Involvement of AMPK in regulating slow-twitch muscle atrophy during hindlimb unloading in mice

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Egawa T, Goto A, Ohno Y, Yokoyama S, Ikuta A, Suzuki M, Sugiura T, Ohira Y, Yoshioka T, Hayashi T, Goto K. Involvement of AMPK in regulating slow-twitch muscle atrophy during hindlimb unloading in mice. Am J Physiol Endocrinol Metab 309: E651–E662, 2015. First published August 4, 2015; doi:10.1152/ajpendo.00165.2015.—AMPK is considered to have a role in regulating skeletal muscle mass. However, there are no studies investigating the function of AMPK in modulating skeletal muscle mass during atrophic conditions. In the present study, we investigated the difference in unloading-associated muscle atrophy and molecular functions in response to 2-wk hindlimb suspension between transgenic mice overexpressing the dominant-negative mutant of AMPK (AMPK-DN) and their wild-type (WT) littermates. Male WT (n = 24) and AMPK-DN (n = 24) mice were randomly divided into two groups: an untreated preexperimental control group (n = 12 in each group) and an unloading (n = 12 in each group) group. The relative soleus muscle weight and fiber cross-sectional area to body weight were decreased by ~30% in WT mice by hindlimb unloading and by ~20% in AMPK-DN mice. There were no changes in pyruvycin-labeled protein or Akt/70-kDa ribosomal S6 kinase signaling, the indicators of protein synthesis. The expressions of ubiquitinated proteins and muscle RING finger 1 mRNA and protein, markers of the ubiquitin-proteasome system, were increased by hindlimb unloading in WT mice but not in AMPK-DN mice. The expressions of molecules related to the protein degradation system, phosphorylated forkhead box class O3a, inhibitor of kBa, microRNA (miR)-1, and miR-23a, were decreased only in WT mice in response to hindlimb unloading, and 72-kDa heat shock protein expression was higher in AMPK-DN mice than in WT mice. These results imply that AMPK partially regulates unloading-induced atrophy of slow-twitch muscle possibly through modulation of the protein degradation system, especially the ubiquitin-proteasome system.

AMP-activated protein kinase; protein degradation; autophagy; ubiquitin-proteasome; microRNA; heat shock protein

SKELETAL MUSCLE IS THE LARGEST TISSUE IN THE BODY, accounting for ~40% of the total body mass, and has a crucial role in metabolism as well as locomotion. Skeletal muscle has a high ability to adapt to multiple stimuli. Increased loading, such as resistance training and mechanical stretching, leads to skeletal muscle hypertrophy (18, 75). In contrast, aging, poor nutrition, several diseases such as diabetes, cancer, sepsis, and chronic renal failure, and decreased loading, such as inactivity, lead to skeletal muscle atrophy (23, 34, 39). Skeletal muscle atrophy occurs as a result of changes in protein turnover: decreased protein synthesis, increased protein degradation, or a combination of both (17). The coordination of protein turnover in the atrophic process is regulated by complicated molecular responses, and the molecular mechanism involved in this process in skeletal muscle is not yet completely understood and remains to be elucidated.

5′-AMP-activated protein kinase (AMPK) is well established as a metabolic sensor that helps maintain cellular energy homeostasis by modulating glucose, lipid, and protein metabolism (16, 26, 28). AMPK is a heterotrimeric kinase comprising a catalytic α-subunit and two regulatory subunits, the β- and γ-subunits. Two distinct α-isofoms (α1 and α2) exist in mammalian cells; α1 is expressed ubiquitously, whereas α2 is dominant in skeletal muscle, heart, and liver (69). Binding of AMP to a Bateman domain of the γ-subunit of AMPK induces the allosteric activation of AMPK and phosphorylation of the Thr172 residue of the α-subunit, which is essential for full kinase activity (27, 59).

Several studies in the past decade suggest that AMPK has a potential role in regulating skeletal muscle mass. Gordon et al. (21) and Thomson and Gordon (72) provided evidence that elevated AMPK activity was associated with diminished capacity for hypertrophy of fast-twitch skeletal muscle in aged rat. Moreover, Paturi et al. (55) suggested that impaired hypertrophy of slow-twitch skeletal muscle during overloading in the diabetic rat was attributed partly to increased AMPK phosphorylation. A recent study using a knockout mouse model demonstrated that overload-induced muscle hypertrophy was accelerated in AMPKα1-deficient mice compared with the wild-type mice (50). Correspondingly, we (13) showed in vitro that stimulation with a pharmacological AMPK agonist on cultured skeletal muscle cells inhibited myotube hypertrophy, and this response was attenuated in the AMPKα1/α2-double-knockdown condition. In addition, skeletal muscle-specific AMPKα1/α2 double-knockout mice exhibit higher muscle mass than the wild-type mice in normal growth conditions (37, 38). Taken together, it is suggested that AMPK is involved in the modulation of skeletal muscle mass during hypertrophic and growth conditions.

Recently, it was shown that the AMPK pathway was activated in atrophic gastrocnemius muscle of mice at the early...
stage (3 days) of hindlimb unloading (11). However, it was not revealed whether the increase of AMPK by hindlimb unloading was associated directly with the progress of muscle atrophy. Therefore, we first aimed to evaluate the potential function of AMPK in skeletal muscle atrophy in response to hindlimb unloading.

