICAR had synergistic effects on Akt and GSK-3β phosphorylation. The reason may be due to enhanced phosphorylation by upstream protein kinases or an inhibition of protein phosphatases that target Akt and GSK-3β. It has been demonstrated that protein phosphatase 2A (PP2A) dephosphorylates Akt [Andjelkovic et al., 1996] and GSK-3β [Lin et al., 2007]. Therefore, we treated myotubes with PP2A inhibitor okadaic acid (50 nM) in the presence or absence of IGF-1. As shown in Figure 2D, blocking PP2A neither increased basal phosphorylation, nor IGF-1 stimulated phosphorylation of Akt (Fig. 2D), suggesting that inhibition of dephosphorylation by PP2A is not a major mechanism enhancing Akt phosphorylation.

AMPK SUPPRESSED MTOR PHOSPHORYLATION DESPITE AKT ACTIVATION

mTOR is a critical downstream effector of PI3K/Akt pathway. Akt activates mTOR through direct inhibition of tuberous sclerosis 2 (TSC2), which is the upstream negative regulator of mTOR [Wan et al., 2007], while AMPK inhibits mTOR signaling through the phosphorylation of TSC2 at different sites [Inoki et al., 2003]. As shown in Figure 3A, mTOR phosphorylation at Ser 2448 was increased in response to IGF-1 treatment. Intriguingly, in spite of enhanced Akt phosphorylation, AMPK activation by higher doses (1.0 mM at 4 h and 0.3 and 1.0 mM at 24 h) of AICAR treatment reduced the phosphorylation of mTOR. To confirm changes in mTOR activity, we examined the phosphorylation of eukaryotic initiation factor 4E binding protein1 (4E-BP1), because 4E-BP1 is recognized as the surrogate for mTOR activity [Hay and Sonenberg, 2004]. The phosphorylation of 4E-BP1 was also decreased by higher doses of AICAR (Fig. 3B). These data showed that AMPK activation inhibited mTOR activity regardless of Akt phosphorylation.

EFFECT OF MTOR INHIBITION ON THE EXPRESSION OF MAFBX AND MURF1

In order to examine the potential role of mTOR in the expression of ULs in C2C12 myotubes, we treated myotubes with 100 nM rapamycin in the presence and absence of 50 ng/ml IGF-1 for 24 h. As shown in Figure 4, inhibition of mTOR activity by rapamycin resulted in the increase of basal expression of MAFbx and MuRF1 proteins. Rapamycin suppressed the IGF-1-induced inhibition of ULs. Our observation suggests that mTOR activity is negatively correlated to UL expression in C2C12 myotubes.

EFFECT OF AMPK AND IGF-1 ON THE EXPRESSION OF MAFBX AND MURF1

Both MAFbx and MuRF1 protein contents decreased after 4 h exposure to 50 ng/ml IGF-1 compared to those of control samples.

