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Original article

2,6-Dimethoxy-1,4-benzoquinone increases skeletal muscle mass and performance by regulating AKT/mTOR signaling and mitochondrial function

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ABSTRACT

Background: 2,6-Dimethoxy-1,4-benzoquinone (DMBQ), a natural phytochemical present in fermented wheat germ, has been reported to exert anti-cancer, anti-inflammatory, and anti-adipogenic effects. However, the effect of DMBQ on muscle hypertrophy and myoblast differentiation has not been elucidated.
Purpose: We investigated the effect of DMBQ on skeletal muscle mass and muscle function and then determined the possible mechanism of DMBQ.
Methods: To examine myogenic differentiation and hypertrophy, confluent C2C12 cells were incubated in differentiation medium with or without various concentrations of DMBQ for 4 days. In animal experiments, C57BL/6 mice were fed DMBQ-containing AIN-93 diet for 7 weeks. Grip strength, treadmill, microscopic evaluation of muscle tissue, western blotting, and quantitative real-time PCR were performed.
Results: DMBQ significantly increased fusion index, myotube size, and the protein expression of myosin heavy chain (MHC). DMBQ increased the phosphorylation of protein kinase B (AKT) and p70 ribosomal protein S6 kinase (S6K), whereas the phosphorylation of these proteins was abolished by the phosphoinositide 3-kinase inhibitor LY294002 in C2C12 cells. In addition, DMBQ treatment increased peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α), which programs mitochondrial biogenesis, protein levels compared with control C2C12 cells. DMBQ significantly increased maximal respiration and spare respiratory capacity in C2C12 cells. In animal experiments, DMBQ increased skeletal muscle weights and skeletal muscle fiber size compared with the control group values. In addition, the DMBQ group showed increased grip strength and running distance on an accelerating treadmill. The protein expression of total MHC, MHC1, MHC2A, and MHC2B in skeletal muscle was upregulated by DMBQ supplementation. We found that DMBQ increased the phosphorylation of AKT and mammalian target of rapamycin (mTOR), as well as downstream S6K and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) in skeletal muscle. DMBQ also stimulated mRNA expression of PGC1α, accompanied by an increase in mitochondrial DNA content, oxidative phosphorylation (OXPHOS) proteins, and oxidative enzyme activity.

Abbreviations: : AIN, American Institute of Nutrition; AKT, protein kinase B; Atrogin1, muscle-specific ubiquitin E3-ligases, F-box protein; CSA, cross-sectional areas; DAPI, 4′,6-diamidino-2-phenylindole; DMBQ, 2,6-dimethoxy-1,4-benzoquinone; DMSO, dimethyl sulfoxide; 4E-BP1, eIF4E-binding protein 1; eIF4E, eukaryotic translation initiation factor 4E; ERRγ, estrogen-related receptor gamma, FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; FNDC5, fibronectin type III domain-containing protein 5; FOXO, forkhead box O; H&E, hematoxylin and eosin; IGF1, insulin/insulin-like growth factor-1; MHC, myosin heavy chain; mtDNA, mitochondrial DNA; mTORC1, mammalian target of rapamycin complex 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MuRF1, muscle RING-finger protein1; Nampt, phosphoribosyltransferase; NRF1, nuclear respiratory factor 1; NRF2, nuclear factor erythroid 2-related factor 2; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PBS, phosphate-buffered saline; PI3K, phosphoinositide 3-kinase; PGC1α, PPAR gamma coactivator 1 alpha; PPAR, peroxisome proliferator-activated receptor; S6K1, p70 ribosomal protein S6 kinase 1; SESN, sestrin; SUnSET, surface sensing of translation; TA, tibialis anterior; TFAM, mitochondrial transcription factor A.

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Conclusion: Collectively, DMBQ was shown to increase skeletal muscle mass and performance by regulating the AKT/mTOR signaling pathway and enhancing mitochondrial function, which might be useful for the treatment and prevention of skeletal muscle atrophy.

Introduction

Skeletal muscle is the dominant organ in maintaining posture, body position, and locomotion, and it plays a critical role in energy metabolism. Age-related reductions in skeletal muscle mass and functional capacity lead to limited physical activity, increased frailty and risk of falls, metabolic problems, and mortality. Therefore, maintaining muscle mass and strength is essential for maintaining the quality of human life, especially in elderly people (Bowen et al., 2015; Rolland et al., 2008; Vijg and Campisi, 2008). Skeletal muscle mass responds to various environmental factors such as nutrients, exercise, training, endocrine disorders, and aging (Denison et al., 2015). It is also dynamically regulated by the balance between protein synthesis and degradation. When the protein synthesis rate exceeds the degradation rate, muscle hypertrophy occurs (Glass, 2005; Gordon et al., 2013). Muscle atrophy is characterized by a decrease in protein content, fiber diameter, and muscle function. Previous studies have demonstrated that mammalian target of rapamycin complex 1 (mTORC1) regulates cellular responses to nutrients and hormones and plays an important role in peripheral metabolism, such as muscle oxidative metabolism and white adipose differentiation. Notably, mTORC1 plays a critical role in protein synthesis in skeletal muscle through changes in the phosphorylation of its downstream p70 ribosomal protein S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (Bodine et al., 2001b; Deldicque et al., 2008; Goodman et al., 2011; Laplante and Sabatini, 2012). In contrast, inactivation of mTORC1 in muscle leads to severe myopathy. mTORC1 is also a key modulator of mitochondrial biogenesis by regulating the expression of peroxisome proliferator-activated receptor (PPAR) gamma coactivator 1 alpha (PGC1α) and resistance exercise-induced changes (Cunningham et al., 2007; Ogasawara et al., 2016). Increased PGC1α in skeletal muscle protects against diabetes, obesity, and sarcopenia that accompanies aging. Protein kinase B (AKT) is a serine/threonine protein kinase that plays an important role in cell survival and proliferation (Shiojima and Walsh, 2006). Moreover, AKT signaling in skeletal muscle, which is primarily activated in response to resistance training, is not activated by endurance training (Atherton et al., 2005; Nader and Esser, 2001), demonstrating that AKT signaling plays a critical role in promoting skeletal muscle hypertrophy. In addition to the regulation of protein metabolism, different fiber types affect the functional and metabolic properties of skeletal muscle during exercise (Izumiya et al., 2008). The expression of myosin heavy chain (MHC) isoforms is the standard for classification of muscle fiber types; MHC types I, IIA, and IIB are the major isoforms expressed in adult rat skeletal muscle (Pandorf et al., 2010). In contrast to endurance exercise, which increases type I muscle fibers, resistance training increases protein synthesis and the content of type II fibers.

