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Activation of the Nrf2/HO-1 signaling pathway contributes to the protective effects of platycodin D against oxidative stress-induced DNA damage and apoptosis in C2C12 myoblasts

Yung Hyun Choi[1,2]

[1] *Anti-Aging Research Center, Dong-eui University, Busan, Republic of Korea*
[2] *Department of Biochemistry, Dong-eui University College of Korean Medicine, Busan, Republic of Korea*

Abstract. Myoblast damage by oxidative stress has been proposed as one of the main causes of skeletal muscle loss due to induction of muscle damage. Platycodin D, a triterpenoid saponin found in the root of *Platycodon grandiflorum* (Jacq.) A. DC., has been known to possess strong antioxidant activity. However, whether platycodin D can defend myoblasts against oxidative injury remains to be elucidated. Therefore, this study was conducted to investigate the potential protective effects of platycodin D against oxidative stress in mouse myoblast C2C12 cells. The results demonstrated that platycodin D inhibited hydrogen peroxide (H_2O_2)-induced cytotoxicity and DNA damage by blocking abnormal reactive oxygen species (ROS) generation. Furthermore, platycodin D protected cells from the induction of mitochondria-mediated apoptosis by oxidative stress. In addition, platycodin D markedly promoted the activation of nuclear factor-erythroid-2-related factor 2 (Nrf2), which was associated with the enhanced expression of heme oxygenase-1 (HO-1) in the presence of H_2O_2. However, inhibiting the expression of HO-1 by Nrf2 siRNA significantly attenuated the protective effect of platycodin D, indicating that platycodin D activates the Nrf2/HO-1 signaling pathway to protect against oxidative stress. Based on current data, platycodin D may be useful as a potential therapeutic agent against various oxidative stress-related muscle disorders.

Key words: Platycodin D — ROS — DNA damage — Apoptosis — Nrf2/HO-1

Introduction

Muscles require a large amount of oxygen due to the high energy demand for contractile activity. Myoblasts are embryonic precursors of muscle cells produced by tissue-resident stem cells called satellite or muscle stem cells. They differentiate into muscle cells through myogenesis, a process that is fused to multi-nucleated myotubes (Chang and Rudnicki 2014; Sambasivan and Tajbakhsh 2015). Oxidative stress is characterized by the overproduction of reactive oxygen species (ROS) in cells or tissues. The role of ROS in myogenic differentiation is complicated by the wide spectrum of cellular responses depending on the ROS level. At an appropriate level, ROS regulate many cellular signaling pathways, including myogenic differentiation, but high levels of ROS are closely related to impaired myogenesis in response to aging and injury repair (Hansen et al. 2007; Sestili et al. 2009; Sandiford et al. 2014). In addition, enhanced ROS generation has been shown to correlate with the degree of skeletal myoblast damage during muscle atrophy, which was related to the acceleration of myoblast apoptosis (del Río et al. 2006). Excess ROS levels also lead to the formation of protein-protein crosslinks, and the oxidation of amino acid residues that cause various pathological phenomena including skeletal muscle atrophy (Kerkweg et al. 2007; Sachdev and Davies 2008). Moreover, abnormal accumulation of ROS in myoblasts, as well as in many other cell types, could increase chromosomal aberration and DNA strand breakage, leading to DNA damage and apoptosis (Caporossi et al. 2003; Santa-Gonzalez et al. 2016). These observations indicate that ROS levels should

Correspondence to: Yung Hyun Choi, Department of Biochemistry, Dong-eui University College of Korean Medicine, 52-57 Yangjeong-ro, Busan 47227, Republic of Korea
E-mail: choiyh@deu.ac.kr

be tightly regulated because high levels of ROS contribute to the loss of myoblast function, promote myoblast death, and further exacerbate muscle function. Therefore, the study of novel antioxidants and their mechanisms has been recognized as a strategy for the prevention and treatment of muscle injuries by elevated ROS.

Platycodin D is a triterpene saponin, one of the main bioactive components contained in Platycodi Radix, the roots of *Platycodon grandiflorum* (Jacq.) A. DC., of the *Campanulaceae* family, which has traditionally been used to treat a variety of diseases in Korea, China, and Japan (Zhang et al. 2015; Ji et al. 2020). Recently, platycodin D has been shown to have multiple pharmacological properties, including anti-inflammatory (Fu et al. 2018b; Ye et al. 2019), immunostimulatory (Xie et al. 2008), anti-cancer (Seo et al. 2018; Huang et al. 2019), antioxidant (Cho et al. 2018; Wang et al. 2018; Shi et al. 2020), and inhibitory effects against various cardiovascular and metabolic diseases (Lin et al. 2018; Liu et al. 2020). The antioxidant activity of platycodin D has been primarily attributed to increased ROS scavenging activity and involves the activation of oxidative stress defense systems. Several previous researchers have reported that activated nuclear factor-erythroid 2-related factor 2 (Nrf2) plays a key role in the antioxidant efficacy of platycodin D. Among them, Gao et al. (2017) reported that platycodin D protected pulmonary inflammation due to cigarette smoke by inhibiting inflammatory and oxidative response through increased expression of heme oxygenase-1 (HO-1), a downstream gene of Nrf2. In addition, increased expression of HO-1 by platycodin D was associated with protecting hepatocytes against acetaminophen-induced hepatotoxicity and suppressing the development of atopic dermatitis-like skin symptoms (Choi et al. 2014; Fu et al. 2018a). However, whether platycodin D can weaken oxidative stress-induced injury in muscle cells has not been studied. Therefore, in this study, the antioxidant potential of platycodin D against oxidative stress (H_2O_2)-induced cytotoxicity in immortalized mouse C2C12 myoblasts was evaluated. In addition, the relevance of the Nrf2 signaling pathway to the potential mechanism of platycodin D-mediated antioxidant protective capacity was investigated.

