AICAR-induced activation of AMPK negatively regulates myotube hypertrophy through the HSP72-mediated pathway in C2C12 skeletal muscle cells

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SKELETAL MUSCLE HAS A GREATER CAPACITY to adapt to various stimuli. Increased loading, such as resistance training and mechanical stretching, stimulates protein synthesis and reduces protein degradation, thereby inducing muscle hypertrophy (10). On the other hand, decrease of use, such as immobilization, denervation, aging, and/or various pathological conditions, attenuates protein synthesis and increases protein degradation, resulting in atrophy (12, 32). Although the process of skeletal muscle adaptation to hypertrophic and atrophic stimuli has been studied, the molecular mechanism involved in this process is not fully understood yet.

5′-AMP-activated protein kinase (AMPK) is well known as a sensor for cellular energy status and metabolic stress, such as muscle contraction, fasting, hypoxia, ischemia, and/or oxidative and osmotic stresses and as a signaling intermediary that controls the use of glucose and fatty acids in skeletal muscle (8, 14, 15). In addition, several studies in the past decade have suggested that AMPK plays an important role in the regulation of skeletal muscle mass. It has been reported that activation of AMPK inhibits myogenesis (9, 27, 41) and hypertrophy of skeletal muscle cells (22, 28, 30) and rodent skeletal muscle (22, 28). Negative correlation between AMPK activity and the degree of hypertrophy in rat muscle have also been reported (35, 39). These responses appear to be related to the deactivation of the signals in the protein synthesis pathway, mammalian target of rapamycin (mTOR)/p70 S6 kinase (p70S6K) (2, 22, 28), and to the activation of the signals in the protein degradation pathway, Forkhead box O transcription factors (Foxo) (31, 36, 41) and muscle-specific E3 ubiquitin ligases, such as muscle RING-finger 1 (MuRF1) and atragon-1/muscle atrophy F-box (MAFbx) (19, 30). Taken together, AMPK would act as a negative regulator for skeletal muscle mass through downregulation of protein synthesis and upregulation of protein degradation pathways.

Heat shock proteins (HSPs) are stress-induced molecular chaperones that play crucial roles in maintaining correct folding and intracellular transport of proteins and in regulation of cell signaling and that exhibit varieties of cytoprotective functions (20, 24, 34). It has also been suggested that HSPs have roles for controlling skeletal muscle mass. We previously showed that administration of the HSP inducer geranylgeranyllactone upregulated the expression of inducible 70-kDa HSP (HSP70 and so-called HSP72) and increased protein content in skeletal muscle cells (11). In addition, overexpression of HSP72 in skeletal muscle prevented immobilization-induced atrophy in rat (38) and improved the structural and functional recovery from atrophy of mouse muscle (26). It was also demonstrated that transfection of 27-kDa HSP (HSP27 and so-called HSP25) attenuated the disuse atrophy of rat muscle (4). The molecular mechanism, by which HSPs regulate skeletal muscle mass, is associated with inhibition of the Foxo pathway and decrease of MuRF1 and atragon-1 expressions (4, 22).
21, 37, 38). In short, HSPs would act as a positive regulator for skeletal muscle mass through downregulation of protein degradation pathways. Considering that AMPK and HSPs have opposite effects on regulation of muscle mass through similar pathways, it raises the possibility that there might be an interrelationship between AMPK and HSPs in the control of muscle mass. Interestingly, some recent studies revealed that inhibition of AMPK upregulated HSP72 expression in a human hepatoma cell line (40) and that activation of AMPK downregulated HSP72 expression in a human malignant epithelial cell line (16). Therefore, we hypothesized that AMPK negatively controls skeletal muscle mass by modulating HSP expression. In this study, we investigated whether AMPK suppresses hypertrophy through downregulation of HSPs in skeletal muscle cells.

