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Caffeine affects myotube size as well as regulates protein degradation and protein synthesis pathways in C2C12 skeletal muscle cells

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Running head: Regulation of skeletal muscle size by caffeine

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ABSTRACT

Background: Caffeine has been implicated in energy metabolism regulation in skeletal muscle. However, it is unclear whether caffeine affects the regulation of skeletal muscle size. In the present study, we evaluated the effect of caffeine on muscle size as well as accompanied changes of ubiquitin-proteasome system and Akt/mammalian target of rapamycin/p70 s6 kinase (p70S6K) signaling.

Methods: Differentiated C2C12 myotubes were incubated with caffeine (0, 0.1, 1.0, 3.0 mM) for 24 h. We then estimated the protein content, myotube diameter, and the expression levels of muscle RING finger 1 (MuRF1) mRNA, atrogin-1/muscle atrophy F-box (MAFbx) mRNA, K48-linked polyubiquitin, phosphorylated 5′AMP-activated protein kinase (AMPK) α Thr^{172}, 72-kDa heat shock protein (HSP72), HSP72 mRNA, inhibitor κBα (IκBα), phosphorylated forkhead box class O3a (FoxO3a) Ser^{253}, myogenin mRNA, microRNA (miR)-23a, phosphorylated Akt Ser^{473}, and phosphorylated p70 s6 kinase (p70S6K) Thr^{389}.

Results: Protein content and myotube diameter were lower in myotubes treated with caffeine (≥ 1 mM) compared with untreated cells. The expression levels of MuRF1 and atrogin-1/MAFbx mRNA and K48-linked polyubiquitin were increased by caffeine treatment. However, phosphorylated AMPKα Thr^{172}, HSP72 protein and mRNA, IκBα, phosphorylated FoxO3a Ser^{253}, and miR-23a expression were not affected by caffeine treatment. Myogenin mRNA expression was upregulated in response to caffeine treatment. The expressions of phosphorylated Akt Ser^{473} and p70S6K Thr^{389} were suppressed by caffeine.

Conclusions: Caffeine might affect muscle size by stimulating ubiquitin-proteasome system and inhibiting Akt/mTOR/p70S6K signaling, partly via an AMPK-independent

mechanism.

INTRODUCTION

Caffeine is a xanthine alkaloid, which has been implicated in energy metabolism regulation in skeletal muscle. Particularly, 5′AMP-activated protein kinase (AMPK) is considered to be a crucial mediator of caffeine-mediated metabolic changes in skeletal muscle. Several studies have suggested that caffeine (≥1 mM) acutely stimulates AMPK in rat skeletal muscle[1-8] and that caffeine-stimulated glucose transport is reduced in AMPK kinase-dead mice[1]. Ding et al.[9] found that caffeine (1 and 5 mM) treatment for 4 days (5 h/day) increased mitochondrial biogenesis via an AMPK-dependent mechanism in skeletal muscle cells. Furthermore, Mathew et al.[10] demonstrated that caffeine (10 mM) treatment for 6 h stimulated autophagy in skeletal muscle cells, which was attenuated in the presence of an AMPK inhibitor.

Recently, AMPK has been considered to be a key factor that modulates skeletal muscle mass. It was reported that elevated AMPK activity was involved in a diminished capacity for hypertrophy in aged rat skeletal muscle[11,12] and that overload-induced muscle hypertrophy was accelerated in AMPKα1-deficient mice compared with the wild-type mice[13]. Akt/mammalian target of rapamycin (mTOR)/p70 S6 kinase (p70S6K), a central signaling modulator of protein synthesis[14], is considered to be a potent signaling pathway for AMPK-mediated regulation of muscle hypertrophy. On the other hand, in our previous study[15], we demonstrated that AMPK-associated suppression of muscle hypertrophy in skeletal muscle cells was through rather activating protein catabolic pathway than inhibiting protein synthesis pathway.