Quinone derivatives have been reported to have various physiological activities such as immune enhancement, anti-oxidant, anti-inflammatory, and anti-adipogenesis activities (Hidvegi et al., 1999; Huang et al., 1985; Szent-Györgyi, 1982). 2,6-Dimethoxy-1,4-benzoquinone (DMBQ, Fig. 1), a type of benzoquinone, is a natural phytochemical mainly present in fermented wheat germ. The content of DBMQ is 0.1, 0.2, 0.001, and 0.07 mg/g in wheat germ, fermented wheat germ, germinated wheat, and barley germ, respectively (Tömösközi-Farkas and Daood, 2004). Wheat germ fermented using the yeast *Saccharomyces cerevisiae* has been developed and is currently sold worldwide as a product called "Avemar," which is standardized to contain approximately 200 μg/g of DMBQ (Heimbach et al., 2007). Previous studies have demonstrated that fermented wheat germ extract has anticancer properties in human cancer cell lines (Otto et al., 2016) and wheat germ supplementation amerliorates insulin resistance in diet-induced obese mice (Ojo et al., 2017). DMBQ also has been reported to inhibit 3T3L1 adipocyte differntiation (Son et al., 2019) and to reduce the growth of gastric cancer (Zu et al., 2020). Because it is well known that skeletal muscle plays an important role in metabolic diseases such as cancer and obesity, we hypothesized that DMBQ would affect skeletal muscle hypertrophy. Thus, we investigated the effect of DMBQ on muscle mass and muscle function and to clarify the possible mechanism of DMBQ.

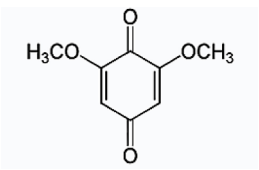

Fig. 1. Chemical structures of DMBQ.

Materials and Methods

Chemicals

DMBQ (97%) and LY294002 [≥ 98%, phosphoinositide 3-kinase (PI3K)/AKT inhibitor] were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Calbiochem (San Diego, California, USA). Antibodies against total MHC, MHC1, and MHC2B were purchased from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA, USA). Antibodies against MHC2A, PGC1α, PPARδ, oxidative phosphorylation (OXPHOS), muscle RING-finger protein1 (MuRF1), muscle-specific ubiquitin E3-ligases, F-box protein (MAFbx/Atrogin1), sestrin (SESN)1, and SESN2 were obtained from Abcam (Cambridge, MA, USA). Antibodies against p-AKT, AKT, p-S6K, S6K, p-mTOR, mTOR, p-4EBP1, and 4EBP1 were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-puromycin antibodies were obtained from Millipore (Billerica, MA, USA). Anti-estrogen-related receptor gamma (ERRγ) and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture

C2C12 murine myoblasts were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere with 5% CO_2. Confluent cells were differentiated into myotubes in the presence of 2% horse serum for 4 days, replacing the differentiation medium daily. To test its effect, DMBQ was added to the differentiation medium over the 4 days. C2C12 cells were pre-treated with or without 10 μM of LY294002 for 30 min and then treated with DMBQ for 4 days.

MTT assay

C2C12 cells were seeded at a density of 1×10^4 per well in a 96-well plate. After 24 h of incubation, the cells were treated with various concentrations of DBMQ and cultured for 24 h at 37 °C. Twenty microliters of MTT was added into each well and incubated for 3 h. Then,

MTT was removed and replaced with 200 μl of dimethyl sulfoxide (DMSO) until the crystals had dissolved. Absorbance at 570 nm was determined in each well with a 96-well plate reader.

Surface sensing of translation (SUnSET) assay

SUnSET is a nonradioactive method for the quantification of protein synthesis in mammalian cells. C2C12 cells were differentiated for 4 days. The myotubes were then treated with DMSO or DMBQ for 24 h, with or without puromycin (1 μM) for the last hour. Puromycin was detected by western blot analysis using a puromycin antibody. Band density was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence and quantification of myotubes

Myotubes were fixed with 4% formaldehyde for 30 min, permeabilized with 0.05% saponin, blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS), and then stained with total MHC antibody overnight at 4 °C. The myotubes were rinsed with PBS, incubated for 30 min at room temperature with Alexa Fluor 488-conjugated anti-mouse IgG (1:500 dilution, Cell Signaling Technology), and then incubated for 1 min at room temperature with 4′,6-diamidino-2-phenylindole (DAPI, 1:10,000 dilution). Images were acquired and analyzed using the Olympus IX71 microscope and Olympus DP controller 3.1.1 software, respectively.

Oxygen consumption rate (OCR) measurement

Mitochondrial OCR in cultured muscle cells was measured using the Seahorse Bioscience XF96 platform (Seahorse Bioscience, North Billerica, MA, USA). Briefly, C2C12 cells were seeded in XF96 cell culture microplates and treated with or without DMBQ for 24 h. After replacing the cell culture medium with assay medium, the cells were transferred to a CO_2-free incubator and maintained at 37 °C for 1 h before the assay. The injection ports were loaded with 1.5 μM oligomycin, 2 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and 1 μM rotenone. Following calibration, OCR measurements were recorded at every cycle of mixing (3 min) and measurement (3 min) for 75 min. OCR values were normalized to the number of cells present in each well, quantified by Hoechst staining (Hoechst 33342, Thermo Scientific, USA) as measured using a Cytation5 reader and Gen5 software (BioTek, USA).

Animals

Seven-week-old male C57BL/6 mice were maintained at a stable temperature (22 ± 2 °C) and humidity (50–60%) and kept on a 12-h light/12-h dark cycle with free access to food and water. After acclimating for 1 week, the mice were randomly divided into two groups. The experimental diets were based on the American Institute of Nutrition (AIN)-93 diet (Dyets, Bethlehem, PA, USA). The DMBQ groups received the AIN-93 diet with 0.05% DMBQ for 7 weeks. Body weight and food intake were measured weekly. At the end of the experiment, all mice were sacrificed, and each tissue weight was measured. All animal experiments were conducted according to procedures approved by the Institutional Animal Care and Use Committee of the Korea Food Research Institute (KFRI-M-17029).

Grip strength measurement

Forelimb grip strength performance was determined using a grid bar attached to a grip strength meter (Bioseb, Chaville, France). Five consecutive measurements were recorded for each test to obtain the peak value. All grip strength readings (measured in grams) were normalized to body weight, and the mean of three consecutive trials was calculated except for maximum and minimum force as an index of forelimb muscle strength.

Exercise capacity

Prior to exercise, the male C57BL/6 mice were acclimated to a motor-driven open treadmill with a shock grid (Daejong Instrument Industry, Seoul, Korea) for 20 min per day for 2 days. During acclimation, the treadmill speed was set at 10 m/min, and the treadmill incline was set at 15°. On the third day, exercise tolerance was tested with the shock grid set at 50 V and the treadmill incline set at 15°. For the first 20 min of testing, the treadmill speed was set at 10 m/min. Every 2 min thereafter, the treadmill speed was increased by 2 m/min. Running was terminated when the mice contacted the shock grid for 10 s.

Hematoxylin and eosin (H&E) staining

C57BL/6 mouse gastrocnemius, tibialis anterior (TA), and quadriceps muscles were fixed in 4% neutral-buffered formaldehyde, embedded in paraffin, sectioned at a defined thickness, and stained with H&E. The images of stained sections were captured with an Olympus BX51 microscope, and the cross-sectional area was quantified using IMT iSolution DT 9.2 software.