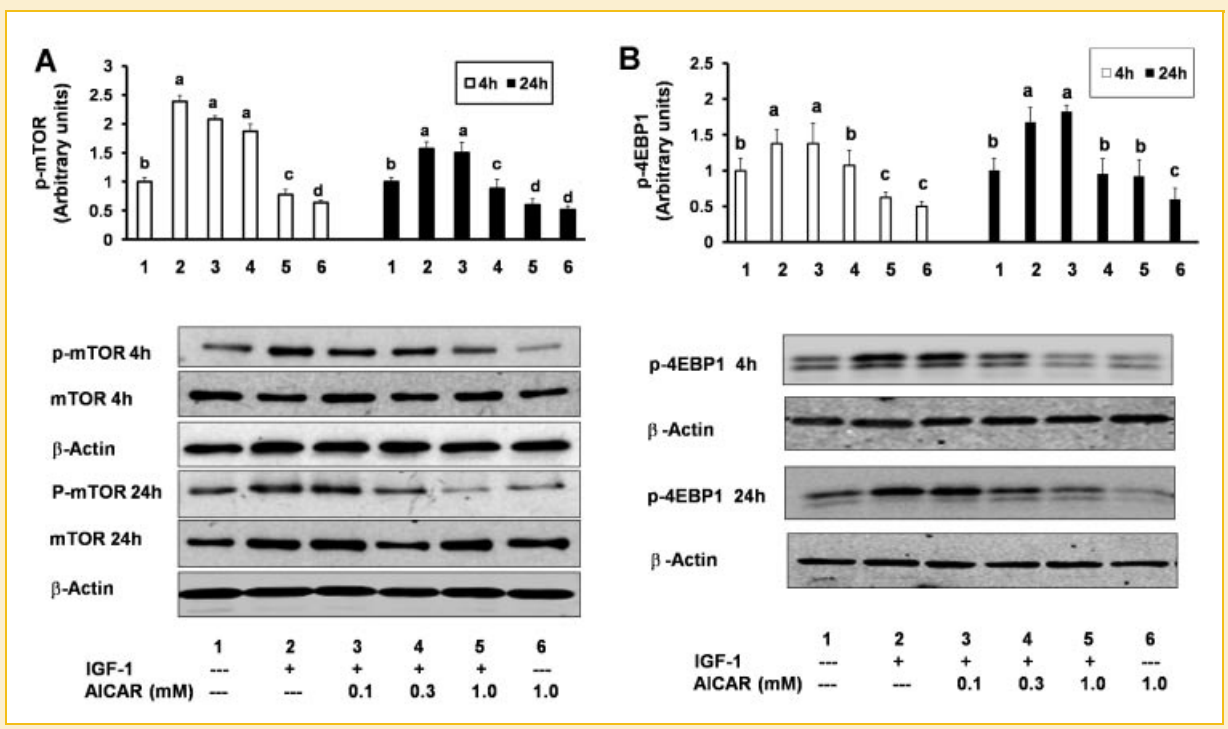

Fig. 3. Effect of AICAR and IGF-1 treatments on the phosphorylation of mTOR at Ser 2448 and 4E-BP1 at Thr 36/47 in C2C12 Myotubes. C2C12 myotubes were incubated with various concentrations of AICAR (0.1, 0.3, and 1.0 mM) in the presence and absence of 50 ng/ml IGF-1 for indicated durations. A: Total and phosphorylated mTOR at Ser 2448; (B) phosphorylated 4E-BP1 at Thr 36/47. Bars depict means ± SE of three independent experiments. Data from each time point (4 or 24 h) were analyzed independently. Different letters indicate significant differences between treatments at $P < 0.05$.