The ubiquitin-proteasome system is a crucial regulatory mechanism for protein catabolism in skeletal muscle. The key enzymes in this pathway are E3 ubiquitin ligases, which are responsible for protein ubiquitination. Two muscle-specific ubiquitin ligases,

i.e., muscle RING finger 1 (MuRF1) and atrogin-1/muscle atrophy F-box (MAFbx), are considered to be involved in skeletal muscle atrophy[16,17]. Previous studies have demonstrated that AMPK activation stimulates protein degradation, which is accompanied by the upregulation of MuRF1 and atrogin-1/MAFbx mRNA expression in skeletal muscle cells[18,19]. In accordance with these researches, we[15] demonstrated that pharmacological AMPK activation altered muscle size through ubiquitin-proteasome system and that these responses were not induced in AMPK-knockdown cells in vitro. Furthermore, our recent study in vivo[20] has shown that the ubiquitin-proteasome system was not activated during unloading-induced muscle atrophy in muscle, where the AMPK activity decreased. Overall, AMPK-induced activation of ubiquitin-proteasome system appears to be involved in the regulation of muscle size.

Thus, the results of previous studies strongly suggest that caffeine also activates the ubiquitin-proteasome system and that it affects the skeletal muscle size through AMPK activation. Therefore, in the present study, we aimed to evaluate the effect of caffeine on muscle size and accompanied changes of the ubiquitin-proteasome system in skeletal muscle cells. In addition, we have previously shown that caffeine inhibited Akt/mTOR/p70S6K signaling in rat skeletal muscle[7,21], indicating that caffeine may inhibit protein synthesis pathway. Therefore, to confirm whether caffeine affects protein synthesis pathway, we also evaluate the effect of caffeine on Akt/mTOR/p70S6K signaling.

MATERIALS AND METHODS

Cell culture and treatment.

Cell culture was performed as described previously[15]. Briefly, mouse myoblast C2C12 cells were cultured on 6-well culture plates with type I collagen-coated surface (Biocoat, Becton-Dickinson Labware, Franklin Lakes, NJ). Cells were maintained in growth medium comprising 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) to allow proliferation under a humidified atmosphere with 95% air and 5% CO_2. After reaching confluence, the culture medium was replaced with differentiation medium comprising 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 differentiation. The medium was replaced with freshly prepared differentiation medium at 2-day intervals over the course of differentiation for 5 days. Five days after the initiation of differentiation, myotubes were incubated in differentiation medium containing caffeine (Wako, Tokyo, Japan), 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR, Sigma, St. Louis, MO), or metformin (Sigma). To investigate the dose-dependent effects of caffeine, the myotubes were incubated in differentiation medium with or without caffeine (0, 0.1, 1.0, 3.0 mM) for 24 h. Subsequently, the cells were harvested for western blot or real-time RT-PCR analyses.

Myotube diameter measurement.

Images of myotubes were visualized at 40× magnification using an inverted light microscope and captured with a camera (Olympus, Tokyo, Japan). The diameter of

myotubes was measured using a modified version of the method described by Williamson et al.[22]. Four fields were chosen randomly and 150 myotubes were measured using ImageJ. The average diameter per myotube was calculated as the mean of three short-axis measurements taken along the length of the myotube.

Sample preparation.

Samples were prepared as described previously with some modifications[23]. Briefly, the cells in each well were rinsed twice with ice-cold phosphate-buffered saline. Next, the cells were scraped from each well into CelLytic™ M cell lysis reagent (Sigma) with 1% (v/v) protease inhibitor cocktail (Sigma) and 1% (v/v) phosphatase inhibitor cocktail (Calbiochem, San Diego, CA). The cell lysate was sonicated and centrifuged for 15 min at $15,000 \times g$ and 4°C. The supernatant was collected to determine the protein content using the Bradford technique (protein assay kit; Bio-Rad, Hercules, CA). The protein contents of the supernatant were expressed as mg/ml.

Western blot analyses.

Western blot analyses were performed as described previously[7,23]. Extracted samples in 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 internal controls for the transfer process or electrophoresis.

After SDS-PAGE, the proteins were transferred to polyvinylidene fluoride 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. Next, the membranes were blocked for 1 h using Blocking One-P (Nacalai Tesque, Kyoto, Japan) or ECL blocking reagent (RPN418, GE Healthcare). The membranes were then incubated overnight at 4°C with primary antibodies: Akt Ser[473] (9271, Cell Signaling Technology, Danvers, MA), Akt (9272, Cell Signaling Technology), AMPKα Thr[172] (2531, Cell Signaling Technology), AMPKα (2532, Cell Signaling Technology), forkhead-box class O (FoxO) 3a Ser[253] (9466, Cell Signaling Technology), FoxO3a (2497, Cell Signaling Technology), 72-kDa heat shock protein (HSP72, ADI-SPA-812, Enzo Life Sciences, Farmingdale, NY), inhibitor of κBα (IκBα, 9242, Cell Signaling Technology), K48-linkage specific polyubiquitin (4298, Cell Signaling Technology), p70S6K Thr[389] (9206, Cell Signaling Technology), p70S6K (9202, Cell Signaling Technology), and β-actin (4967, Cell Signaling Technology). The membranes were washed with Tris-buffered saline containing 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 a final wash with TBS-T, the protein bands were visualized using chemiluminescence (Wako). The signal density was measured using a Light-Capture system (AE-6971, ATTO Corporation, Tokyo, Japan).

