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Research Article

Ginsenoside compound K ameliorates palmitate-induced atrophy in C2C12 myotubes via promyogenic effects and AMPK/autophagy-mediated suppression of endoplasmic reticulum stress

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ABSTRACT

Background: Compound K (CK) is among the protopanaxadiol (PPD)-type ginsenoside group, which produces multiple pharmacological effects. Herein, we examined the effects of CK on muscle atrophy under hyperlipidemic conditions along with its pro-myogenic effects. Further, the molecular pathways underlying the effects of CK on skeletal muscle have been justified.

Methods: C2C12 myotubes were treated with palmitate and CK. C2C12 myoblasts were differentiated using CK for 4–5 days. For the *in vivo* experiments, CK was administered to mice fed on a high-fat diet for 8 weeks. The protein expression levels were analyzed using western blotting analysis. Target protein suppression was performed using small interfering (si) RNA transfection. Histological examination was performed using Jenner-Giemsa and H&E staining techniques.

Results: CK treatment attenuated ER stress markers, such as eIF2α phosphorylation and CHOP expression and impaired myotube formation in palmitate-treated C2C12 myotubes and skeletal muscle of mice fed on HFD. CK treatment augmented AMPK along with autophagy markers in skeletal muscle cells *in vitro* and *in vivo* experiments. AMPK siRNA or 3-MA, an autophagy inhibitor, abrogated the impacts of CK in C2C12 myotubes. CK treatment augmented p38 and Akt phosphorylation, leading to an enhancement of C2C12 myogenesis. However, AMPK siRNA abolished the effects of CK in C2C12 myoblasts.

Conclusion: These findings denote that CK prevents lipid-induced skeletal muscle apoptosis via AMPK/autophagy-mediated attenuation of ER stress and induction of myoblast differentiation. Therefore, we may suggest the use of CK as a potential therapeutic approach for treating muscle-wasting conditions associated with obesity.

1. Introduction

Age-related sarcopenia causes metabolic syndrome [1]. As a result of various factors, including obesity, skeletal muscle atrophy occurs when intramuscular proteolysis exceeds muscle protein synthesis [2]. Therefore, maintaining skeletal muscle mass is required to interrupt the metabolic vicious cycle. Skeletal muscle degenerative diseases are closely associated with muscle stem cell dysfunction and decline of muscle regenerative capacity [3]. Especially, obesity or excess intake of fat intake negatively influences the skeletal muscle mass and structure [4]. Low levels of type I muscle fibers and high levels of type IIb fibers have been reported in obese individuals [5]. Furthermore, animal models of obesity, such as leptin knock-out rats and ob/ob mice had skeletal muscle mass less than lean groups [6]. These reports reveal that

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diet-induced obesity possibly may induce hyperlipidemia, which may promote the development of skeletal muscle atrophy.

The endoplasmic reticulum (ER) maintains protein homeostasis in skeletal muscle cells. The accumulation of unfolded proteins in the ER or physiological stimuli can cause ER stress, resulting in unfolded protein response (UPR). UPR serves as a defense mechanism against ER stress through suppression of protein synthesis and proteolytic activation [7]. As maintaining calcium homeostasis in the sarcoplasmic reticulum lumen (ER in muscle cells) is crucial, disorders of the ER negatively affect muscle contraction. ER stress and apoptosis have been observed in the skeletal muscle of type 2 diabetes rats [8]. Moreover, several *in vitro* studies have found that palmitate treatment induces ER stress and apoptosis in C2C12 myocytes [4]. Furthermore, improvement of ER stress ameliorates palmitate-induced apoptosis in myotubes [9]. Thus, ER stress contributes to the development of muscle atrophy in obese subjects.

Compound K (CK) is among the protopanaxadiol (PPD)-type ginsenoside group producing diverse pharmacological properties, such as anti-carcinogenic [10], anti-inflammatory [11], anti-allergic [12], anti-aging [13], neuroprotective [14], and anti-diabetic [15]. These studies suggest that CK can be used for treatment of various conditions. Ginsenoside Rg1 ameliorated starvation-induced atrophy in C2C12 myotubes through repression of protein degradation regulated by the Akt/mTOR/FoxO pathway [16], whereas ginsenoside Rb1 and Rb2 induce muscle hypertrophy through Akt/mTOR signaling [17]. However, the effects of CK on lipid-induced muscle atrophy have not been addressed directly.

Here, we found that CK enhanced AMP-activated protein kinase (AMPK) phosphorylation and the autophagy pathway, preventing ER stress-medicated apoptosis in lipid-treated myotubes. Further, we found that CK stimulates the differentiation of C2C12 myoblasts. Our novel findings suggest that CK ameliorates apoptosis via AMPK/autophagy-dependent repression of ER stress and stimulation of myogenesis in myotubes under hyperlipidemic conditions. Therefore, CK might be considered as a therapeutic approach for treating obesity-induced muscle weakness and wasting.

2. Materials and methods

2.1. C2C12 cell culture and treatments

Mouse skeletal muscle cell line C2C12 (ATCC, Manassas, VA, USA) was cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), antibiotics (penicillin [100 units/mL], and streptomycin [100 μg/mL]) (Invitrogen). C2C12 myoblasts were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. **Differentiation**: C2C12 myoblasts were treated with 2% horse serum for 4–5 days. It was confirmed that there was no mycoplasma contamination in C2C12 myoblasts. All our experimental works on myoblasts were performed at passages 7–15. CK (Sigma, St Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma). Sodium palmitate (Sigma) was conjugated with 2% BSA (fatty acid-free grade; Sigma) and dissolved in DMEM. 3-methyladenine (3-MA; Sigma) was dissolved in culture media. Tunicamycin (Sigma) and 4-Phenylbutyric acid (4-PBA; Sigma) were dissolved in DMSO.

2.2. Animal experiments

This study was approved by the institutional animal review board of the Institutional Animal Care and Use Committee of Chung-Ang University, Seoul, Republic of Korea (2020–00040). **Experiment 1**: a control and two experimental groups of 8-week-old male C57BL/6J (B6) mice were fed on a normal diet (ND; Cat. No. Altromin 1324; Brogaarden, Gentofte, Denmark) or a high-fat diet (HFD composed of 60% fat and 20% carbohydrates; Cat. No. D12492; Research Diets, New Brunswick, NJ, USA) for 8 weeks. Mice of HFD + CK group were injected intravenously (tail vein) with CK at a dose rate of 1 mg/kg/day for 8 weeks. **Experiment 2**: a control and one treated group of 8-week-old male B6 mice were provided with a normal diet for 8 weeks. The mice of CK group were administered CK intravenously at a dose of 1 mg/kg/day for 8 weeks. Before treatment, CK was dissolved in a DMSO: saline (2:8, *v/v*) mixture [18]. DMSO: saline (2:8, *v/v*) mixture was used as a vehicle. The mouse skeletal muscle (gastrocnemius) was collected following the animal experiment. . All experimental mice were sacrificed under anesthesia after an overnight fasting.