AMPK is known to modify several signaling molecules that engage protein synthesis and degradation. It is accepted that AMPK inhibits the mammalian target of rapamycin (mTOR) signaling pathway (5), which is the major signaling pathway regulating protein synthesis. Indeed, the interaction of AMPK and mTOR signaling in regulating muscle hypertrophy was reported previously (50). On the other hand, AMPK seems to control protein degradation via two major catabolic systems in skeletal muscle; the ubiquitin-proteasome (35, 52) and autophagy systems (62) are activated by pharmacological AMPK stimulation. We (13) also demonstrated previously that AMPK inhibits hypertrophy partly through 72-kDa heat shock protein (HSP72)-mediated activation of the ubiquitin-proteasome system in skeletal muscle cells. In addition, our data in the study suggested that a posttranscriptional regulation by microRNA (miRNA) might be associated with the activating process of the ubiquitin-proteasome system. The discovery of miRNAs has suggested that a posttranscriptional regulation by microRNA (miRNA) might be associated with the activating process of the ubiquitin-proteasome system. The discovery of miRNAs has provided a new aspect that could expand our knowledge to understand the mechanisms of skeletal muscle atrophy (68, 77). Therefore, we also aimed to evaluate the possible involvement of these molecules in AMPK-mediated regulation of muscle mass during hindlimb unloading.

For these purpose, we examined the alterations of muscle mass and molecular responses after 2-wk hindlimb unloading using transgenic mice that overexpress the muscle-specific dominant-negative mutant of AMPKα1 (AMPK-DN) (44). AMPK-DN mice exhibit almost complete depletion in AMPKα2 activity and moderate depletion in AMPKα1 activity (15, 32, 44, 70). We found that loss of muscle mass in slow-twitch soleus muscle but not fast-twitch gastrocnemius-plantaris (GAS-PLA) complex and extensor digitorum longus (EDL) muscles of AMPK-DN mice during hindlimb unloading was less than that of wild-type (WT) littermate mice. Furthermore, we found that the adaptive responses of the molecules related to protein degradation during hindlimb unloading were attenuated in atrophic soleus muscle of AMPK-DN mice compared with WT mice. Our findings give new insights into the molecular processes involved in the skeletal muscle adaptation under atrophic conditions.

MATERIALS AND METHODS

Animals. Transgenic (AMPK-DN) mice expressing a dominant negative mutant of AMPKα1 in the skeletal muscle (44) were obtained from the JCRB (Japanese Collection of Research Bioresources Cell Bank) Laboratory Animal Resource Bank at the National Institute of Biomedical Innovation (Osaka, Japan). This strain expresses transgene-introduced D157A mutation into cDNA encoding the full-length amino acid sequence of rat AMPKα1 subunit under the control of the human α-actin promoter on $-2,000$ bp. The heterozygous AMPK-DN mice were backcrossed with C57BL/6Ncr mice, and 24 male AMPK-DN mice (age 13.2 ± 3.2 wk, body weight 24.4 ± 1.5 g; means ± SD) and 24 WT mice (age 13.5 ± 3.5 wk, body weight 23.2 ± 2.9 g; means ± SD) were used. All mice were housed in an animal room maintained at 22–24°C with a 12:12-h light-dark cycle and fed a standard laboratory diet and water ad libitum. All animal protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Bethesda, MD) and were approved by the Animal Use Committee at Toyohashi SOZO University (A2012001, A2013003, and A2014003). All treatments of animals were performed under anesthesia with intraperitoneal injections of sodium pentobarbital (50 mg/kg), and all efforts were made to prevent discomfort and suffering.

Procedure of hindlimb unloading. Both AMPK-DN and WT mice were randomly divided into two groups: untreated preexperimental control ($n = 12$ in each group) and unloading ($n = 12$ in each group) groups. Mice in the unloading group were subjected to continuous hindlimb suspension for 2 wk. Hindlimb suspension was performed as described previously (43). Briefly, tails of the mice were cleaned and loosely surrounded by adhesive tapes cross-sectionally, fixing a string on the dorsal side of the tail, to keep the blood flow intact. The string was fastened to the roof of the cage at a height allowing the forelimbs to support the weight yet preventing the hindlimbs from touching the floor and the sides of the cage. The mice could reach food and water freely by using their forelimbs. The body weights of each mouse were recorded at the end of the experiment.

Protein synthesis measurements. Protein synthesis was measured by the surface sensing of translation method, as described previously (20). Briefly, 30 min before tissue collection, some mice ($n = 4$ in each group) were injected intraperitoneally with puromycin (0.04 μmol/g) dissolved in 100 μl of phosphate-buffered saline under anesthesia. The expression of puromycin-labled proteins was analyzed by Western blot analyses, as described below.

Tissue collection. Under anesthesia, soleus, EDL, and GAS-PLA muscles were dissected from each mouse and weighed. We analyzed each molecule in each soleus muscle dissected from each mouse. Briefly, left soleus muscle were cross-sectionally cut into halves at the midbelly region, and proximal half of the left muscles were immediately frozen in 2-methylbutane cooled with liquid nitrogen and stored at $-80°C$ for muscle fiber cross-sectional area (CSA) analyses. Distal half of the left muscles for real-time RT-PCR analyses and right muscles for Western blot analyses and AMPK assay were frozen in liquid nitrogen and stored at $-80°C$.