Real-time RT-PCR analyses.

Real-time RT-PCR analyses were performed as described previously[24]. Briefly, total RNA was extracted from muscles using a miRNeasy Mini kit (Qiagen, Hiden, Germany) according to the manufacturer's protocol. To detect MuRF1 (Trim63) mRNA, atrogin-1/MAFbx (Fbxo32) mRNA, and HSP72 (Hspa1a) mRNA, the RNA was reverse-transcribed to cDNA using PrimeScript RT Master Mix (Takara Bio), and the synthesized cDNA was then subjected to real-time RT-PCR (Thermal Cycler Dice Real Time System

IIMRQ, Takara Bio) using Takara SYBR Premix Ex Taq II (Takara Bio). To detect

microRNA (miR)-23a, the RNA was reverse-transcribed to cDNA using a Mir-X^{TM}

miRNA First Strand Synthesis Kit (Clontech Laboratories, Mountain View, CA), and the

synthesized cDNA was then subjected to real-time RT-PCR using a Mir-X^{TM} miRNA

qRT-PCR SYBR Kit (Clontech Laboratories). Relative fold changes in the expression

levels were calculated by the comparative CT method using Takara Thermal Cycler Dice

Real Time System Software Ver. 4.00. To normalize the total amount of RNA present in

each reaction, 18S ribosomal RNA was used as an internal standard for MuRF1 and

HSP72, and U6 for miR-23a.

The following primers were used: Trim63 (MuRF1), 5′-
AGGACTCCTGCAGAGTGACCAA-3′ (forward) and 5′-
TTCTCGTCCAGGATGGCGTA-3′ (reverse); atrogin-1/MAFbx (Fbxo32), 5′-
TGTCCTTGAATTCAGCAAGCAAAC-3′ (forward) and 5′-
TGTGGCCATCCATTATTTCCAG-3′ (reverse); Hspa1a (HSP72), 5′-
CAAGAACGCGCTCGAATCCTA-3′ (forward) and 5′-
TCCTGGCACTTGTCCAGCAC-3′ (reverse); 18S ribosomal RNA, 5′-
ACTCCAAAGGGCACAACTCA-3′ (forward) and 5′-
CCTTTAGGGACCGTTACACTA-3′ (reverse); and miR-23a, 5′-
ATCACATTGCCAGGGATTTCC-3′ (forward). The U6 primer and reverse primers for

miRNA were provided with the kit.

Statistical analyses.

All values were expressed as means ± SEM. Differences between two groups were

compared using the Student's t-test. Multiple means were analyzed using Dunnett's

multiple comparisons test. Differences in the myotube diameter distributions were analyzed using the Chi-squared test. Differences between groups were considered statistically significant at $P < 0.05$. All of the statistical analyses were performed using Ekuseru-Toukei 2012 software (Social Survey Research Information, Tokyo, Japan).

RESULTS

Caffeine inhibited myotube hypertrophy.

To determine whether caffeine affects myotube size, we measured the protein content and myotube diameter in caffeine-treated cells. The dose-response analysis demonstrated that the protein content was decreased by caffeine treatment at 1.0 and 3.0 mM compared with no treatment (Figure 1A). Correspondingly, the myotube diameter was smaller in myotubes treated with caffeine (1 mM) compared with no treatment (Figure 1B). Greater numbers of smaller and lower numbers of larger myotubes were observed following caffeine treatment (Chi-square test: $P < 0.05$, Figure 1C).

Caffeine activated the ubiquitin-proteasome system.