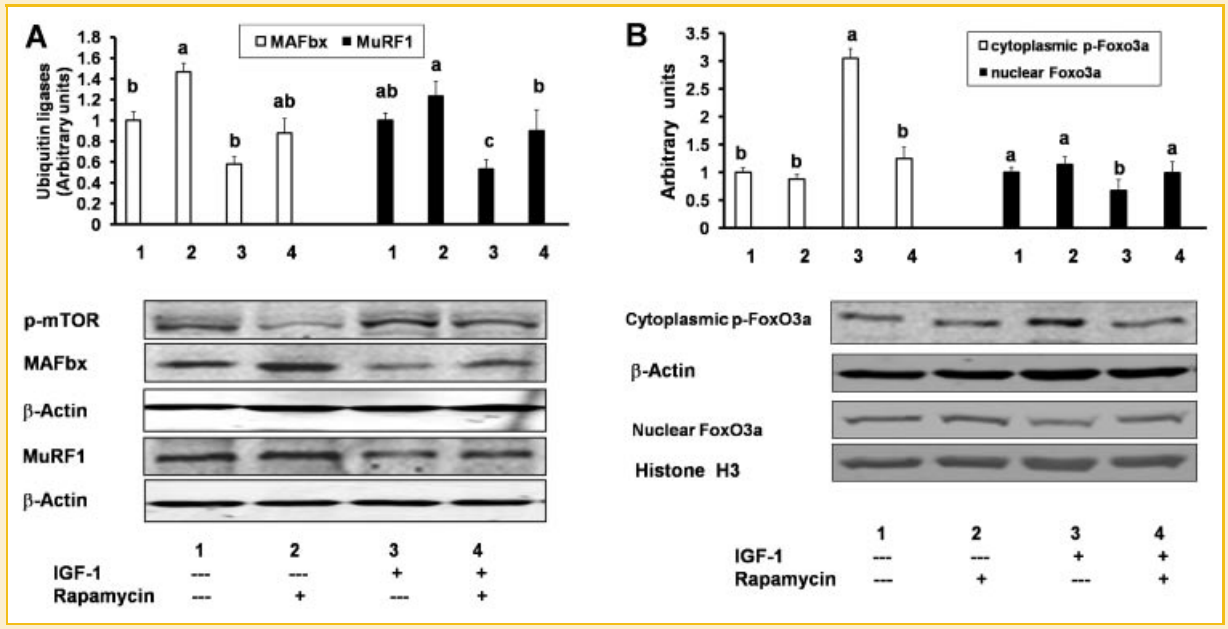

Fig. 4. Effects of IGF-1 and rapamycin treatments on expressions of MAFbx and MuRF1 and subcellular location of FoxO3a in C2C12 myotubes. C2C12 myotubes were incubated with 50 ng/ml IGF-1 and 100 nM rapamycin for 24 h. A: MAFbx and MuRF1 protein contents in the whole cell lysate; (B) cytoplasmic phosphorylation level of FoxO3a at Thr 318/321, and nuclear FoxO3a content. Bars depict means ± SE of three independent experiments. Data of open bars and filled bars were analyzed independently. Different letters indicate significant differences between treatments at $P < 0.05$.

AICAR only treatment significantly increased UL expression (Fig. 5A,B) which is consistent with previous observations [Krawiec et al., 2007; Nakashima and Yakabe, 2007]. Combining IGF-1 with 0.3 and 1.0 mM AICAR treatments increased UL levels despite enhanced Akt phosphorylation. These data showed that AMPK activation stimulated UL protein expression in C2C12 myotubes and 50 ng/ml IGF-1 failed to fully blunt its effect. When myotubes were treated with 1.0 mM AICAR for 24 h, an obvious morphological atrophy was observed as compared to control and IGF-1 treated conditions (Fig. 5E). Compound C treatment attenuated AMPK induced UL up-regulation, further demonstrating that AMPK stimulates UL expression in C2C12 myotubes (Fig. 5F).

To further verify the effects of AMPK and IGF-1 on UL expression, we analyzed MAFbx and MuRF1 mRNA levels after 4 h treatment by Real-time PCR. As shown in Figure 5C,D, C2C12 myotubes incubated with IGF-1 exhibited a decrease for both MAFbx and MuRF1 mRNA levels. But UL mRNA level was enhanced with AICAR supplement. The UL mRNA contents were highest in the samples with 1.0 mM AICAR treatment only (Fig. 5C,D). These observations were consistent with their protein contents.

AICAR REDUCED CYTOPLASMIC FOXO3A PHOSPHORYLATION AND CAUSED ITS NUCLEAR RELOCATION

It has been well demonstrated that MAFbx and MuRF1 expressions are induced by FoxO transcription factors [Sandri et al., 2004]. In order to induce MAFbx and MuRF1 expression, FoxOs need to be translocated to nuclei [Skurk et al., 2005]. Therefore, we further analyzed FoxO contents and their phosphorylation at subcellular levels following IGF-1 and AICAR treatments. At both time points, IGF-1 treatment enhanced cytoplasmic FoxO3a phosphorylaiton at Thr 318/321 and reduced nuclear FoxO3a contents (Fig. 6A–C), suggesting that more FoxO3a was sequestered in the cytoplasm compared to control, consistent with reduced UL expression observed in this study. Increasing AICAR dose reduced IGF-1-induced phosphorylation of cytoplasmic FoxO3a and consequently caused its nuclear relocation (Fig. 6A–C). In addition, we observed that 24 h of AICAR exposure caused a decrease of FoxO3a content in the cytoplasm compared with IGF-1 only treatment (Fig. 6A). The subcellular expression of FoxO1 followed a similar pattern with FoxO3a following IGF-1 and AICAR treatments (data not shown).

