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Platycodon grandiflorum-derived saponin enhances exercise function, skeletal muscle protein synthesis, and mitochondrial function

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ABSTRACT

Lower physical performance is an important risk factor in hypokinetic-related chronic disease, metabolic syndrome, and muscle atrophy. Our previous research demonstrated that *Platycodon grandiflorum*-derived saponin (PS) protects against eccentric exercise-induced muscle damage and mitochondrial function-related peroxisomal acyl-coenzme A oxidase (ACOX-1) and carnitine palmitoyltransferase (CPT-1) in high-fat diet-induced non-alcoholic steatohepatitis, and it inhibits osteoclast differentiation. However, the effects of PS on physical performance remain unknown. Therefore, we investigated whether PS enhances physical activity and skeletal muscle function. Supplementation with PS (2 mg/kg for 4 weeks) increased grip strength, wheel running repetition, and time to exhaustion in treadmill and swimming exercises. Marked increases in the synthesis of skeletal muscle proteins and muscle stem cell-related paired-box 7 (PAX7) were observed, and a decrease in the negative regulator myostatin was associated with enhanced muscle regeneration. Furthermore, PS induced expression of mitochondrial function proteins, including OXPHOS-III and -IV, *in vivo* and *in vitro*. These results suggest that PS enhances exercise function by ameliorating skeletal muscle protein synthesis and mitochondrial function.

1. Introduction

Increased muscular strength and muscle endurance can enhance physical activity. Muscle performance is associated with muscle mass and muscle metabolism. Muscles are weakened by exposure to pathological conditions such as sepsis, cachexia, trauma, burns, and sarcopenia, and recovery after muscle damage becomes more difficult (Rodriguez et al., 2017). The muscle damage repair and muscle maintenance systems require muscle regeneration processes that include an inflammatory response (Almada and Wagers, 2016; Charge and Rudnicki, 2004) and subsequent muscle protein synthesis (Haegens et al., 2012; Ives et al., 2017; Kato et al., 2016). Physical activity and nutritional interventions, such as resistance exercise and branched chain amino acids, increase muscle protein synthesis via the anabolic mammalian target of rapamycin (mTOR) signaling cascade (Liao et al., 2017; Moberg et al., 2016). Muscle protein synthesis is the driving force behind muscle volume and strength (Atherton and Smith, 2012).

Skeletal muscle mitochondrial dysfunction occurs with many disease conditions (Romanello and Sandri, 2015) and enhanced oxidative stress via the activation of NAD(P)H oxidase (Dai et al., 2014). Persistent skeletal muscle mitochondrial malfunction is associated with metabolic disorders such as obesity with diabetes (Di Meo et al., 2017). Sedentary behavior and muscle weakness decrease adenosine triphosphate (ATP) production by lowering mitochondrial function (Rabol et al., 2006). Physical activity and nutritional interventions, such as aerobic exercise and resveratrol, stimulate mitochondrial function via the AMPK signaling cascade (Di Meo et al., 2017; Haramizu et al., 2017). Skeletal muscle mitochondrial function is the driving force behind muscle metabolism and muscle endurance (Gonzalez-Franquesa and Patti, 2017). In many health conditions, physical inactivity is a major contributing factor to decreased muscle mass and muscle metabolism (Bogdanis, 2012). Thus, good health and physical fitness require increased muscle protein synthesis and muscle mitochondrial function (Dai et al., 2014; English et al., 2016; Robinson et al., 2017).

The biological actions of herbal medicine have recently received considerable attention. A previous study reported that *Platycodon*

Abbreviations: CPT-1, carnitine palmitoyltranserase; ERK, extracellular-signal-regulated kinases; mTOR, mammalian target of rapamycin; MHC, myosin heavy chain; OXPHOS, Oxidative phosphorylation; PAX7, paired-box 7; PS, *Platycodon grandiflorum* derived saponin; SDH, succinate dehydrogenase; 70S6K, p70 ribosomal protein S6 kinase

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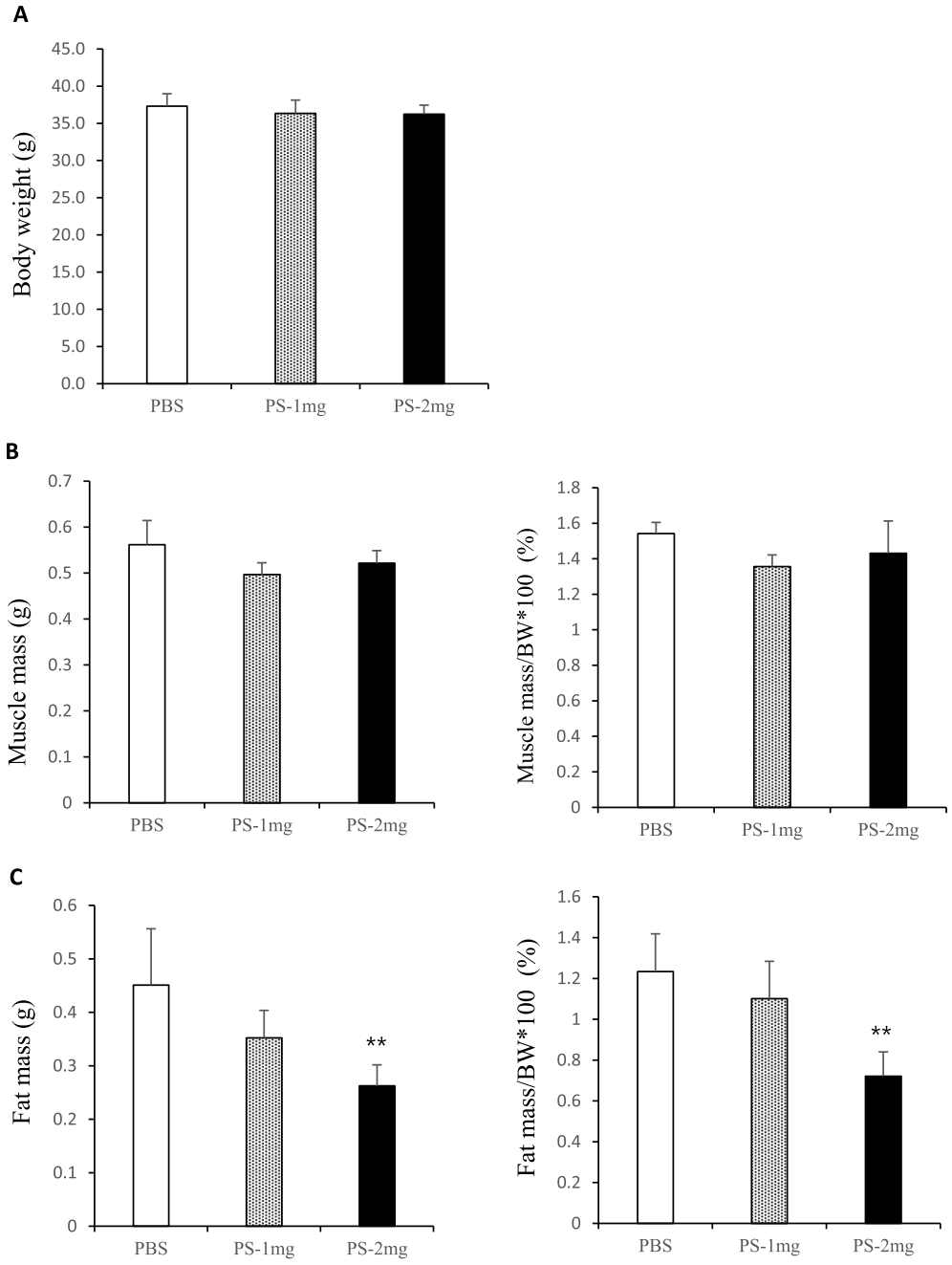