To identify whether caffeine activates the ubiquitin-proteasome system, we analyzed the expression levels of MuRF1 and atrogin-1/MAFbx mRNA and K48-linked polyubiquitin in myotubes treated with or without caffeine (1 mM). The expression levels of MuRF1 mRNA (Figure 2A), atrogin-1/MAFbx mRNA (Figure 2B) and K48-linked polyubiquitin (Figure 2C) were increased by caffeine treatment.

Caffeine did not affect AMPK activity or HSP72 expression.

In a previous study[15], we showed that the AMPK-induced suppression of hypertrophy is partly mediated by the downregulation of HSP72 protein. Therefore, we investigated whether caffeine affects the phosphorylation of AMPKα Thr[172], which is the primary site responsible for AMPK activation[25], and the expression levels of HSP72 protein and mRNA. The expression level of phosphorylated AMPKα Thr[172] increased (Figure 3A) whereas the expression levels of HSP72 protein (Figure 3B) and mRNA (Figure 3C)

decreased after treatment with the pharmacological AMPK activators AICAR (0.5 mM) and metformin (2 mM). However, there were no changes in AMPKα Thr^{172} phosphorylation and the expression levels of HSP72 protein and mRNA under caffeine treatment (Figure 3).

Caffeine increased myogenin mRNA expression but not the expression levels of IκBα, phosphorylated FoxO3a Ser^{253}, and miR-23a.

Next, we examined the effects of caffeine on the expression levels of molecules that directly or indirectly target MuRF1 and atrogin-1/MAFbx transcriptions. The expression levels of IκBα (Figure 4A), phosphorylated FoxO3a Ser^{253}, a key regulatory site for the transcription activity[26] (Figure 4B), and miR-23a (Figure 4C) were not altered by caffeine treatment. By contrast, myogenin mRNA expression was upregulated by caffeine (Figure 4D).

Caffeine inhibited the protein synthesis pathway.

To confirm whether caffeine affects the protein synthesis pathway, we evaluated the expression levels of phosphorylated Akt Ser^{473} and p70S6K Thr^{389}, which are the primary site responsible for activations of Akt[27] and p70S6K[28], respectively, in myotubes treated with or without caffeine (1 mM). Treatment with caffeine decreased phosphorylation of both Akt Ser^{473} (Figure 5A) and p70S6K Thr^{389} (Figure 5B).

DISCUSSION

In the present study, we obtained the following novel findings related to the effects of caffeine on the regulation of skeletal muscle size. First, caffeine ($\geq$1 mM)-treated myotubes had a lower protein content and smaller myotube diameter compared with untreated myotubes (Figure 1). Second, caffeine treatment increased the expression levels of MuRF1 and atrogin-1/MAFbx mRNA and K48-linked polyubiquitin (Figure 2). Third, treatment with AICAR and metformin increased the phosphorylation of AMPKα Thr^{172} but decreased the expression levels of HSP72 protein and mRNA. Conversely, caffeine treatment had no effects on the phosphorylation of AMPKα Thr^{172} and the expression levels of HSP72 protein and mRNA (Figure 3). Fourth, caffeine treatment increased myogenin mRNA expression but not the phosphorylation of FoxO3a Ser^{253}, IκBα, and miR-23a (Figure 4).

A recent study demonstrated that caffeine promotes autophagy in skeletal muscle cells[10]. Autophagy is a crucial cell proteolytic system, which controls protein turnover in skeletal muscle[29]. In the present study, we found that caffeine increased the expression levels of MuRF1 and atrogin-1/MAFbx mRNA and K48-linked polyubiquitin (Figure 2), suggesting that caffeine also activates another proteolysis pathway, the ubiquitin-proteasome system. K48-linked polyubiquitination is the best-studied type of poly-ubiquitination, which typically targets proteins for proteasomal degradation[30], and it is synthesized by MuRF1[31] and/or atrogin-1/MAFbx[32]. Several signaling molecules are involved in the regulation of MuRF1 and atogin-1/MAFbx transcriptions.

AMPK stimulates myofibrillar protein degradation via the upregulation of MuRF1 and atrogin-1/MAFbx[18,19]. We hypothesized that caffeine may increase MuRF1 and atrogin-1/MAFbx expressions via an AMPK-dependent mechanism since caffeine is

known to be a potent activator of AMPK. However, caffeine did not affect the phosphorylation of AMPKα Thr^{172} (Figure 3A). A previous study found that caffeine acutely (approximately 4 h) increased the phosphorylation of AMPKα Thr^{172}, but it returned to the control level by 12 h in skeletal muscle cells[33]. Our results support these findings, and it is suggested that AMPK signaling was transiently activated following caffeine treatment but returned to the basal state at 24 h in the present study.