MATERIALS AND METHODS

Cell culture. Cell culture was performed as described previously (11, 33). Mouse myoblast C2C12 cells were cultured on six-well culture plates with type I collagen-coated surface (Biocat, Becton-Dickinson Labware, Franklin Lakes, NJ). Cells were maintained in 2 ml of growth medium consisting of Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum containing high glucose (4.5 g/l glucose, 4.0 mM l-glutamine, without sodium pyruvate) for proliferation under a humidified atmosphere with 95% air and 5% CO2. On reaching confluence, the culture medium was changed to the same amount of differentiation medium consisting of DMEM supplemented with 2% heat-inactivated horse serum containing low glucose (1.0 g/l glucose, 4.0 mM l-glutamine, and 110 mg/l sodium pyruvate) to initiate the differentiation. Medium was exchanged with freshly prepared differentiation medium with 2-day intervals during the 5 days of differentiation.

AICAR treatment. Five days after the initiation of differentiation, myotubes were incubated with AMPK activator 5-aminomidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR; Sigma, St. Louis, MO). To investigate the dose-dependent effects of AICAR on AMPK activation (phosphorylation level of AMPKα Thr172), myotubes were incubated in 2 ml of fresh differentiation medium with or without AICAR (0.1–2 mM) for 24 h. Furthermore, time-dependent effects were also investigated by culturing myotubes in 2 ml of medium with or without 0.5 mM AICAR for 0, 1, 4, 16, or 24 h. The parameters measured were phosphorylation level of acetyl-CoA carboxylase (ACC) Ser216, protein content, myotube diameter, the expression levels of HSP25, 70-kDa heat shock cognate (HSC70), HSP72, and heat shock transcription factor 1 (HSF1) in differentiated C2C12 myotubes. Control samples were incubated without AICAR. Subsequently, cells were harvested for Western blot or real-time RT-PCR analyses.

RNA interference. Three days after the onset of differentiation, RNA oligos were transfected into differentiated myotubes using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, siRNA and Lipofectamine were separately diluted in Opti-MEM (Life Technologies). The diluted Lipofectamine reagent was added to the siRNA mixture and allowed to complex with siRNA for 10 min. Lipid/siRNA complexes were then added to the differentiation medium, and myotubes were incubated for 24 h in the medium containing the transfection mixture. The final concentration of siRNA was set at 10 nM. Transfection medium was changed with freshly prepared differentiation medium following 24 h of the transfection, and myotubes were incubated in differentiation medium for an additional 24 h prior to AICAR treatment (0.5 mM, 24 h). Then, myotubes were harvested for Western blot or real-time RT-PCR analyses. The siRNA oligonucleotides designed against mouse AMPKα1 (targeting sequence 5’-GCAUCAAGCAGUUUGAUU-3’), AMPKα2 (targeting sequence 5’-GAAUGCAUCAACCUUCUG-3’), HSP72 (targeting sequence 5’-CGAUAUCUGCAAGGUUAU-3’), and nontargeting control siRNA (nontarget siRNA) were obtained from Takara Bio (Takara Bio, Otsu, Japan).

Sample preparation. Sample preparation was performed with some modification of the previously reported method (33). Briefly, the cells in each well were rinsed twice with 1 ml of ice-cold phosphate-buffered saline. Then, the cells in each well were scraped off into 0.3 ml of Celllytic TM-M cell lysis reagent (Sigma) with 1% (vol/vol) protease inhibitor cocktail (Sigma) and 1% (vol/vol) phosphatase inhibitor cocktail (Calbiochem, San Diego, CA). The cell lysate was sonicated and centrifuged at 15,000 g at 4°C for 15 min. The supernatant was collected for the determination of protein content. Protein content was determined using the Bradford technique (protein assay kit; Bio-Rad, Hercules, CA) with bovine serum albumin (Sigma) as the standard. Protein contents in the supernatant were expressed as milligrams per milliliter.

