Original Article

3-(4-Hydroxy-3-methoxyphenyl) propionic acid mitigates dexamethasone-induced muscle atrophy by attenuating Atrogin-1 and MuRF-1 expression in mouse C2C12 skeletal myotubes

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(Received 11 August, 2023; Accepted 24 June, 2024; Released online in J-STAGE as advance publication 25 July, 2024)

3-(4-Hydroxy-3-methoxyphenyl) propionic acid is an *in vivo* metabolite of 4-hydroxy-3-methoxycinnamic acid which is abundantly found in coffee bean, rice bran, fruits, and vegetables. Previous studies reported that polyphenols and their metabolites exhibit positive effects on muscle health. Thus, the effect of 3-(4-hydroxy-3-methoxyphenyl) propionic acid on muscle atrophy induced by dexamethasone was investigated using mouse C2C12 skeletal myotubes. Dexamethasone treatment (10 μM) reduced the diameter and myosin heavy chain protein expression in C2C12 myotubes; it also increased muscle atrophy-associated ubiquitin ligases, such as muscle atrophy F-box protein 1/Atrogin-1 and muscle ring finger protein-1, along with their upstream regulator Krüppel-like factor 15. Dexamethasone dephosphorylated FoxO3a transcription factor and increased total FoxO3a expression. Interestingly, 10 μM 3-(4-hydroxy-3-methoxyphenyl) propionic acid treatment significantly attenuated dexamethasone-induced reduction in myotube thickness and muscle protein degradation and suppressed muscle atrophy-associated ubiquitin ligases. 3-(4-Hydroxy-3-methoxyphenyl) propionic acid also prevented dexamethasone-induced Krüppel-like factor 15 and FoxO3a expression. In conclusion, these results suggest that *in vivo* metabolite of polyphenols per se could be the real origin of the anti-muscular atrophy activity, as 3-(4-hydroxy-3-methoxyphenyl) propionic acid ameliorated glucocorticoid-induced muscle atrophy by suppressing Atrogin-1 and MuRF-1.

***Key Words*:** **muscle atrophy, 3-(4-hydroxy-3-methoxyphenyl) propionic acid, mouse C2C12 skeletal myotubes, ubiquitin ligases, glucocorticoids**

Commonly known as ferulic acid, 4-hydroxy-3-methoxycinnamic acid (HMCA) is a hydroxycinnamic acid-derived polyphenol that is most abundantly present in coffee beans, rice bran, cereals, nuts, fruits, and vegetables.[1] Various studies reported that HMCA contributes to the improvement of metabolic abnormalities, cardiovascular diseases, hyperlipidemia, bone remodeling, dyslipidemia, and neurodegenerative conditions.[2,3] It also attenuates myopathic abnormalities in rats.[4] Moreover, 3-(4-hydroxy-3-methoxyphenyl) propionic acid (HMPA) is a metabolite of HMCA produced when metabolized by the gut microbiota.[5] It is also found in cocoa and cocoa products, coffee, tea, fruits, legumes, and nuts.[6] HMPA shows potent antioxidant, anti-inflammatory, anti-obesity, hepatoprotective, neuroprotective, and antidiabetic effects.[5,7,8] Coffee, chlorogenic acid or HMCA are extensively metabolized into HMPA and detected in cecum, urine, and plasma after their intake by rodents.[5,7] A previous study reported that the anti-obesity effect of HMCA is ascribable to its metabolite HMPA which was absorbed into the body and showed lipid metabolizing and insulin sensitivity activity.[5] The same study found that; feeding 1% HMCA in food to the mice for 4 weeks produces a vast proportion of HMPA that was found in cecum and urine. The above facts suggest that HMCA fed orally could metabolize into HMPA in the intestine by gut microbiota which is bioavailable to the body. Therefore, HMPA could be a potential therapeutic substance in health and disease. Although the pharmacokinetics[9] and pharmacodynamic effects of HMPA were investigated in various disease models, but its role in attenuating muscle atrophy has not yet been revealed.

Glucocorticoids are immunomodulatory drugs generally used in treating inflammatory diseases.[10] However, their higher dose or prolonged use may cause serious side effects, including osteoporosis, and muscle atrophy. Dexamethasone (Dex), a potent synthetic glucocorticoid, causes muscle atrophy by retarding muscle protein synthesis and encouraging muscle protein breakdown via the ubiquitin-proteasome system (UPS).[11,12] Mechanistically, Dex interacts with glucocorticoid receptors (GR) and induces the expression of Krüppel-like factor 15 (KLF15), a member of the zinc finger transcription factor family of proteins.[13] KLF15 regulates carbohydrate, lipid, and protein metabolism,[14] and its expression is directly induced by GR.[15] It is a catabolic modulator of skeletal muscle via the direct transcriptional upregulation of the ubiquitin ligases muscle atrophy F-box protein 1 (MAFbx1)/Atrogin-1 and muscle ring finger protein-1 (MuRF-1), which are associated with muscle atrophy.[16] The upregulation of ubiquitin ligases by KLF15 can be mediated directly or indirectly via FoxO3a activation.[17] As glucocorticoid has a role in instigating muscle atrophy during sepsis and burn injury, Dex-mediated muscle atrophy is a suitable model for investigating muscle atrophy.

Previous studies suggest that polyphenols and their metabolites are effective in attenuating Dex-induced muscle atrophy.[18] Thus, this study aimed to investigate the effects of HMPA treatment on Dex-induced skeletal muscle atrophy, as well as to evaluate possible mechanisms responsible for HMPA effects.

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doi: 10.3164/jcbn.23-70

Materials and Methods

Chemicals. Dex and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). HMCA, its precursor, caffeic acid (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), their metabolites HMPA (Tokyo Chemical Industry, Tokyo, Japan) were used. All other chemicals and reagents used were of standard quality.

Measurement of antioxidant capacity. The free radical scavenging capacity of HMPA and its parent compounds (HMCA and caffeic acid) was assessed using an antioxidant assay kit, with 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a substrate (Dojindo Laboratories, Kumamto, Japan). Shortly, the optimum concentration range of samples showing 50% scavenging potency was calculated. Next, IC_{50} was determined by plotting the regression line based on the obtained range of sample concentrations for 50% scavenging. The results are expressed as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC).