Materials and Methods

Cell culture

C2C12 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum and antibiotics mixture (WelGENE, Inc., Gyeongsan, Republic of Korea) in a water-saturated humidified incubator at 5% CO_2 and 37°C. Platycodin D was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical Co.), and diluted with cell culture medium to adjust the final treatment concentrations before use in experiments.

Cell viability assay

Cells were treated with various concentrations of platycodin D for 24 h or pretreated with 30 μM platycodin D or 10 mM N-acetyl-L-cysteine (NAC, Sigma-Aldrich Chemical Co.) for 1 h and then incubated with or without 1 mM H_2O_2 (Sigma-Aldrich Chemical Co.) for 24 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical Co.) solution was then added to a final concentration of 0.5 mg/ml at 37°C for 3 h. At the end of the incubation, the formed formazan crystals were dissolved in DMSO. Optical density was measured at 570 nm absorbance using an enzyme-linked immunosorbent assay (ELISA) reader (Dynatech Laboratories, Chantilly, VA, USA). The optical density of the formazan crystals formed in untreated control cells was used to represent 100% viability (Kim et al. 2019). Data were obtained from three independent experiments.

Measurement of ROS generation

To measure the amount of intracellular ROS generated in cells, cells were treated with or without platycodin D or NAC for 1 h, before another 1 h culture with the addition of H_2O_2. The cells were washed with phosphate-buffered saline (PBS), and lysed with PBS containing 1% Triton X-100 for 10 min at 37°C. The cells were then stained with 10 μM 2',7'-dichlorofluorescein diacetate (DCF-DA, Eugene, OR, USA) for 30 min at room temperature (RT) in the dark, and washed with PBS. Intracellular ROS generation was immediately recorded at 515 nm by a flow cytometer (Becton Dickinson, San Jose, CA, USA). The results were expressed as the percentage increase relative to untreated cells (Yoon et al. 2019). It was also analyzed the levels in ROS by fluorescence microscopy. To this end, cells cultured in glass cover slips were treated with H_2O_2 in the absence or presence of platycodin D or NAC. After 1 h of treatment, the cells were incubated in a medium containing 10 μM DCF-DA at 37°C for 20 min. Stained cells were washed twice with PBS and observed with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). In same condition, to evaluate the levels of mitochondrial-derived ROS were detected in cells stained for 20 min with 5 μM MitoSOX™ Red (Thermo Fisher Scientific, Waltham, MA, USA). Stained images were acquired with a fluorescence microscope (Carl Zeiss).

Comet assay

After cells were exposed to H_2O_2 with or without platycodin D, the extent of DNA damage was analyzed using a comet assay. The cells were suspended in 0.5% low melting point agarose (LMA) at 37°C, aliquoted and then spread onto fully frosted glass microscope slides precoated with 1% normal melting agarose. After the agarose are solidified in the dark, the slide was covered with 0.5% LMA and submerged in lysis solution (2.5 M of NaCl, 100 mM Na-ethylenediaminetetraacetic acid (Na-EDTA), 1% Trion X-100, 10 mM of Tris, and 10% DMSO, pH 10) for 1 h at 4°C. The slides were then incubated for 30 min in a gel electrophoresis device containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) and electrophoresed at 30 V (1 V/cm) and 300 mAmp for 20 min to draw negatively charged DNA toward the anode. Slides were subsequently washed three times with neutralizing buffer, 0.4 M of Tris (pH 7.5) at 4°C, and stained with 20 μg/ml of propidium iodide (PI, Sigma-Aldrich Chemical Co.). The slides were observed under a fluorescence microscope and the resulting images were analyzed as previously described (Aristizabal-Pachon and Castillo 2019).

Western blot analysis

The collected cells were lysed in ice-cold lysis buffer (5 mM Na-EDTA, 25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% nonidet-P40, and 5 mM dithiothreitol) for 30 min. In a parallel experiment, the mitochondrial and cytosolic proteins were isolated using a mitochondrial fractionation kit (Active Motif, Inc., Carlsbad, CA, USA) according to manufacturer's procedure. Protein concentration of the collected supernatants was measured using the Bradford assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's protocol. Subsequently, the same amount of protein from each sample was separated by sodium-dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bedford, MA, USA). The membranes were blocked with Tris-buffered saline (10 mM Tris-Cl, pH 7.4) containing 5% skim milk and 0.5% Tween-20 for 1 h at RT and then incubated over-night at 4°C with primary antibodies, which were purchased from Abcam, Inc. (Cambridge, MA, UK), Cell Signaling Technology (Danvers, MA, USA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). After washing with PBS, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Life Science, Arlington Heights, IL, USA) for 2 h at RT. Protein bands were detected using an enhanced chemiluminescence (ECL) detection system (R&D Systems Inc., Minneapolis, MN, USA) and a chemiluminescence imager (Azure Biosystems, Inc., Dublin, CA, USA).