Western blot analysis. Western blot analysis was performed as described previously (5, 33). Extracted samples in the cell lysis reagents were solubilized in Laemmli’s sample buffer and boiled for 5 min. The samples (10 μg of protein) were separated by SDS-PAGE using 10% polyacrylamide gel at a constant current of 35 mA/gel for 90 min. Bio-Rad Precision Markers (Bio-Rad Laboratories, Hercules, CA) were applied to both sides of the gel as the internal controls for transfer process or electrophoresis.

After SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes (Hybond-P, GE Healthcare, Buckinghamshire, UK) using trans-blot cell (Bio-Rad) at a constant voltage of 100 V for 1 h at 4°C. After the transfer, the membranes were blocked for 1 h using ECL blocking reagent (RPN418, GE Healthcare). Then, the membranes were incubated overnight at 4°C with primary antibody [AMPKα Thr172 (2531; Cell Signaling Technology, Danvers, MA), AMPK (2532, Cell Signaling Technology), ACC Ser79 (3661, Cell Signaling Technology), ACC (3662, Cell Signaling Technology), HSP72 (AD1-SPA-812; Enzo Life Sciences, Farmingdale, NY), HSP25 (AD1-SPA-801, Enzo Life Sciences), HSC70 (AD1-SPA-816, Enzo Life Sciences), HSF1 (4356, Cell Signaling Technology), p70S6K Thr389 (9234, Cell Signaling Technology), p70S6K (2708, Cell Signaling Technology), Akt Ser473 (9271, Cell Signaling Technology), Akt (9272, Cell Signaling Technology), and β-actin (4967, Cell Signaling Technology)]. The membranes were then washed with Tris-buffered saline with 0.1% Tween 20 (TBS-T, pH 7.5) and reacted with anti-rabbit IgG (Cell Signaling Technology) for 1 h at room temperature. After the final wash with TBS-T, protein bands were visualized using chemiluminescence (GE Healthcare), and signal density was measured using Light-Capture (AE-6971; ATTO, Tokyo, Japan) with ImageJ software (National Institutes of Health, Bethesda, MD).

Real-time RT-PCR analysis. Real-time RT-PCR analysis was performed as described previously (42). Briefly, total RNA was extracted from muscles by using the miRNeasy Mini kit (Qiagen, Hiden, Germany) according to the manufacturer’s protocol. For the detection of MuRF1 and atrogin-1, the RNA was reverse-transcribed to cDNA using PrimeScript RT Master Mix (Takara Bio), and then synthesized cDNA was applied to real-time RT-PCR (Thermal Cycler Dice Real Time System IIMRQ, Takara Bio) with bovine serum albumin (BSA) as the standard. Protein contents in the supernatant were expressed as milligrams per milliliter.
The following primers were used: MuRF1 (Trim63), 5′-AG-GACTCCCTGCAAGTGAACAA-3′ (forward) and 5′-TTCGTC-CAGGATGCCGTA-3′ (reverse); atrin-1 (Pbox32), 5′-TGCTCT- TAGATTACCAGAAGCACCAC-3′ (forward) and 5′-TGTTGCATC-CATTATTTCCAG-3′ (reverse); rpS18, 5′-TTCGCGCAAGCTCTAGAC-3′ (forward) and 5′-CCAGGTCTTGTTGCTGCTGA-3′ (reverse); miR-1, 5′-TGAATTCAGCAAGCAAAC-3′ (forward); miR-133a, 5′-TTGGTCTCCCTCAACCACGCTG-3′ (forward); miR-206, 5′-TGGAATGGAAGTATGTAT-3′ (forward); MuRF1 (Trim63), 5′-CCAGTGGTCTTGGTGTGCTGA-3′ (forward) and 5′-TGTCCTACGGTGCCATCGTGTGCTGA-3′ (forward). The U6 primer and reverse primers for miRNA were provided with the kit.

Measurements of myotube diameter. Images of myotubes were visualized at ×40 magnification using an inverted light microscope and captured with a camera (Olympus, Tokyo, Japan). Myotube diameter was measured by using ImageJ. The average diameter per myotube was calculated as the mean of three short-axis measurements taken along the length of the myotube.