Cell culture. C2C12 myoblasts of mouse origin were obtained from the American Type Culture Collection and cultured in 10% fetal bovine serum (FBS) solution, which was prepared using Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) with 10% FBS (Thermo Fischer Scientific, Waltham, MA) and 1% penicillin-streptomycin mixed solution (Nacalai Tesque, Kyoto, Japan). Myoblasts were maintained at 37°C in 5% CO_2 humidified air to reach 100% confluency. Then, a differentiation medium (DMEM containing 2% horse serum) (Thermo Fischer Scientific) was added by replacing the culture medium to differentiate myoblasts into myotubes. Every 48 h, the medium was changed with a fresh one. When myotubes were completely differentiated, they were treated with 10 μM HMPA for 1 h as a pretreatment, followed by 10 μM Dex treatment for 24 h to induce muscle atrophy. DMSO (0.01%) and milli-Q water were used as vehicles for HMPA and Dex, respectively. The dose of HMPA (10 μM) was selected based on our random study of its four independent doses (1, 10, 50, and 100 μM) and their effectiveness against Dex-induced atrophic markers (Supplemental Fig. 1*). Finally, cells were harvested, processed, and subjected to quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis.

Selection of HMPA to investigate against Dex-induced muscle atrophy. To determine the effectiveness of the aforementioned polyphenols against Dex-mediated muscle atrophy, caffeic acid, HMCA, and HMPA were randomly treated at different doses for 1 h, followed by 10 μM Dex treatment for 24 h. Cells were harvested, and the expression of muscle atrophy ubiquitin ligases Atrogin-1 and MuRF-1 was investigated.

Cytotoxicity assay. To investigate the cytotoxicity effect of HMPA at different doses, 0.05×10^5 C2C12 cells were seeded in each well in 96 well plates, grown and differentiated into myotubes as shown above. Then, myotubes were treated with 10 μM Dex for 24 h in the presence or absence of 1, 10, 50, and 100 μM HMPA as 1 h pretreatment. Subsequently, the LDH test was carried out using Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories) according to the manufacturer's protocol.

Measurement of myotube diameter. The diameter of C2C12 myotubes was measured by capturing their images using a phase-contrast microscope (BIOREVO, BZ-9000; Keyence, Osaka, Japan) following a previously published protocol.[19,20] Briefly, 10 images per group of myotube culture were captured at 20× magnification. In total, 100 myotubes/group were measured randomly from 10 myotubes' micrographs. Diameter changes were analyzed using a BZ-II analyzer software (Keyence). Each myotube's thickest portion was selected for diameter measurement.

Quantitative (RT) PCR. RT-PCR was done using Agilent Brilliant III Ultra-Fast SYBR Green QPCR Master Mix™ (Agilent Technologies, Santa Clara, CA) on a StepOnePlus™ Real-Time PCR system (Thermo Fischer Scientific). Briefly, cells were harvested using ISOGEN™ (Nippon Gene, Tokyo, Japan) to collect complete RNA from cells. Then, the isolated RNA was quantified by a Nanodrop 1000 Spectrophotometer (Thermo Fischer Scientific), followed by reverse transcription to cDNA. The sequences of primers used in this experiment are shown in Table 1; 18S ribosomal RNA was assigned as an internal standard.

Western blotting. Cells for protein analysis were harvested using a lysis buffer with 5 mM EDTA, 50 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 2 mM Na_3VO_4, 10 mM NaF, 1% Triton-X-100, a protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland), and 10 μM MG-132. Protein concentration was determined using a Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific), with bovine serum albumin as the standard, following the manufacturer's protocol.

Western blotting was done to analyze the protein expression of myosin heavy chain (MyHC) (fast and slow type) and total and phosphorylated FoxO3a using ProteinSimple™ WES, a fully automated Western blotting system, following the protocol discussed in Ulla *et al.*[21] In brief, 1 μg protein was mixed with a simple western sample buffer and 5X Fluorescent Master Mix and denatured at 95°C for 5 min. Protein samples, primary and secondary antibodies, and chemiluminescent substrates were dispensed to microplate according to the manufacturer's protocol and subjected to the WES machine. After approximately 3 h, software-generated results were analyzed to determine protein

Table 1. Primers used for PCR

Target gene		Sequence	Length (bp)
MAFbx1/Atrogin-1	S	GGCGGACGGCTGGAA	101
	AS	CAGATTCTCCTTACTGTATACCTCCTTGT	
MuRF-1	S	TGTCTGGAGGTCGTTTCCG	183
	AS	CTCGTCTTCGTGTTCCTTGC	
KLF15	S	CCAGGCTGCAGCAAGATGTACAC	125
	AS	TGCCTTGACAACTCATCTGAGCGG	
18Sr	S	CATTCGAACGTCTGCCCTA	119
	AS	CCTGCTGCCTTCCTTGGA	
GR	S	CAAAGCCGTTTCACTGTCC	296
	AS	ACAATTTCACACTGCCAC	

18Sr, 18S ribosomal RNA; GR, glucocorticoid receptor; KLF15, Krüppel-like factor 15; MuRF-1, muscle ring finger protein-1.

*See online. https://doi.org/10.3164/jcbn.23-70

expression levels. Antibodies against fast- or slow-type myosin heavy chain (MyHC), β-actin (Sigma-Aldrich), total FoxO3a, phosphorylated-FoxO3a, and rabbit IgG (Cell Signaling Technology, Danvers, MA) were used.

Measurement of reactive oxygen species. Myotubes cultured and treated on 96-well plates were washed with medium and incubated with 5 μM H_2-DCFDA reagent (Thermo Fischer Scientific) in the medium for 1 h at 37°C. Afterwards, myotubes were washed with Hank's Balanced Salt Solution (HBSS) twice and fluorescence was measured using a microplate reader (INFINITE M NANO™; Tecan, Mannedorf, Switzerland) at an excitation and emission wavelengths of 495 nm and 527 nm respectively according to the manufacturer's protocol.

Small interfering RNA and transfections. The siRNA technique for specific knockdown of GR, KLF15 and FoxO3a mRNA expression was used. Myotubes were transfected with the GR siRNA (Thermo Fischer Scientific), FoxO3a siRNA (Silencer® Select siRNA FoxO3; ID - s80658) or non-targeting (NT) siRNA (Thermo Fischer Scientific) using the transfection reagent lipofectamine RNAiMAX (Thermo Fisher Scientific). The siRNA used for mouse GR was 5'-GCAUGUAUGACCAAUGUAAtt-3'. The siRNA for FoxO3a and NT (used as negative control), was from the supplier (Thermo Fischer Scientific) and their sequence was not opened. The final concentration of the siRNA construct was 50 nM with a medium in combination with lipofectamine, according to the manufacturer's protocol. After 24 h of transfection, the medium was replaced with fresh medium, and myotubes were treated with Dex (10 μM) and Dex with HMPA as mentioned in the Cell Culture section.