Citrate synthase and mitochondrial respiratory complex I and II activity

To measure citrate synthase activity, homogenized gastrocnemius muscle tissues were extracted with RIPA buffer and measurements were performed using a Citrate Synthase Assay Kit (Sigma-Aldrich). Mitochondria were prepared using a Mitochondria Isolation Kit (Thermo Scientific, Rockford, IL, USA). Mitochondrial complex I and II activities were measured in the gastrocnemius muscle tissues using a Complex I, II Enzyme Activity Microplate Assay Kit (Abcam). All protocols were followed according to the kit manufacturer's instructions.

ATP assay

Homogenized gastrocnemius muscle tissues were extracted with RIPA buffer and the ATP contents measured using an ATP luminescence detection assay system (ATPlite, PerkinElmer, Waltham, MA, USA).

RNA extraction and quantitative RT-PCR

Total RNA was extracted from C2C12 myotubes and mouse skeletal muscle tissue using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany), and cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix kit (Toyobo, Osaka, Japan). Quantitative RT-PCR was performed using SYBR Green Master Mix (Toyobo) according to the manufacturer's instructions. PCR was performed using a ViiA7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Western blot assay

The protein from cells and mouse skeletal muscle was extracted with RIPA buffer (Thermo Fisher), and total protein concentration was determined using a bicinchoninic acid (BCA) protein assay. Equal quantities of proteins were subjected to SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% skim milk in Tris-buffered saline containing Tween 20 for 1 h at room temperature and then incubated with the indicated primary antibodies overnight at 4 °C. After washing, the membranes were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase, and a chemiluminescence detection kit (Amersham Phamacia Biotech, Piscataway, NJ, USA) was then used to detect the protein bands.

Quantification of mitochondrial DNA (mtDNA) content

Total DNA was extracted using the AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea), and qPCR was performed using mtDNA- or non-coding DNA (ncDNA)-specific primers (Lagouge et al., 2006).

Statistical analysis

The data are expressed as the means ± SEM for *in vivo* assays and means ± SD for *in vitro* assays. Statistical analyses were performed using GraphPad Prism Version 8.0 software (GraphPad Software, San Diego, CA, USA). Student's unpaired *t*-tests were utilized to assess any differences between groups ($p < 0.05$). One-way analysis of variance was used to compare more than two groups, followed by Dunnett's multiple comparison tests.

Results

DMBQ stimulates myoblast differentiation and increases myotube size

During differentiation, myotubes express myotube-specific structural proteins such as MHC (Devlin and Emerson Jr, 1978). The sizes of individual myotubes depend on their net protein balance, and an increase in net protein leads to hypertrophy (White et al., 2010). We first performed MTT assays to analyze the effect of DMBQ on the viability of C2C12 myoblasts. The results show that DMBQ did not affect cell viability at a concentration of up to 5 μM (Fig. S1A). To examine the effect of DMBQ on myotube differentiation and hypertrophy, C2C12 myoblasts were differentiated in the presence or absence of DMBQ for 4 days. The diameter of the myotubes was significantly increased by DMBQ treatment as assessed by immunofluorescence with total MHC antibody. The ratio of the average number of DAPI-stained nuclei localized in MHC-positive myotubes to total nuclei was calculated and called the fusion index. DMBQ treatment significantly increased this fusion index (Fig. 2A and S1B). Furthermore, western blot analysis showed that DMBQ increased the expression of total MHC, MHC1, MHC2A, and MHC2B protein (Fig. 2B and S1C). AKT is an upstream kinase of mTOR-S6K that plays a central role in signal transduction pathways. The phosphorylation state of AKT increases throughout muscle differentiation and hypertrophy. Phosphorylation of AKT and S6K was observed in the presence of DMBQ (Fig. 2C). Treatment with LY294002 inhibited the phosphorylation of AKT, mTOR, S6K, and 4EBP1, and consequently reduced the protein expression of total MHC. Moreover, DMBQ treatment did not reverse the phosphorylation of AKT, mTOR, S6K, 4EBP1, and total MHC in LY294002-treated cells (Fig. 2D). Furthermore, we quantified protein synthesis using the SUnSET assay. As a result, we found that DMBQ significantly increased the protein expression of puromycin, indicating that the synthesis of new proteins was increased (Fig. 2E).

DMBQ improves mitochondrial function in C2C12 myotubes

Next, we investigated the effect of DMBQ on mitochondrial function by analyzing mitochondrial function-related gene expression in DMBQ-treated C2C12 cells. DMBQ increased the protein levels of PGC1α, which programs mitochondrial biogenesis, compared to the levels in control C2C12 cells (Fig. 3A and S1D). We also examined the expression levels of PGC1α and fibronectin type III domain-containing protein 5 (FNDC5), which are both induced by exercise. Furthermore, nicotinamide phosphoribosyltransferase (Nampt), nuclear respiratory factor 1 (NRF1), nuclear factor erythroid 2-related factor 2 (NRF2), mitochondrial transcription factor A (TFAM), ERRγ, and PPARδ are known to be regulated by PGC1α. As shown in Fig. 3B and 3C, the mRNA expression levels of *PGC1α, Nampt, NRF1, NRF2, TFAM, ERRγ*, and *PPARδ* were significantly elevated by DMBQ treatment, and the expression of *FNDC5* mRNA tended to increase. Next, we measured OCR to examine the effect of DMBQ treatment on the cellular respiration rate. DMBQ significantly increased FCCP-induced maximal respiration and spare respiratory capacity (maximal respiration−ATP production) (Fig. 3D).

DMBQ increases skeletal muscle mass and improves muscle performance in mice

To assess the effect of DMBQ on skeletal muscle weight and function in mice, male C57BL/6 mice were fed AIN-93diet *ad libitum* with or without 0.05% (w/w) DMBQ for 7 weeks. There was no difference in body weight and food intake between DMBQ-fed and control mice (Fig. 4A and 4B). However, DBMQ-fed mice showed a tendency to increase the weight of TA, extensor digitorum longus (EDL), and soleus, and significantly increased triceps, gastrocnemius, and quadriceps muscles compared to those of control mice (Fig. 4C). Next, we performed H&E staining of skeletal muscle and assessed cross-sectional areas (CSA) to examine the effect of DMBQ on muscle size. As shown in Fig. 4D and S2, the size of the skeletal muscle fibers was larger in the mice that received DMBQ than in the control mice. To determine whether DMBQ altered muscle fiber type, MHC protein levels in the skeletal muscle were quantified by western blotting. As shown in Fig. 4E, total MHC, MHC1, MHC2A, and MHC2B protein levels in the skeletal muscle were significantly higher in the DMBQ group than in the control group. The expression levels of *MHC* mRNAs were also elevated in the skeletal muscle of DMBQ-fed mice compared with the corresponding levels in control mice (Fig. S3A). Skeletal muscle size and strength are highly correlated, and skeletal muscle hypertrophy explains the increase in strength following resistance exercise (Maughan et al., 1983). Therefore, we performed a grip strength test and treadmill exercise to investigate whether DMBQ affects skeletal muscle performance. As shown in Fig. 4F and 4G, DMBQ supplementation significantly increased grip strength, as well as running distance and total running time on an accelerating treadmill compared to that of the control. These results indicate that skeletal muscle hypertrophy induced by DMBQ is related to increased skeletal muscle mass and performance.