2.3. Western blotting and antibodies

C2C12 muscle cells and skeletal muscle tissues were harvested, and total proteins were extracted using a lysis buffer (PRO-PREP; Intron Biotechnology, Seoul, Republic of Korea) for 60 min at 4 °C. A protein concentration of 30 μg was applied to SDS-PAGE (10 or 12%), transferred to a nitrocellulose membrane (Amersham Bioscience, Westborough, MA, USA), and probed with the indicated primary antibody, followed by a secondary antibody conjugated with horseradish peroxidase (Vector, Burlingame, CA, USA). The *signals* derived from the *protein bands* were detected using enhanced chemiluminescence (ECL) kits (BIO-RAD, Hercules, CA, USA). Anti-IRE-1 (Cat. No. sc-390960; 1:2,500), anti-phospho Akt (Cat. No. sc-7985; 1:1,000), anti-Akt (Cat. No. sc-81434; 1:1,000), anti-MyoD (Cat. No. sc-377460; 1:1,000), anti-myogenin (Cat. No. sc-12732; 1:1,000), anti-MHC (Cat. No. sc-59199; 1:1,000), anti-CHOP (Cat. No. sc-7351; 1:1,000), anti-ATF6 (Cat. No. sc-22799; 1:1,000), anti-MyH7 (Cat. No. sc-53089; 1:1,000), anti-MyH4 (Cat. No. sc-32732; 1:1,000), anti-atrogin-1 (Cat. No. sc-166806; 1:1,000), anti-MuRF1 (Cat. No. sc-398608; 1:1,000), anti-myostatin (Cat. No. sc-393335; 1:1,000), and anti-beta actin (Cat. No. sc-47778; 1: 5,000) were secured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho eIF2α (Cat. No. #9721; 1:1,000) and anti-cleaved caspase 3 (Cat. No. #9664; 1:1,500) antibodies (Cell Signaling Technology, Beverly, MA, USA) were used. Anti-phospho IRE-1 (Cat. No. ab48187; 1:2,500) antibody was procured from Abcam (Cambridge, MA, USA). The dilution factors used for Western blotting analysis are shown in parentheses next to the Cat. No.

2.4. Transfection for gene knock-down

Small interfering (si) RNA oligonucleotides (20 nmol/L) for AMPKα1 (Cat. No. sc-29674) were acquired from Santa Cruz Biotechnology. To silence gene expression, cell transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions.

2.5. Jenner-Giemsa staining

C2C12 skeletal muscle cells were washed with PBS and fixed with absolute methanol for 10 min. The Jenner and Giemsa staining solutions were diluted with distilled water (1:1 and 1:9, respectively). Cells were incubated with diluted Jenner staining solution for 20 min and washed twice with PBS. Next, they were stained for 60 min and washed twice with PBS. The images of newly formed myotubes were captured using a digital camera adapted to an inverted microscope; they can be identified by a darker brown color.

2.6. Histopathological analysis

Mouse skeletal muscle sections were collected and embedded in paraffin after fixation with 10% paraformaldehyde for 1 week. H&E

staining was then performed to detect myotubes in skeletal muscle tissue of mice, by Labcore (Seoul, Republic of Korea).

2.7. Statistical analyses

The SPSS/PC statistical program (version 12.0 for Windows; SPSS, Chicago, IL, USA) was used for all statistical analyses. Results are expressed as the fold of the highest value (mean ± SEM). *In vitro* experiments were performed at least three times. Student's t-test or one-way ANOVA were used to determine statistical significance.

3. Results

3.1. CK alleviates skeletal muscle apoptosis in vitro and in vivo under hyperlipidemic conditions

First, cell toxicity induced by CK was examined. Treatment with CK at a dose rate of 100 μM for 24 h dramatically decreased cell viability in C2C12 myotubes assessed by MTT (Fig. S1). Muscle apoptosis likely contributes to muscle atrophy [19]. To examine the effects of CK on myotubes under hyperlipidemic conditions, differentiated C2C12 cells were treated with 350 μM palmitate and various concentrations (0–20 μM) of CK for 24 h. To quantify the extent of myotube formation, the thickness of Jenner-Giemsa-positive myotubes was measured [20] using Image J software. Treatment of C2C12 myotubes with palmitate decreased the myotube thickness and increased apoptosis (that was assessed by MTT assay and caspase 3 activity assay) compared to control. CK treatment reversed dose-dependently the palmitate-induced changes (Fig. 1A and B). Decreased myotube thickness and upregulated cleaved caspase 3 expression and caspase 3 activities were detected in skeletal muscle of HFD-fed mice. However, similar to *in vitro* results, the addition of CK reversed all these changes in HFD-fed mice (Fig. 1C and D). Significant decreases in the expression of MyH4, as a marker for *type IIb "fast twitch" fibers,* was observed in skeletal muscle of HFD-fed mice. However, CK treatment reversed this change (Fig. 1E). CK administration did not affect the expression of MyH7 (a marker for type I "slow-twitch" fibers) in skeletal muscle of HFD-fed mice (Fig. 1E).