When expressed into ratio of phosphorylated at Thr 318/321 to total Foxo3a, AMPK activation reduced this ratio (Fig. 7A), showing that AMPK activation decreased Foxo3a phosphorylation by Akt. At the same time, Foxo3a phosphorylation at Ser 413/588, two sites phosphorylated by AMPK, was increased (Fig. 7B).

DISCUSSION

In the present study, we examined the effects of AMPK and IGF-1 on UL expression in C2C12 myotubes and the potential underlying mechanisms. Akt plays a critical role in mediating various effects of insulin/IGF-1 [Kido et al., 2001; Matsuzaki et al., 2003]. Activation of Akt by IGF-1/PI3K pathway promotes SM hypertrophy through enhancing mTOR signaling [Lai et al., 2004; Wullschleger et al., 2006] and inhibition of GSK-3β activity [Cross et al., 1995]. In addition to enhance protein synthesis, activation of Akt antagonizes protein degradation by regulating its downstream target FoxO transcription factors [Stitt et al., 2004]. Akt phosphorylates FoxO at

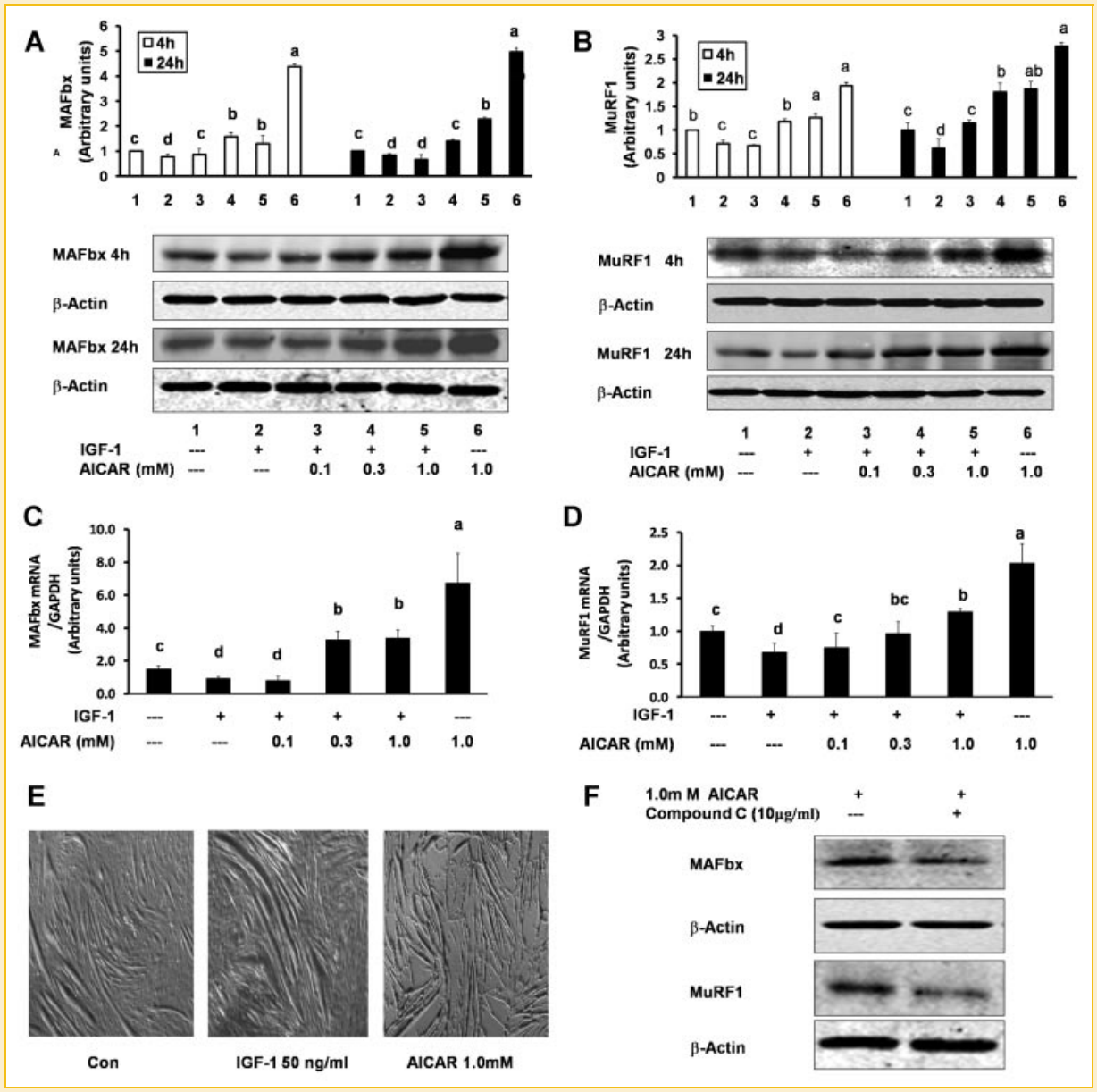

Fig. 5. Effect of AICAR and IGF-1 treatments on MAFbx and MuRF1 expression in C2C12 myotubes. C2C12 myotubes were incubated with various concentrations of AICAR (0.1, 0.3, and 1.0 mM) in the presence and absence of 50 ng/ml IGF-1 for indicated durations. A,B: MAFbx and MuRF1 protein contents; (C,D) MAFbx and MuRF1 mRNA levels; (E) the morphology of C2C12 myotubes after 24 h treatments, 40× magnification; (F) MAFbx and MuRF1 protein contents following 10 μg/ml of Compound C treatment. Bars depict means ± SE of three independent experiments. Data from each time point (4 or 24 h) were analyzed independently. Different letters indicate significant differences between treatments at $P < 0.05$.