Statistical analysis. All values are expressed as means ± SE. Statistical significance was analyzed by using Dunnett’s multiple comparison test (Figs. 1 and 2) or two-way ANOVA followed by post hoc comparison with Tukey’s test (Figs. 3–8). Differences in the distribution of myotube diameter were analyzed by using χ² test (Figs. 3 and 6). The differences between groups were considered statistically significant at P < 0.05.

RESULTS

AICAR-induced AMPK activation inhibited myotube hypertrophy and HSP72 expression. The rate of phosphorylation of AMPKα Thr172, an indicator of AMPK activation, was greater in myotubes incubated in 0.5 and 2 mM AICAR for 24 h than those of myotubes incubated with 0 mM AICAR (Fig. 1A). The rate of phosphorylation of the Ser79 site of ACC, a downstream target of AMPK in skeletal muscle, paralleled the increase in AMPK Thr172 phosphorylation (Fig. 1B). In accord with these changes, protein content and myotube diameter were lower in myotubes incubated in 0.5 and 2 mM AICAR compared with control (0 mM) (Fig. 1, C and D). In addition, the expression level of HSP72 was reduced by treatment with 0.5 and 2 mM AICAR (Fig. 1E). The expressions of the other HSP-associated proteins, HSP25, HSC70, and HSF1, did not change by AICAR treatment (Fig. 1, F–H).

Fig. 1. Dose-dependent effects of AICAR on (A) phosphorylated 5′-AMP-activated protein kinase (AMPKα Thr172 (p-AMPK)/AMPKα, (B) phosphorylated acetyl-CoA carboxylase Ser29 (p-ACC)/ACC, (C) protein content, (D) diameter, (E) heat shock protein (HSP72, (F) HSP25, (G) heat shock cognate (HSC)70, and (H) heat shock transcription factor (HSF)1 in C2C12 myotubes. Myotubes were incubated in differentiation medium either without (open bars) or with (filled bars) indicated concentration of AICAR for 24 h. Representative immunoblots are also shown. Values are means ± SE; n = 6 per group. *P < 0.05.
The rate of phosphorylation of AMPKα Thr172 was increased by incubation with AICAR for over 4 h compared with those of control myotubes (0 h; Fig. 2A). The rate of phosphorylation of ACC Ser79 displayed a pattern similar to that for phosphorylation of AMPK Thr172 (Fig. 2B). The protein content and diameter in myotubes incubated with AICAR for 24 h were less than in control (Fig. 2, C and D). HSF72 expression was decreased by 24-h incubation with AICAR (Fig. 2E). However, HSP25, HSC70, and HSF1 expressions were not influenced by AICAR treatment at any time points (Fig. 2, F–H).

Suppression of AMPK expression attenuated the AICAR-induced downregulation of myocyte hypertrophy and HSP72. Expression of AMPKα protein was suppressed in myotubes transfected with AMPKα1/2-specific siRNA compared with that in myotubes transfected with nontarget siRNA (Fig. 3A). There was a significant interaction between AICAR treatment and AMPKα siRNA transfection in the expression of phosphorylated AMPK Thr172 (Fig. 3B), the rate of phosphorylation of ACC Ser79 (Fig. 3C), and myotube diameter (Fig. 3D). AICAR treatment enhanced the expression of phosphorylated AMPK Thr172 and the rate of phosphorylation of ACC Ser79 in nontarget siRNA-transfected myotubes, but not in AMPKα siRNA-transfected myotubes (Fig. 3, B and C). In nontarget siRNA-transfected myotubes, the diameter of AICAR-treated myotubes was less than that of control, but such an effect of AICAR treatment was not seen in AMPKα siRNA-transfected myotubes (Fig. 3D). More and fewer numbers of smaller and larger myotubes, respectively, were seen following treatment with AICAR (χ² test: *P < 0.05; Fig. 3E). On the other hand, treatment with AICAR did not affect the diameter distribution of AMPK siRNA-transfected myotubes (χ² test: *P = 0.56; Fig. 3F).