Nuclear staining

To determine apoptosis, changes in nuclear morphology were examined using 4,6-diamidino-2-phenylindole (DAPI) staining. Briefly, cells were harvested after treatment with H_2O_2 in the absence or presence of platycodin D, washed with PBS, and fixed with 3.7% paraformaldehyde (Sigma-Aldrich Chemical Co.) in PBS for 10 min at RT. The cells were washed with PBS again and stained with 2.5 μg/ml DAPI (Sigma-Aldrich Chemical Co.) solution for 10 min at RT. The cells were observed *via* a fluorescence microscope.

Detection of apoptosis by annexin V staining

The magnitude of apoptosis was detected using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (R&D Systems Inc.) according to manufacturer instructions. In brief, the collected cells were washed with cold PBS, fixed in 75% ethanol at 4°C for 30 min and then stained with annexin V-FITC and PI for 20 min at RT in the dark. Using a flow cytometer, the fluorescence intensities of the cells were quantified as percentages of annexin V-positive and PI-negative (annexin V^+/PI^-) cell populations, as indicators of apoptotic cells, while the V^-/PI^- cell population was considered normal (Zhang et al. 2020).

Measurement of mitochondrial membrane potential (MMP, $\Delta\psi_m$)

Mitochondrial function was identified by membrane potential fluorescence staining using a mitochondrial potential sensor 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethyl-imidacarbocyanune iodide (JC-1). Briefly, after treatment with H_2O_2 in the absence or presence of platycodin D, the cells were trypsinized. The collected pellets were suspended in PBS, and incubated with 10 μM JC-1 (Sigma-Aldrich Chemical Co.) at 37°C for 20 min. The cells were then washed with cold PBS and analyzed using a flow cytometer.

Small interfering RNA (siRNA) transient transfection

siRNA transient transfection for Nrf2 gene inhibition was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were seeded at a density of 3×10^4 cells *per* well in 6-well plates with fresh cell culture medium. The cells were transfected with targeted Nrf2 siRNA, and control siRNA as a negative control, which were purchased from Santa Cruz Biotechnology, Inc., using Lipofectamine 2000 reagent in serum-free medium for 6 h. Following transfection, the reagent was replaced with normal growth medium, and the cells were incubated for an additional 24 h,

and then treated with H_2O_2 in the absence or presence of platycodin D for the indicated periods.

Determination of caspase-3 enzymatic activity

The caspase-3-like activity (DEVDase) was determined by using a colorimetric activity assay kit (R&D Systems Inc.) according to the manufacturer's instructions. Briefly, the cells were incubated in a supplied lysis buffer on ice for 15 min. The supernatants were collected, and a total of 200 μg protein lysate was incubated with 5 μl of caspase-3 (Acetyl (Ac)-Asp (D)-Glu (E)-Val (V)-Asp (D)-*p*-nitroanilide (*p*NA)) substrate in the dark for 2 h at 37°C, according to the kit protocol. The concentrations of *p*NA released from the substrate by caspase-3 were calculated from the absorbance values at 405 nm. According to their concentration curve, the results of three independent experiments were expressed as fold change, compared with the untreated control cells.

Statistical analysis

The results were expressed as the mean ± standard deviation (SD) of at least three independent experiments. Statistical analyses were performed using the SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). The statistical significance was analyzed by one-way ANOVA. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

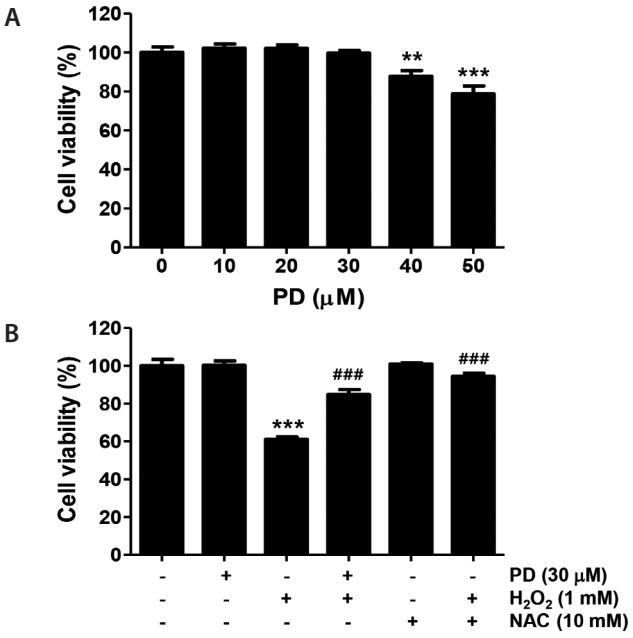

Figure 1. Protective effect of platycodin D (PD) on H_2O_2-induced cytotoxicity in C2C12 cells. Cells were treated with the various concentrations of PD for 24 h (**A**) or pretreated with or without 30 μM PD or 10 mM N-acetyl-L-cysteine (NAC) for 1 h and then cultured in the presence of 1 mM H_2O_2 for 24 h (**B**). The cell viability was determined by MTT reduction assay. The results are expressed as the mean ± SD obtained from three independent experiments; ** $p < 0.01$ and *** $p < 0.001$ compared with the control group, ### $p < 0.001$ compared with the H_2O_2-treated group.