multiple sites, which leads to its nuclear exclusion and blocks its transcriptional function [Skurk et al., 2005].

In this study, we observed that IGF-1 in combination with AICAR caused a further marked increase of Akt phosphorylation at Ser 473 compared to IGF-1 treatment only, suggesting that AMPK synergized Akt activity induced by IGF-1 in C2C12 myotubes. The reason may be through sensitization of insulin/IGF-1 signaling by AMPK [Jakobsen et al., 2001; Kim et al., 2004; Aguilar et al., 2007]. mTOR feedback inhibits insulin/IGF-1 signaling [O'Reilly et al., 2006; Tesseraud et al., 2007; Wan et al., 2007]. AMPK inhibits mTOR, sensitizing insulin/IGF-1 signaling. Another possibility is due to the inhibition of dephosphorylation of Akt and GSK-3β. Protein phosphotase 2A (PP2A) dephosphorylates and inhibits Akt [Andjelkovic et al., 1996] and GSK-3β [Lin et al., 2007]. To test this notion, we used okadaic acid to inhibit PP2A activity. Data showed that IGF-1 in combination with okadaic acid did not further increase Akt phosphorylation at Ser 473, suggesting that PP2A might not be involved.

Since AMPK synergizes IGF-1 induced Akt phosphorylation, we would expect AMPK activation to reduce UL expression in C2C12 myotubes compared to the samples with IGF-1 treatment only. However, our data revealed a more complicated picture. At 0.1 mM,