Statistical analysis. Results are presented as means ± SEM. One-way or Two-way ANOVA followed by Tukey post hoc test, was used for the comparison of differences between the groups. Student's *t* test was used to compare the difference between the two groups. All analyses were performed using GraphPad Prism ver. 9.3.1 (GraphPad Software, San Diego, CA). *P* value <0.05 was considered statistically significant.

Results

Free radical scavenging activity of phenolic compounds. The antioxidant effects of the mentioned polyphenols were evaluated based on DPPH reduction. Their TEAC and IC_{50} values are presented in Table 2. Caffeic acid, the precursor of HMCA, was found to be the most efficient antioxidant entity followed by its metabolite HMCA.

HMPA more effectively suppressed ubiquitin ligases than its parent compounds. HMPA was found to be the most effective agent in attenuating both Atrogin-1 and MuRF-1 at lower doses (Supplemental Fig. 1*). In contrast, HMCA could suppress MuRF-1, with no effect on Atrogin-1 expression. Caffeic acid was ineffective in suppressing both the ubiquitin ligases (Supplemental Fig. 1*). As HMPA was the most efficient phenol in suppressing both the ubiquitin ligases, so HMPA was used for further experiments.

Cytotoxicity effect of HMPA. The LDH test was performed to explore the cytotoxicity of HMPA treatment on C2C12 myotubes. HMPA at higher doses (50 and 100 μM) significantly induced cytotoxicity as indicated by increased release of LDH level (Supplemental Fig. 2*).

Effect of HMPA on Dex-mediated myotube diameter. C2C12 myotubes thickness was significantly decreased by Dex treatment compared to vehicle-treated control myotubes (Fig. 1). Interestingly, HMPA pretreatment effectively mitigated Dex-induced decrease in C2C12 myotubes thickness. The diameter of myotubes after HMPA administration alone was comparable to that of the control group.

Table 2. Antioxidant capacity of polyphenolic compounds

Sample	IC50 (μM)	TEAC (μM)
Caffeic Acid	124	2.03
HMCA	164	1.53
HMPA	249	1.01

DPPH assay was used for the measurement of antioxidant capacity. IC_{50} and Trolox equivalent antioxidant capacity (TEAC) values were calculated using the following formulas: inhibition ratio of the sample (%) = (absorbance of the blank – absorbance of the sample)/absorbance of the blank × 100; and TEAC = IC_{50} of Trolox/IC_{50} of the sample. HMCA, 4-hydroxy-3-methoxycinnamic acid; HMPA, 3-(4-hydroxy-3-methoxyphenyl) propionic acid.

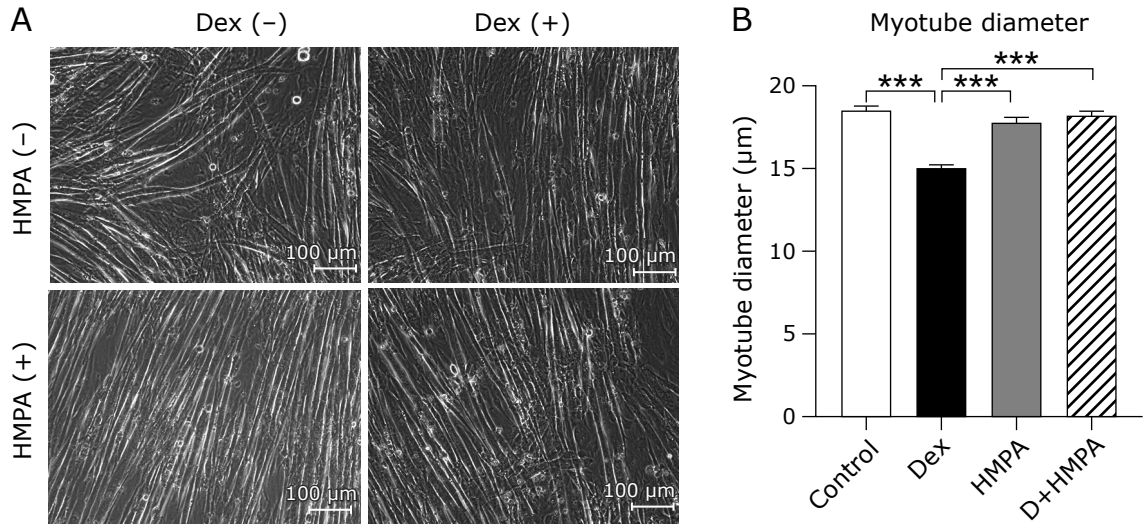

Fig. 1. HMPA mitigated Dex-mediated atrophy of C2C12 myotubes. C2C12 myotubes were pretreated with or without 10 μM HMPA for 1 h, followed by treatment with 10 μM Dex for 24 h. (A) Myotube morphology and (B) myotube diameter. The diameter was calculated as described in the Material and Method section. The results are presented as means ± SEM ($n = 100$ per group). Dex, dexamethasone; HMPA, 3-(4-hydroxy-3-methoxyphenyl) propionic acid; D+HMPA, Dex + HMPA. *p<0.05, **p<0.01, ***p<0.001 indicates a significant difference between indicated groups. Scale bar, 100 μm.