DMBQ increases the phosphorylation of AKT and mTOR

The regulation of muscle protein synthesis by the AKT/mTOR signaling pathway is a critical mediator of skeletal muscle hypertrophy (Bodine et al., 2001a). To understand the mechanism responsible for the increased skeletal muscle mass, size, and performance in the DMBQ-fed mice, we evaluated the phosphorylation of proteins involved in the AKT/mTOR signaling pathway by western blot assay. DMBQ supplementation increased the levels of p-AKT and p-mTOR in skeletal muscle (Fig. 5A and S3B). Two families of mTOR substrates have been characterized: S6K1 and 4EBP1. Both protein families interact with the translational machinery. S6K1, which is phosphorylated and activated by mTOR, regulates ribosomal biogenesis. 4EBP1 is inactivated by mTOR phosphorylation, but its hypophosphorylated form binds to and inactivates eIF4E, which is responsible for CAP-dependent translation. As shown in Fig. 5A and S3B, DMBQ increased the phosphorylation of S6K and 4EBP1, two major substrates of mTOR. In addition, DMBQ supplementation decreased the protein expression of MuRF1 and Atrogin1 in skeletal muscle (Fig. 5B). The protein levels of SESN1 and SESN2 increased in skeletal muscle of DMBQ-fed mice compared with control mice (Fig. 5C). These results suggest that DMBQ supplementation increases skeletal muscle mass through the AKT/mTOR pathway.

DMBQ improves mitochondrial function in mice

PGC1α is the prime regulator of mitochondrial content and oxidative metabolism and is important for maintaining muscle energy homeostasis. Therefore, we next investigated whether DMBQ affects the gene expression of total PGC1α, PGC1α isoforms, and FNDC5 *in vivo*. As

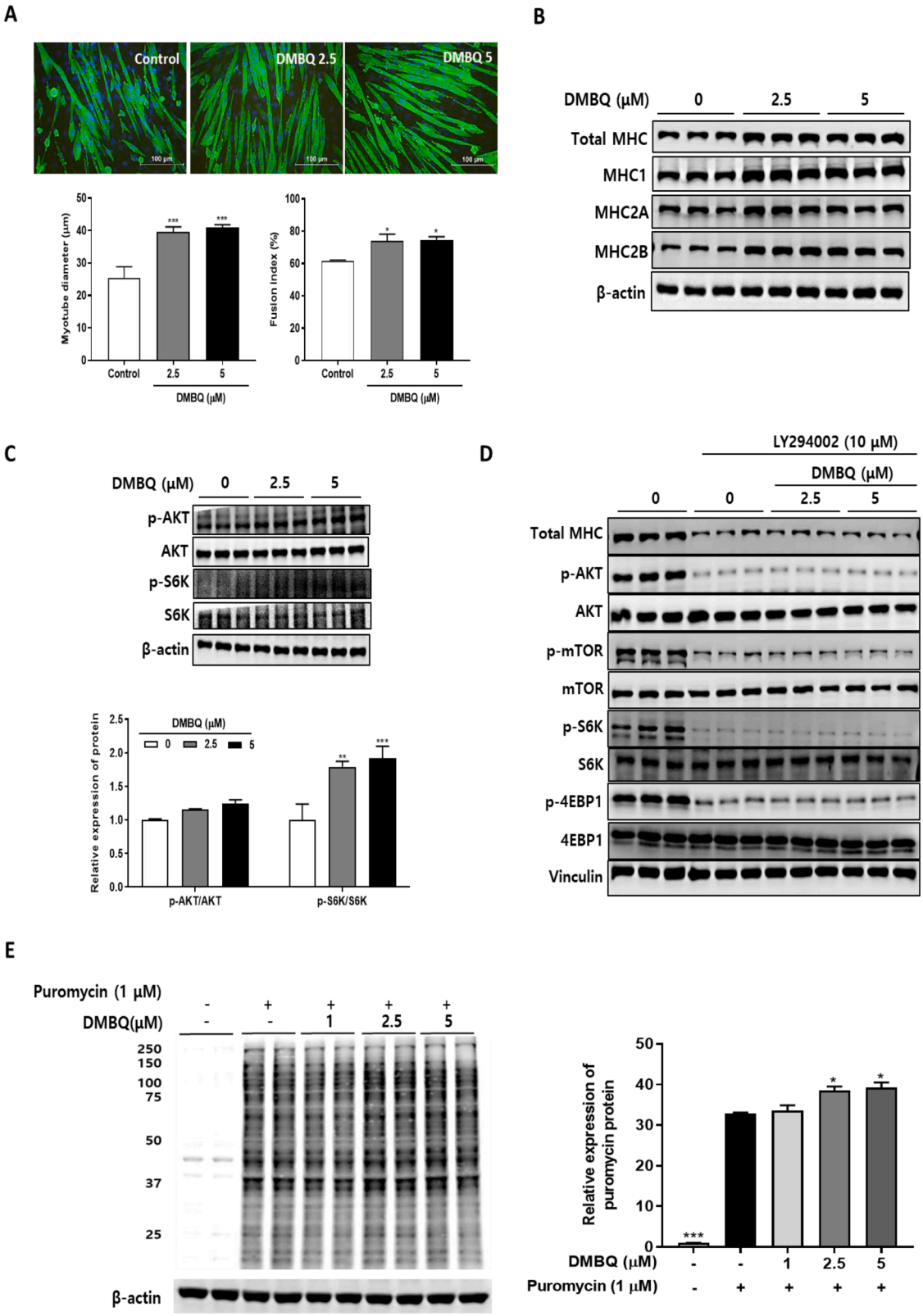

Fig. 2. Effect of DMBQ on myogenic differentiation and hypertrophy. (A) C2C12 cells were differentiated in the presence or absence of DMBQ for 4 days. After 4 days of differentiation, cells were fixed and stained with total MHC antibody. Images are representative of three independent experiments, each of which was performed in triplicate (scale bar, 100 μm). Images were captured from three different sites per well. Myotube diameter was calculated as the average diameter of MHC-positive multinucleated myotubes. Fusion index was calculated as the average number of nuclei in MHC positive multinucleated cells above total nuclei. (B) Protein expression of total MHC, MHC1, MHC2A, and MHC2B. (C) Protein expression of p-AKT/AKT and p-S6K/S6K. (D) Protein expression of total MHC, p-AKT/AKT, p-mTOR/mTOR, p-S6K/S6K, and p-4EBP1/4EBP1 in PI3K/AKT-inhibited cells. (E) Newly synthesized protein after 24 h of DMBQ treatment was assessed by SUnSET assays, and band density was analyzed using ImageJ software (National Institutes of Health). Results are expressed as mean ± SD of three independent experiments, each of which was performed in triplicate (duplicate for the SUnSET assays). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs*. control myotubes.