Fig. 1. Effects of *Platycodon grandiflorum*-derived saponin (PS) supplementation on body composition and exercise function. Mice were provided ad libitum access to a control diet supplemented with PS for 4 weeks. Body weight (BW) (A), muscle/BW ratio (B), and fat/BW ratio (C) in the three groups of mice are shown. Each group consisted of eight animals (n = 8). Exercise function was determined based on grip strength/BW (D), wheel running distance (E), exhausted treadmill exercise time (F), exhausted swimming time, and glucose level (G). Data are shown as the mean ± standard deviation (SD). *p < 0.05 and **p < 0.01 vs. phosphate-buffered saline (PBS) group; #p < 0.05 vs. PS-1 mg/kg group.

grandiflorum contains triterpenoid saponins, such as deapioplatycodin D3, platycodin D3, polygalcin D2, platyconic acid A, platycodin D2, platycodin D, and 2′-O-acetylpolygalacin D2 (Choi et al., 2014). The roots of *Platycodon grandiflorum* have been used as a food, in traditional oriental medicine to treat adult diseases such as asthma, bronchitis, and pulmonary tuberculosis, and even as a sedative (Zhang et al., 2015). In our previous work, PS suppressed the eccentric exercise-induced muscle damage, indicating that PS has potential for enhancing the exercise function (Kim et al., 2018). In the present study, both *in vivo* and *in vitro* experiments were employed to investigate the role of *Platycodon grandiflorum* in protein synthesis and mitochondrial function in skeletal muscles. Our results provide the first evidence that the administration of PS effects exercise function, protein synthesis signaling, and skeletal muscle mitochondrial function.

2. Materials and methods

2.1. Animal and treatments

Eight-week-old male ICR mice were obtained from Daehan Biolink (Seoul, South Korea) and acclimatized to the experimental facility for 1 week. Mice were housed in a controlled environment (22–23 °C, 12/12-h light/dark cycle) in accordance with the guidelines of the Chungnam National University Animal Ethics Committee (CNU-0068).

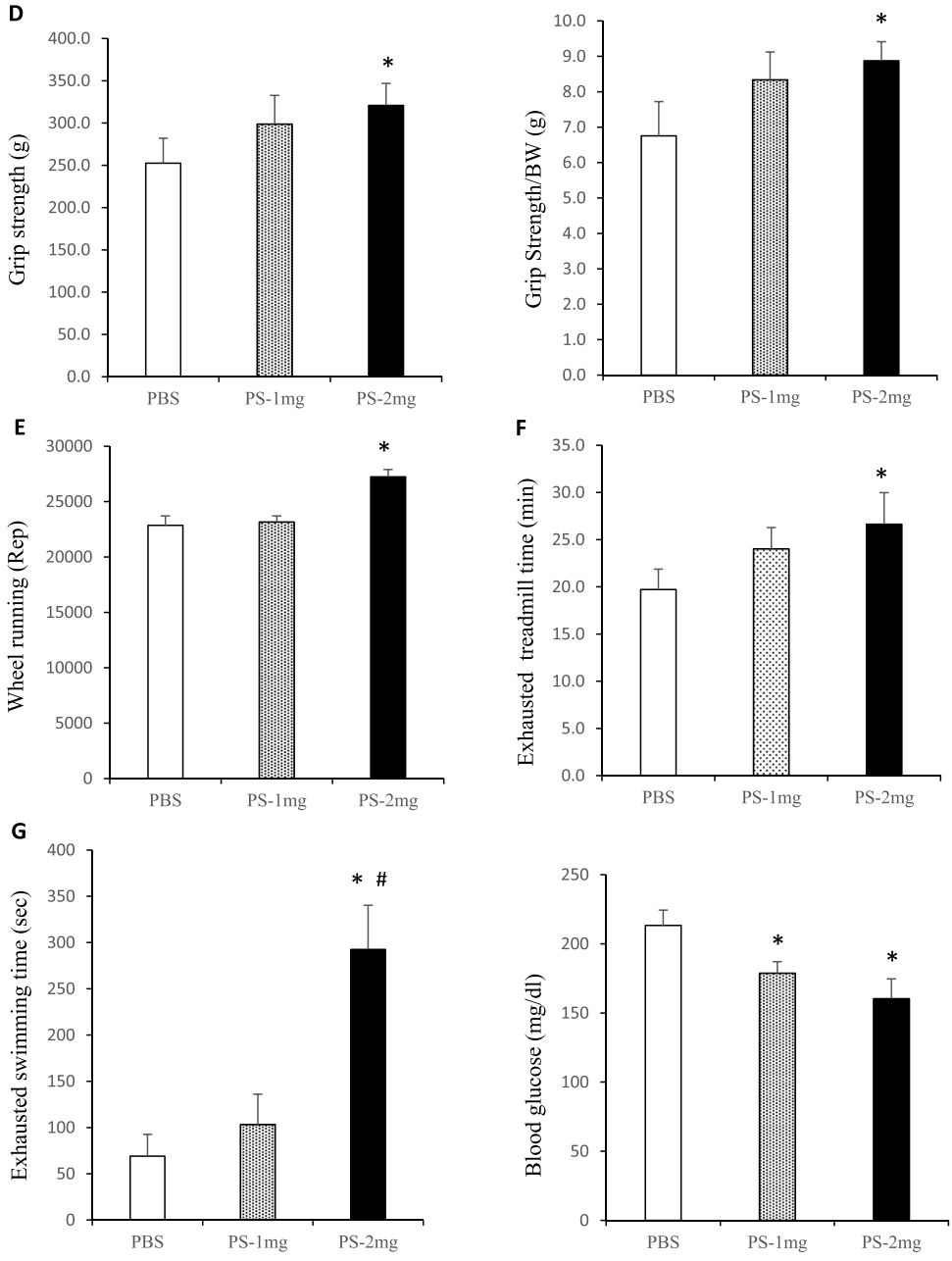


Fig. 1. (*continued*)