Western blot analyses. Sample preparation and Western blot analyses were performed with some modification of the previously reported method (14, 53). Briefly, the soleus muscles (>4 mg) were homogenized in 60 times their weight of ice-cold lysis buffer (Cel-Lytic MT; Sigma-Aldrich, St. Louis, MO) with a protease/phosphatase inhibitor cocktail (no. 5872; Cell Signaling Technology, Danvers, MA) that contained inhibitors against the major classes of endogenous proteases and phosphatases. The homogenates were then sonicated and centrifuged at 16,000 g at 4°C for 15 min. The supernatants were collected for the determination of protein contents by using the Bradford technique. Protein contents in the supernatants were >420 μg in each muscle. The supernatants were solubilized in Laemmli’s sample buffer containing mercaptoethanol and boiled. Samples (10 μg of protein) were separated by SDS-PAGE using a 7.5, 10, or 12% polyacrylamide gel. Proteins were then transferred to polyvinylidene difluoride membranes by using the Trans-Blot Turbo transfer system (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked for 1 h at room temperature in Blocking One-P (Nacalai Tesque, Kyoto, Japan) and then incubated overnight at 4°C with primary antibodies [acetyl-CoA carboxylase (ACC) Ser79 (no. 3661; Cell Signaling Technology, Danvers, MA), ACC (no. 3662; Cell Signaling Technology, Danvers, MA), heat shock transcription factor 1 (HSF1; no. 4356; Cell Signaling Technology), Akt (no. 9272; Cell Signaling Technology), forkhead box class O3a (FoxO3a) Ser253 (no. 9271; Cell Signaling Technology), Akt (no. 9272; Cell Signaling Technology), forkhead box class O3a (FoxO3a) Ser253 (no. 9466; Cell Signaling Technology), microtubule-associated protein 1 light chain 3 (LC3; no. 2775; Cell Signaling Technology), muscle RING finger 1 (MuRF1; GTX110475; Gene Tex, Irvine, CA), puromycin...
signals was quantified using ImageJ software (National Institutes of Cambridge, UK) for 1h at room temperature. After the final wash with Cell Signaling Technology), or anti-mouse IgG2a (ab97245, Abcam, in Tris buffered saline with 0.1% Tween 20 (TBS-T). The membranes performed as was described previously (81). Briefly, total RNA was performed with some modification of the previously reported (forward); miR-208b, 5'-ACTCAACACGGGAAACCTCA-5' (reverse); 18S (forward) and 5'-TGTCCTTGAATTCAGCAAGCAAAC-3' (reverse); MAFbx), 5'-ACTCACATTGCCAGGGATTTCC-3' (forward) and 5'-TGTCCTTGAATTCAGCAAGCAAAC-3' (reverse); 18S rRNA, 3'-ACTCAACAGCGGGAACCTCA-5' (forward) and 3'-AACACGAGAAATCGTCCAC-5' (reverse); miR-1, 5'-TGGAAATGTAAGAAGATGTAT-3' (forward); miR-23a, 5'-ATCACATTGCCAGGATTTC-3' (forward); miR-133a, 5'-TTGGTTGCCCCCTCAACCCGCT-3' (forward); miR-206, 5'-TGGAAATGTAAGAAGATGTATGTTG-3' (forward); miR-208b, 5'-ATAAGACGAAACAAGGTTTGT-3' (forward); and miR-499, 5'-TTAGAGCTTGACGGATGT-3' (forward). The U6 primer and reverse primers for miRNA were provided with the kit.

Isoform-specific AMPK activity assay. The AMPK activity assay was performed with some modification of the previously reported method (74). Muscles were homogenized as described in Western blot analyses, and resultant supernatants (50 μg of protein) were immunoprecipitated with isoform-specific antibodies directed against the α or α2 catalytic subunits of AMPK (kindly gifted by Dr. Licht Miyamoto) and protein A-Sepharose beads (GE Healthcare, Buckinghamshire, UK). Immunoprecipitates were washed twice in wash buffer (240 mM LiCl and 480 mM NaCl). Kinase reactions were performed in the presence of synthesized and purified SAMS peptide (HMRSAMSGLHILVRK) as AMPK substrate (74) and 40 μM HEPES (pH 7.0), 0.2 mM AMP, 80 mM NaCl, 0.8 mM dithiothreitol, and 5 mM MgCl2, 0.2 mM ATP (10 μCi of [γ-32P]ATP/sample) (Perkin-Elmer, Wellesley, MA) in a final volume of 40 μL for 20 min at 30°C. At the end of the reaction, a 15μL aliquot was removed and spotted onto Whatman P81 paper (Whatman International, Maidstone, UK). The papers were washed six times in 1% phosphoric acid and once in acetone. 32P incorporation was quantitated with a scintillation counter, and kinase activity was expressed as fold increases relative to the basal samples.

Fiber CSA analyses. To measure the CSA of individual fibers, muscle cryostat sections were stained for laminin, a major component of the basal lamina, by the standard immunohistochemical technique (33). Briefly, serial transverse cryosections (7 μm thick) of the frozen samples were cut at −20°C and mounted on the glass slides. Cross sections were fixed with paraformaldehyde (4%) and then postfixed in ice-cold methanol. After blocking by using a reagent (1% Roche Blocking Regent; Roche Diagnostics, Penzberg, Germany), samples were incubated with the primary antibodies for rabbit polyclonal anti-laminin (Z0097; Dako Cytomation, Glostrup, Denmark). Sections were also incubated with the second primary antibodies for fluorescein isothiocyanate-conjugated anti-rabbit IgG. Fiber CSA (~100 fibers/muscle) was automatically measured as the internal laminin-unstained area by using ImageJ. The relative fiber CSA to body size was normalized by body weight two-thirds power (BW0.33) according to the method of Jaric et al. (30).

Plasma corticosterone measurement. Blood samples were collected from the carotid artery into tubes containing heparin as anticoagulant, and plasma was separated by centrifugation at 8,000 g for 15 min and kept at −80°C until use. Plasma level of corticosterone was measured using an ELISA kit (Assaypro, St. Charles, MO).