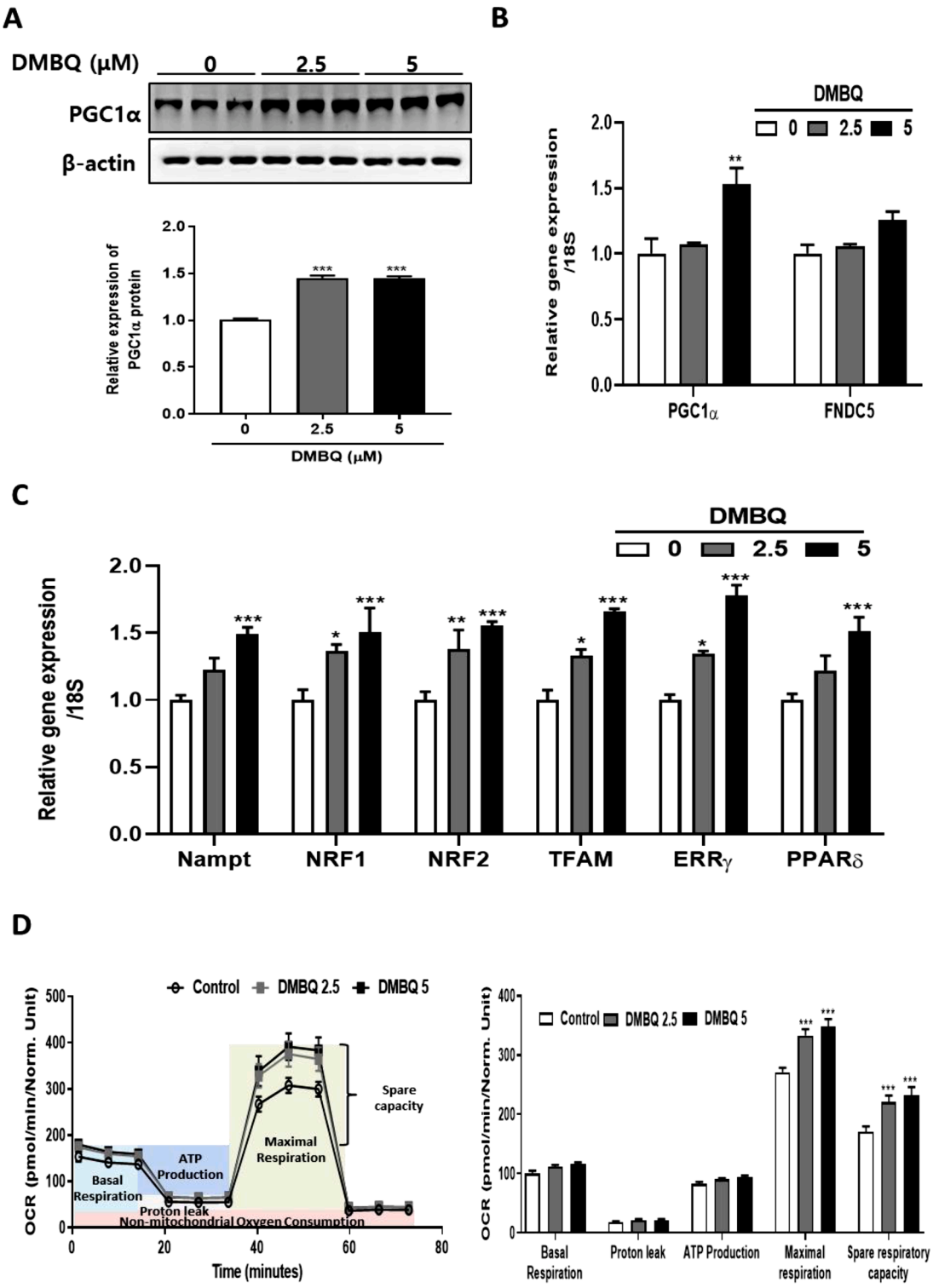

Fig. 3. Effect of DMBQ on mitochondrial function in C2C12 cells. (A) Western blotting analysis of PGC1α in cells, and band density was analyzed using ImageJ software. (B) Expression levels of *PGC1α* and *FNCD5* genes in C2C12 cells by qRT-PCR. (C) qRT-PCR analyses of *Nampt, NRF1, NRF2, TFAM, ERRγ*, and *PPARδ* mRNA expression in cells. (D) OCR in C2C12 cells. Results are expressed as mean ± SD of three independent experiments, each of which was performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* control myotubes.

shown in Fig. 6A, S3C, and 6F, DMBQ enhanced the mRNA and protein expression of total PGC1α and the mRNA expression of *FNDC5* and *PGC1α* isoforms 1 and 4. Next, to investigate the effect of DMBQ on mitochondrial function, we measured mtDNA content and the activities of citrate synthase and mitochondrial respiratory complexes I and II in the skeletal muscle. As shown in Fig. 6B, the mtDNA content was significantly higher in the DMBQ-treated mice than in the control mice. The citrate synthase, complex I and II subunit activities, and ATP content also increased significantly in the skeletal muscle of the DMBQ group (Fig. 6C-6E). In addition, we examined the mRNA expression levels of *Nampt, NRF1, NRF2, TFAM, ERRγ*, and *PPARδ* in skeletal muscle, which are known to be regulated by PGC1α. The expression