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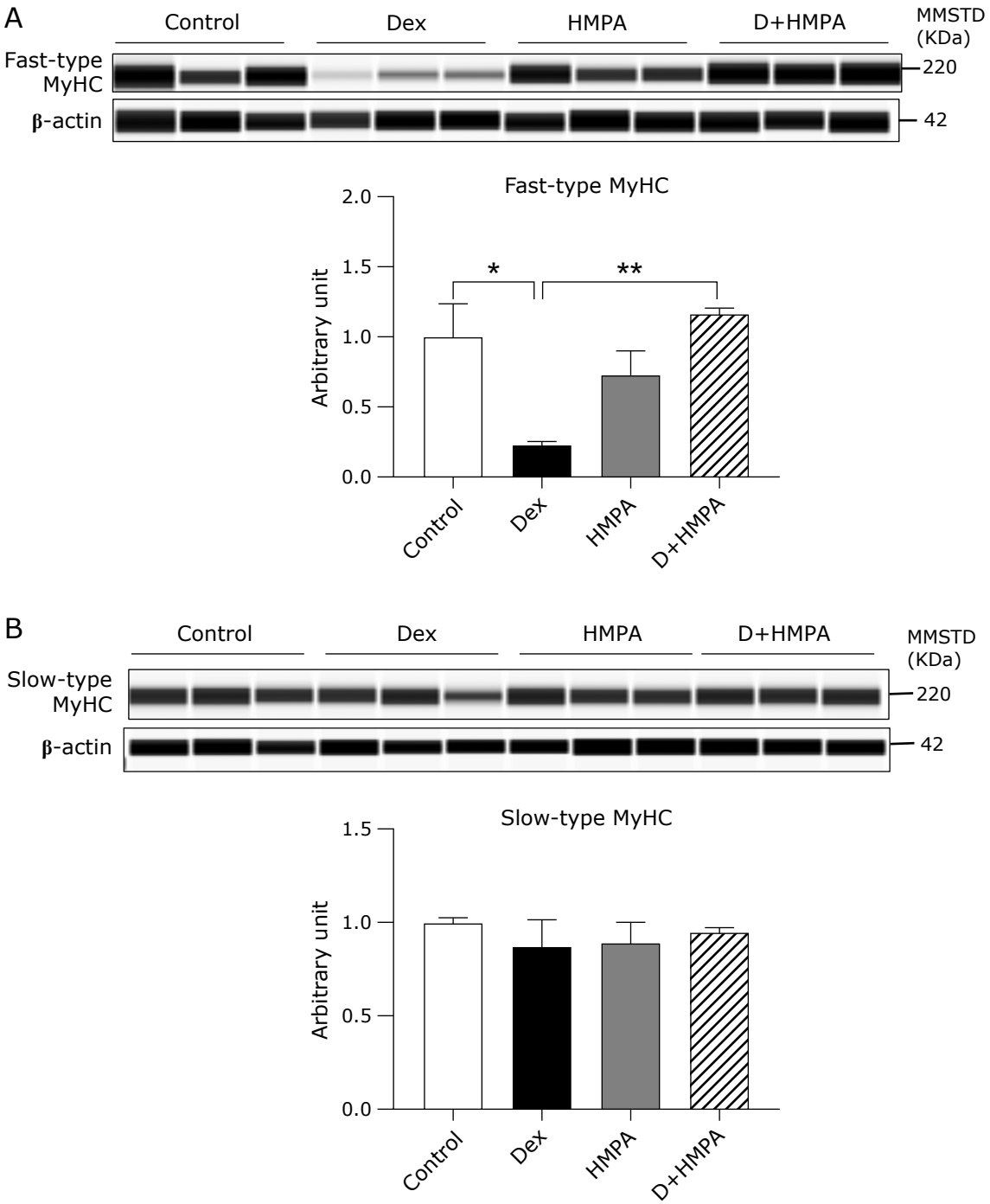

Fig. 2. HMPA prevented Dex-mediated protein degradation. Myotubes were treated as mentioned above, and protein levels were measured for the assessment of (A) fast-type MyHC and (B) slow-type MyHC. The results are expressed as means ± SEM ($n = 3$ per group). Dex, dexamethasone; HMPA, 3-(4-hydroxy-3-methoxyphenyl) propionic acid; D+HMPA, Dex + HMPA. *$p<0.05$, **$p<0.01$, ***$p<0.001$ indicates a significant difference between indicated groups. MMSTD, molecular mass standard.

Effect of HMPA on Dex-mediated muscle protein degradation. Dex induces muscle protein breakdown, especially in glycolytic muscle fiber. Therefore, in the next experiment, the effects of Dex, and/or HMPA on fast- and slow-type MyHC proteins in C2C12 myotubes were investigated using the Western blot technique. Dex 10 μM treatment for 24 h significantly reduced fast-type MyHC protein expression compared to vehicle-treated C2C12 myotubes (Fig. 2A). Interestingly, HMPA treatment successfully prevented the downregulation of fast-type MyHC induced by Dex; however, HMPA treatment alone showed no significant changes compared to the control group.

Neither Dex nor HMPA treatment showed any remarkable changes in slow-type MyHC protein expression compared to vehicle-treated C2C12 myotubes (Fig. 2B). These results suggest that HMPA can be an effective agent for the suppression of Dex-mediated muscle protein degradation, especially that of fast type.