Results

Platycodin D inhibits H_2O_2-induced cytotoxicity in C2C12 cells

To investigate the protective effect of platycodin D against oxidative stress using H_2O_2 in C2C12 cells, the effect of platycodin D on the survival rate of C2C12 cells was evaluated. Figure 1A shows that in C2C12 cells treated with platycodin D below 30 μM, there was no significant difference in cell viability compared with the control, but platycodin D over the concentration of 40 μM significantly decreased C2C12 cell viability. Therefore, to study the cytoprotective effects of platycodin D against H_2O_2-induced oxidative stress, platycodin D was used less than 30 μM that has no cytotoxicity. The concentration of H_2O_2 inducing oxidative stress was set at 1 mM, which was approximately 60% of cell viability compared to the untreated control cells (Fig. 1B). Subsequently, the ability of platycodin D to counteract H_2O_2-induced cytotoxicity was investigated. The result showed that platycodin D significantly inhibited the H_2O_2-mediated loss of C2C12 cell viability (Fig. 1B). It was found that pretreatment of NAC, a well-established ROS scavenger, had a complete inhibitory effect on H_2O_2-induced cytotoxicity (Fig. 1B).

Platycodin D suppresses H_2O_2-induced ROS generation in C2C12 cells

Next, it was examined whether platycodin D abolished H_2O_2-induced ROS accumulation using a fluorescent probe, DCF-DA, and found that the production of ROS in H_2O_2-treated C2C12 cells peaked within 1 h (approximately 9.7 times more than the control) and gradually decreased over time (data not shown). However, pretreatment with platycodin D significantly reduced the effect of H_2O_2 on ROS overproduction, and NAC also almost completely eliminated the accumulation of ROS (Fig. 2).

Platycodin D reduces H_2O_2-induced apoptosis in C2C12 cells

To validate the ability of platycodin D to prevent H_2O_2-induced apoptosis, DAPI staining and annexin V-FITC/PI double staining assays were performed. As presented in Figure 3A, C2C12 cells exposed to H_2O_2 alone demonstrated characteristic apoptotic features, including nuclear condensation and fragmentation. However, pretreatment with platyco-