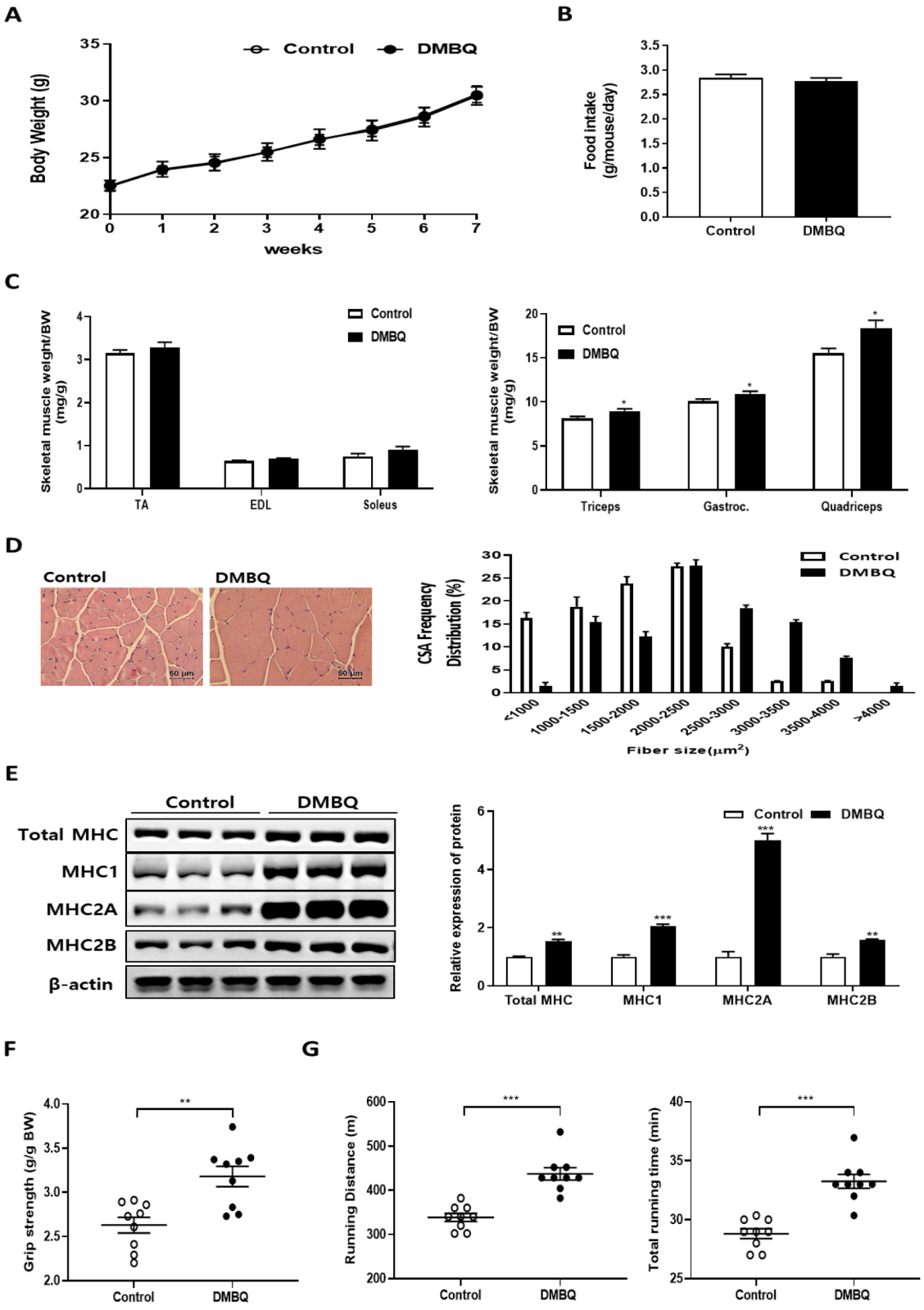

Fig. 4. Effect of DMBQ on skeletal muscle mass and muscle performance in mice. Male C57BL/6 mice were provided ad libitum access to standard diet or standard diet supplemented with 0.05% DBMQ for 7 weeks. (A and B) Body weight and food intake. (C) Tibialis anterior, extensor digitorum longus, triceps, gastrocnemius, soleus, and quadriceps muscle weight. (D) Representative image of myofiber cross-section of gastrocnemius muscle (hematoxylin and eosin staining; scale bar, 50 μm). Images were analyzed by light microscopy, and CSA was measured in at least three different sites (n = 5 per group). Distribution of muscle fiber CSA. (E) Expression of total MHC, MHC1, MHC2A, and MHC2B in quadriceps muscle as determined by western blotting. Band density was analyzed using ImageJ software: each band in the western blotting represents pooled sample of three muscles (F and G). DMBQ enhanced grip strength (g/g BW), running distance (m) and time (min). Results are expressed as mean ± SEM (n = 9). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs*. the control group.

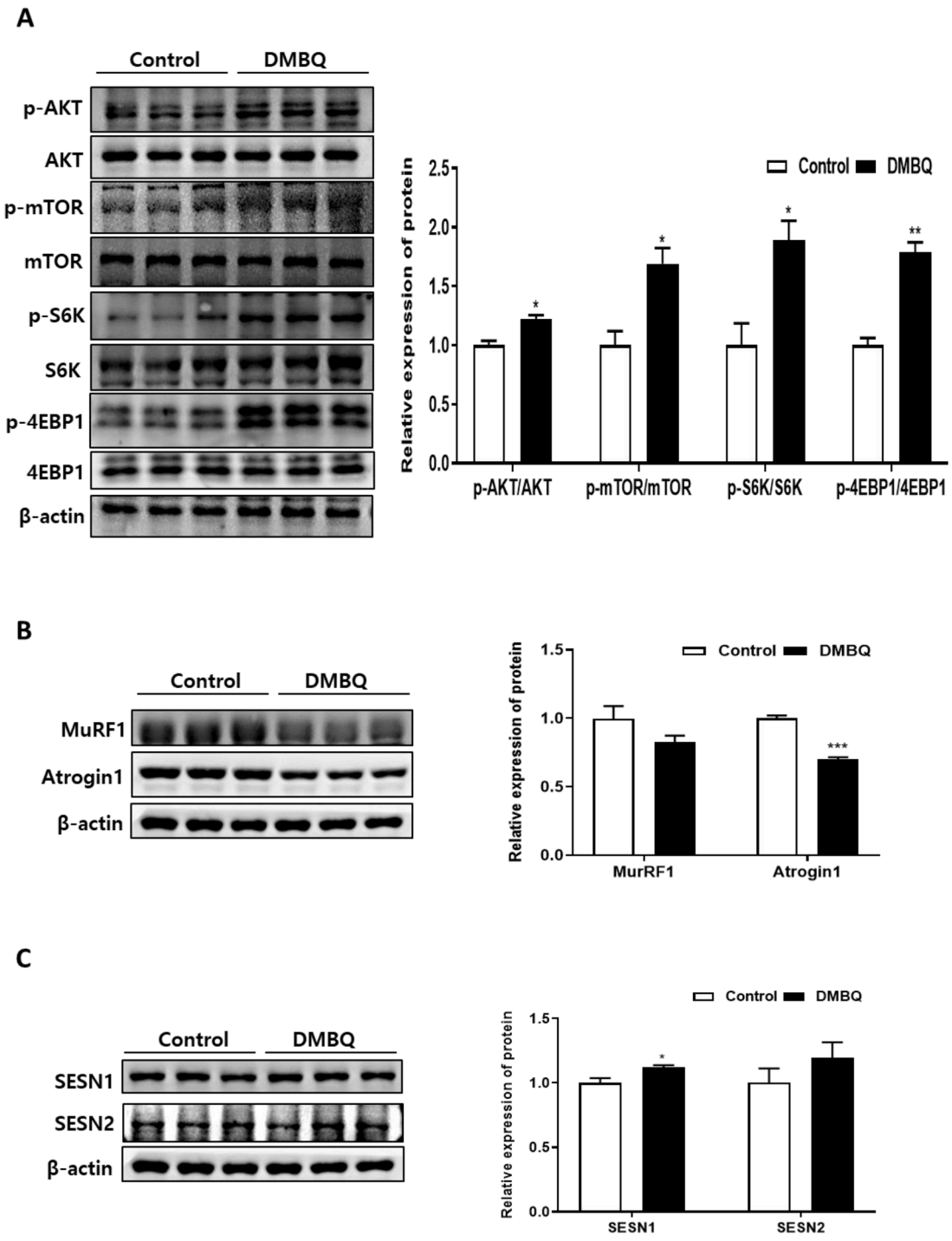

Fig. 5. Effect of DMBQ on the AKT/mTOR signaling pathway. (A) Protein expression of p-AKT, AKT, p-mTOR, mTOR, p-S6K, S6K, p-4EBP1, and 4EBP1 (B) MuRF1, Atrogin1, (C) SESN1, and SESN2 in quadriceps muscle as determined by western blotting. Band density was analyzed using ImageJ software: each band in the western blotting represents a pooled sample of three muscles. Results are expressed as mean ± SEM * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs*. the control group.