Experimental animals for *Platycodon grandiflorum*-derived saponin (PS) administration were randomly divided into three groups, i.e., PS-1 mg/kg, and PS-2 mg/kg and respective treatments were administered via oral gavage once daily for 7 days. Saponin were purified from *Platycodon grandiflorum*, and their composition were previously determined by high-performance liquid chromatography (Kim et al., 2018). PS extract was prepared as previously described (Kim et al., 2018). Mice were randomly divided into three groups to assess skeletal muscle protein synthesis as follows: phosphate-buffered saline (PBS): control group given PBS (n = 8); PS-1: mice given 1 mg/kg PS (n = 8); and PS-2: mice given 2 mg/kg PS (n = 8) for 4 weeks. After sacrifice, the gastrocnemius muscle was collected and weighed.

2.2. Exercise function measurement

Grip strength test: A maximal voluntary force testing system (BIO-G53, BIOSEB, Canada) was used to measure the forelimb grip strength of mice as previously described (Menalled et al., 2010).

Wheel running test: After 3 weeks of PS supplementation, all animals were subjected to voluntary wheel running exercise. Mice were individually housed in cages equipped with a 12.5 × 5-cm mouse metal running wheel coupled to a turn counter. Wheel revolutions were recorded daily by a digital counter between 18:00 and 09:00 h (Banjanin and Mrosovsky, 2000).

Exhaustive treadmill exercise test: An exhaustive running test began at a rate of 10 m/min for 5 min with a 0% slope. The speed was then gradually increased to 15 and then 20 m/min, and then maintained at this speed until exhaustion, which was defined as the inability to run for 10 s (Yu et al., 2014).

Swimming test: In the exhaustion swimming test, mice carried constant loads corresponding to 3% of their body weight (BW) while swimming to analyze endurance time, as previously described (Filgueiras and Manhaes, 2004). Exhausted swimming was defined as a loss of coordinated movements and failure to return to the surface

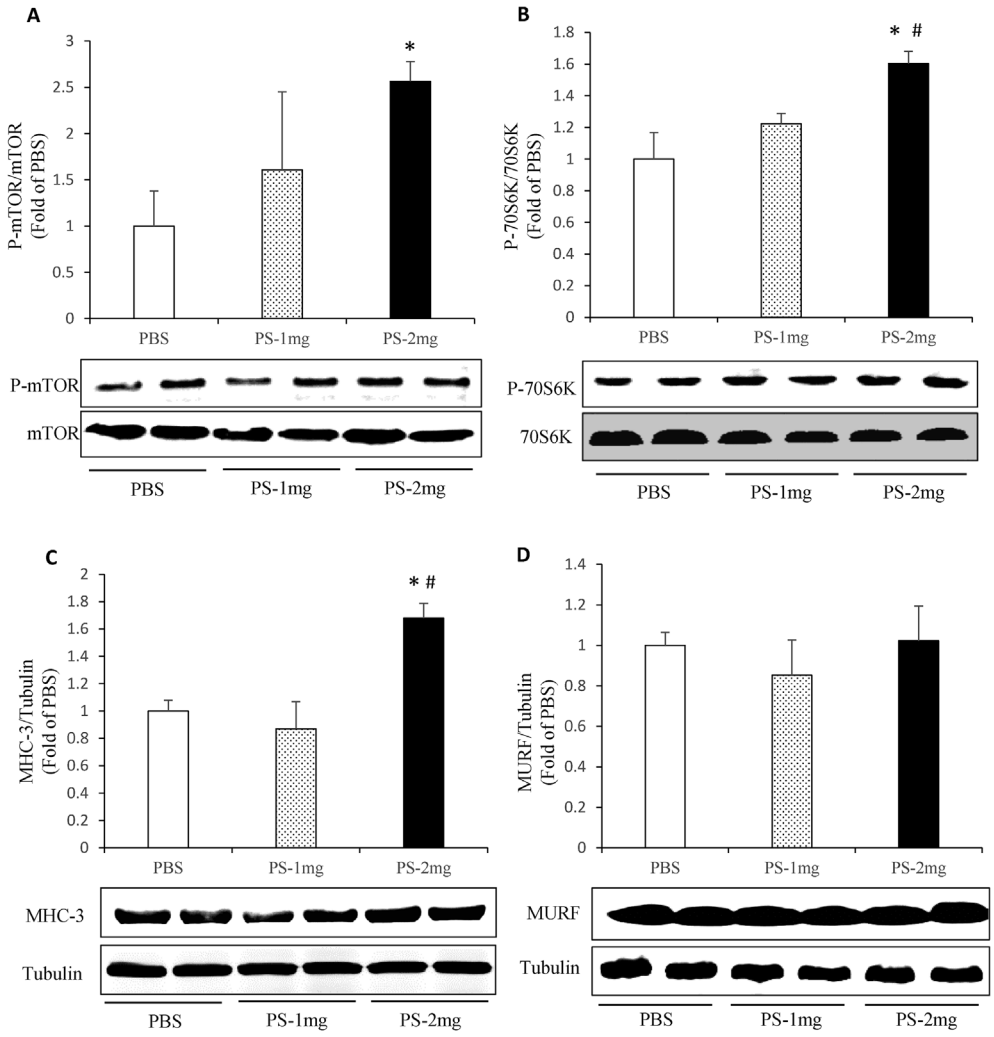


Fig. 2. Effects of PS supplementation on skeletal muscle formation *in vivo*. (A–C) Phosphorylation state of mammalian target of rapamycin (mTOR) and the ratio of total mTOR. Phosphorylation state of p70 ribosomal protein S6 kinase (70S6K) and the ratio of total 70S6K. Protein levels of myosin heavy chain-3 (MHC3) and α-tubulin (loading control). (D and E) Protein expression of MURF and Atrogin. α-Tubulin was used as the loading control. (F and G) Protein expression of paired-box 7 (PAX7) and myostatin. Results are expressed as the mean ± SD. *p < 0.05 vs. PBS group; #p < 0.05 vs. PS-1 mg/kg group.

within 7 s.