Statistical analyses. Values were expressed as means ± SE. In Fig. 3A, values are expressed as means ± SD due to small numbers of animals. Statistical significance was analyzed by using two-way analysis of variance (ANOVA), with mice and treatments as main factors. If there are any significant main effects (mice and/or treatments), post hoc multiple-comparison tests were performed between the factors (WT vs. AMPK-DN and/or untreated control vs. hindlimb unloading), and if there are any significant interactions (mice × treatments), post hoc simple-effects tests were performed among four groups. Post hoc analyses were conducted with Tukey-Kramer’s test. Student’s t-test was used to compare the percentage decrease by hindlimb unloading in muscle weight between WT and AMPK-DN (Fig. 2, C and E). The differences between groups were considered statistically significant at P < 0.05. The effect size (ES) was also calculated to examine whether the effect was meaningful and practically important. We interpreted the magnitude of the ES by using conventional threshold values of 0.1 as the smallest effect, 0.3 as a moderate effect, and 0.5 as a large effect (12).

RESULTS

AMPK activity. The measurement results of AMPK activity and phosphorylation level of ACC Ser79, a marker of AMPK activity, in soleus muscle are shown in Fig. 1. Basal activity of AMPKα1 and AMPKα2 was lower by 20 (P < 0.05, ES = 0.66) and 95% (P < 0.05, ES = 0.97) in AMPK-DN mice than in WT mice, respectively (Fig. 1A). The predominant reduction of AMPKα2 activity rather than AMPKα1 activity in the transgenic mice expressing the inactive α1 mutant was corresponding with the results reported previously (15, 32, 44, 70). Hindlimb unloading did not affect either AMPKα1 (P = 0.06, ES = 0.32) or AMPKα2 (P = 0.96, ES = 0.01) activity (Fig. 1A). Basal expression level of phosphorylated ACC Ser79 was lower by 55% in AMPK-DN mice than in WT mice (P < 0.05, ES = 0.73) and was not affected by hindlimb unloading (P = 0.11, ES = 0.19; Fig. 1B). ACC expression was increased by hindlimb unloading (P < 0.05, ES = 0.37; Fig. 1B).

Plasma corticosterone level. The changes in the plasma corticosterone level, an indicator of stress status, are shown in Table 1. The plasma corticosterone level was increased in response to hindlimb unloading (P < 0.05, ES = 0.45), but
there was no difference in the levels of corticosterone between WT and AMPK-DN mice (P = 0.26, ES = 0.20; Table 1).

**Body weight and muscle mass.** The data of body weight and muscle mass are shown in Fig. 2. The overall differences in the body weight between mice were statistically significant; the body weight of AMPK-DN mice was slightly less compared with WT mice during experimental period (P < 0.05, ES = 0.23; Fig. 2A). The body weight was reduced by hindlimb unloading (P < 0.05, ES = 0.66; Fig. 2A).

Although the absolute soleus weight was reduced by hindlimb unloading (P < 0.05, ES = 0.93), the level after hindlimb unloading in AMPK-DN mice was significantly higher than in WT mice (P < 0.05, ES = 0.29; Fig. 2B). The relative soleus weight to body weight was also decreased by hindlimb unloading (P < 0.05, ES = 0.77), and the level after hindlimb unloading in AMPK-DN mice was significantly higher than in WT mice (P < 0.05, ES = 0.62; Fig. 2C). The degree of percent changes was larger in WT mice (30%) than in AMPK-DN mice (17%) (P < 0.05, ES = 0.62; Fig. 2C). Similarly, the fiber CSA of soleus muscle after hindlimb unloading was smaller than before (P < 0.05, ES = 0.89; Fig. 2D). The relative fiber CSA of soleus muscle to BW\(^{23}\) was decreased in response to hindlimb unloading (P < 0.05, ES = 0.76), and the level after hindlimb unloading in AMPK-DN mice was significantly higher than in WT mice (P < 0.05, ES = 0.63; Fig. 2E). The degree of percent changes was larger in WT mice (28%) than in AMPK-DN mice (16%) (P < 0.05, ES = 0.63; Fig. 2E).

The absolute EDL weight was reduced in response to hindlimb unloading (P < 0.05, ES = 0.49) and was lower in AMPK-DN mice compared with WT mice (P < 0.05, ES = 0.73; Fig. 2F). The relative EDL weight to body weight was lower in AMPK-DN mice than in WT mice (P < 0.05, ES = 0.56), and no change was observed by hindlimb unloading (P = 0.07, ES = 0.27; Fig. 2G). Hindlimb unloading also decreased the absolute GAS-PLA complex weight (P < 0.05, ES = 0.77) and the relative GAS-PLA complex weight to body weight (P < 0.05, ES = 0.56; Fig. 2H). Both the absolute (P < 0.05, ES = 0.53) and relative (P < 0.05, ES = 0.52) GAS-PLA complex weights were lower in AMPK-DN mice compared with WT mice (Fig. 2).

**Protein synthesis pathway.** Figure 3 shows the changes in the expression of puromycin-labeled proteins and molecules associated with protein synthesis in soleus muscle. The expression of puromycin-labeled proteins was diminished by hindlimb unloading (P < 0.05, ES = 0.61; Fig. 3A). Correspondingly, the expressions of phosphorylated Akt Ser\(^{473}\) (P < 0.05, ES = 0.57; Fig. 3C) and phosphorylated p70S6K Thr\(^{389}\) (P < 0.05, ES = 0.37; Fig. 3D) were also decreased in response to hindlimb unloading in both mice. There were no differences in these parameters between WT and AMPK-DN mice (Akt: P = 0.77, ES = 0.04; p70S6K: P = 0.63, ES = 0.11; Fig. 3, C and D). Akt (P < 0.05, ES = 0.65; Fig. 3C) but not p70S6K (P = 0.14, ES = 0.29; Fig. 3D) expression was increased by hindlimb unloading.