levels of all of these mRNAs were significantly elevated in the skeletal muscle of the DMBQ-fed mice compared with the corresponding levels in the control mice (Fig. 6F). DMBQ supplementation also enhanced the protein expression of ERRγ and PPARδ above control levels (Fig. 6G). We then analyzed the expression of OXPHOS genes in skeletal muscle and found both their mRNA (Fig. 6H) and protein (Fig. 6I) levels to be increased in the DMBQ group. These results indicate that DMBQ increases mitochondrial oxidative capacity, leading to improvements in mitochondrial function in mice.

Discussion

The importance of increasing or maintaining skeletal muscle mass and strength with aging has motivated considerable research into muscle hypertrophy and atrophy caused by many mechanisms. Recently, much attention has been given to identifying a natural compound that shows low toxicity and reduces skeletal muscle weakness caused by various problems. For example, tomatidine stimulates mTORC1 signaling and anabolism to induce skeletal muscle hypertrophy and prevent muscle loss (Dyle et al., 2014). Resveratrol has been

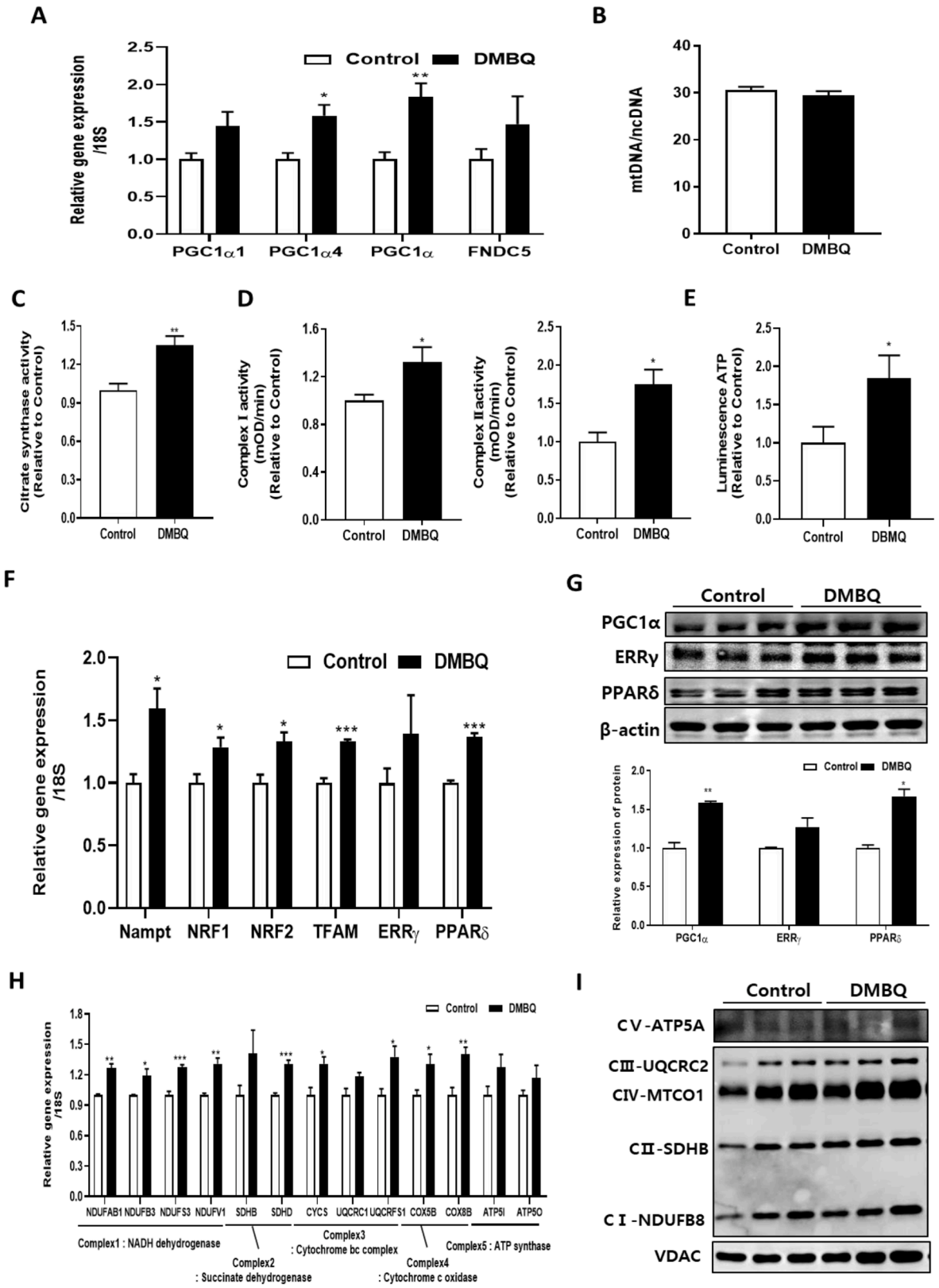

Fig. 6. Effect of DMBQ on mitochondrial function in mice. (A) mRNA expression levels of *PGC1α1, PGC1α4, PGC1α*, and *FNDC5* genes in gastrocnemius muscle quantified by qRT-PCR. (B) mtDNA content, (C) citrate synthase activity in gastrocnemius muscle of mice. (D) Complex I and II enzyme activities in gastrocnemius muscle of mice. (E) ATP content in gastrocnemius muscle of mice. (F) qRT-PCR analyses of *Nampt, NRF1, NRF2, TFAM, ERRγ* and *PPARδ* mRNA expression in gastrocnemius muscle. (G) Expression of PGC1α, ERRγ, and PPARδ in quadriceps muscle as determined by western blotting band density was analyzed using ImageJ software. (H) Relative transcript levels in gastrocnemius muscle of genes encoding proteins involved in OXPHOS. (I) Western blotting analysis of mitochondrial OXPHOS in gastrocnemius muscle: each band represents a pooled sample of three muscles. Results are expressed as mean ± SEM (n = 9). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* the control group.