There was a significant interaction between AICAR treatment and AMPKα siRNA transfection in HSF72, p70S6K Thr389, and MuRF1 (Fig. 4, A, C, and D). HSF72 expression in nontarget siRNA-transfected myotubes was downregulated by treatment with AICAR, but this downregulation was not induced in AMPKα siRNA-transfected myotubes (Fig. 4A). The rate of phosphorylation of Akt Ser473 was not altered by treatment with AICAR in either nontarget siRNA and AMPKα siRNA-transfected myotubes (Fig. 4B). The rate of phosphorylation of p70S6K Thr389 was decreased by treatment with AICAR in both nontarget siRNA- and AMPKα siRNA-transfected myotubes (Fig. 4C). But the greater decrement seen in the nontarget siRNA-transfected myotubes was attenuated in the AMPKα siRNA-transfected myotubes. The expression of MuRF1 mRNA was upregulated by treatment with AICAR in only nontarget siRNA-transfected myotubes (Fig. 4D). A sig-

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**Fig. 2.** Time-dependent effects of AICAR on (A) p-AMPK/AMPKα, (B) p-ACC/ACC, (C) protein content, (D) diameter, (E) HSF72, (F) HSP25, (G) HSC70, and (H) HSF1 in C2C12 myotubes. Myotubes were incubated in differentiation medium either without (open bars) or with (filled bars) 0.5 mM AICAR for the indicated time. Representative immunoblots are also shown. Values are means ± SE; n = 6 per group. *P < 0.05.
Fig. 3. Effects of AICAR on (A) AMPKα, (B) p-AMPK, (C) p-ACC/ACC, and (D) diameter in nontargeting control siRNA (nontarget siRNA) and AMPKα siRNA-transfected C2C12 myotubes. The frequency distributions of the diameter in (E) nontarget siRNA- and (F) AMPKα siRNA-transfected C2C12 myotubes are shown. Myotubes were incubated in differentiation medium either without (control, open bars) or with (filled bars) 0.5 mM AICAR for 24 h. Representative immunoblots and images of myotubes are also shown. Scale bars, 100 μm. Values are means ± SE; n = 6 per group. *P < 0.05; §significant interaction between AICAR treatment and siRNA transfection; †significant effect of AMPKα siRNA by two-way ANOVA.
significant effect of AMPKα siRNA transfection on the expression of atrogin-1 mRNA was noted (Fig. 4E), but not in response to treatment with AICAR (P = 0.075).

AICAR-induced activation of AMPK upregulated expression of miR-1 but not miR-133a and miR-206. There was a significant interaction between AICAR treatment and AMPKα siRNA transfection in miR-1 expression (Fig. 5A). Application of AICAR upregulated miR-1 expression in nontarget siRNA-transfected myotubes but not in AMPKα siRNA-transfected myotubes. Expression of neither miR-133a (Fig. 5B) nor miR-206 (Fig. 5C) changed by AICAR treatment.

AMPK-related inhibition of myotube hypertrophy was not induced in the absence of HSP72. Expression of HSP72 protein was suppressed in myotubes transfected with HSP72 siRNA compared with that in myotubes transfected with nontarget siRNA (Fig. 6A). There was a significant interaction between AICAR treatment and HSP72 siRNA transfection in myotube diameter (Fig. 6B). The diameter in nontarget siRNA-transfected myotubes was significantly greater than in AMPKα siRNA-transfected myotubes (P < 0.05 vs. control).
fected myotubes treated with AICAR was less than in controls, but such phenomena were not observed in HSP72 siRNA-transfected myotubes. More distribution of smaller myotubes was seen following the treatment with AICAR, and the percent distribution of myotubes with larger diameter was less than controls (Fig. 6C). Meanwhile, there was no change by AICAR treatment in the size of HSP72 siRNA-transfected myotubes (Fig. 6D).