2.3. C2C12 cell culture

Mouse C2C12 myoblasts were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). C2C12 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/ml streptomycin up to 70% confluence. During the proliferation phase, 6×10^2 cells/cm^2 were maintained in mitogen-rich growth medium (GM) as single myoblasts up to 90% confluence, at which point, the GM was removed and cells were transferred to DMEM supplemented with 2% horse serum. These differentiating cells were treated with PS 2 μg/ml. The ideal concentration for cell treatment was 2 μg/ml, which was confirmed by applying PS concentrations of 1, 2, 4, and 6 μg/ml (Supplementary Fig. S1). Cells were grown at 37 °C in an atmosphere containing 5% CO_2. A stock solution of PS was prepared in dimethyl sulfoxide (DMSO). Platyconic acid A (PCA) and platycodin D (PCD) were isolated from PS (Choi et al., 2015; Hwang et al., 2013). Control cells were treated with DMSO only. The final DMSO concentration in all experiments was < 0.2%.

2.4. Western blot analysis

Following treatment, isolated gastrocnemius muscles and C2C12 cells were lysed in lysis buffer on ice for 30 min and centrifuged at 22,250 g for 20 min. Supernatants were collected, and protein concentrations were measured using a protein assay kit (Intron Bio, Inc., Korea). Aliquots of the lysates were boiled and electrophoresed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes, which were then incubated with a secondary anti-mouse or anti-rabbit antibody. Finally, the protein bands were detected using an enhanced chemiluminescence detection kit (BIOFACT, Inc., Korea). The integrated optical density of each protein band was calculated using ImageJ software. Values were normalized to the housekeeping gene α-tubulin or to the non-phosphorylated protein.

2.5. Statistical analysis

All data are expressed as the mean ± standard deviation of the mean. Differences among groups were analyzed by one-way analysis of variance with SPSS22 (IBM, Armonk, NY, USA). P < 0.05 was considered statistically significant.

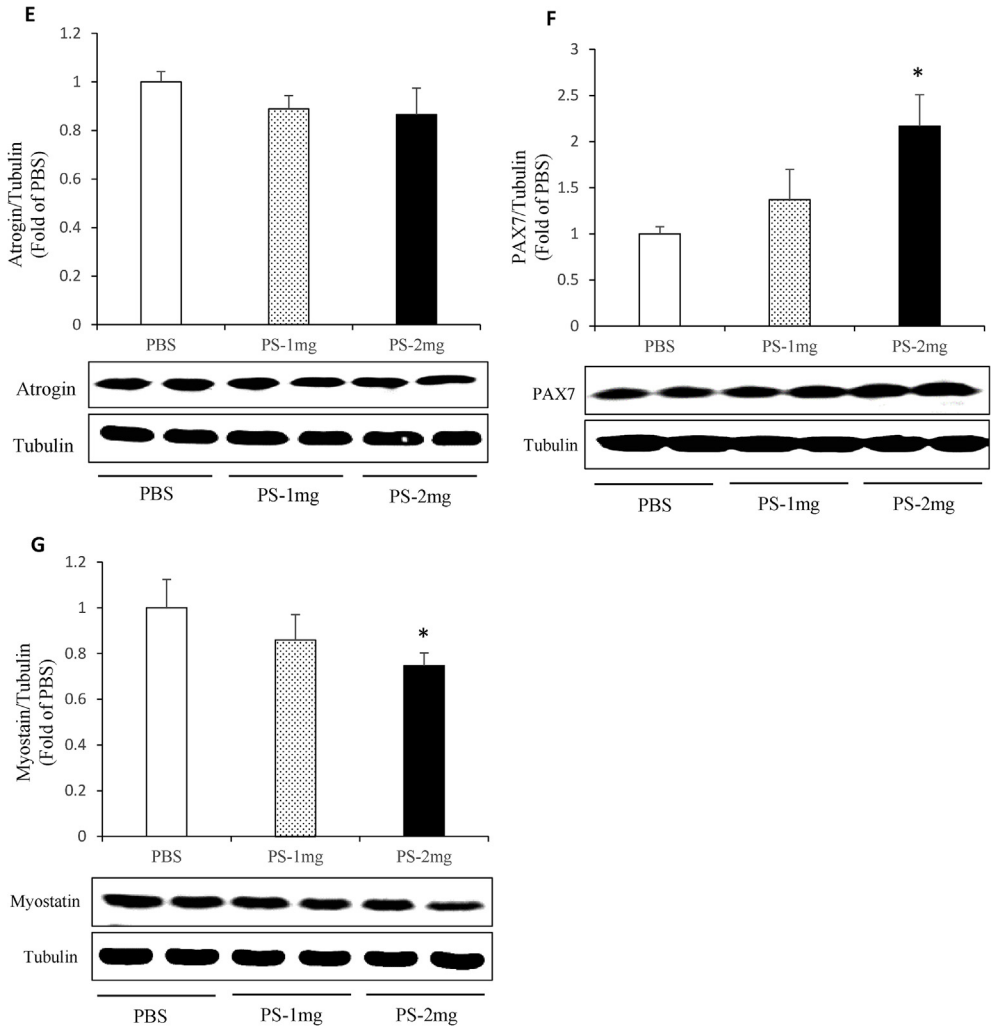


Fig. 2. (*continued*)

3. Results

3.1. PS treatment enhanced exercise function

To evaluate the effects of PS supplementation on physical characteristics, we examined body composition and exercise function. BW and muscle mass following PS treatment did not differ among the groups examined (Fig. 1-A and B). However, fat mass and the fat mass/BW ratio were significantly lower in the PS-2 group than in the PBS group (Fig. 1-C). We then investigated the phenotypic effect of PS treatment on exercise function. As shown in Fig. 1-D, PS supplementation increased mouse grip strength in a dose-dependent manner. Notably, the PS-2 group exhibited significantly increased grip strength and grip strength/BW ratio compared to the PBS group. Additionally, wheel running revolutions and exhausted treadmill exercise time were increased in parallel with grip strength (Fig. 1-E and F). After PS administration, all mice were loaded with 3% of their BW and performed a swimming test until exhaustion. The PS-2 group exhibited a significantly longer time to exhaustion and lower glucose level compared with the PBS group (Fig. 1-G).