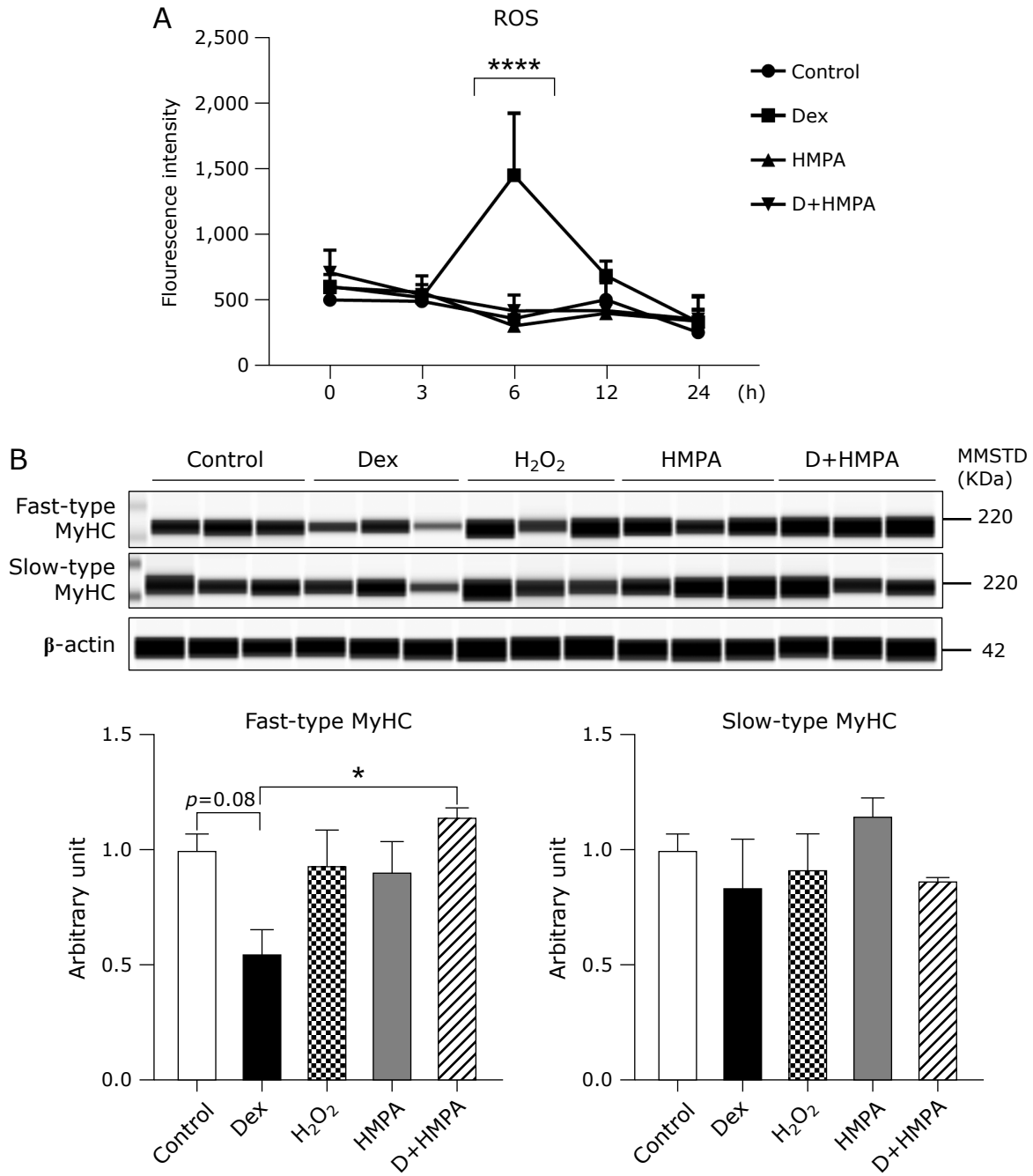

Fig. 3. HMPA suppressed the Dex-induced ROS production in C2C12 myotubes. Myotubes were pretreated with HMPA for 1 h, followed by Dex treatment for different times (0, 3, 6, 12, and 24 h). (A) ROS production was measured as mentioned in the Material and Method section. The results are presented as means ± SEM ($n = 4$ per group). ****$p<0.001$ indicates the significant difference between the indicated groups. (B) Fast and Slow types of MyHC protein. Myotubes were treated as mentioned in Fig. 1 with the addition of the H_2O_2 (100 μM) group. The results are presented as means ± SEM ($n = 3$ per group). *$p<0.05$ indicates the significant difference between indicated groups. MMSTD, molecular mass standard.

Effect of HMPA on Dex-induced ROS production in C2C12 myotubes. The ROS scavenging activity of HMPA against Dex-induced ROS production was analyzed using H_2-DCFDA reagent in C2C12 myotubes. ROS production was significantly increased by Dex treatment (Fig. 3A), especially at 6 h compared to control myotubes. HMPA pretreatment significantly suppressed the Dex-induced ROS production in C2C12 myotubes. Treatment with HMPA alone did not affect ROS level and showed a similar trend as of vehicle-treated control myotubes.

Next, to investigate the sensitivity of ROS/H_2O_2 towards fast or slow types of MyHC, Western blotting was performed after treating the myotubes with Dex and or HMPA as described in the manuscript, and H_2O_2 100 μM for 24 h. Dex decreased the fast type MyHC expression which was attenuated by HMPA (Fig. 3B). However, H_2O_2 at 100 μM did not show any significant changes of either fast or slow type of MyHC protein in our experiment (Fig. 3B). We anticipate that a higher dose of H_2O_2 or a longer duration of H_2O_2 treatment is required to induce the degradation of fast-type MyHC protein. Moreover, various previous reports suggest that Dex significantly increases ROS pro-

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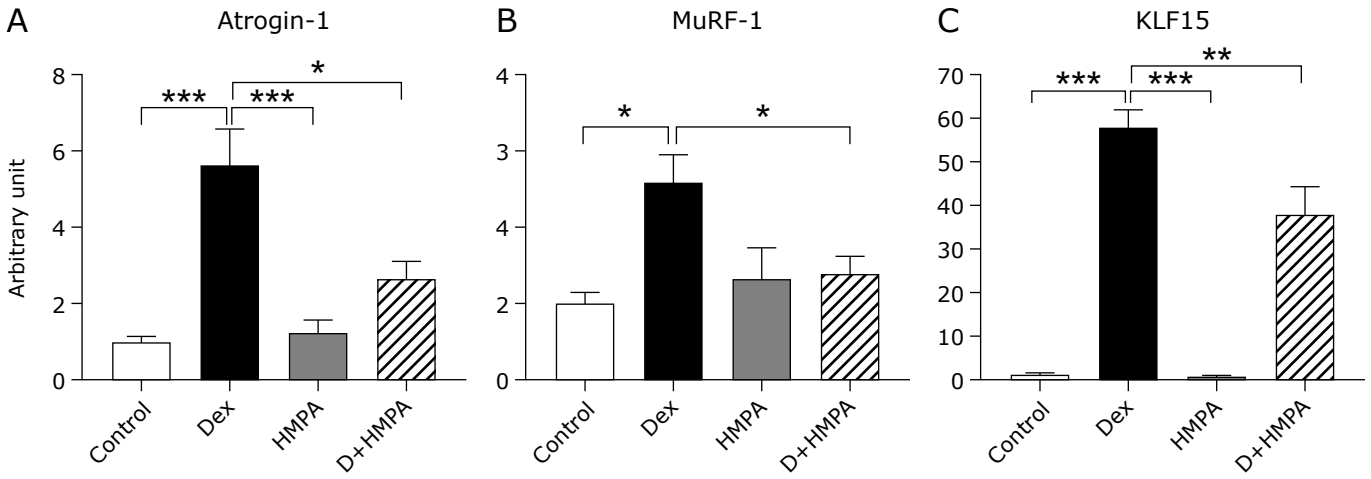

Fig. 4. HMPA attenuated Dex-induced ubiquitin ligases expression. C2C12 myotubes were treated as mentioned in Fig. 1, followed by the analysis of mRNA expression of ubiquitin ligases MAFbx1/Atrogin-1, MuRF-1, and KLF15 by qRT-PCR. (A) indicates Atrogin-1, (B) MuRF-1, and (C) KLF15 expression. The results are presented as means ± SEM. n = 3–4/group. Dex, dexamethasone; HMPA, 3-(4-hydroxy-3-methoxyphenyl) propionic acid; D+HMPA, Dex + HMPA. *p<0.05, **p<0.01, ***p<0.001 indicates a significant difference between indicated groups.

duction in C2C12 cells and mice.[19,22–24] We previously found that Dex-induced ROS production is mediated via GR, as GR knockdown abolished the ROS production and myotube atrophy.[19] As Dex is a strong ROS inducer in muscle cells, it may also affect fast-type MyHC by ROS-mediated pathway. Therefore, HMPA could exert its antioxidative effect to suppress muscle protein degradation as it suppresses ROS level (Fig. 3A). However, for an in-depth mechanism, more studies are needed.