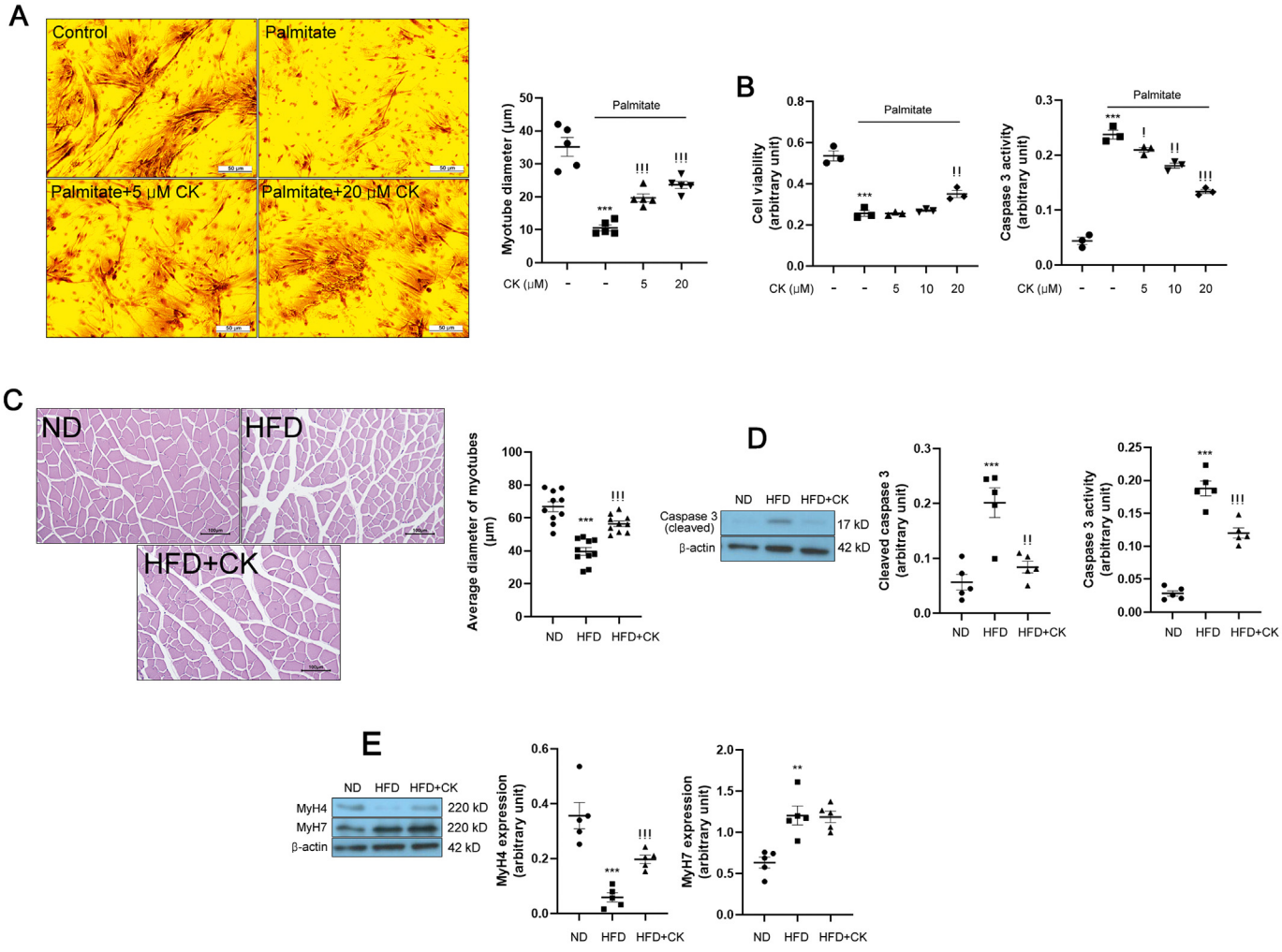

Fig. 1. CK treatment attenuates apoptosis and impairment of myogenic differentiation in myotubes and mouse skeletal muscle under hyperlipidemic conditions. Jenner-Giemsa staining (A), as well as the MTT assay and caspase 3 activity assay (B) of C2C12 myotubes treated with palmitate (200 μM) and CK (0–20 μM) for 24 h. The myotube diameter levels were analyzed using image J. (C) H&E staining of skeletal muscle of experimental mice. (D) Western blotting of cleaved caspase 3, as well as caspase 3 activity assay of skeletal muscle of experimental mice treated with HFD and/or CK for 8 weeks. (E) Western blotting of myH4 and myH7 of skeletal muscle of experimental mice treated with HFD and/or CK for 8 weeks. The results are presented as the mean ± SEM, calculated from three independent *in vitro* experiments and from five or seven independent *in vivo* experiments. ***P < 0.001 and **P < 0.01 compared to control or ND. $^{!!!}P < 0.001$, $^{!!}P < 0.01$, and $^{!}P < 0.05$ compared to palmitate or HFD treatment.

3.2. AMPK/autophagy signaling involves in the inhibitory effects of CK on myotube ER stress-induced apoptosis

Palmitate causes ER stress and apoptosis in various cell types, including myotubes [21]. Therefore, we then investigated the effects of CK on ER stress in palmitate-treated myotubes. We found that palmitate-induced ER stress markers, such as IRE-1 and eIF2α phosphorylation and cleaved ATF6 and CHOP expression, were dose-dependently suppressed using CK treatment in C2C12 myotubes (Fig. 2A). Moreover, CK treatment attenuated tunicamycin-induced eIF2α phosphorylation and cleaved ATF6 and CHOP expression (Fig. S2). In line with *in vitro* results, CK treatment also alleviated eIF2α phosphorylation, cleaved ATF6, and CHOP expression in skeletal muscle of HFD-fed mice (Fig. 2B). It was documented that AMPK attenuates ER stress via autophagy-dependent signaling [22]. Thus, we next examined whether CK treatment affect levels of AMPK phosphorylation and autophagy in C2C12 myotubes and mouse skeletal muscle. Treatment with CK dose-dependently augmented the AMPK phosphorylation and induced the levels of autophagy assessed by LC3 conversion and the degradation of p62 as along with autophagosome stained by MDC (Fig. 2C and D). To verify the role of AMPK or autophagy in CK-mediated effects, siRNA transfection was performed to inhibit the expression of AMPK or 3-MA, an inhibitor of autophagy treatment.