**Autophagy system.** The changes in the expression of proteins related to autophagy system in soleus muscle are shown in Fig. 4. Ulk1 expression was increased by hindlimb unloading in WT mice (P < 0.05, ES = 0.63), whereas it was not altered in AMPK-DN mice (P = 0.90, ES = 0.03; Fig. 4B). The expression level of LC3-I in AMPK-DN mice was same as that in WT mice (P = 0.41, ES = 0.15) and was not altered during hindlimb unloading (P = 0.17, ES = 0.25; Fig. 4C). LC3-II expression was upregulated in WT mice (P < 0.05, ES = 0.50) by hindlimb unloading but not in AMPK-DN mice (P = 0.25, ES = 0.88; Fig. 4C). The relative expression of LC3-II to LC3-I was increased in response to hindlimb unloading by eightfold in WT mice (P < 0.05, ES = 0.58) but by twofold in AMPK-DN mice (P = 0.19, ES = 0.50; Fig. 4C). p62 expression was increased by hindlimb unloading in WT mice.
In WT mice, the accumulation of ubiquitinated proteins was observed after hindlimb unloading (Fig. 5). The expression of ubiquitin-proteasome system in soleus muscle are shown in ES/H11005 0.65) but not in AMPK-DN mice (P < 0.05, ES = 0.07; Fig. 5C). The protein expression of MuRF1 was also higher after hindlimb unloading in WT mice (P < 0.05, ES = 0.46), whereas there was no alteration in AMPK-DN mice (P = 0.85, ES = 0.06; Fig. 5C). Signaling molecules associated with protein degradation systems. The changes in the expression of proteins involved in autophagy and ubiquitin-proteasome systems in soleus muscle are shown in Fig. 6. The expression of phosphorylated FoxO3a Ser253 was decreased by hindlimb unloading in WT mice (P < 0.05, ES = 0.65), whereas in AMPK-DN mice was not affected by hindlimb unloading (P = 0.30, ES = 0.14; Fig. 6B). The expression of FoxO3a was not different between the mice (P = 0.46, ES = 0.12) and was not changed by hindlimb unloading (P = 0.17, ES = 0.26; Fig. 6B). The decrease in the expression level of IκBα by hindlimb unloading was observed in WT mice (P = 0.07, ES = 0.70), whereas there was no alteration in AMPK-DN mice (P = 0.85, ES = 0.04; Fig. 6C).

Fig. 2. Changes in the body weight, soleus weight, and muscle fiber cross-sectional area (CSA) in response to hindlimb unloading. A: body weight (BW). B: soleus weight. C: relative soleus weight to BW. D: fiber CSA in soleus. E: relative fiber CSA to BW two-thirds power (BW²/³). F: extensor digitorum longus (EDL) weight. G: relative EDL weight to BW. H: gastrocnemius-plantaris (GAS-PLA) complex weight. I: relative GAS-PLA complex weight to BW. Representative images of immunofluorescence are shown. Scale bars, 20 μm. Values are means ± SE; n = 6–12/group. *Post hoc simple-effects tests following 2-way ANOVA or Student’s t-test showed that the differences were statistically significant between the indicated groups; †post hoc multiple-comparison tests following 2-way ANOVA showed that the overall differences were statistically significant between the C and HU groups.

(P < 0.05, ES = 0.71) but not in AMPK-DN mice (P = 0.59, ES = 0.12; Fig. 4D).

Ubiquitin-proteasome system. The variations in the expression of ubiquitinated protein and molecules related to the ubiquitin-proteasome system in soleus muscle are shown in Fig. 5. The accumulation of ubiquitinated proteins was observed after hindlimb unloading in WT mice (P < 0.05, ES = 0.65) but not in AMPK-DN mice (P = 0.77, ES = 0.07; Fig. 5A). MuRF1 mRNA expression was significantly increased in response to hindlimb unloading fourfold in WT mice (P < 0.05, ES = 0.77) but not in AMPK-DN mice (P = 0.28, ES = 0.32; Fig. 5B). The atrogin-1/MAFbx mRNA expression was upregulated in response to hindlimb unloading (P < 0.05, ES = 0.26; Fig. 5B). The protein expression of MuRF1 was also higher after hindlimb unloading in WT mice (P < 0.05, ES = 0.46), whereas there was no alteration in AMPK-DN mice (P = 0.85, ES = 0.06; Fig. 5C).
The IkBα expression level after hindlimb unloading was higher in AMPK-DN mice than in WT mice \((P < 0.05, ES = 0.51);\) Fig. 6C. The expression of HSF72 was constantly greater in AMPK-DN mice compared with WT mice during experiment \((P < 0.05, ES = 0.57)\) and was reduced in response to hindlimb unloading by one-half in WT mice and by 20\% in AMPK-DN mice \((P < 0.05, ES = 0.35);\) Fig. 6D. The expression of HSF1 was downregulated by hindlimb unloading \((P < 0.05, ES = 0.52);\) Fig. 6E.

miRNA. Figure 7 shows the changes in the expression of miRNAs related to skeletal muscle atrophy in soleus muscle. The expressions of miR-1 \((P < 0.05, ES = 0.85)\) and miR-23a \((P < 0.05, ES = 0.76)\) were downregulated by hindlimb unloading in WT mice, whereas those in AMPK-DN mice were not altered \((miR-1: P = 0.20, ES = 0.38; miR-23a: P = 0.64, ES = 0.13);\) Fig. 7, A and B. The expressions of miR-133a \((P < 0.05, ES = 0.55);\) Fig. 7C, miR-208b \((P < 0.05, ES = 0.64);\) Fig. 7E, and miR-499 \((P < 0.05, ES = 0.69);\) Fig. 7F were reduced in response to hindlimb unloading. There was no effect by hindlimb unloading on miR-206 expression \((P = 0.17, ES = 0.28);\) Fig. 7D.