A significant effect of AICAR treatment was seen in the rate of phosphorylation of AMPK Thr172 (Fig. 7A), ACC Ser79 (Fig. 7B), and p70S6K Thr389 (Fig. 7D). The mean levels of phosphorylated AMPKα Thr172 and ACC Ser79 in AICAR-treated myotubes were greater (Fig. 7, A and B) and that of p70S6K Thr389 was less than in controls (Fig. 7D). However, the phosphorylation of Akt Ser473 was not influenced by AICAR (Fig. 7C). There was a significant interaction between AICAR treatment and siRNA transfection in MuRF1 and atrogin-1 mRNA expressions (Fig. 7, E and F). MuRF1 and atrogin-1 mRNA expressions were upregulated by AICAR treatment in nontarget siRNA-transfected myotubes, whereas there were not significant changes in HSP72 siRNA-transfected myotubes.

Suppression of HSP72 expression had no effect on AICAR-induced upregulation of mir-1 expression. A significant effect of AICAR was seen in the expression of miR-1 (Fig. 8A). The expressions of miR-133a and miR-206 were not affected by AICAR treatment in these myotubes (Fig. 8, B and C).

DISCUSSION

The present study shows the following novel findings related to the stimulation of muscle hypertrophy by AMPK. First, the treatment with AICAR decreased HSP72 expression in myotubes (Figs. 1 and 2), and such responses were not induced in the AMPKα knockout condition (Fig. 4). Second, the treatment with AICAR increased miR-1 expression in the

Fig. 6. Effects of AICAR on (A) HSP72 expression and (B) diameter in nontarget siRNA- and HSP72 siRNA-transfected C2C12 myotubes. Also, the frequency distribution of diameter in (C) nontarget siRNA- and (D) HSP72 siRNA-transfected C2C12 myotubes are shown. Myotubes were incubated in differentiation medium either without (control, open bars) or with (filled bars) of 0.5 mM AICAR for 24 h. Representative immunobLOTS and images of myotubes are also shown. Scale bars, 100 μm. Values are means ± SE; n = 6 per group. *P < 0.05; §significant interaction between AICAR treatment and siRNA transfection by two-way ANOVA.
The presence of AMPKα (Fig. 5) and HSP72 (Fig. 8). This change was also seen in the HSP72 knockdown condition (Fig. 8) but not in the AMPKα knockdown condition (Fig. 5). Third, AICAR-induced inhibition of myotube hypertrophy was not seen in the HSP72 knockdown condition (Fig. 6). Fourth, AICAR-induced upregulations of MuRF1 and atrogin-1 were attenuated in the HSP72 knockdown condition but not of p70S6K deactivation was (Fig. 7).

AMPK is a crucial regulator of skeletal muscle plasticity. Previous reports revealed that overloading-induced muscle growth was accompanied by an increase in p-AMPK/AMPK ratio. In the present study, we found that AICAR-induced upregulation of p-AMPK/AMPK was not observed in the HSP72 knockdown condition (Fig. 7A).

AICAR-induced upregulations of MuRF1 and atrogin-1 were attenuated in the HSP72 knockdown condition but not of p70S6K deactivation was (Fig. 7).

Fig. 7. Effects of AICAR on (A) p-AMPK/AMPKα, (B) p-ACC/ACC, (C) p-Akt/Akt, (D) p-p70S6K/p70S6K, (E) MuRF1 mRNA, and (F) atrogin-1 mRNA in nontarget siRNA and HSP72 siRNA-transfected C2C12 myotubes. Myotubes were incubated in differentiation medium either without (control, open bars) or with (filled bars) of 0.5 mM AICAR for 24 h. Representative immunobots are also shown. Values are means ± SE; n = 6–9 per group. *P < 0.05; §significant interaction between AICAR treatment and siRNA transfection; ¶significant effect of AICAR revealed by two-way ANOVA.