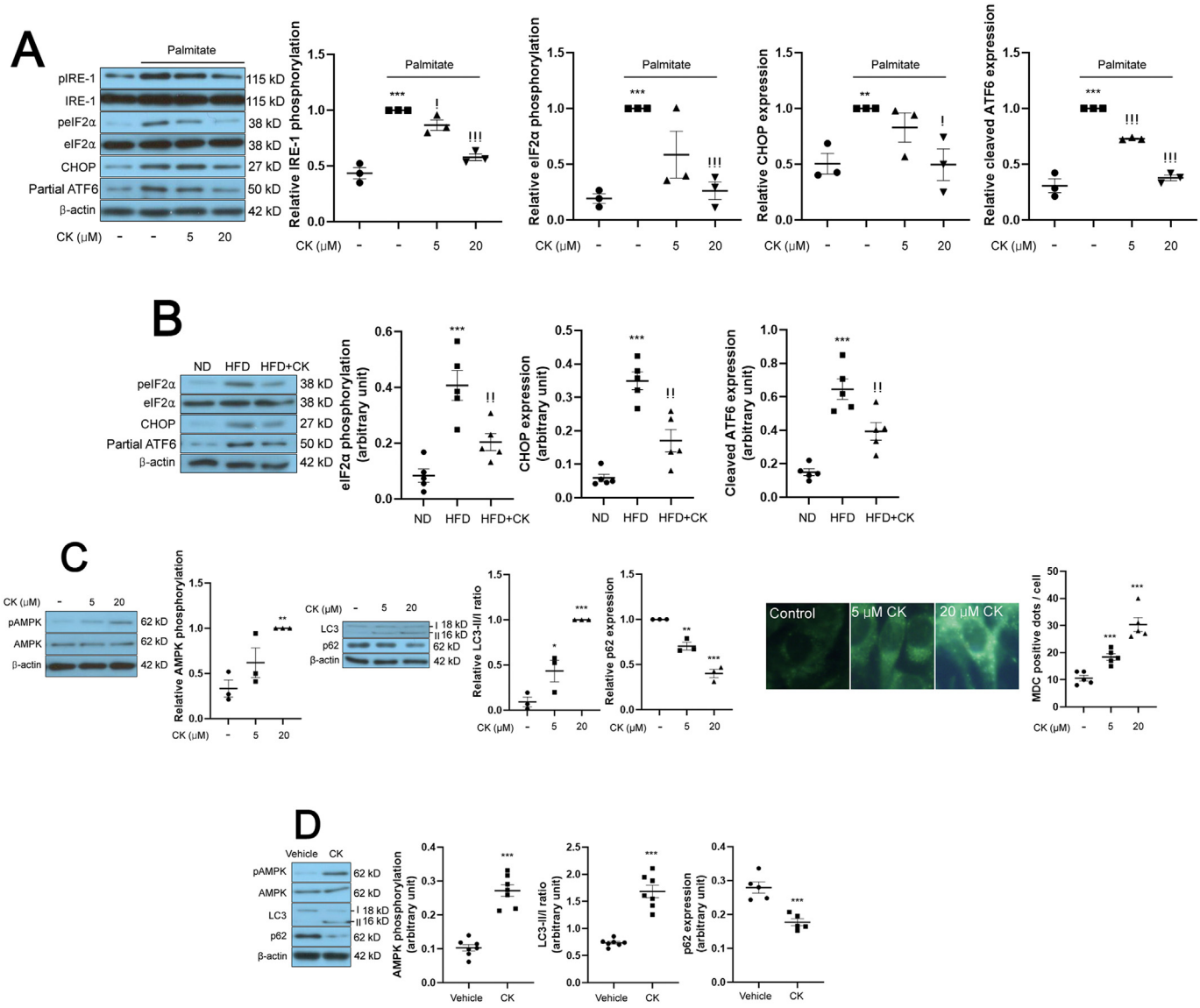

Fig. 2. CK treatment alleviates ER stress in skeletal muscle through AMPK/autophagy-mediated signaling under hyperlipidemic conditions. (A) Western blotting of phosphorylation of IRE-1 and eIF2α, as well as partial ATF6 and CHOP expression of C2C12 myotubes treated with palmitate (200 μM) and CK (0–20 μM) for 24 h. (B) Western blotting of eIF2α phosphorylation, partial ATF6, and CHOP expression in skeletal muscle of experimental mice treated with HFD and/or CK for 8 weeks. (C) Western blotting of AMPK phosphorylation, LC3, and p62 expression as well as MDC staining of C2C12 myotubes treated with CK (0–20 μM) for 24 h. (D) Western blotting of AMPK phosphorylation, as well as LC3 and p62 expression in skeletal muscle of experimental mice treated with CK for 8 weeks. (E) Western blotting of phosphorylation of IRE-1 and eIF2α, as well as partial ATF6 and CHOP expression of C2C12 myotubes treated with palmitate, CK (20 μM), and AMPK siRNA (20 nM) for 24 h. (F) Western blotting of phosphorylation of IRE-1 and eIF2α, as well as partial ATF6 and CHOP expression of C2C12 myotubes treated with palmitate, CK (20 μM), and 3-MA (5 mM) for 24 h. MTT assay and caspase 3 activity assay (G), as well as Jenner-Giemsa staining (H) of C2C12 myotubes treated with palmitate, CK (20 μM), AMPK siRNA (20 nM), and/or 3-MA (5 mM) for 24 h. (I) Western blotting of LC3 and p62 of C2C12 myotubes treated with CK (20 μM) and AMPK siRNA (20 nM) for 24 h. The results are presented as the mean ± SEM, calculated from three independent *in vitro* experiments and from five or seven independent *in vivo* experiments. ***P < 0.001, **$P < 0.01$, and *$P < 0.05$ compared to the control or ND. $^{!!!}P < 0.001$, $^{!!}P < 0.01$, and $^{!}P < 0.05$ compared to palmitate, HFD, or CK treatment. $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared to CK + palmitate treatment.

AMPK siRNA or 3-MA abrogated the effects of CK on palmitate-induced ER stress (Fig. 2E and F) and apoptosis (Fig. 2G) in C2C12 myotubes. Moreover, the effect of CK on palmitate-induced suppression of myotube formation was abolished by AMPK siRNA or 3-MA (Fig. 2H). As AMPK directly promotes autophagy via ULK1-dependent signaling pathway [23], we confirmed whether autophagy is involved in AMPK-mediated signaling. We found that AMPK siRNA suppressed CK-induced LC3 conversion and p62 degradation as shown in Fig. 2I.

3.3. CK suppresses palmitate-induced atrogin-1 expression via myostatin signaling, but not ER stress signaling

Proteolytic pathway plays a pivotal role in maintaining muscle mass [2]. Therefore, we inspected the effects of CK on the expression of E3 ubiquitin ligases and their upstream regulator, myostatin. In line with a previous report [24], palmitate up-regulated the expression of atrogin-1 and MuRF1 as along with myostatin (Fig. 3A). The increase in expression levels of atrogin-1, MuRF1, and myostatin were observed in skeletal muscle of HFD-fed mice (Fig. 3B). However, CK treatment reversed these changes (Fig. 3A and B). Unexpectedly, 4-PBA, an ER stress inhibitor, did not affect the expression levels of atrogin-1 and MuRF1 as well as myostatin in palmitate-treated C2C12 myotubes (Fig. 3C).

3.4. CK treatment enhances C2C12 myotube thickness

Because myogenesis plays a principal role in wasting and maintaining muscle mass [25], we next assessed whether CK affects C2C12 myotube thickness. Treatment of C2C12 myoblasts with CK increased the thickness of myotubes in a dose-dependent manner (Fig. 4A). Because MyoD (myoblast determination protein 1) and myogenin play a crucial role in myoblast differentiation and MHC has been identified as a specific marker of late stage myoblast