Effect of HMPA on Dex-induced muscle atrophy ubiquitin ligases. The effect of HMPA on the expression of muscle-atrophy-associated ubiquitin ligases in response to Dex administration was investigated. Dex treatment significantly elevated the mRNA expression of ubiquitin ligases Atrogin-1 and MuRF-1 compared to those in vehicle-treated myotubes (Fig. 4A and B). HMPA treatment effectively diminished Dex-mediated upregulation of these ubiquitin ligases in C2C12 myotubes, whereas HMPA treatment alone did not affect their expression (Fig. 4A and B).

KLF15 is an upstream transcriptional regulator that induces Atrogin-1 and MuRF-1 expression in response to Dex treatment. Therefore, to identify the molecular mechanism of ubiquitin ligases suppression by HMPA, KLF15 expression was measured. Dex remarkably increased the KLF15 expression, similar to other studies.[25] Meanwhile, HMPA treatment suppressed Dex-induced KLF15 upregulation (Fig. 4C). These findings indicate that HMPA attenuates muscle atrophy and related anomalies by suppressing KLF15-mediated ubiquitin ligase suppression.

Effect of HMPA on muscle atrophy-associated transcriptional factors in C2C12 myotubes. As dephosphorylation of FoxO3a stimulates the expression of muscle atrophy-associated ubiquitin ligases, such as MAFbx1/Atrogin-1 and MuRF-1, we next examined the total and phosphorylated FoxO3a protein level in C2C12 myotubes treated with Dex with or without HMPA. Dex significantly increased the total FoxO3a expression in C2C12 myotubes compared to control myotubes, which was noticeably decreased by HMPA (Fig. 5A). Moreover, Dex-significantly induced FoxO3a dephosphorylation (Fig. 5B). Interestingly, HMPA treatment alone or with Dex increased FoxO3a phosphorylation by almost 8–10 folds compared to Dex-alone treated myotubes (Fig. 5B). These results indicate that Dex-mediated upregulation of ubiquitin ligases was also contributed via FoxO3a activation, and the suppressive effects of HMPA led to a decreased FoxO3a-induced ubiquitin ligase expression.

Effect of HMPA in GR/KLF15 and FoxO3a transfected myotubes. To elaborate on the possible role of KLF15 and FoxO3a in regulating HMPA's protective effect against Dex-induced muscle atrophy, we treated Dex and or HMPA in KLF15 and FoxO3a knockdown myotubes. GR siRNA transfection significantly reduced the expression of GR as well as KLF15 mRNA expression compared to non-targeting (NT) siRNA treated control (Fig. 6A and B). Similarly, transfection with FoxO3a siRNA significantly reduced the expression of FoxO3a mRNA level (Fig. 6C).

Next, the mRNA expression of ubiquitin ligases was investigated in NT, GR/KLF15 and FoxO3a-siRNA transfected myotubes after Dex and Dex with HMPA treatment. In NT siRNA-transfected myotubes, treatment of Dex significantly increased the expression of muscle atrophy-associated ubiquitin ligases Atrogin-1 and MuRF-1 (Fig. 6D and E), which were effectively suppressed by HMPA treatment.

In GR/KLF15 and FoxO3a knockdown myotubes, although treatment of Dex increased the expression of Atrogin-1 and MuRF-1, HMPA was found ineffective in mitigating the Dex-mediated increase of Atrogin-1 and MuRF-1 (Fig. 6D and E). Considering the above results, it can be suggested that HMPA mediates its beneficial effect via KLF15 and FoxO3a as their knockdown abolished the beneficial effects of HMPA.

Discussion

Recently, profound interest has been paid to develop therapies for muscle atrophy. Natural extracts or compounds with anti-atrophic effects are also being studied. Polyphenols or their metabolites[18] are continuously investigated as alternative treatment agents to alleviate muscle atrophy due to their easy availability, low cost, fewer side effects, and target specificity.[26,27] Consistent to this effort, this study investigated the role of HMPA against Dex-mediated skeletal muscle atrophy using C2C12 skeletal myotubes.

Skeletal muscle being an important tissue for movement, metabolic homeostasis, and thermogenesis constitutes almost 40% of the total body weight and 50% of total protein.[28] A decreased skeletal muscle mass and function by catabolic and anabolic imbalance referred a muscle atrophy which has multifarious cause like chronic disease, inactivity, aging, cachexia, and glucocorticoids use.[29] UPS is the proteolytic system chiefly