The AMPK-mediated activation of the ubiquitin-proteasome system is partly dependent on HSP72. In a previous study[15], we demonstrated that the AMPK-mediated upregulation of MuRF1 mRNA is suppressed in HSP72-knockdown cells. HSP72 is one of the most prominent members of the HSP family, and it is considered to have an important role in controlling skeletal muscle mass[34,35]. HSP72 targets FoxOs, a transcriptional factor of MuRF1[36]. In the present study, there were no changes in the expression levels of HSP72 protein and mRNA (Figure 3), or in the phosphorylation of FoxO3a Ser^{253} (Figure 4B). Overall, in contrast to our expectations, our results suggest that caffeine upregulates the ubiquitin-proteasome system via an AMPK-independent mechanism. However, it cannot be excluded the possibility that the transient activation of AMPK might affect the caffeine-mediated responses in the present study. In this regard, further investigations, using a pharmacological blocker or cells with knockdown of AMPK, are necessary to understand the role of AMPK in caffeine-mediated regulation of muscle size.

NF-κB, miR-23a, and myogenin are potent molecules that transcribe MuRF1 and/or atrogin-1/MAFbx. NF-κB is a transcriptional factor, which is sequestered in the cytoplasm by a family of inhibitory proteins called IκBα[37]. The IκB kinase complex phosphorylates IκBα, thereby resulting in its degradation, which leads to the nuclear

translocation of NF-κB and its activation. It has been reported that NF-κB sites are required for the transcriptional activation of MuRF1 during the regulation of muscle mass[38]. However, in the present study, we found that IκBα expression was not affected by caffeine (Figure 4B).

miRs comprise a novel class of small, non-coding endogenous RNAs, which regulate gene expression by directing their target mRNAs for degradation or translational repression[39,40]. Recently, it was shown that miR-23a suppresses post-transcriptional MuRF1 and atrogin-1/MAFbx expressions in vitro and that the forced expression of miR-23a in skeletal muscle leads to resistance against atrophy by regulating MuRF1 and atrogin-1/MAFbx genes[41]. In the present study, caffeine did not affect the expression level of miR-23a (Figure 4C).

Myogenin is an essential transcription factor for skeletal muscle development[42], which binds and activates the promoter regions of the MuRF1 and atrogin-1/MAFbx genes[43]. It has been demonstrated that the upregulation of MuRF1 and atrogin-1/MAFbx in denervated skeletal muscle was impaired in myogenin null mice[43]. In the present study, caffeine upregulated the expression level of myogenin mRNA (Figure 4D), which suggests that caffeine might activate the ubiquitin-proteasome system by increasing myogenin-mediated MuRF1 and atrogin-1/MAFbx transcriptions.

Skeletal muscle mass is regulated by the rate of protein turnover, namely the balance between protein synthesis and degradation[44]. In previous studies[7,21], we showed that caffeine treatment acutely inhibits the Akt/mTOR/p70S6K signaling in rat skeletal muscle. Correspondingly, in the present study, we confirmed that caffeine decreased phosphorylation of Akt Ser[473] (Figure 5A) as well as p70S6K Thr[389] (Figure 5B) in caffeine-treated cells, suggesting that caffeine also affects protein synthesis pathway in

addition to proteolysis pathway. Taken together, the data obtained the present study suggests that the decreased muscle size (Figure 1) by caffeine might be attributed to the disturbance of protein turnover, including suppression of protein synthesis and stimulating protein degradation.

In terms of exercise, caffeine ingestion elicits high performance with respect to the endurance capacity and muscle strength[45]. However, caffeine might also inhibit protein synthesis and stimulate protein degradation, given that caffeine suppresses the Akt/mTOR/p70S6K signaling pathway as well as promoting the autophagy[10] and ubiquitin-proteasome pathways. Therefore, caffeine ingestion may possibly affect muscle size: inhibiting muscle hypertrophy or promoting muscle atrophy. Furthermore, it has been shown that caffeine ingestion acutely lowers glucose tolerance in skeletal muscle[46] by preventing insulin signal transduction[7]. Therefore, caffeine is not necessarily beneficial for muscle functions. To the best of our knowledge, the present study is the first to demonstrate the involvement of caffeine in the regulation of muscle size and the ubiquitin-proteasome system in skeletal muscle. Further research should clarify the effects of caffeine on skeletal muscle size in physiological conditions to obtain a better understanding of the roles of caffeine in skeletal muscle functions.