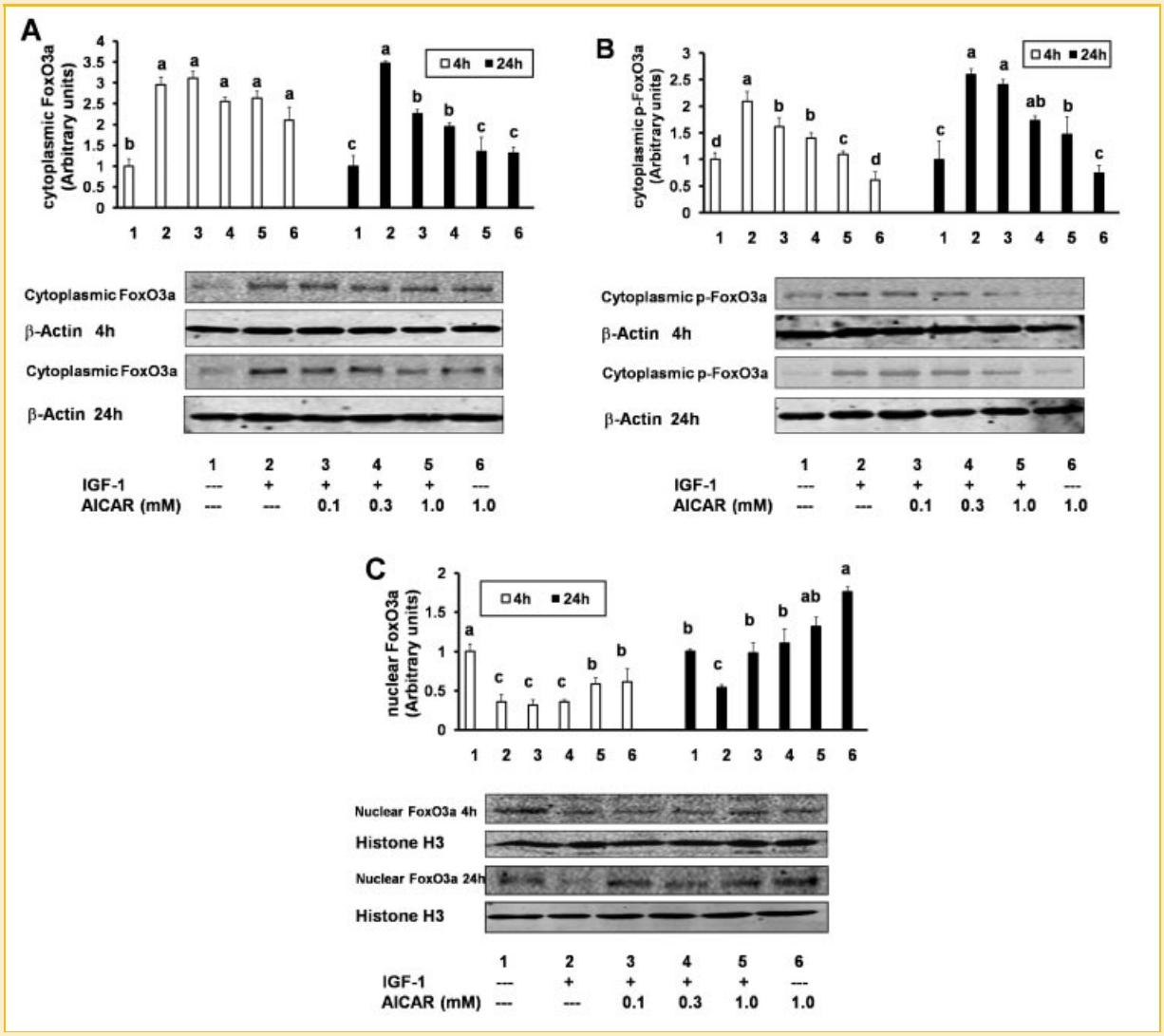

Fig. 6. Effect of AICAR and IGF-1 treatments on total FoxO3a, phospho-FoxO3a contents in the cytoplasm and nucleus of C2C12 myotubes. C2C12 myotubes were incubated with various concentrations of AICAR (0.1, 0.3, and 1.0 mM) in the presence and absence of 50 ng/ml IGF-1 for indicated durations. A: Total FoxO3a content in the cytoplasm; (B) phosphorylation levels of FoxO3a at Thr 318/321; (C) total FoxO3a content in the nucleus. Bars depict means ± SE of three independent experiments. Data from each time point (4 or 24 h) were analyzed independently. Different letters indicate significant differences between treatments at $P < 0.05$.

AICAR slightly stimulated AMPK activity, and UL expression was not increased. But higher AICAR doses (0.3 and 1.0 mM) led to enhanced UL expression, surpassing the inhibitory effect induced by IGF-1. 1.0 mM AICAR treatment increased UL expression both at mRNA and protein levels, and a myotube atrophy was observed morphologically. Such activation of UL expression by AMPK seems independent on Akt.