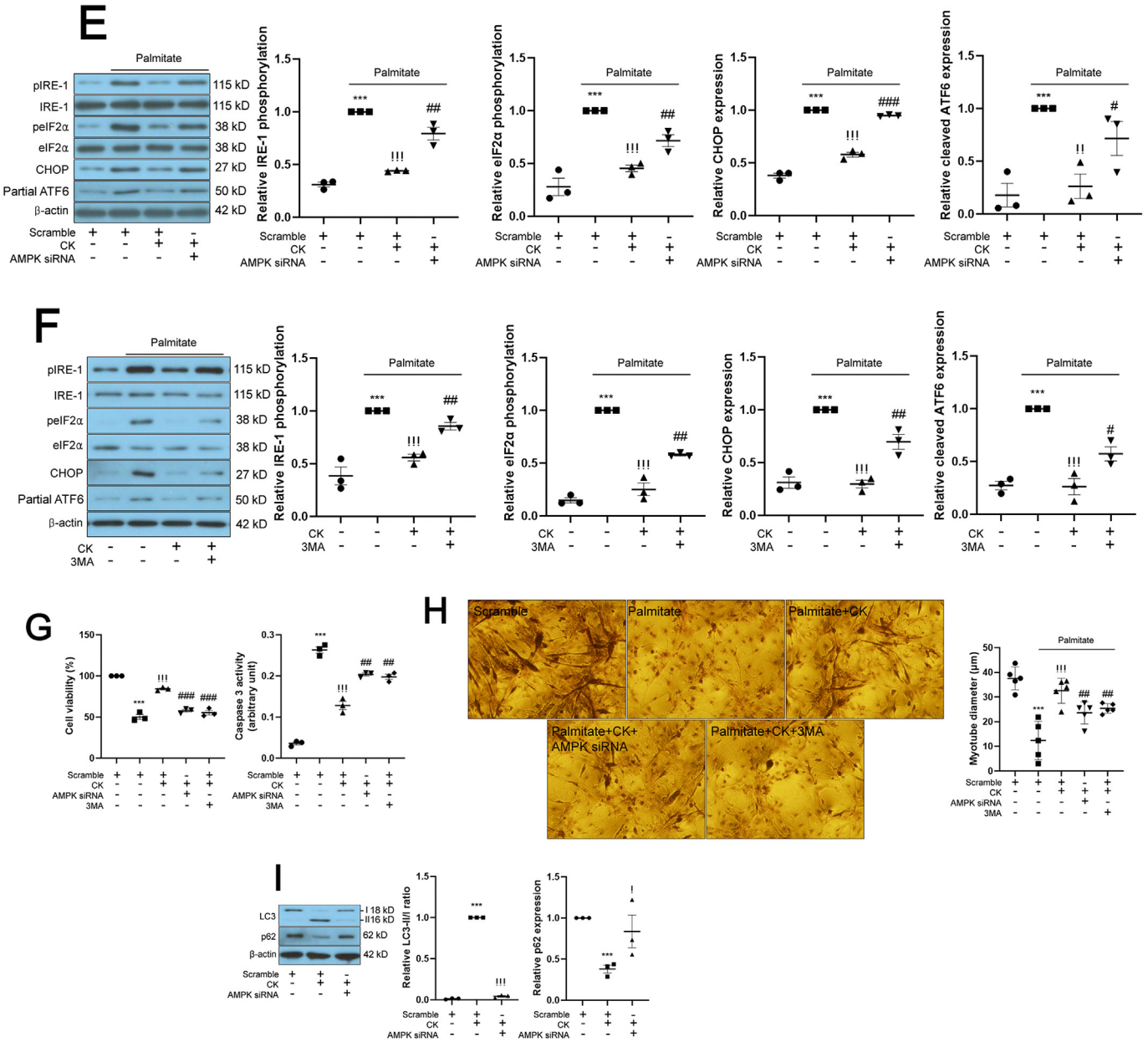

Fig. 2. (*continued*).