3.2. PS treatment enhanced skeletal muscle formation in vivo

To obtain a better understanding of the effect of PS on exercise function in mice, we measured muscle formation *in vivo* and *in vitro*. The mammalian target of rapamycin (mTOR) phosphorylation ratio in the gastrocnemius muscle was significantly increased in the PS-2 group compared with the PBS group (Fig. 2-A). Additionally, the p70 ribosomal protein S6 kinase (70S6K) phosphorylation ratio was significantly increased in parallel with the mTOR phosphorylation ratio (Fig. 2-B). The muscle contraction protein myosin heavy chain 3 (MHC3) was significantly increased in the PS-treated group compared with the PBS group (Fig. 3-C). However, there was no significant difference in the muscle protein breakdown-related proteins MURF or Atrogin in the PS-treated group compared with the PBS group (Fig. 2-D and E). Notably, PS supplementation may affect muscle regeneration because paired-box 7 (PAX7), a stem cell marker in skeletal muscle, was significantly increased in the PS-2 group compared with the PBS group (Fig. 2-F), and the muscle negative regulator myostatin was suppressed in the PS-2 group compared with the PBS group (Fig. 2-G).

3.3. PS treatment enhanced myotube formation in vitro

We hypothesized that PS treatment exerts a biological function in skeletal muscle protein synthesis, so we examined the effects of PS on muscle generation. We demonstrated *in vitro* that PS promoted protein synthesis and hypertrophy. We also investigated morphological features and protein synthesis in the late differentiation phase to determine whether the mature form was promoted in C2C12 cells after exposure to PS-2 μg/ml for the indicated number of days. Morphological changes were also observed in the cells treated with PS-2 μg/ml. PS appeared to promote the typical fiber shape characteristic of C2C12 cells in the

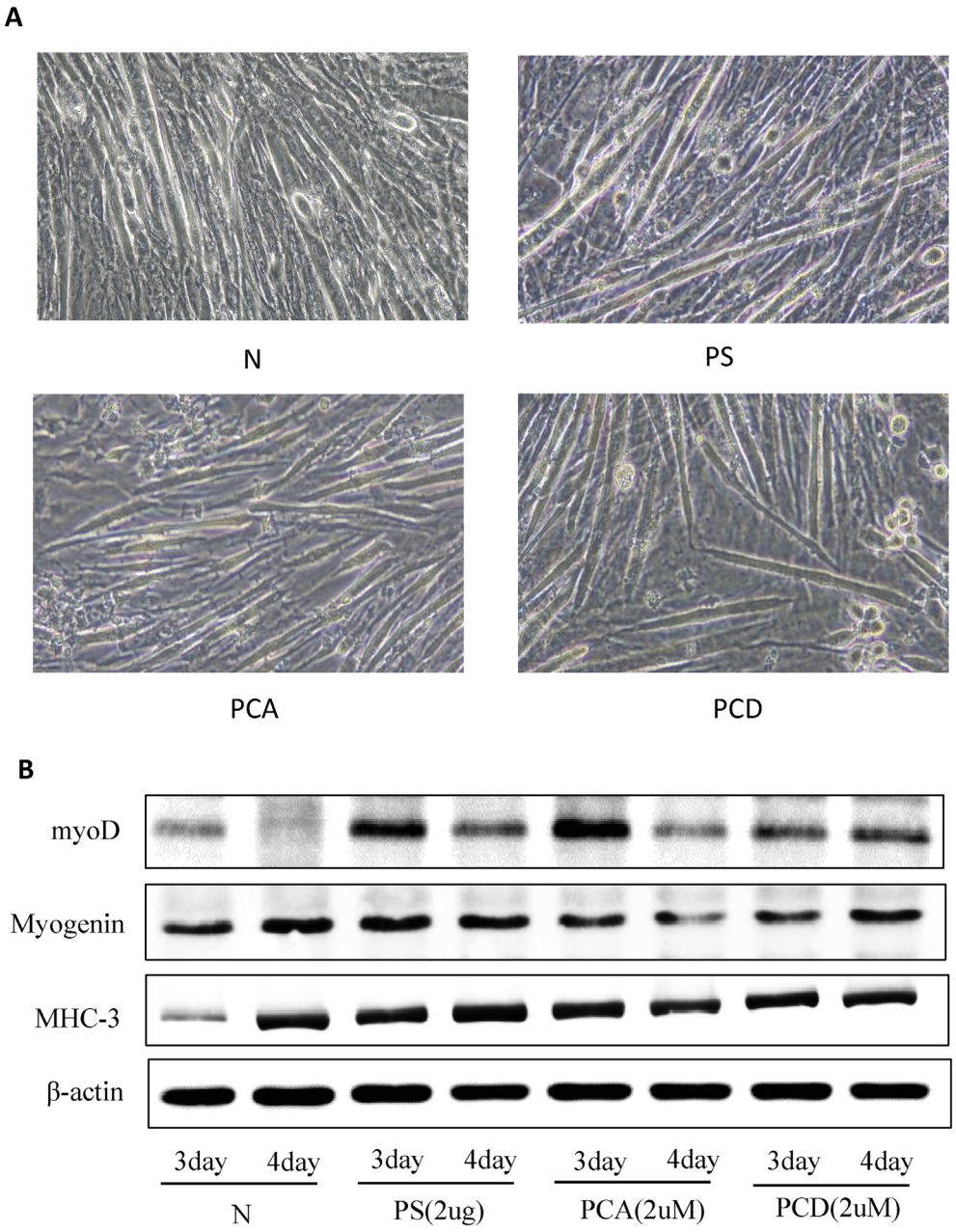
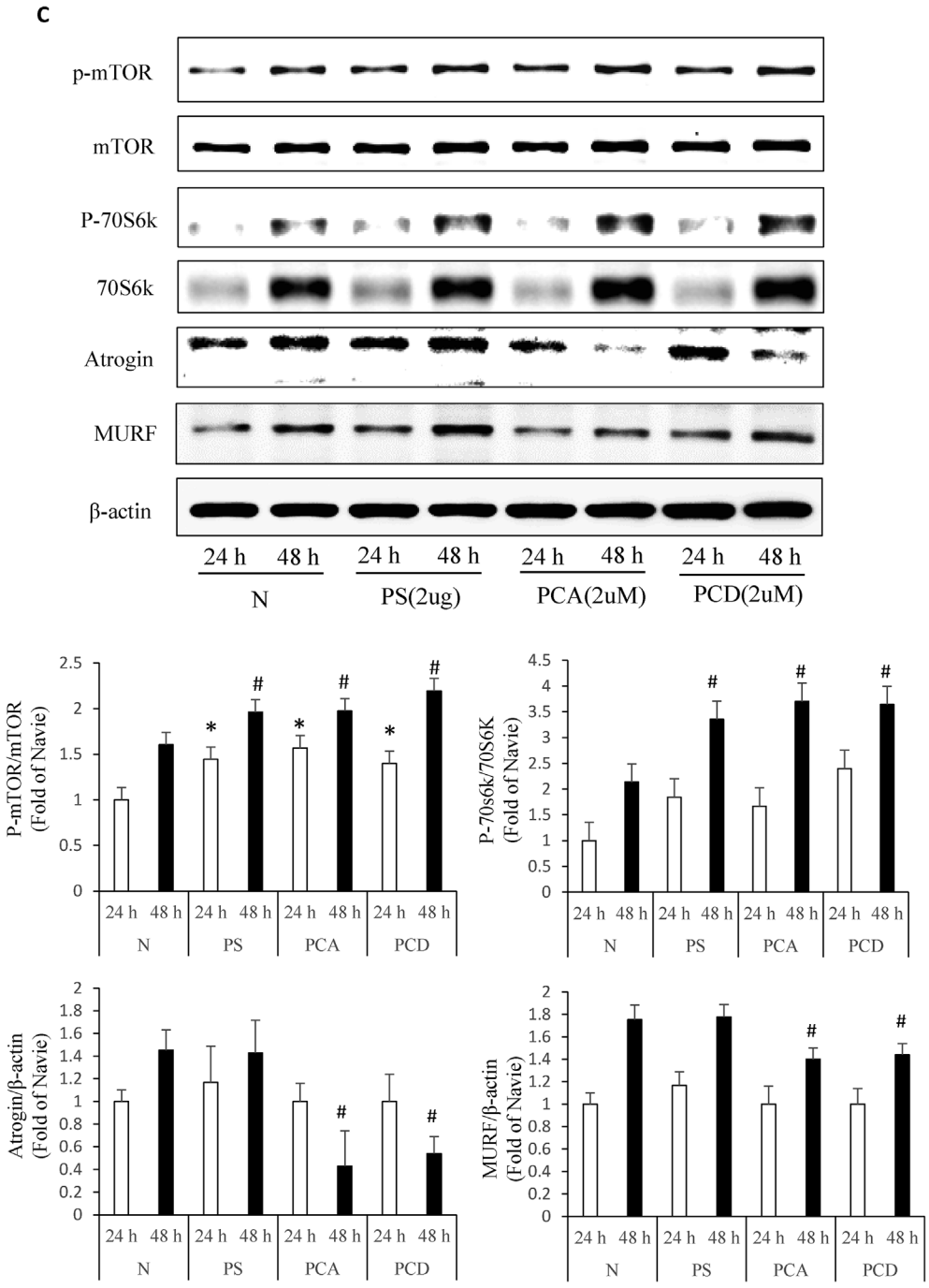