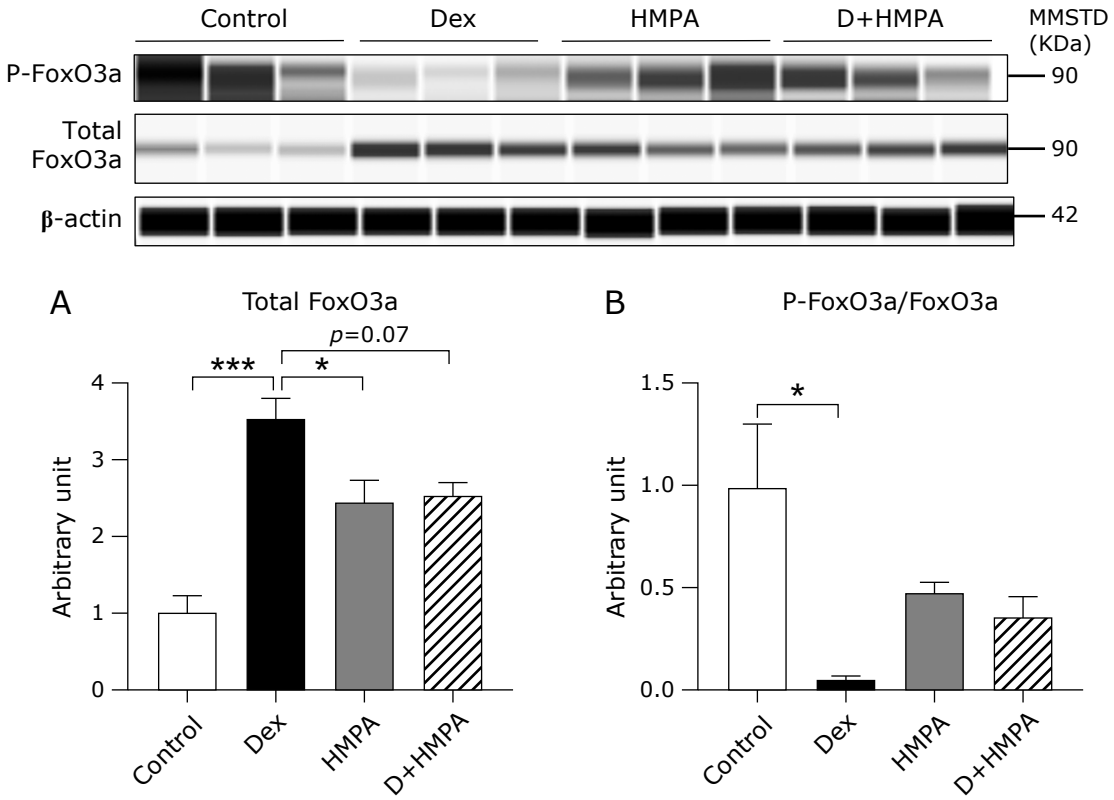

Fig. 5. HMPA suppressed Dex-induced FoxO3a expression. Myotubes were treated as discussed above, followed by the measurement of total and phosphorylated FoxO3a by Western blotting. Western blot, with the protein levels of (A) Total FoxO3a and (B) phosphorylated FoxO3a. The results are expressed as means ± SEM ($n = 3$ per group). Dex, dexamethasone; HMPA, 3-(4-hydroxy-3-methoxyphenyl) propionic acid; D+HMPA, Dex + HMPA; P-FoxO3a, phosphorylated FoxO3a. *p<0.05, **p<0.01, ***p<0.001 indicates a significant difference between indicated groups. MMSTD, molecular mass standard.

involved in glucocorticoid-mediated muscle atrophy.[30] In UPS, a substrate protein is ubiquitinated by ubiquitin ligases Atrogin-1 and MuRF-1, leading to muscle proteolysis. Dex activates UPS by increasing the expression of ligases, such as Atrogin-1 and MuRF-1,[30] which are upregulated by KLF15 and FoxOs activation.[16] KLF15 is a direct downstream target of glucocorticoid receptor (GR) whose expression is regulated by Dex.[16] Dex binds to GR and translocates it from the cytoplasm to the nucleus.[31] Nuclear GR triggers transcription of muscle-specific E3 ubiquitin ligases, Atrogin-1 and MuRF-1 by activating KLF15 and forkhead transcription factor 3 alpha (FoxO3a) causing muscle atrophy. Phosphorylation negatively regulates FoxO3a.[17] In the current study, Dex significantly reduced the phosphorylation of FoxO3a facilitating its entry into the nucleus (Fig. 5A and B). In contrast, HMPA stimulated phosphorylation of FoxO3a and also suppressed GR-mediated KLF15 upregulation that directly triggers Atrogin-1 and MuRF-1 expression. Interestingly, the preventive effect of HMPA was lost in GR/KLF15 and FoxO3a knockdown myotubes (Fig. 6D and E). Therefore, HMPA decreased the nuclear translocation of FoxO3a and also suppressed KLF15 expression which may be possibly by interfering with the translocation of GR as previous literature reported that isoflavones and other antioxidative phytochemical compounds interfere with the nuclear translocation of GR in Dex-treated C2C12 cells and animals.[32–34] However, to elaborate on the precise mechanism of action of HMPA, further experiments are warranted. Based on these findings, it was suggested that HMPA can mitigate muscle atrophy by suppressing the expression of ubiquitin ligases through KLF15 and FoxO3a modulation.

Dex treatment significantly reduced the thickness/diameter of myotubes. It also led to significant degradation of fast-type MyHC proteins, similar to previous reports.[35] Dex preferentially degrades fast-twitch type (type II) than slow-twitch type (type I) muscle fibers.[11,35] The specificity of Dex against type II fibers may be due to its higher expression of GR. Interestingly, in our study, HMPA treatment significantly reduced Dex-induced decrease in myotube diameter and fast-type MyHC protein degradation. The anti-proteolytic effects of HMPA may be credited to its attenuating effects on ubiquitin ligases Atrogin-1 and MuRF-1.

Previous reports suggest that HMCA extensively metabolized into HMPA.[5] It further elaborated that hypolipidemic effect of HMCA is due to the *in vivo* metabolite HMPA. Moreover, the colonic metabolites HMPA was reported to modulate platelet activation more strongly than their phenolic precursors caffeine, 5-caffeoylquinic acid and 3,5-dicaffeoylquinic acid, indicating to increase the effectiveness with the metabolism of the phenolic compounds.[36] The microbial conversion of polyphenols in the colon often produces metabolites with higher bioavailability than their precursor, and therefore with better activity. Consistently in our study, HMCA could suppress MuRF-1 whereas HMPA successfully attenuated the upregulation of both Atrogin-1 and MuRF-1 against Dex-induced upregulation. An *in vivo* study is required to clearly illustrate the effect of HMCA and HMPA along with their bioavailability, however the current study suggest that HMPA could be more potential anti-atrophic agent than its precursor HMCA and caffeic acid.