CONCLUSIONS

In this study, we showed that caffeine treatment reduced in myotube size in cultured C2C12 cells and stimulated the ubiquitin-proteasome system. In addition, caffeine treatment did not promote AMPK signaling or its associated molecules, whereas it upregulated myogenin mRNA expression. On the other hand, the protein synthesis pathway was inhibited by caffeine. The results obtained in the present study indicate that caffeine might affect muscle size by stimulating ubiquitin-proteasome system and inhibiting Akt/mTOR/p70S6K signaling, partly via an AMPK-independent mechanism.

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DISCLOSURES

The authors state that they have no conflicts of interest to declare.

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FIGURE LEGENDS

Figure 1

Effects of caffeine on the: (A) protein content, (B) myotube diameter, and (C) C2C12 myotube diameter distribution. Myotubes were incubated in differentiation medium without or with caffeine (A: indicated concentration; B and C: 1 mM) for 24 h. Representative images of myotubes are also shown. Scale bars indicate 100 μm. Values represent the mean ± SEM. n = 6 per group. *: $P < 0.05$ vs. no treatment.

Figure 2

Effects of caffeine on the expression levels of: (A) MuRF1 mRNA, (B) atrogin-1/MAFbx mRNA and (C) K48-linked polyubiquitin (K48-Ub) in C2C12 myotubes. Myotubes were incubated in differentiation medium without or with caffeine (1 mM) for 24 h. Representative immunoblots are also shown. Values represent the mean ± SEM. n = 3-6 per group. *: $P < 0.05$ vs. no treatment.

Figure 3

Effects of caffeine, AICAR, or metformin on the expression levels of: (A) phosphorylated AMPK Thr^{172} (p-AMPK)/AMPK, (B) HSP72, and (C) HSP72 mRNA in C2C12 myotubes. Myotubes were incubated in differentiation medium without or with caffeine (1 mM), AICAR (0.5 mM), and metformin (2 mM) for 24 h. Representative immunoblots are also shown. Values represent the mean ± SEM. n = 6 per group. *: $P < 0.05$ vs. no treatment.