Fig. 3. Effects of PS treatment on the differentiation and hypertrophy of C2C12 cells. (A) Microscopy shows hypertrophic morphological changes in myotube thickness (10 × magnification). (B) Differentiation: protein expression of MyoD, myogenin, MHC3, and β-actin (loading control). (C) Protein synthesis: phosphorylation state of mTOR and the ratio of total mTOR. Phosphorylation state of 70S6K and the ratio of total 70S6K (loading control). Protein breakdown: protein expression of Atrogin and MURF. β-Actin was used as the loading control. (D) Negative regulator: protein expression of myostatin. Myostatin downstream signaling: extracellular signal-regulated kinase (ERK), p38, and SMAD2/3. Results are expressed as the mean ± SD. *p < 0.05 vs. naïve cells at 24 h; #p < 0.05 vs. naïve cells at 48 h.

active differentiation phase, which is necessary to achieve a newly elongated morphology. Phase-contrast images collected at day 4 of growth to the mature form confirmed these morphological features: changes in fiber thickness and shape were compared in detail, emphasizing the analogy between differentiation control (DM) cells and PS-2 μg-treated cells (Fig. 3-A and B). Expression of the protein synthesis-related proteins mTOR and 70S6K increased significantly with the onset of late differentiation in PS-, PCA-, and PCD-treated cells compared with naïve cells (Fig. 3-C). The muscle-specific E3 ubiquitin ligase proteins MURF and Atrogin were potently suppressed in PCA- and PCD-treated cells compared with naïve cells (Fig. 3-C). To confirm the data obtained from thick fibers, we examined the expression of myostatin and its downstream proteins SMAD, extracellular signal-regulated kinase (ERK), and p38. Notably, PCA and PCD suppressed the expression of myostatin and reduced the phosphorylation of p38 and SMAD2/3 (Fig. 3-D).

3.4. PS treatment enhanced skeletal muscle OXHPHOS in vivo and in vitro

To obtain a better understanding of the effect of PS on endurance exercise function in mice, we measured skeletal muscle mitochondrial function *in vivo* and *in vitro*. OXPHOS-III in the gastrocnemius muscle was significantly increased in the PS-2 group compared with the PBS group. However, there was no significant difference in OXPHOS-III in the PS-1 group compared with the PS-2 group. OXPHOS-IV was significantly increased in parallel with OXPHOS-III. Additionally,