found to promote myogenesis and hypertrophy in murine myoblasts (Montesano et al., 2013). Ursolic acid, a natural compound in apples, reduces muscle atrophy and stimulates muscle hypertrophy by enhancing insulin/insulin-like growth factor-1 (IGF1) signaling (Kunkel et al., 2011). Collectively, these results show that supplementation with food derivatives can help minimize the side effects of pharmaceuticals for the prevention of muscle loss. In the current study, DMBQ stimulated myoblast differentiation and increased myotube size in C2C12 cells (Fig. 2). Furthermore, DMBQ supplementation increased skeletal muscle mass *in vivo* without affecting body weight and food intake. In fact, DMBQ was found to decrease the weight of adipose tissue compared to the control group (data not shown). This increase in muscle mass in the DMBQ group may be partially affected by a decrease in the weight of adipose tissue, and further studies are required to clarify this issue, including crosstalk between skeletal muscle and adipose tissue. DMBQ also improved exercise capacity, such as grip strength and treadmill running, compared with control mice.)()()() In addition, DMBQ increased the skeletal muscle fiber size and the levels of MHC muscle fiber isoforms in mice. Improved exercise performance and increased MHC gene expression promote skeletal muscle hypertrophy. Endurance training leads to an increase in the proportion of type I muscle fibers, referred to as slow/oxidative fibers, and stimulation of mitochondrial content. However, resistance training leads to an increase in muscle size and the proportion of type II muscle fibers. Our results showed that DMBQ-treated mice exhibited an increase in muscle mass and size as well as total MHC, MHC1, MHC2A, and MHC2B protein expression levels. Furthermore, DMBQ promoted endurance and resistance exercise. These results demonstrated the role of DMBQ as a pharmaceutical supplement for skeletal muscle hypertrophy. Avemar, the activity of which is attributed to two quinones, DMBQ and 2-methoxy-1,4-benzoquinone (2-MBQ), has been used in Hungary since 1998 as a dietary supplement and was approved in 2002 as a "medical nutriment for cancer patients." In the United States, Avemar pulvis is intended to be used as a dietary supplement at 8.5 g/dose (or 121 mg/kg bw/day for a 70 kg individual) for an average adult (Heimbach et al., 2007).

AKT plays a role in inducing protein synthesis and blocking the transcriptional upregulation of key mediators of skeletal muscle atrophy. AKT is activated by a series of intracellular signaling cascades, including IGF1 and PI3K, and regulates skeletal muscle hypertrophy by activating mTOR (Leger et al., 2006). On the other hand, AKT inhibits forkhead box O (FOXO)-dependent atrogene expression, specifically MuRF1 and Atrogin1, by phosphorylating FOXO transcription factors and disrupting their nuclear activity (Stitt et al., 2004). In addition, the activation of AKT by SESN blocks ubiquitin-proteasome-dependent proteolysis through inhibition of FOXO signal (Segales et al., 2020). In the present study, it was observed that DMBQ increased the AKT phosphorylation, thereby increasing protein synthesis and decreasing skeletal muscle atrophy *in vivo*. In addition, increase of AKT phosphorylation by DMBQ was abolished by PI3K/AKT inhibitor in C2C12 cells.

Based on the present study, mTORC1 activation should lead to an increase in muscle mass and muscle fiber size. This belief is based on the finding that activation of the mTORC1 upstream kinase AKT causes an increase in muscle mass (Bodine et al., 2001a). In addition, it has been observed that mtDNA copy number and the expression of many genes encoding proteins involved in oxidative metabolism are increased by mutations that activate mTORC1 signaling. mTORC1 controls the transcriptional activity of PGC1α, a transcriptional coactivator that plays a key role in mitochondrial biogenesis and oxidative metabolism, by directly altering its physical interaction with another transcription factor (Cunningham et al., 2007). In the current study, DMBQ enhanced PGC1α expression, accompanied by an increase in mtDNA content, mitochondrial OXPHOS proteins, and oxidative enzyme activity. Meanwhile, our previous study showed that DMBQ negatively regulates mTORC1 activity, thereby inhibiting adipocyte differentiation (Son et al., 2019). DMBQ has also recently been reported as a potent mTOR inhibitor that reduces the growth of gastric cancer (Zu et al., 2020). These results are inconsistent with the data presented in this study. mTOR plays a central role in regulating the inhibition of catabolic pathways such as autophagy while increasing the production of proteins, lipids, and nucleotides for growth and division in various tissues (Saxton and Sabatini, 2017). In addition, resveratrol and ursolic acid, known to increase mTOR activity in skeletal muscle (Kim et al., 2015; Wang et al., 2014), have been reported to inhibit mTOR activity in other tissues, especially cancer tissues (He et al., 2011; Meng et al., 2015). DMBQ also seems to play a different role depending on the tissue.

Four transcripts of *PGC1α* (1, 2, 3, and 4), which regulate the expression of different downstream genes, are produced from the *PGC1α* gene (Millay and Olson, 2013). PGC1α1 functions as a major regulator of mitochondrial biogenesis, fiber-type switching, angiogenesis, and resistance to muscle atrophy, whereas PGC1α4 is mostly known to promote hypertrophy by activating the expression of IGF1 and suppressing myostatin in skeletal muscle (Ruas et al., 2012). Little is known about the functions of PGC1α2 and PGC1α3. In the present study, DMBQ enhanced *PGC1α1* and *PGC1α4* gene expression, thereby increasing mitochondrial biogenesis and muscle fiber size. PGC1α regulates many biological programs related to energy metabolism and stimulates an increase in FNDC5 expression, which in turn leads to increased brown adipose tissue, insulin sensitivity, and glucose tolerance (Boström et al., 2012). Furthermore, NRF1 and NRF2 regulate mitochondrial biogenesis through co-activation with PGC1α and expression of TFAM, which is involved in the transcription and replication of mtDNA (Kang et al., 2007). ERRγ and PPARδ, which are PGC1α transcriptional partners, are associated with the expression of nuclear-encoded mitochondrial proteins that are involved in OXPHOS and fatty acid oxidation (Handschin and Spiegelman, 2006). In the current study, DMBQ increased PGC1α mRNA levels and protein expression in C2C12 cells and mouse skeletal muscle. Furthermore, the DMBQ-fed mice showed increased mtDNA content, as well as citrate synthase and mitochondrial respiratory complex I and II activities, which in turn would increase ATP production in the skeletal muscle.

In conclusion, we showed that DMBQ stimulates myoblast differentiation and increases myotube size in C2C12 cells. DMBQ supplementation also increased skeletal muscle mass and performance *in vivo* through the AKT/mTOR signaling pathway. Furthermore, this beneficial effect involved the improvement of mitochondrial function both *in vitro* and *in vivo*. Our findings indicate that DMBQ is valuable as a leading compound for skeletal muscle hypertrophy and function and could have significant potential in the prevention and treatment of skeletal muscle atrophy.

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CRediT authorship contribution statement

Ahyoung Yoo: Investigation, Writing – original draft. **Young Jin Jang:** Methodology. **Jiyun Ahn:** Data curation. **Chang Hwa Jung:** Data curation. **Tae Youl Ha:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2021.153658.

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