Akt was traditionally shown to inhibit UL expression [Stitt et al., 2004; Glass, 2005], but there are several contradictory observations. In QT6 fibroblasts, increase of Akt phosphorylation was concomitant with increase of MAFbx/Atrogin-1 gene expression [Tesseraud et al., 2007]. Mechanical ventilation (MV) results in a rapid onset of diaphragmatic fiber atrophy. But the antioxidant attenuates MV induced atrophy, which is independent of Akt's effect on FoxO transcription of MAFbx and MuRF-1 ubiquitin ligases [McClung et al., 2007]. In patients with chronic obstructive pulmonary disease, muscle atrophy was induced through up-regulation of MAFbx/Atrogin-1 and MuRF1 which might occur independent of Akt [Doucet et al., 2007]. In hepatocytes, kinases distinct from Akt phosphorylate FoxO1 at Thr 24 [Nakae et al., 2001]. These findings and our data in C2C12 myotubes suggest that a mechanism independent of Akt may be responsible for the up-regulation of UL expression by AMPK.

Numerous reports show UL expression is mediated by FoxO, and Akt phosphorylation of FoxO leads to the exclusion of phosphorylated FoxO proteins from the nuclei and inhibition of their transcriptional functions [Brunet et al., 1999]. We further analyzed the subcellular contents of total and phosphorylated FoxO in C2C12 myotubes. As expected, following the treatment with IGF-1, the phosphorylation of FoxO3 at Thr 318/321 in the cytoplasm was enhanced and FoxO3a was excluded from nuclei, which is consistent with Akt activation by IGF-1. Conversely, treatment with 0.3 and