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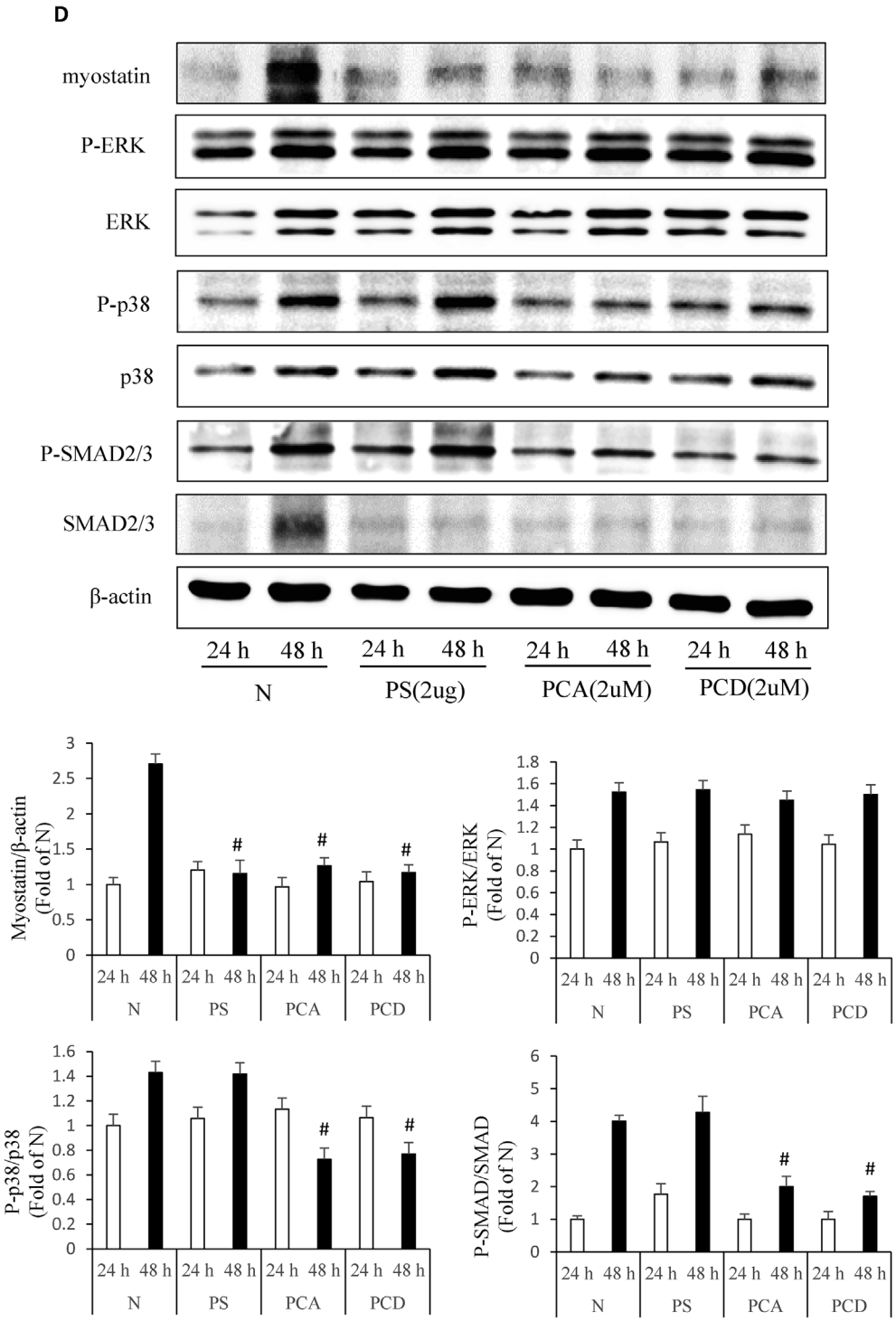
OXPHOS-IV was significantly increased, by 3 fold, in the PS-2 group compared with the PS-1 group (Fig. 4-A). OXPHOS-III in C2C12 myotubes was significantly increased in the PS-2 compared with the naïve (N) group. OXPHOS-IV was significantly increased, by 2.5 fold, in the PS-2 group compared with the naïve group, consistent with OXPHOS-III (Fig. 4-B).

4. Discussion

In this study, we first demonstrated that PS supplementation enhanced exercise function, muscle protein synthesis, and muscle mitochondrial function both *in vivo* and *in vitro*. *In vivo* experiments in PS-treated mice revealed enhancement of exercise function. We also showed that PS supplementation reduced fat mass and increased protein synthesis. We used differentiated C2C12 cells to obtain more insights into the molecular mechanisms of the effects of PS in muscle.

The most important finding of this study was that the chronic administration of PS increased muscle protein synthesis both *in vivo* and *in vitro*. Muscle protein synthesis is the driving force behind muscle volume and strength (Atherton and Smith, 2012). mTOR, a central anabolic metabolism sensor, plays a pivotal role in muscle protein synthesis (Yuan et al., 2013). Thus, improved exercise function may be due to the increased synthesis of muscle proteins, including mTOR and 70S6K, and proteins involved in muscle contraction, such as MHC-3 (Wells et al., 1996). MHC is responsible for the power stroke movement that breaks down ATP, converting it into mechanical energy that pulls the actin filament across the myosin filament (Varkuti et al., 2012). Aberrant distribution of MHC isoforms has been observed in muscular dystrophy



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(Beedle, 2016; Hamida et al., 1994), exercise training-induced hypertrophy (Herda et al., 2016), and muscle damage (Kim et al., 2012). In contrast, the effect of PS was not consistent with the signaling associated with muscle protein breakdown *in vivo* and *in vitro* (MURF and Atrogin). Expression of both MURF and Atrogin were highly increased in skeletal muscles of rats and mice upon immobilization, denervation or hind-limb suspension (a muscular disuse approach) induced atrophy (Bodine et al., 2001; Gomes et al., 2001). In mice, gene deficiencies for either MURF or Atrogin lead to a strong reduction in denervation induced skeletal muscle weight loss and the reduction of muscle fiber diameter decrease. The elaborate maintenance of muscle mass is achieved by balancing protein synthesis and degradation. Thus, an increase protein synthesis with no difference in protein breakdown can lead to an increase in muscle protein. Notably, PS administration may affect muscle regeneration because PAX7, a stem cell marker of skeletal muscle, was increased, and myostatin, a muscle negative regulator, was simultaneously suppressed both *in vivo* and *in vitro*. PAX7 is regulated during myoblast proliferation and differentiation. It is well accepted that high levels of PAX7 compromise myogenic differentiation due to excess and prolonged proliferation, whereas low levels of PAX7 result in precocious differentiation (Riuzzi et al., 2012). After muscle injury, activated satellite cells give rise to the myogenic progenitors needed to