DISCUSSION

The present study showed that the suppression of skeletal muscle-specific AMPK activity, mainly AMPKα2 activity, partially attenuated unloading-induced atrophy of slow-twitch soleus muscle along with suppressed activation of the protein degradation pathways, including the ubiquitin-proteasome system. To our knowledge, our findings are the first evidence that demonstrates the direct role of AMPK in skeletal muscle atrophy in vivo.

It is well known that diminished loading results in skeletal muscle atrophy (7). The hindlimb suspension rodent model was developed to mimic spaceflight-associated skeletal muscle atrophy. Furthermore, antigravitational slow-twitch soleus muscle has been used the most frequently in the experiment of unloading (22, 43, 71, 81) because it is comprised of more than 50\% slow-twitch fiber (78), which is more affected by reduced weight-bearing. The molecular profiling of soleus muscle in response to hindlimb suspension has significance for understanding the underlying mechanism of unloading- and disuse-associated muscle atrophy. The hindlimb suspension has also proven to be useful for investigating the physiological responses to unloading as well as disuse (47), but the suspension is a stressful procedure (48). In fact, the high corticosterone levels in plasma (Table 1) and the decrease in body weight (Fig. 2A) were observed after hindlimb unloading, although the changes in plasma corticosterone and body weight in both types of mice were identical.

When the muscle weight was normalized to body weight to correct for the loss of weight after hindlimb unloading, the relative weight of soleus (Fig. 2C) and GAS-PLA (Fig. 2F) muscles to body weight was decreased following hindlimb unloading. Notably, soleus muscle was atrophied by ∼30\% in WT mice during hindlimb unloading, whereas the deficiency of skeletal muscle AMPKα2 activity weakened the progress of atrophy by almost one-half (∼17\%; Fig. 2, C and E). In contrast, there was no difference in the degree of atrophy in...
GAS-PLA muscle between WT and AMPK-DN mice. These data indicate that AMPK, mainly AMPKα2, may be a crucial molecule regulating unloading-induced skeletal muscle atrophy, especially in slow-twitch muscle.

On the other hand, a previous study demonstrated that AMPKα1, rather than AMPKα2, is important for regulating skeletal muscle mass in overload-induced hypertrophy (50). Although we cannot exclude the involvement of AMPKα1 in the present study because AMPKα1 activity was also lower by 20% in AMPK-DN mice (Fig. 1), the different α-isofoms might play a major role in regulating skeletal muscle mass under hypertrophic or atrophic stimuli.

A recent study reported that skeletal muscle AMPK signaling was upregulated at the early stage (3 days) of hindlimb unloading and returned to the basal state at 7 days in mice (11). Correspondingly, our findings showed no activation of AMPK signaling after 2-wk hindlimb unloading (Fig. 1). Although we did not examine the time course changes of AMPK activity, it

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**Fig. 4. Changes in the autophagy system in response to hindlimb unloading.** A: representative immunoblots of Unc-51-like kinase 1 (Ulk1), microtubule-associated protein 1 light chain 3 (LC3), and p62. B: Ulk1. C: LC3. D: p62. Values are means ± SE; n = 8/group. *Post hoc simple-effects tests following 2-way ANOVA or Student’s t-test showed that the differences were statistically significant between the indicated groups.

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**Fig. 5. Changes in the ubiquitin-proteasome system in response to hindlimb unloading.** A: ubiquitinated protein. The signal intensity of full-molecular-weight ubiquitinated proteins was quantified. B: mRNA expression of muscle RING finger 1 (MuRF1) and atrogin-1/muscle atrophy F-box (MAFbx). C: protein expression of MuRF1. Representative immunoblots are shown. Values are means ± SE; n = 8/group. *Post hoc simple-effects tests following 2-way ANOVA or Student’s t-test showed that the differences were statistically significant between the indicated groups; ¶post hoc multiple-comparison tests following 2-way ANOVA showed that the overall differences were statistically significant between the C and HU groups.
Unloading-induced skeletal muscle atrophy is considered to be contributed partly by decreased protein synthesis (63). The Akt/mTOR/p70S6K pathway is well established as a central regulator of protein synthesis (45), and the negative correlation of AMPK with mTOR signaling has been shown (5). Moreover, in vivo research has demonstrated that AMPK deficiency induces overgrowth in overloaded skeletal muscle through upregulation of the mTOR signaling pathway in mice (38, 50). In the present study, however, we found that the expression of puromycin-labeled proteins, an indicator of protein synthesis (20), was downregulated in response to unloading, and this downregulation occurred independent of the AMPK activity (Fig. 3A). Although the downregulation was statistically significant (P = 0.001), there was some concern that high variability of the expression in the untreated control group of WT mice was observed (Fig. 3A) and that this experiment was performed with small sample size (n = 4/group). With regard to this point, we consider these data to be reliable because the effect size, a standardized index that is independent of sample size, was large (ES = 0.61). Moreover, the phosphorylated levels of Akt (activated Akt) and p70S6K (activated p70S6K) were suppressed by unloading in both mice (Fig. 3, B and C) as correspondence with the data of puromycin-labeled proteins. Taken together, it is suggested that AMPK-mediated modulation of skeletal muscle mass during unloading is controlled by another process from the protein synthesis pathway.