This study has been carried out *in vitro* using C2C12 myotubes. For an in-depth mechanistic illustration and further

doi: 10.3164/jcbn.23-70

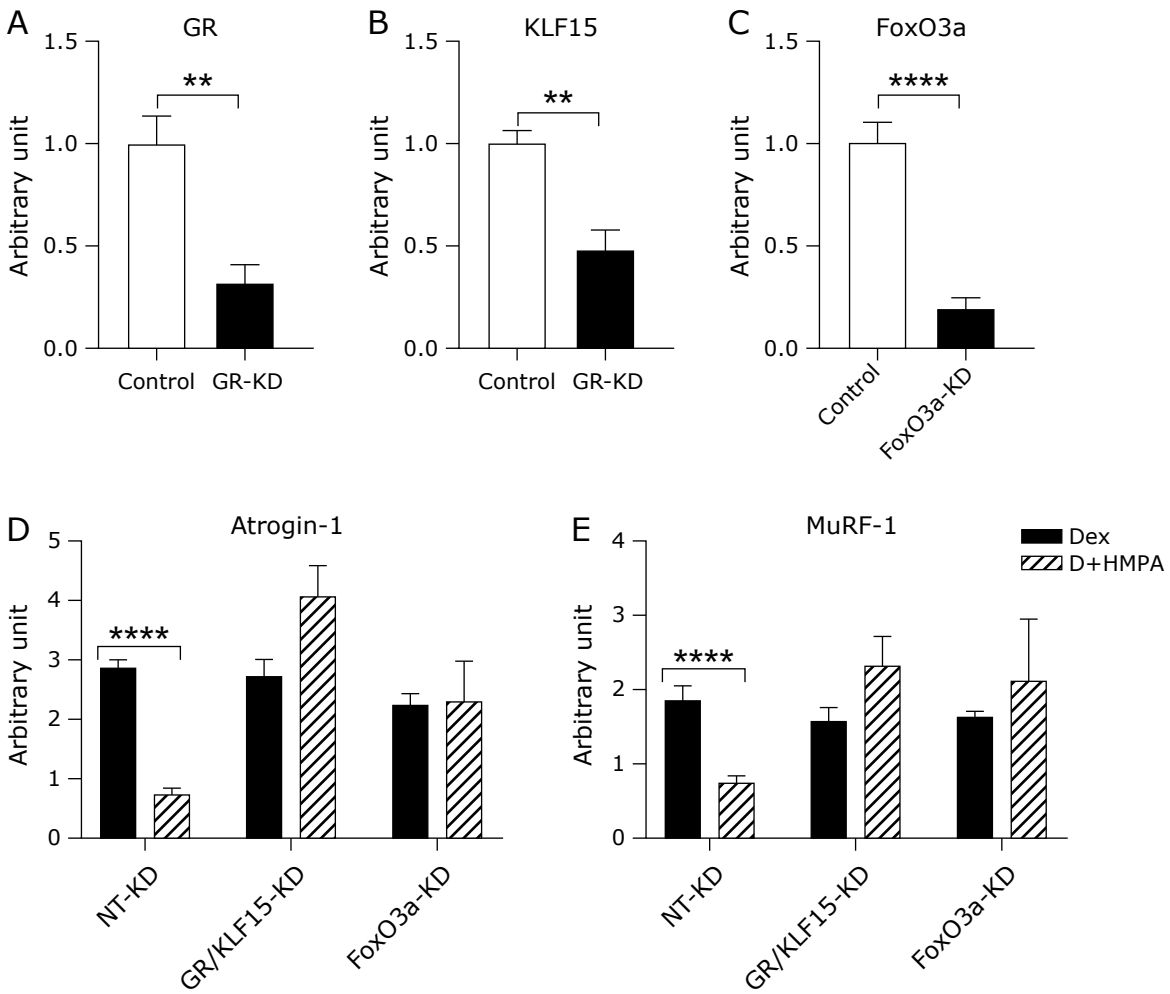

Fig. 6. Knockdown of KLF15 and FoxO3a diminished protective effects of HMPA. Myotubes were transfected with GR, FoxO3a and NT siRNA as described in the Materials and Methods section, followed by the analysis of (A) GR, (B) KLF15 and (C) FoxO3a mRNA expression. The results are expressed as means ± SEM ($n = 4$ per group). *$p<0.05$, **$p<0.01$, ***$p<0.001$ indicates the significant difference between indicated groups. (D, E) Ubiquitin ligases Atrogin-1 and MuRF-1 expression in NT, GR/KLF15 and FoxO3a-siRNA transfected myotubes. Transfected myotubes were treated with HMPA for 1 h as pretreatment followed by Dex for 24 h. The results are expressed as means ± SEM ($n = 4$ per group). *$p<0.05$, **$p<0.01$, ***$p<0.001$ indicates the significant difference between indicated groups. siRNA, small interfering non-targeting RNA; GR, Glucocorticoid receptor; NT, non-targeting.

explanation, an *in vivo* experiment is warranted. In this study, we conclude that the metabolite HMPA mitigates Dex-induced muscle atrophy in C2C12 myotubes. HMPA reduced the expression of genes associated with muscle atrophy and prevented Dex-induced protein degradation of MyHC. These effects were mediated by suppressing the upstream transcription factors KLF15 and FoxO3a. Therefore, we suggest that HMPA could be a potential functional food with positive effects on muscle health.

Author Contributions

Study concept and design, TN, AU, HKayaki, and HKuwahara; methodology, AU, YN, and SY; validation and analysis, AU, TU, MMR; investigation, AU, TU, MMR; data acquisition, AU and TU; writing – original draft, AU; writing – review and editing TN, HKayaki), HKuwahara, YN, and SY; visualization, TN; supervision, TN; project administration, TN; funding acquisition, TN. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

We thank Enago for the English language editing and Maruzen Pharmaceuticals Co. Ltd. for providing the investigational polyphenolic compounds. This work was supported by Cabinet Office, Government of Japan, Cross ministerial Moonshot Agriculture, Forestry and Fisheries Research and Development Program, "Technologies for Smart Bio-industry and Agriculture" (funding agency: Bio-oriented Technology Research Advancement Institution), Grant Number JPJ009237.

Abbreviations

Dex	dexamethasone
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
GR	glucocorticoid receptor
FoxO3a	forkhead transcription factor 3 alpha
HMPA	3-(4-hydroxy-3-methoxyphenyl) propionic acid
HMCA	4-hydroxy-3-methoxycinnamic acid
KLF15	Krüppel like factor 15
MAFbx1	muscle atrophy F-box protein 1

MuRF-1	muscle ring finger protein-1
MyHC	myosin heavy chain
TEAC	trolox equivalent antioxidant capacity
UPS	ubiquitin proteasome system

Conflict of Interest

HKayaki, YN, SY, and HKuwahara are employed in Maruzen Pharmaceuticals Co., Ltd. All other authors have no conflicts of interest.

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doi: 10.3164/jcbn.23-70