Figure 4

Effects of caffeine on the expression levels of: (A) IκBα, (B) phosphorylated FoxO3a Ser^{253} (p-FoxO3a)/FoxO3a, (C) miR-23a, and (D) myogenin mRNA in C2C12 myotubes. Myotubes were incubated in differentiation medium without or with caffeine (1 mM) for 24 h. Representative immunoblots are also shown. Values represent the mean ± SEM. n = 6 per group. *: $P < 0.05$ vs. no treatment.
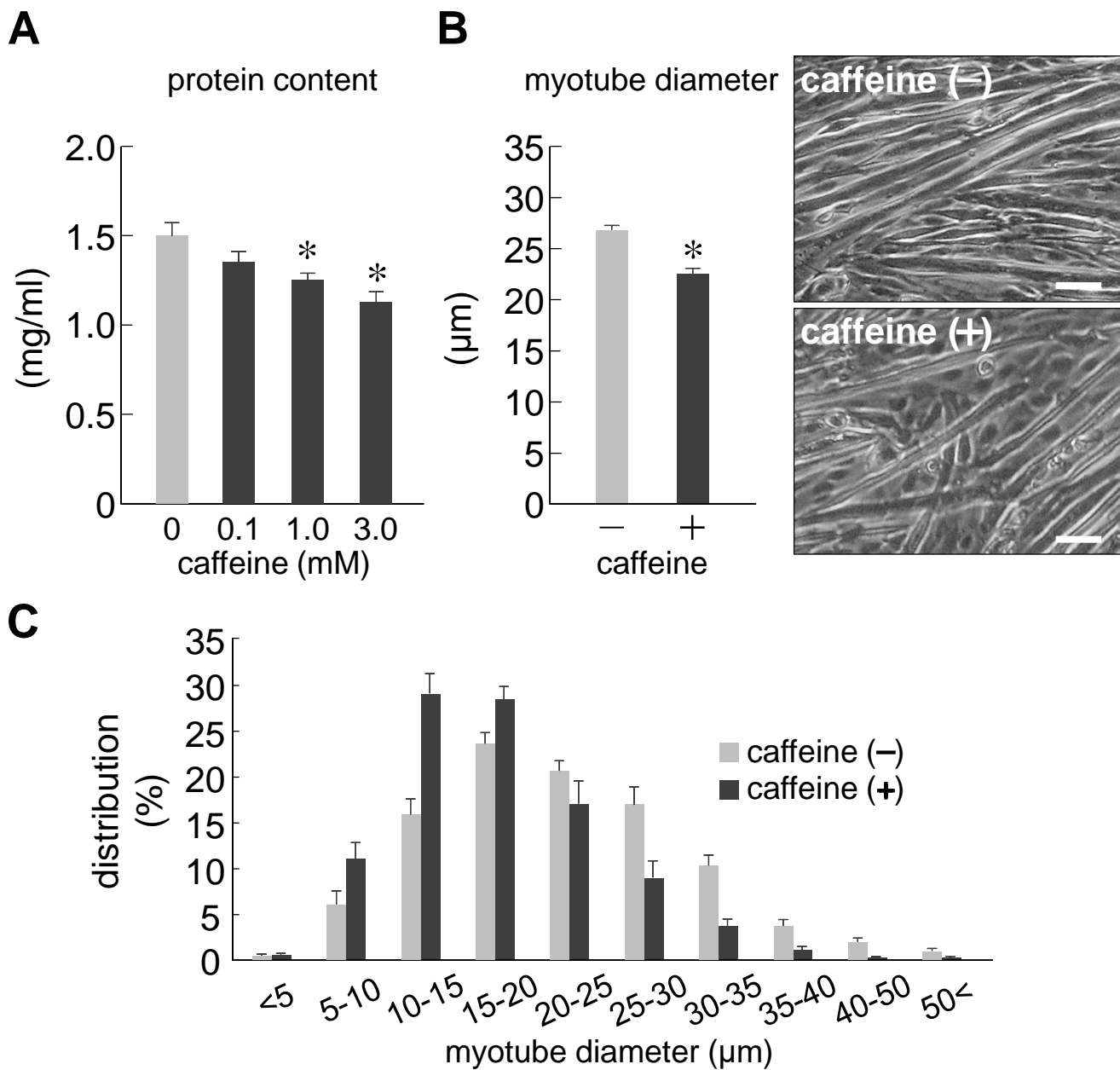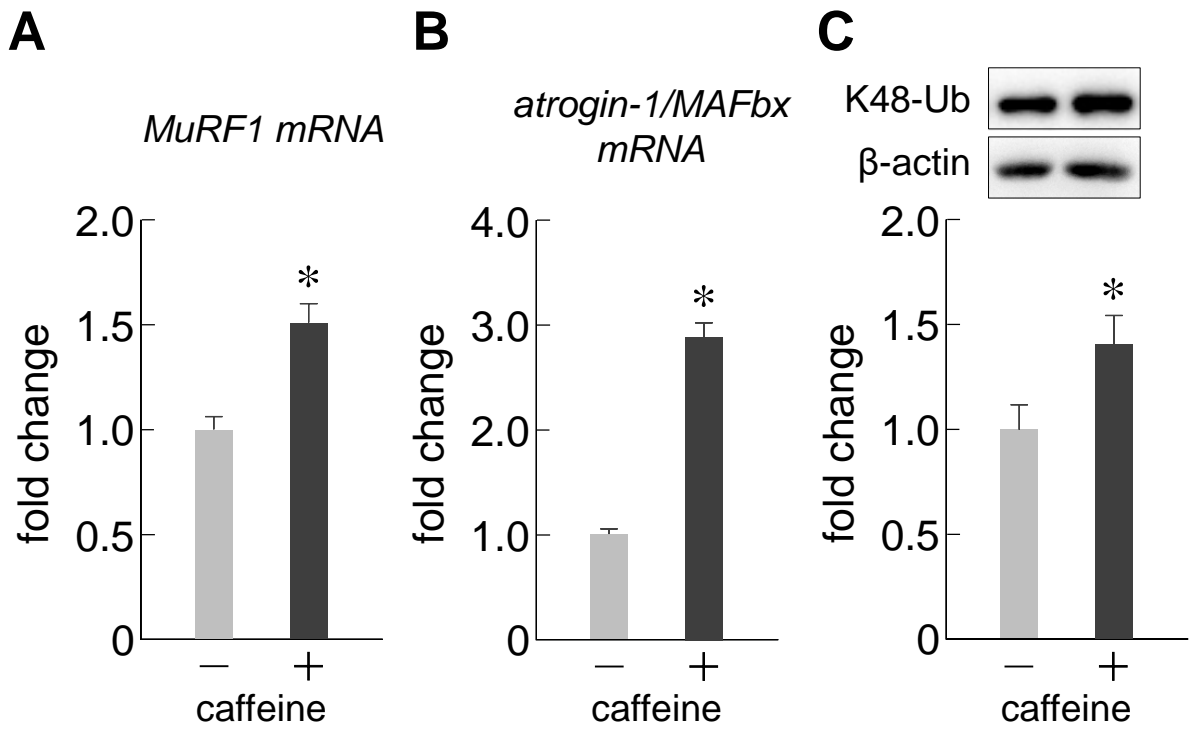
Figure 5