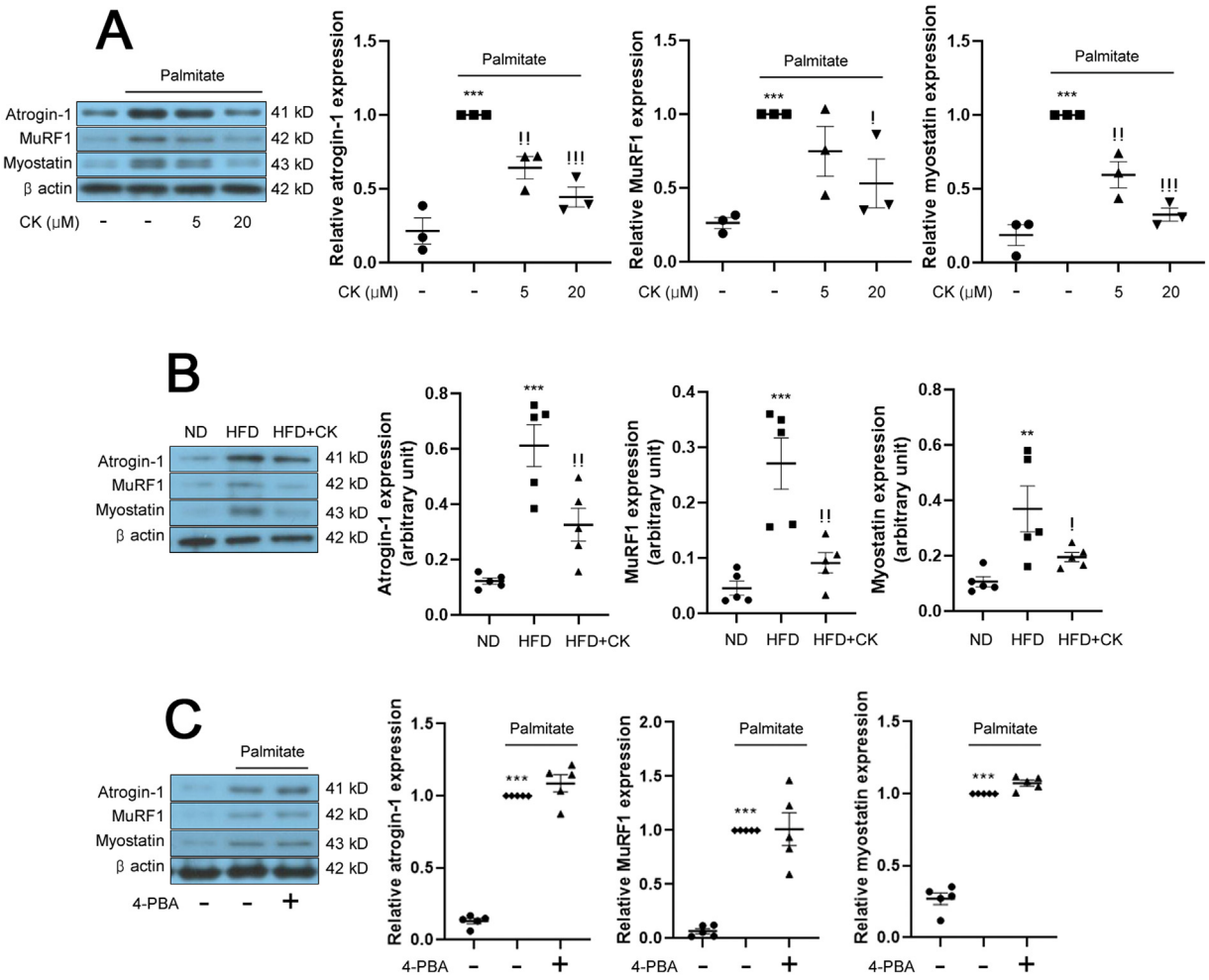

Fig. 3. CK treatment ameliorates proteolytic degradation in skeletal muscle cells under hyperlipidemic conditions in an ER stress-independent manner. (A) Western blotting of atrogin-1, MuRF1, and myostatin expression of C2C12 myotubes treated with palmitate (200 μM) and CK (0–20 μM) for 24 h. (B) Western blotting of atrogin-1, MuRF1, and myostatin expression in skeletal muscle of experimental mice treated with HFD and/or CK for 8 weeks. (C) Western blotting of atrogin-1, MuRF1, and myostatin expression of C2C12 myotubes treated with palmitate (200 μM) and 4-PBA (2 mM) for 24 h. The results are presented as the mean ± SEM, calculated from three or five independent *in vitro* experiments and from five independent *in vivo* experiments. ***$P < 0.001$ and **$P < 0.01$ compared to control or ND. $^{!!!}P < 0.001$, $^{!!}P < 0.01$, and $^{!}P < 0.05$ compared to palmitate or HFD treatment.

differentiation [26], we then determined the effects of CK on the expression of muscle-specific markers, such as MyoD, myogenin, and MHC in myoblasts. Treatment with CK t dose-dependently upregulated the expression of MyoD, myogenin, and MHC (Fig. 4B). Consistent with the *in vitro* results, CK treatment also upregulated the expression of MyoD, myogenin, and MHC in mouse skeletal muscle (Fig. 4C). However, significant induction of myotube thickness via CK treatment was not detected (Fig. 4D).

3.5. Akt and p38 mainly contribute to CK-mediated induction of myogenesis

Akt and p38 MAPK enhance myogenic differentiation of myoblasts [27,28]. To explore the CK-mediated molecular signaling in promyogenic effect, C2C12 myoblasts were treated with various concentrations (0–20 μM) of CK for 24 h and were evaluated for p38 and Akt phosphorylation. CK treatment augmented dose-dependently p38 and Akt phosphorylation (Fig. 5A). In accordance with the results from *in vitro* experiments, administration of CK enhanced p38 and Akt phosphorylation in mouse skeletal muscle (Fig. 5B). Each inhibitor of Akt or p38 abolished the effects of CK on the expression of MyoD, myogenin, and MHC in C2C12 myoblasts (Fig. 5C). AMPK has a promyogenic effect [29]. Thus, we further tested the role of AMPK in CK-stimulated myogenesis in C2C12 myoblasts. AMPK siRNA mitigated the CK-induced myotubes thickness (Fig. 5D). Interestingly, AMPK siRNA selectively suppressed CK-induced myogenin expression, whereas it does not affect the expression of MHC and MyoD (Fig. 5E).

4. Discussion

ER stress has been reported to contribute to the development of obesity-mediated muscle atrophy [30], which is closely associated with metabolic disorders, such as insulin resistance [31]. Therefore, it is important to find potential substances that can be used properly to attenuate ER stress or maintain the skeletal muscle mass through induction of myogenesis. Here, for the first time, we found that CK attenuates fatty acid-induced muscle atrophy through AMPK/autophagy-associated inhibition of ER stress and induction of myogenesis using *in vitro* and *in vivo* experimental models. We demonstrated the following novel findings: 1) treatment of C2C12 myotubes with CK suppressed palmitate-induced ER stress by activating AMPK/autophagy-dependent signaling, thereby attenuating myotube apoptosis; 2) treatment of C2C12 myotubes with CK

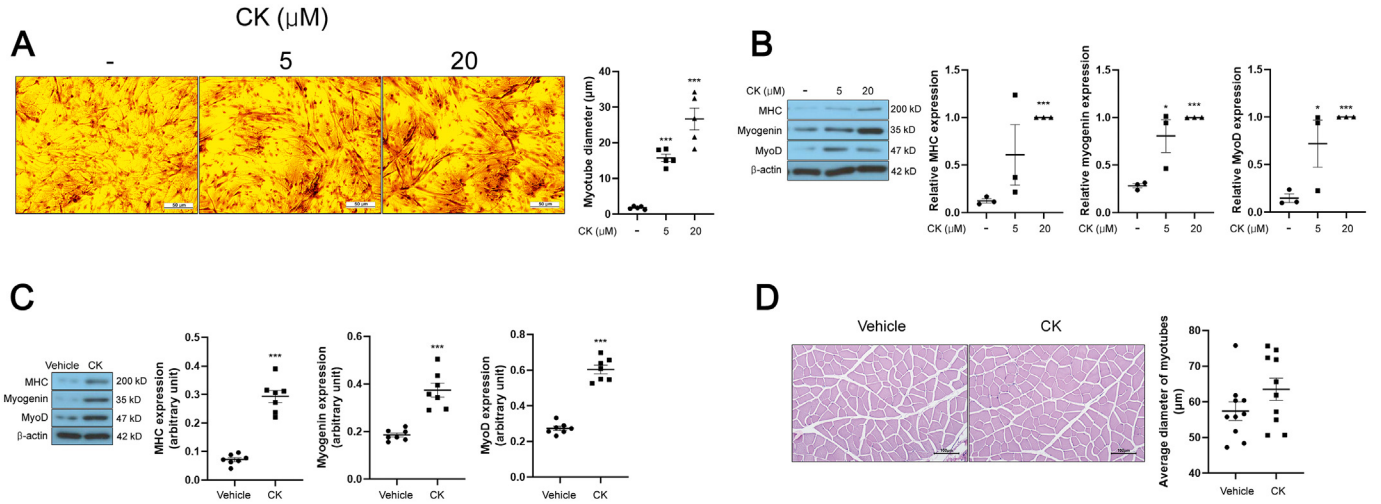

Fig. 4. CK treatment promotes myogenic differentiation in myoblasts. Jenner-Giemsa staining (A) as well as western blotting analysis of MHC, myogenin, and MyoD (B) of C2C12 myoblasts treated with CK (0–20 μM) for 48 h. Western blotting of MHC, myogenin, and MyoD (C), as well as H&E staining (D) of skeletal muscle of experimental mice treated with CK for 8 weeks. The results are expressed as the mean ± SEM, calculated from three independent *in vitro* experiments and from five or seven independent *in vivo* experiments. ***P < 0.001 and *P < 0.05, compared to control or vehicle.

upregulated Akt and p38 phosphorylation, leading to the formation of myotubes; 3) similar to the *in vitro* results, CK administration augmented Akt and p38 phosphorylation, resulting in thickening of myotubes. Further, CK administration enhanced AMPK phosphorylation and autophagy, thereby ameliorating obesity-induced ER stress and atrophy in mouse skeletal muscle.