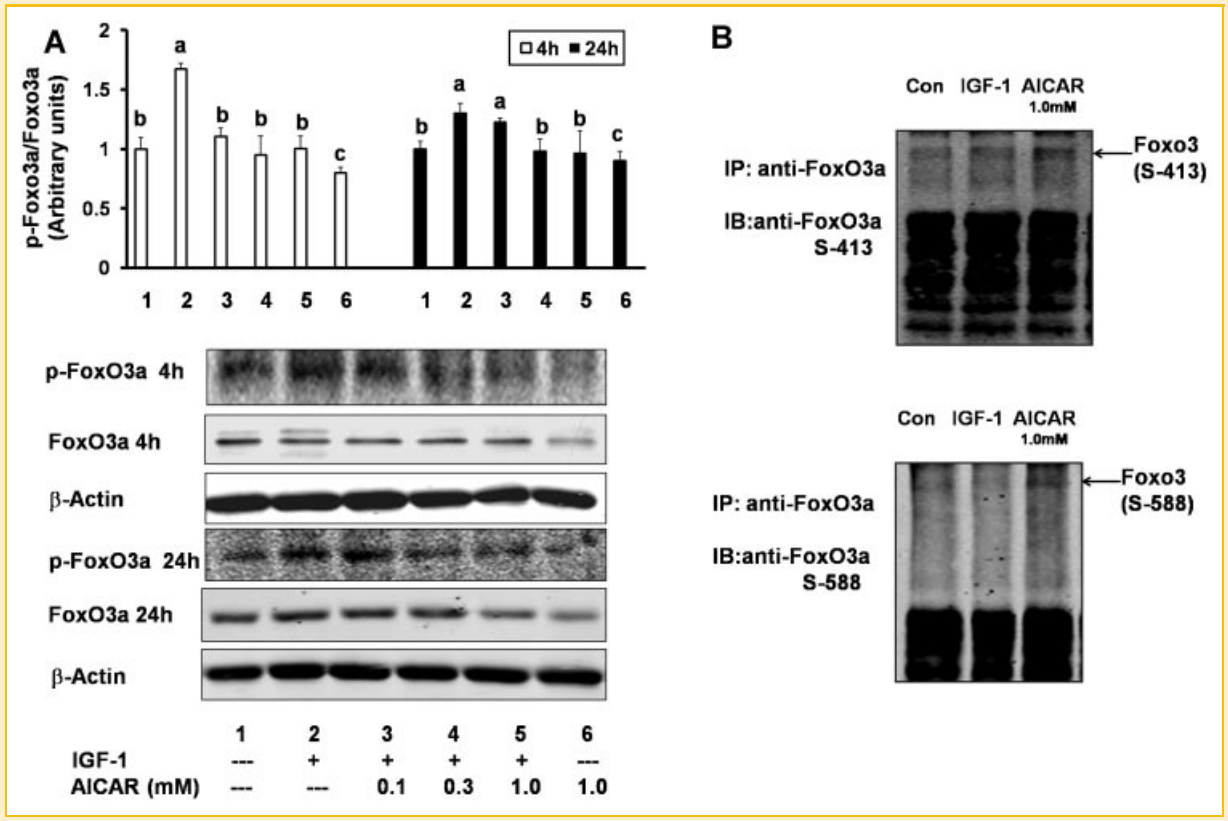

Fig. 7. Effect of AICAR and IGF-1 treatments on the ratio of phospho-FoxO3a to FoxO3a contents and AMPK-mediated phosphorylation of FoxO3a in C2C12 myotubes. C2C12 myotubes were incubated with various concentrations of AICAR (0.1, 0.3, and 1.0 mM) in the presence and absence of 50 ng/ml IGF-1 for indicated durations. A: Ratio of FoxO3a phosphorylation at Thr 318/321 to total FoxO3a contents. Bars depict means ± SE of three independent experiments; (B) phosphorylation level of FoxO3a at Ser 413 and Ser 588 after immunoprecipitation with anti-FoxO3a antibody. C2C12 myotubes were incubated with 50 ng/ml IGF-1 or 1.0 mM AICAR for 24 h. Different letters indicate significant differences between treatments at $P < 0.05$.

1.0 mM AICAR promoted FoxO3a relocation to nuclei even with the presence of IGF-1 and synergizes of Akt phosphorylation by AMPK; this relocation was associated with the reduction of FoxO3a phosphorylation at Thr 318/321 in the cytoplasm following AICAR treatment. The mechanism leading to the inhibition of FoxO3a phosphorylation at Thr 318/321 by AMPK is unclear. One possibility is that phosphorylation of FoxO3a by AMPK inhibits its phosphorylation by Akt; we observed that FoxO3a phosphorylation at Ser 413 and Ser 588, two sites phosphorylated by AMPK, were increased after AICAR exposure [Greer et al., 2007].

In the current study, we observed that mTOR inhibition increased the basal expression of MAFbx and reversed the inhibitory effect of IGF-1 on UL expression. These results suggest that mTOR may be involved in the regulation of E3 ligases expression in C2C12 myotubes. It has been demonstrated that blockade of mTOR is sufficient to inhibit perturbation of most of the genes regulated by IGF-1 in C2C12 myobubes [Latres et al., 2005]. We detected that mTOR phosphorylation decreased substantially by AMPK, which may contribute to the up-regulation of UL by AMPK, since inhibition of mTOR increased the expression of UL independent of FoxO [Latres et al., 2005].

In conclusion, our observations in this study include: (1) AMPK synergizes with IGF-1 to activate Akt; (2) a high level of AMPK activation promotes UL expression, which abrogates the inhibitory effect of IGF-1 on UL expression; (3) AMPK activation reduced phosphorylation of FoxO3a at Thr 318/321, and caused FoxO3a nuclear relocation which may partially explain the enhancement of UL expression by AMPK; and (4) AMPK inhibits mTOR which may provide an additional explanation for up-regulation of UL expression by AMPK. Of course, other mechanisms linking AMPK and UL expression may exist. In skeletal muscle, AMPK enhances SIRT1 activity, thereby increasing FoxO1 and FoxO3a transcriptional activity [Canto et al., 2009]. Additional studies will be needed to clarify whether SIRT-mediated deacetylation of FoxO is involved in up-regulation of UL in AICAR treated myotubes.

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