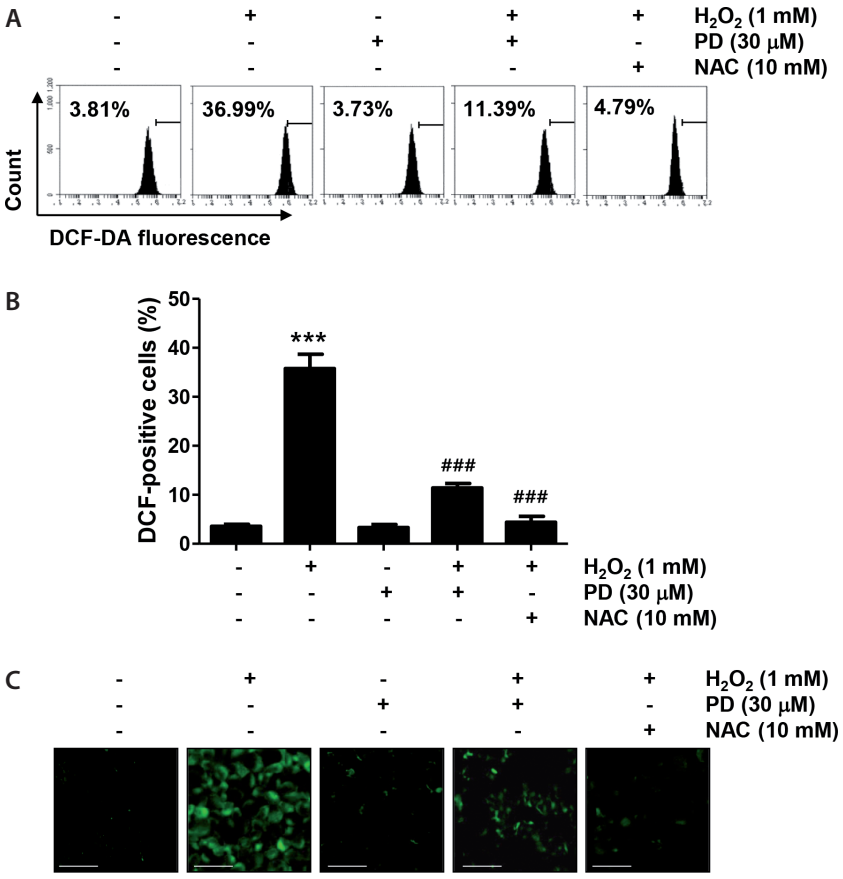

Figure 2. Inhibition of H_2O_2-induced ROS generation by platycodin D (PD) in C2C12 cells. Cells were treated with 30 μM PD or 10 mM N-acetyl-L-cysteine (NAC) for 1 h and then stimulated with or without 1 mM H_2O_2 for an additional 1 h. The medium was removed, and the cells were incubated with a medium containing DCF-DA. **A.** ROS production was measured using a flow cytometer, and representative profiles are shown. **B.** The measurements were made in triplicate, and the values are expressed as the mean ± SD; *** $p < 0.001$ compared with the control group, ### $p < 0.001$ compared with the H_2O_2-treated group. **C.** DCF fluorescence images of cells cultured under the same conditions were captured by a fluorescence microscope (scale bar; 25 μM). Each panel is representative image selected from those making at least from three independent experiments.

din D markedly reduced these morphological changes. In addition, flow cytometric analysis using annexin V/PI staining showed that H_2O_2 triggered more apoptosis than did the controls (approximately 9 times more; Fig. 3C). However, after pretreatment with platycodin D, the percentage of apoptotic cells significantly decreased.

Platycodin D protects H_2O_2-induced mitochondrial dysfunction in C2C12 cells

To analyze whether inhibition of mitochondrial impairment is a mechanism involved in the cytoprotective effect of platycodin D, JC-1 dye to estimate the MMP was used. As shown by flow cytometry analysis in Figure 4A and B, a significant decrease in the ratio of JC-1 monomers with an increase in JC-1 aggregates in cells exposed to H_2O_2, indicating that H_2O_2 reduced MMP was observed. In addition, the expression of cytochrome *c* in H_2O_2-treated cells decreased in the mitochondrial fraction and increased in the cytoplasmic fraction (Fig. 4D), indicating that cytochrome *c* was released from the mitochondria to the cytosol. The expression of anti-apoptotic Bcl-2 protein decreased and that of apoptosis Bax increased, and the degradation of caspase-3 and poly (ADP-ribose) polymerase (PARP) protein in H_2O_2-treated cells (Fig. 4E) that was associated with the activation of caspase-3 (Fig. 4F) was observed. However, pretreatment with platycodin D reversed these changes. Furthermore, it was assessed whether platycodin D-mediated mitochondrial damage involve mitophagy in C2C12 cells. To investigate the amount of mitochondrial-derived ROS, the cells stained with MitoSOX red dye. The result showed that H_2O_2 markedly increased the population of MitoSOX-positive cells, whereas this H_2O_2-induced increasing was significantly decreased by platycodin D treatment (Fig. 4C). Moreover, expression of the mitophagy regulators, including Parkin and NIX/BNIP3L, was greatly down-regulated by platycodin D in H_2O_2-treated cells (Fig. 4E).

Platycodin D activates the Nrf2/HO-1 signaling pathway in C2C12 cells

It was further investigated whether the antioxidant activity of platycodin D correlated with the activation of the Nrf2/HO-1 signaling pathway. Immunoblotting results indicated that the expression of Nrf2 and its phosphorylation level (p-Nrf2) was slightly higher in the H_2O_2 alone