Interestingly, total Akt expression was increased following hindlimb unloading despite the phosphorylated form of Akt being decreased (Fig. 3C). Upregulation of total Akt expression by 3-wk hindlimb unloading in mouse muscle was reported previously (41). However, it has been shown that total Akt expression was not changed (67) or decreased (4) by 2-wk hindlimb unloading in rat muscle. Total Akt alterations in response to hindlimb unloading have been controversial, and thus further investigation is required on this matter.

Increased protein degradation contributes to unloading-induced atrophy in skeletal muscle (71). Autophagy is an important cell proteolytic system that controls protein turnover in skeletal muscle (42). The process of autophagy is regulated by multiple autophagy-related proteins. ULK1, also known as Atg1, is considered to be a key serine/threonine protein kinase acting at the early step of autophagosome formation (79). During autophagosome formation, LC3-I is converted to LC3-II through lipidation that allows for LC3 to become associated with autophagic vesicles. The presence of LC3 in autophagosomes and the conversion of LC3 to the lower
migrating form LC3-II have been used as indicators of autophagy activity (31). Recently, it has been reported that AMPK activation stimulates autophagosome formation in skeletal muscle cells (62), and thus it is possible to involve a modulation of the autophagy process in the AMPK-mediated regulation of protein degradation during unloading. In the present study, the expression of Ulk1 (Fig. 4B) and relative expression of LC3-II to LC3-I (Fig. 4C) were upregulated by unloading in WT mice, whereas the upregulation was attenuated in the suppression of AMPK activity. These findings indicate that AMPK mediates autophagosome formation during unloading-induced skeletal muscle atrophy.

The ubiquitin-binding protein p62, which binds to LC3, is preferentially degraded by autophagy (54), and thus breakdown of p62 is generally used as a marker of autophagy flux (46). In the present study, accumulation of p62 after hindlimb unloading was also observed in WT mice but not in AMPK-DN mice (Fig. 4D). This is consistent with the previous findings that p62 mRNA is upregulated in mouse soleus (10) and gastrocnemius muscle (11) following 3-day hindlimb unloading and that p62 protein is increased by 4-wk hindlimb unloading in mouse tibialis anterior and gastrocnemius muscle (40). Accumulation of p62 generally indicates an impairment of autophagy flux (46), but p62 hyperexpression was also observed in cancer cachexia-induced skeletal muscle atrophy despite the autophagy induction (56). In addition, a recent study reported the accumulation of p62 in atrophic muscle of aged mice (60). Although our findings indicate that AMPK modulates the expression of autophagy-related proteins during unloading-induced muscle atrophy, we cannot ascertain whether AMPK-mediated autophagy regulation is associated with the progress of muscle atrophy in response to hindlimb unloading.

The ubiquitin-proteasome system is also well known as a major protein degradation pathway (6). The key enzyme in this pathway is the E3 ubiquitin ligase, which is responsible for protein ubiquitination. The two muscle-specific ubiquitin ligases MuRF1 and atrogin-1/MAFbx have been considered to be master regulators of skeletal muscle atrophy, because these genes are upregulated in different models of muscle atrophy and have an important role in increasing protein degradation through the ubiquitin-proteasome system (3, 19). Previous studies have reported that agonist-induced activation of AMPK enhances protein degradation accompanied by increased MuRF1 and atrogin-1/MAFbx mRNA expressions in cultured myotubes (35, 52). In addition, we have demonstrated recently that pharmacological activation of AMPK upregulates MuRF1 mRNA expression, and this upregulation is abolished in AMPK-knockdown cells (13). Thus, AMPK appears to be associated with activation of the ubiquitin-proteasome system, and it is possible that AMPK regulates protein degradation through the ubiquitin-proteasome system during unloading. In the present study, the unloading-induced activation of the ubiquitin-proteasome system was attenuated in the suppression of AMPK (Fig. 5). Therefore, it is suggested that AMPK regulates ubiquitin-proteasome system-mediated protein degradation during skeletal muscle atrophy in response to unloading.

Our findings suggest a role of AMPK that regulates unloading-induced skeletal muscle atrophy through modulating protein degradation systems. In this context, there are some possible mechanisms by which AMPK activates protein degradation systems during unloading. FoxOs are transcriptional factors that regulate transcription of genes associated with skeletal muscle homeostasis, including skeletal muscle atrophy (61, 65). Previous reports have suggested that AMPK-mediated modulation of FoxO3a expression and/or nuclear translocation contributes to activation of ubiquitin-proteasome and autophagy systems in skeletal muscle cells (52, 62, 73). Thus, it is possible that AMPK regulates protein degradation systems in unloaded-associated skeletal muscle atrophy through a FoxO3a-dependent mechanism. Phosphorylation of FoxO3a at Ser253 results in exclusion from the nucleus and thereby inhibits the transcription activity (8). In the present study, the phosphorylation level of FoxO3a at Ser253 was decreased by unloading in WT mice (Fig. 6B), indicating increased FoxO3a activity. This result is consistent with previous reports (25, 65). On the other hand, no change in the expression of phosphorylated FoxO3a at Ser253 was observed during unloading in the suppression of AMPK activity (Fig. 6B), suggesting that AMPK participates in the activation of FoxO3a during skeletal muscle unloading. Therefore, FoxO3a is a possible molecule related to AMPK-mediated upregulation of protein degradation systems in response to unloading.