Fig. 8. Effects of AICAR on the expressions of (A) miR-1, (B) miR-133a, and (C) miR-206 in nontarget siRNA- and HSP72 siRNA-transfected C2C12 myotubes. Myotubes were incubated in differentiation medium either without (control, open bars) or with (filled bars) of 0.5 mM AICAR for 24 h. Values are means ± SE; n = 9 per group. ¶Significant effect of AICAR revealed by two-way ANOVA.
AMPK INHIBITS MYOTUBE HYPERTROPHY BY HSP72 DOWNREGULATION

hypertrophy was accelerated in AMPKα1 knockout mice (28) and that muscle mass and fiber cross-sectional area were greater in muscle-specific AMPKα1/2-deficient mice than in transgenic control mice (22). Furthermore, in vitro studies demonstrated that AMPK negatively regulated the hypertrophy of muscle cells (22, 28, 30). Consistent with these observations, the levels of protein content and myotube diameter, indicators of hypertrophy, were less in AMPK-activated myotubes than in controls (Figs. 1 and 2). Furthermore, it was confirmed that AICAR-induced inhibition of myotube hypertrophy did not occur in the AMPKα knockdown condition (Fig. 3). Therefore, it is suggested that AMPK plays a role in inhibition of muscle hypertrophy. On the other hand, more recent reports indicate that AMPK is a key molecular target for promoting myogenesis (6, 7). These are not consistent with previous studies that demonstrated that AMPK has inhibitory effects on myogenesis (9, 27, 41). Although we and other researchers have shown that AMPK has negative function for muscle hypertrophy, even more additional studies will be required to elucidate a precise role of AMPK on myogenesis.

Data obtained in the present study show that AICAR-induced AMPK activation decreased the expression level of HSP72 protein in myotubes (Figs. 1 and 2) and that HSP72 was not suppressed in the AMPKα knockdown condition (Fig. 4). These results indicate the novel function of AMPK in the modulation of HSP72 expression in skeletal muscle cells. HSPs, especially HSP72 and HSP25, are inducible molecular chaperones that are upregulated when cells are exposed to stress (24). The upregulations of HSP72 and HSP25 prevent muscle atrophy, which is caused by disuse and/or immobilization (4, 26, 38). The transcription of HSPs is mediated by HSF1, which binds to heat shock elements located on HSP genes and induces their expressions (24). The results obtained from this study demonstrate that activation of AMPK affected not only HSP72, but not HSP25 and HSC70, a constitutive form of the HSP70 family (Figs. 1 and 2). These observations raise the possibility that AMPK modulates the expression of HSPs through HSF1-independent regulation and may be due to posttranslational mechanisms. In fact, no change of HSF1 protein expression in AICAR-treated myotubes was seen (Figs. 1 and 2).

There is a possibility that miRNAs regulate AMPK-mediated HSP72 expression. miRNAs are short (~20–25 nucleotides) and noncoding RNA molecules, which repress gene expression by binding to the 3′-untranslated region of target mRNAs and either inhibit translation or promote cleavage of the transcript (1, 13). Recently, muscle-specific miRNAs miR-1, miR-133a, and miR206 have been shown to participate in the regulation of muscle mass (3, 17, 18, 25, 29). Some studies have reported that these miRNAs promote proliferation and differentiation of muscle cells (3, 18, 29), whereas miR-1 and miR-133a seem to be downregulated during skeletal muscle hypertrophy (17, 25). Furthermore, upregulations of miR-1 in dexamethasone-mediated muscle atrophy in mice and atrophic signals, which are integrated by miR-1, have been reported (19). In addition, it has been reported that miR-1 targets HSP72 during dexamethasone-mediated myotube atrophy (21). In the present study, it is shown that AICAR-induced AMPK activation upregulated miR-1, and this alteration was not seen in the AMPKα knockdown condition (Fig. 5), suggesting that AICAR-induced AMPK activation may modulate the expression of HSP72 protein via mediating miR-1. The limitation in the present study is that there is no direct evidence of a causal link between upregulation of miR-1 and downregulation of HSP72 by AICAR treatment. Further investigations, using muscle tissue or cells with knockdown and/or overexpression of miR-1, are necessary for further understanding of the involvement of miR-1 in AMPK-induced downregulation of HSP72.