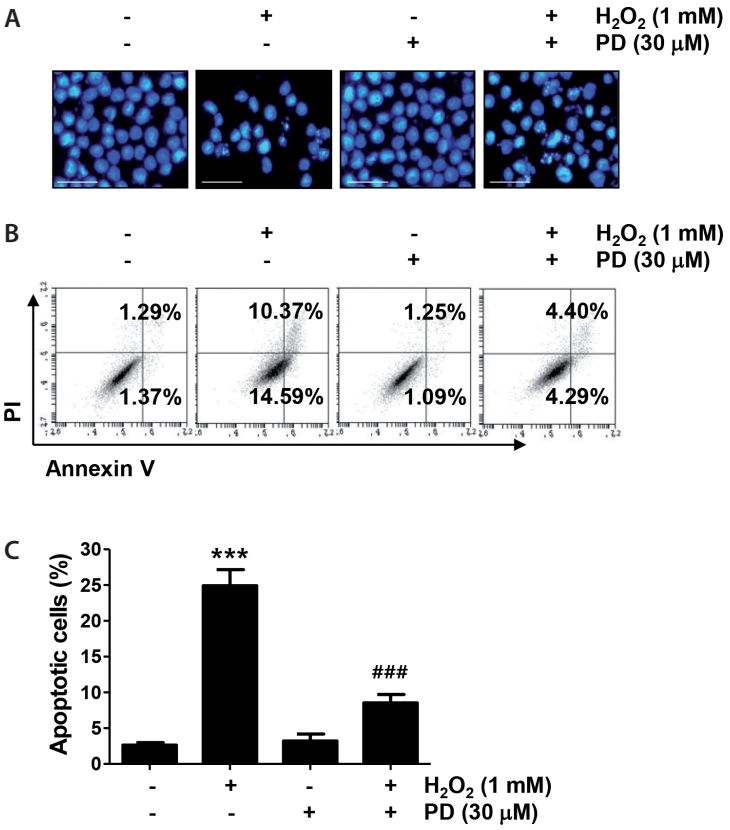

Figure 3. Inhibitory effect of platycodin D (PD) on H_2O_2-induced apoptosis in C2C12 cells. Cells were treated with or without 30 μM PD for 1 h before treatment with 1 mM H_2O_2 for 24 h. **A.** The cells were stained with DAPI solution, and stained nuclei were observed using a fluorescence microscope (scale bar; 25 μM). Each image represents at least three independent experiments. **B** and **C.** The cells were fixed and stained with annexin V-FITC and propidium iodide (PI) for flow cytometry analysis. B. The results show early apoptosis, defined as annexin V^+ and PI^- cells (lower right quadrant), and late apoptosis, defined as annexin V^+ and PI^+ (upper right quadrant) cells; representative profiles are shown. C. The percentages of apoptotic cells were determined by expressing the numbers of annexin V^+ cells as percentages of all the present cells. The data represent the mean ± SD of three independent experiments; *** $p < 0.001$ compared with the control group, ### $p < 0.001$ compared with the H_2O_2-treated group.

group than in the untreated controls, but their expression greatly increased in cells co-treated with H_2O_2 and platycodin D (Fig. 5A). At the same time, HO-1 expression was similarly upregulated in cells treated with both H_2O_2 and platycodin D compared with H_2O_2 alone or control. In contrast, the expression of Kelch-like ECH-associated protein-1 (Keap1), a negative regulator of Nrf2, was lower in cells treated with platycodin D and H_2O_2.

Nrf2/HO-1 signaling pathway is involved in mitigating H_2O_2-mediated apoptosis by platycodin D in C2C12 cells

To clarify whether the increased expression of HO-1 by platycodin D is Nrf2-dependent, Nrf2 expression was blocked by Nrf2 siRNA transfection. Figure 5B indicates that the knockdown of Nrf2 efficiently decreased its expression, as well as HO-1 promoted by platycodin D (Fig. 5B). Therefore, it was finally examined whether the Nrf2/HO-1 signaling pathway was directly involved in the anti-apoptotic effects of platycodin D in C2C12 cells. Figures 5C and D show that the anti-apoptotic effect of platycodin D was significantly lost by treatment with Nrf2 siRNA in H_2O_2-insulted cells. At the same time, Nrf2 siRNA transfection eventually eliminated the protective effect of platycodin D against the H_2O_2-induced reduction of cell viability (Fig. 5E).

Discussion

In the present study, it was investigated whether platycodin D can protect C2C12 myoblasts from oxidative stress. For this purpose, oxidative damage was induced using H_2O_2, which is widely used as a representative ROS for establishing various oxidative stress models, and showed that H_2O_2 induced cytotoxicity by triggering DNA damage and mitochondria-mediated apoptosis through the promotion of ROS production. However, platycodin D was found to have the ability to revise H_2O_2-induced cytotoxicity while scavenging ROS.

Apoptosis, a programmed cell death, is tightly regulated by a variety of factors and can be broadly divided into extrinsic and intrinsic pathways. The extrinsic pathway is activated by extracellular ligands that bind to death receptors on the cell surface, while activation of the intrinsic pathway is associated with intracellular apoptotic signals that cause mitochondrial dysfunction (Popgeorgiev et al. 2018; Bock and Tait 2020). In particular, ROS overload by mitochondrial dysfunction causes free radical attack of the phospholipid bilayer of the mitochondria, which depolarizes the mitochondrial membrane, resulting in the loss of MMP (Xiong et al. 2014; Kiraz et al. 2016). During this process, the permeability of the mitochondrial membranes increases,

allowing apoptogenic factors in mitochondrial intermembrane space, especially cytochrome *c*, to be released into the cytoplasm. Thus, the loss of MMP and the cytosolic release of cytochrome *c* indicate impaired mitochondrial function and are evident early phenomena in the onset of intrinsic apoptosis (Er et al. 2006; Bock and Tait 2020). To evaluate the preventive effect of platycodin D on mitochondrial dysfunction, MMP values and cytochrome *c* expression were examined. The results indicated that the loss of MMP and cytosolic cytochrome *c* expression were markedly increased in H_2O_2-treated C2C12 cells. However, platycodin D pretreatment prevented the reduction of MMP and maintained the expression of cytochrome *c* in mitochondria during H_2O_2 exposure, demonstrating that platycodin D can inhibit mitochondrial damage caused by oxidative stress. Mitochondrial damage often contributes to mitophagy and mitochondrial apoptosis (Kubli and Gustafsson 2012). Damaged mitochondria are selective removed by