On the other hand, a recent study has suggested that nuclear factor-κB (NF-κB) signaling is more important than FoxO signaling in disuse muscle atrophy (80), since NF-κB sites, but not FoxO sites, are required for the transcription of MuRF1 during hindlimb unloading. NF-κB is a transcriptional factor that is sequestered in the cytoplasm by a family of inhibitory proteins called IκBα (51). The IκB kinase complex phosphorylates IκBα, resulting in its degradation, thereby leading to nuclear translocation of NF-κB and activation. It has been reported that disruption of NF-κB prevents skeletal muscle atrophy induced by hindlimb unloading (29). In the present study, the expression of IκBα tended to decrease during muscle atrophy in WT mice, and the expression was high in AMPK-DN mice compared with WT mice after hindlimb unloading (Fig. 6C). These results suggest that AMPK regulates NF-κB signaling via the expression of IκBα during unloading-associated muscle atrophy, and this might affect the different activation of the ubiquitin-proteasome system, including MuRF1 expressions. To our knowledge, this is the first report to show the association of AMPK with NF-κB signaling in muscle mass regulation.

HSP72 might be another candidate molecule involved in the regulation of AMPK-mediated protein degradation systems during unloading. HSP72 is one of the most prominent members of the HSP family and is considered to have an important role in preventing skeletal muscle atrophy (64). In the present study, it was observed that HSP72 expression in AMPK-DN mice was high and decreased less by unloading compared with WT mice (Fig. 6D). It has been reported that overexpression of HSP72 in skeletal muscle prevents immobilization-induced atrophy in the rat (66). Furthermore, a molecular mechanism of the resistance to skeletal muscle atrophy by HSP72 seems to be that HSP72 directly prevents FoxO3a activation during unloading (65, 66). We have also demonstrated previously that AMPK negatively regulates HSP72 expression in skeletal muscle cells and that HSP72 controls AMPK-mediated activation of the ubiquitin-proteasome system (13). Considering these findings, it is suggested that a high expression of HSP72 due to...
the suppression of AMPK activity is a possible mechanism that attenuates the unloading-induced activation of the protein degradation system partly through FoxO3a deactivation.

It has been unclear how AMPK regulates HSP72 expression. We obtained data showing that HSF1, a major transcriptional factor of HSPs in mammalian skeletal muscle (82), was down-regulated following hindlimb unloading in both mice (Fig. 6E), suggesting that AMPK does not modulate HSF1 expression in unloading-induced skeletal muscle atrophy. This is supported by previous data showing that pharmacological activation of AMPK did not affect HSF1 expression in skeletal muscle cells (13). Thus, we consider that AMPK-mediated regulation of HSP72 during hindlimb unloading is independent of HSF1.

There has been evidence shown that a multiple miRNAs are involved in regulation of skeletal muscle atrophy (77). We have also shown that AMPK-mediated inhibition of skeletal muscle hypertrophy is accompanied by upregulation of miR-1 (13). Therefore, we considered that miRNAs might be potent mediators of AMPK-associated regulation of muscle mass during unloading. miRNAs are short and noncoding RNA molecules ~20–25 nucleotides in length that suppress gene expressions by binding to the 3′-untranslated region of target miRNAs and either inhibit translation or promote cleavage of the transcript (1, 24). Recently, it has been shown that miR-23a posttranscriptionally suppresses MuRF1 and atrogin-1/MAFbx expression in vitro and that miR-23a overexpression in mouse skeletal muscle counteracts dexamethasone-induced muscle atrophy (76). Moreover, several reports have suggested that muscle-enriched miRNAs (miR-1, miR-133a, miR-206, miR-208b, and miR-499) are associated with modulation of skeletal muscle mass. miR-1 and miR-133a are downregulated by unloading in human skeletal muscles (57). miR-1 appears to interact with FoxO3a activity through HSP72 during dexamethasone-mediated muscle atrophy (36). miR-206 is upregulated by denervation-induced atrophy in mice skeletal muscle, and inhibition of miR-206 partially protects the atrophy (68). miR-208b (9) and miR-499 (2) can potentially repress the expression of myostatin, a well-known negative regulator of muscle growth (58). In the present study, miR-1 and miR-23a were downregulated during unloading in WT mice, but these responses did not occur in the suppression of AMPK activity (Fig. 7). Thus, these two miRNAs, miR-1 and miR-23a are potentially involved in the AMPK-mediated adaptation of muscle mass and its related molecules during unloading-induced atrophy. This is the first study to show the involvement of AMPK with miRNAs during atrophic condition, but knowledge about functions of the miRNAs during muscle atrophy is still limited and controversial. Our findings would help establish the function of miRNAs in regulating skeletal muscle mass.

In conclusion, we showed that the suppression of muscle-specific AMPK activity (mainly AMPKα2) partially attenuated unloading-induced atrophy of slow-twitch soleus muscle. The protective effect of muscle atrophy might be attributed to attenuation of the activity of the ubiquitin-proteasome-mediated protein degradation. This is supported by the alterations of signaling molecules, including FoxO3a, IκBα, HSP72, and miRNAs (miR-1 and miR-23a), although there is the limitation that these changes do not reflect the dynamics of protein degradation directly. Overall, we suggest that AMPK is required for proper adaptation of muscle mass and its related molecules during skeletal muscle unloading. Further study is expected to clarify the effect of AMPK attenuation on physiological parameters such as muscle strength for a better understanding of the role of AMPK in physiological muscle functions.

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DISCLOSURES
The authors state that there are no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

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