AMPK plays a central role in maintaining energy homeostasis and ameliorating ER stress [32]. It was reported that activation of AMPK by AICAR, a AMPK activator, alleviates ER stress in human bronchial cells via FoxO1-mediated induction of the expression of ER chaperone ORP150 [33]. Moreover, Jung et al. have reported that AMPK activation by CTRP9 attenuates ER stress via autophagy-dependent pathway in palmitate-treated hepatocytes [34]. At variance, exacerbated ER stress was confirmed in endothelial cells from AMPK-deficient mice [35]. AMPK has been documented to be activated by several ginsenosides in various cell types. Rg3 suppresses 3T3-L1 adipocyte differentiation [36] and lipid accumulation in hepatocytes [37] through AMPK-mediated signaling. In this occasion, Zhang et al. have reported that Rg1 ameliorates lipid accumulation and inflammation in palmitate-treated hepatocytes via AMPK/NFκB signaling [38]. Rb1 treatment attenuates hypoxia-induced apoptosis in cardiomyocytes [39] and hepatic steatosis in HFD-fed rats [40] through an AMPK-mediated pathway. Treatment of hepatocytes with Rb2 activates AMPK, leading to suppression of ER stress-mediated gluconeogenesis [41]. Hwang et al. have demonstrated that CK ameliorated glucose intolerance and hepatic steatosis via activation of AMPK in animal models of type 2 diabetes [42]. In addition, CK-mediated AMPK activation suppresses lipid accumulation in hepatocytes [43]. Therefore, we hypothesized that CK, as an AMPK activator, can block ER stress-induced apoptosis in myotubes under hyperlipidemic conditions. We found that treatment of C2C12 myotubes with CK dose-dependently augmented AMPK phosphorylation. Moreover, CK administration increased AMPK phosphorylation in mouse skeletal muscle. Elevated ER stress responses and apoptosis in skeletal muscle cells under hyperlipidemic conditions were reversed via CK treatment both in *in vitro* and *in vivo* models. siRNA for AMPK mitigated the effects of CK on ER stress and apoptosis. These results reveal that AMPK markedly participates in CK-mediated protection against hyperlipidemia-induced ER stress, resulting in inhibition of apoptosis in muscle cells.

Autophagy is a physiological process participating in the degradation and recycling of excessive or misfolded proteins and damaged organelles through autophagosome-lysosome fusion, contributing to cell survival [44]. This cellular process has been well-reported as a cellular defense mechanism to ameliorate ER stress [45]. Rapamysin, an mTOR inhibitor, ameliorates ER stress through activation of autophagy in palmitate-treated adipocytes [46]. Chang et al. have demonstrated that intermittent hypoxia-induced autophagy alleviates ER stress and apoptosis in cardiomyocytes [47]. Herein, we found that CK induces LC3 conversion and p62 degradation in a dose-dependent manner. Furthermore, 3-MA treatment mitigates the effects of CK against palmitate-induced ER stress and apoptosis in C2C12 myotubes. Similar to *in vitro* results, CK administration increased autophagic markers in mouse skeletal muscle. 3-MA exposure abrogated the effects of CK on ER stress and apoptosis in palmitate-treated C2C12 myotubes. Consistently with previous reports, AMPK has been involved in CK-mediated induction of autophagy [48,49]. These results suggest that AMPK-regulated autophagy plays a key role in CK-dependent suppression of skeletal muscle ER stress and apoptosis under hyperlipidemic conditions.

We further investigated whether CK affects skeletal muscle proteolysis under hyperlipidemic conditions. CK treatment alleviated atrogin-1 and MuRF1 as well as myostatin expression in palmitate-treated C2C12 myotubes and skeletal muscle of HFD-fed mice. However, 4-PBA did not change CK-mediated repression of these proteins expression. These results suggest that CK attenuates obesity-related ER stress independent proteolytic degradation in skeletal muscle. Since TNFα augments expression of myostatin, which enhances proteolysis in C2C12 myotubes [50], inflammation, but not ER stress is involved in palmitate-induced atrogin-1, MuRF1, and myostatin expression.

Interestingly, CK treatment reversed the suppressed MyH4 expression. However, CK treatment did not affect HFD-induced MyH7 expression in mouse skeletal muscle. Since there is a possibility that CK may not affect myogenesis in type I “slow-twitch” fibers, further studies should be performed regarding the fiber type selectivity of CK.

Myogenesis plays a central role in the onset of muscle wasting or maintenance of muscle mass [25]. Therefore, we then evaluated the effects of CK on myogenesis in C2C12 myotubes. We found that treatment of C2C12 myotubes with CK enhanced the