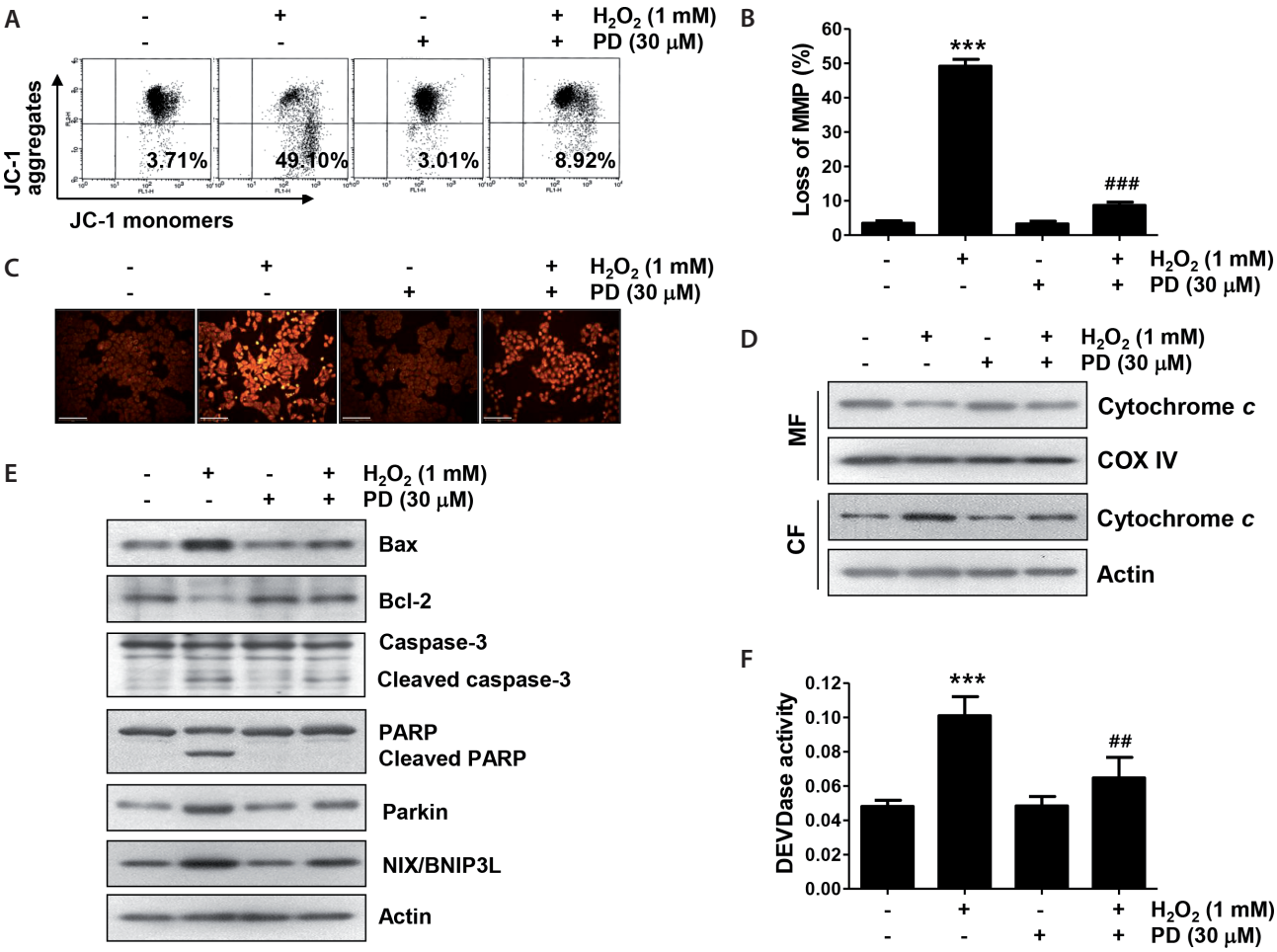

Figure 4. Attenuation of H_2O_2-induced mitochondrial dysfunction by platycodin D (PD) in C2C12 cells. Cells were treated with 30 μM PD for 1 h and then exposed to 1 mM H_2O_2 for 24 h. **A.** The cells were collected and stained with JC-1. JC-1 fluorescence intensity was detected for measuring the MMP changes using a flow cytometer. **B.** Bars indicate the percentages of cells with JC-1 monomers, and the data represent the mean ± SD of triplicate determinations. **C.** The cells were stained with MitoSOX^{TM} red, a mitochondrial ROS indicator. Representative images were acquired with fluorescence microscope (scale bar; 200 μM). Each image represents at least three independent experiments. **D.** Cytochrome *c* levels were analyzed by Western blotting on mitochondrial and cytoplasmic fractions isolated from cells cultured under the same conditions. Actin and cytochrome oxidase subunit VI (COX IV) serve as protein loading controls for the cytosol and mitochondria, respectively. **E.** Whole cell lysates were prepared, and Bax, Bcl-2, caspase-3, PARP, Parkin and NIX/BNIP3L expressions were identified by Western blot analysis. The equivalent loading of proteins in each well was confirmed by actin. **F.** The activity of caspase-3 (DEVDase activity) in cell lysates was measured using the respective substrate peptides, Ac-DEVD-*p*NA. Data are expressed as the mean ± SD obtained from three independent experiments; *** $p < 0.001$ compared with the control group, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ compared with the H_2O_2-treated group.

autophagosomes and degraded before apoptosis or necrosis can be triggered in an effort to prevent cell death (Geisler et al. 2010; Kubli and Gustafsson 2012). The PTEN-induced putative kinase 1 (PINK1)/Parkin pathway is important in regulating mitophagy in cells (Geisler et al. 2010). BNIP3 and NIX/BNIP3L were originally identified as pro-apoptotic BH3-only proteins that cause cell death *via* permeabilization of the outer mitochondrial membrane (Kubli et al. 2007). NIX/BNIP3L is essential for removal of mitochondria in reticulocytes by mitophagy (Zhang and Ney 2009). In present study, oxidative stress markedly increased mitochondrial-derived ROS production, simultaneously up-regulated the expression of mitophagy regulator. However, this activation of H_2O_2-mediated mitophagy clearly suppressed by platycodin D.