Effects of caffeine on the expression levels of: (A) phosphorylated Akt Ser^{473} (p-Akt)/Akt and (B) phosphorylated p70S6K Thr^{389} (p-p70S6K)/p70S6K in C2C12 myotubes. Myotubes were incubated in differentiation medium without or with caffeine (1 mM) for 24 h. Representative immunoblots are also shown. Values represent the mean ± SEM. n = 6 per group. *: $P < 0.05$ vs. no treatment.
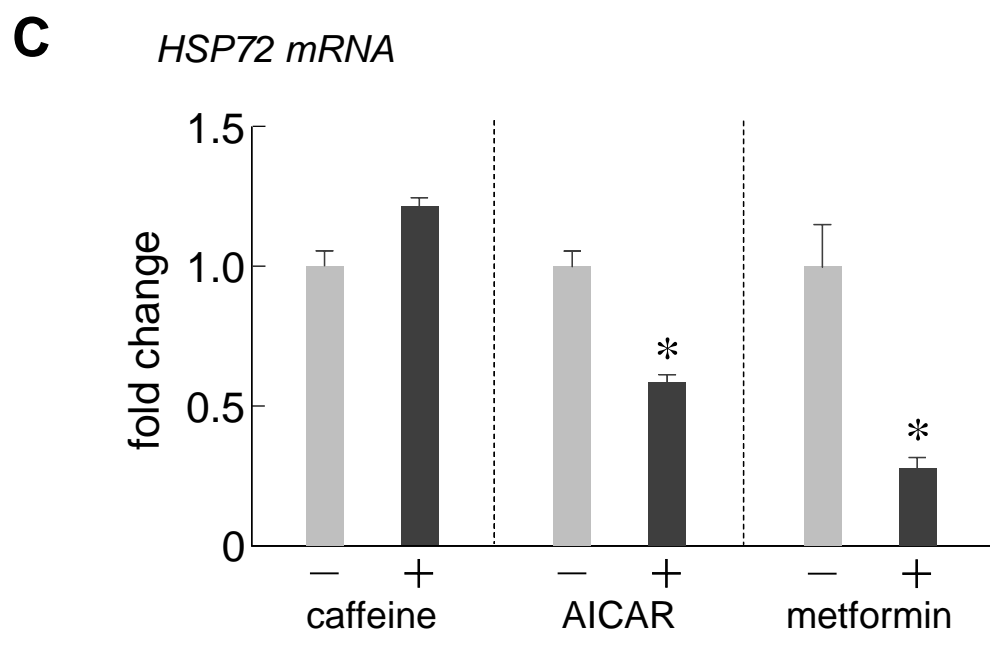
A

protein content

B

myotube diameter

C

Figure 1

A *MuRF1 mRNA*

B *atrogin-1/MAFbx mRNA*

C K48-Ub

β-actin

Figure 2

A

p-AMPK

AMPK

B

HSP72

β-actin

HSP72 mRNA

C

Figure 3

A

IκBα

β-actin

B

p-FoxO3a

FoxO3a

C

miR-23a

D

myogenin mRNA

Figure 4

A

p-Akt

Akt

B

p-p70S6K

p70S6K

fold change

1.5

1.0

0.5

0

*

−   +

caffeine

fold change

1.5

1.0

0.5

0

*

−   +

caffeine

Figure 5