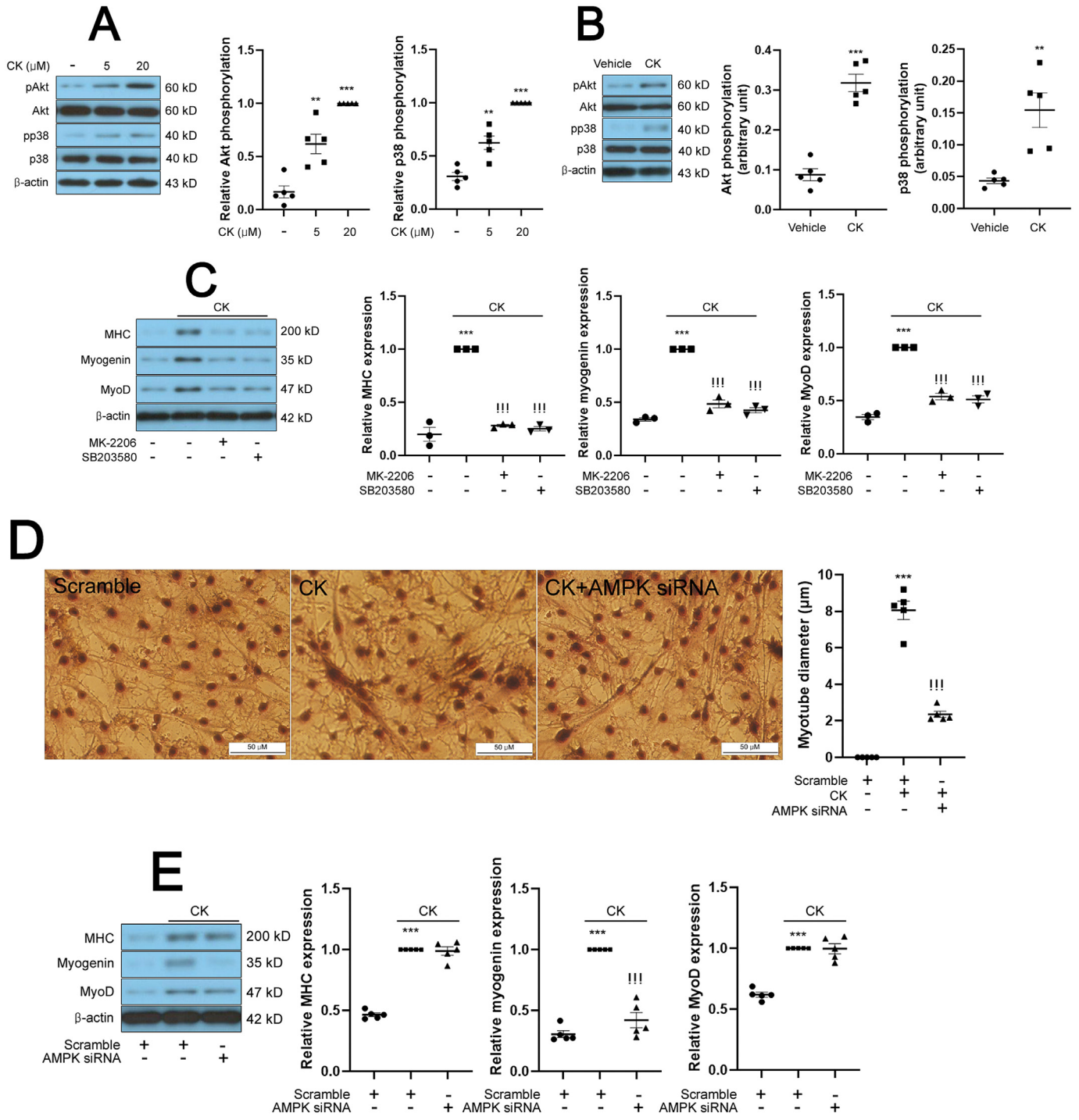

Fig. 5. p38 and Akt principally contribute to CK-mediated promyogenic effect. Western blotting of phosphorylation of p38 and Akt of C2C12 myoblasts treated with CK (0–20 μM) for 24 h (A) and skeletal muscle of experimental mice treated with CK for 8 weeks (B). (C) Western blotting of MHC, myogenin, and MyoD of C2C12 myoblasts treated with CK (20 μM), MK-2206 (10 μM), and/or SB203580 (10 μM) for 24 h. Jenner-Giemsa staining (D) and Western blotting of MHC, myogenin, and MyoD (E) of C2C12 myoblasts treated with CK (20 μM), and AMPK siRNA (20 nM) for 48 h. The results are expressed as the mean ± SEM, calculated from three independent *in vitro* experiments and from five or seven independent *in vivo* experiments. ***P < 0.001 and **P < 0.01 compared to control or vehicle. $^{!!!}P < 0.001$ compared to CK treatment.

phosphorylation of p38 and Akt, known to promote myogenic differentiation [51,52]. The expression profiles of various myogenic markers, such as MyoD, MHC, and myogenin, as well as myotubes thickness, were elevated using a CK treatment. These results suggest that CK exhibits a promyogenic effect through p38 and Akt-mediated signaling.

In this study, CK ameliorated palmitate-induced skeletal muscle apoptosis and stimulated myogenesis in a concentration-dependent manner. Therefore, these results denote that there is a correlation between the myogenic effect and the anti-muscle atrophic effect of CK. Herein; we confirmed that AMPK is uniquely associated; however, further studies are required to justify this issue.

Until now, the clinical doses of CK to improve *obesity*-induced *muscle atrophy* are still largely unknown. In upcoming studies, pharmacokinetics should be carried out in clinical practices to

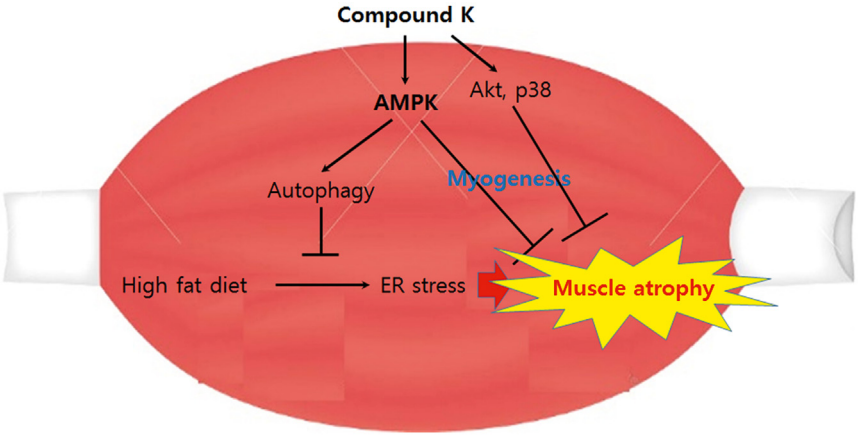

Fig. 6. Schematic diagram of CK effects on muscle atrophy under hyperlipidemic conditions.

optimize the oral dose. This should be conducted in reference to CK serum levels obtained after administration of 1 mg/kg to mouse tail vein for 8 weeks in the current study.

Obesity decreases AMPK activity and exacerbates myogenesis [53]. Further, Fu et al. have declared that AMPK participates in enhancing myogenesis and muscular regeneration [29]. Therefore, we further examined the role of AMPK in CK-associated induction of myogenic markers. AMPK siRNA mitigated the effect of CK on myogenin expression, which is in line with others [29]. However, AMPK siRNA did not influence the expression of MyoD and MHC. These results imply that CK enhances myogenesis through AMPK and unknown molecules. Further studies are needed to clarify this issue.

5. Conclusion

We demonstrated that CK ameliorates muscle ER stress and apoptosis via an AMPK/autophagy-associated pathway and stimulates myogenesis, thereby preventing muscle atrophy under hyperlipidemic conditions (Fig. 6). Our novel results suggest that CK-mediated modulation of lipid-induced ER stress and induction of myogenesis can be used as an effective therapeutic approach for treating obesity-mediated muscle atrophy.

Author contributions

TJK: Conceptualization, Methodology, Investigation; DHP: Conceptualization, Methodology, Investigation; JHJ: Conceptualization, Writing-Review & Editing; TWJ:Conceptualization, Investigation, Writing-Review & Editing, Data Curation, Formal analysis, Funding acquisition; MJK: Investigation; AMA: Writing-Review & Editing. . AMA, JHJ, and TWJ are responsible for the integrity of the work.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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