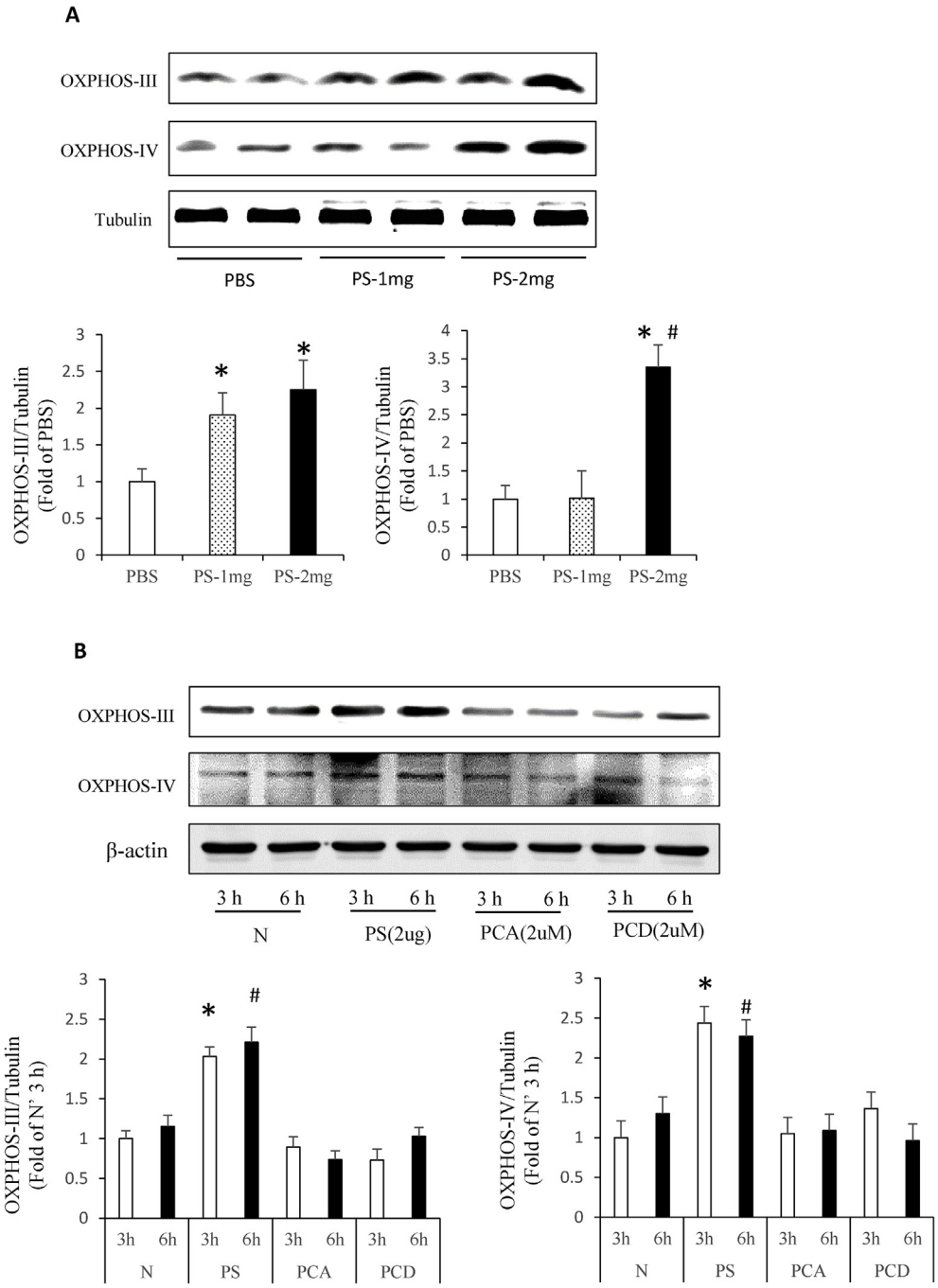

Fig. 4. Effects of PS treatment on mitochondrial function *in vivo* and *in vitro*. (A) Protein expression of OXPHOS-III and -IV in the gastrocnemius muscle. α-Tubulin was used as the loading control. Results are expressed as the mean ± SD. *p < 0.05 vs. PBS; #p < 0.05 vs. PS-1 mg/kg. (B) Protein expression of OXPHOS-III and -IV in C2C12 cells. β-actin was used as the loading control. Results are expressed as the mean ± SD. *p < 0.05 vs. naïve cells at 3 h; #p < 0.05 vs. naïve cells at 6 h.

form new myofibers, but they also need to self to maintain the satellite cell pool (Dumont et al., 2015; Shinin et al., 2006). Previous studies have demonstrated a complete lack of regeneration in adult skeletal muscle depleted of PAX7 expressing satellite cells (Gunther et al., 2013; Lepper et al., 2011; von Maltzahn et al., 2013). Myostatin, a transforming growth factor (TGF)-β superfamily member, negatively regulates satellite cell activation and self-renewal (McFarlane et al., 2008). TGF-β belongs to a family of cytokines whose signals are transduced via the SMAD intracellular signaling cascade (Cohn et al., 2007; Heldin et al., 1997; Hwang et al., 2016). Myostatin binds to type I and type II receptors (ACTRIIB) to activate both canonical SMAD2/3 and non-canonical MAPK (p38, JNK, and ERK1/2) pathways (Egerman et al., 2015). We examined the expression of ERK, p38, and SMAD2/3 to confirm the downstream signal of myostatin, the muscle negative regulator, *in vitro*. Myostatin suppression by PCA and PCD administration acted via p38 and SMAD2/3. Actually, PS has been shown to suppression effect of myostatin on eccentric exercise induced muscle damage (Kim et al., 2018). Therefore, our results suggest that PS administration may promote skeletal muscle regeneration.

In this study, to obtain a better understanding of the effect of PS on endurance exercise function, we measured skeletal muscle mitochondrial function. PS-2 mg/kg treatment increased the expression of OXPHOS-III and -IV compared with the PBS group. Additionally, mitochondrial respiratory chain-related OXPHOS-III and -IV proteins were

increased in the PS-2 μg-treated C2C12 cells compared with naïve cells. The increases in time to exhaustion during the treadmill and swimming exercises and in wheel running revolutions were accompanied by an increase in oxidative skeletal muscle function. Skeletal muscle mitochondrial function is the driving force behind muscle metabolism and muscle endurance (Gonzalez-Franquesa and Patti, 2017). An abundant mitochondrial respiratory chain complex can enhance cellular energy metabolism, resulting in efficient ATP production, which is associated with muscle endurance (Rabol et al., 2006). In addition, the expression of some OXPHOS protein subunits (OXPHOS-III and -IV) were increased upon exposure of cells to PS.

In conclusion, these results suggest for the first time that PS supplementation enhances exercise function by facilitating skeletal muscle protein synthesis and mitochondrial function.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2018.04.062.

Conflicts of interest

None of the authors declares a conflict of interest.

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