AMPK-mediated suppression of myotube hypertrophy was attenuated in the HSP72 knockdown condition (Fig. 6). This observation supports our hypothesis that AMPK negatively regulates skeletal muscle mass by modulation of HSP expression. Therefore, effects of AMPK activation on the molecules involving the protein synthesis pathway (Akt/mTOR/p70S6K) and the molecules involving the ubiquitin proteasome pathway (MuRF1 and atrogin-1) were investigated to identify which downstream target is involved in the AMPK/HSP72-mediated inhibition of myotube hypertrophy. As the results show, AICAR did not affect Akt Ser473 phosphorylation, an indicator of Akt activation, and AICAR-induced p70S6K deactivation and MuRF1 upregulation were attenuated by AMPKα knockdown (Fig. 4). It is suggested that inhibition of myotube hypertrophy by AMPK activation may be mediated by both protein synthesis and degradation pathways. In contrast, AMPK deactivated p70S6K (inhibited the phosphorylation of p70S6K) in both the presence and the absence of HSP72, whereas the upregulations of MuRF1 and atrogin-1 were induced only in the presence of HSP72 (Fig. 7), suggesting that AMPK regulates MuRF1 and atrogin-1 expression through an HSP72-dependent mechanism. It was reported that HSP72 directly regulated Foxo signaling and repressed Foxo-dependent transcription of MuRF1 and atrogin-1 genes (37) and that HSP72 overexpression in mouse muscles attenuated the upregulation of these two genes (38) under the condition inducing muscle atrophy. Considering the interaction between HSP72 and Foxo, there is a possibility that AMPK modulates MuRF1 and atrogin-1 expression through HSP72-mediated modulation of Foxo transcriptional activity. Taken together, it is speculated that the HSP72-mediated protein degradation pathways might be more important for AMPK-induced inhibition of myotube hypertrophy than the protein synthesis pathways.

RNAi-associated knockdown of AMPK per se did not affect myotube diameter (Fig. 3D), HSP72 expression (Fig. 4A), phosphorylation of p70S6K (Fig. 4C), MuRF1 mRNA expression (Fig. 4D), and miR-1 expression (Fig. 5A), even though they were increased or decreased by activation of AMPK. The present study demonstrates that the promoting effects of AICAR on phosphorylation of AMPK Thr172 and ACC Ser79 were completely attenuated by siRNA transfection (Fig. 3, B and C), but basal phosphorylation levels were depressed by ~50 and ~25%, respectively. Therefore, RNAi could not completely inhibit basal AMPK activity. A previous study also did not show the upregulation of phosphorylation level of p70S6K Thr389 by RNAi-induced AMPK suppression in C2C12 cells (23). On the contrary, transgenic knockout of AMPK induced myotube hypertrophy and upregulation of p70S6K phosphorylation by using primary cultured myoblasts under nonstimulated condition (22). Therefore, complete suppression of AMPK activity might lead to alterations of morphology and the related intracellular signals in the basal nonstimulated condition of muscle cells.

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In conclusion, the results obtained from the present study indicate that AICAR-induced AMPK activation inhibited myotube hypertrophy, and these alterations were accompanied with downregulation of HSP72 and upregulation of miR-1. A novel mechanism, in which AMPK negatively regulates skeletal muscle hypertrophy through, in part, a HSP72-associated pathway, is suggested.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.E., Y. Ohno, and K.G. conception and design of research; T.E. and A.G. performed experiments; T.E. analyzed data; T.E., Y. Ohno, A.I., M.S., T.O., S.Y., Y. Ohira, T.Y., and K.G. interpreted results of research; T.E. and A.G. prepared figures; T.E. drafted manuscript; T.E., Y. Ohno, A.I., M.S., T.O., S.Y., Y. Ohira, T.Y., and K.G. approved final version of manuscript.

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