Cytochrome *c* released into the cytoplasm interacts with and activates caspase-9, which sequentially activates downstream effector caspases, such as caspase-3 and caspase-7, eventually completing cell death. This process is accompanied by the degradation of substrate proteins of effector caspases, including PARP, as evidenced that caspase-dependent apoptosis was induced (Hassan et al. 2014; Kiraz et al. 2016). The activation of this caspase cascade is tightly regulated by the expression of various regulators. Among them, Bcl-

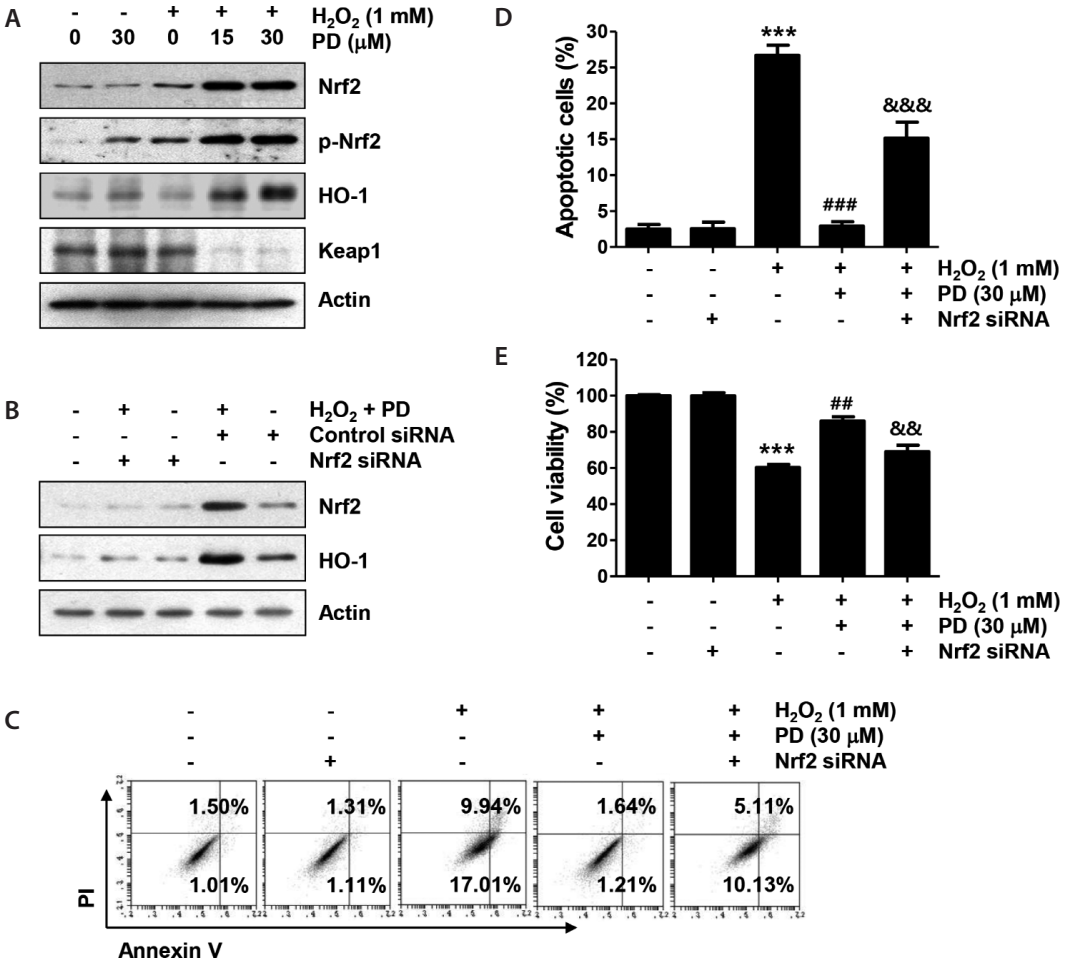

Figure 5. Activation of Nrf2/HO-1 signaling pathway by platycodin D (PD) in C2C12 cells. **A.** Cells were treated with or without the indicated concentrations of PD for 1 h before treatment with 1 mM H_2O_2 for 24 h. **B–E.** Cells transfected with or without control siRNA or Nrf2 siRNA were pretreated with 30 μM PD for 1 h and then treated with or without 1 mM H_2O_2 for 24 h. A and B. Equal amounts of proteins were subjected to Western blot analysis of the listed proteins. Actin was used as an internal control, and the proteins were visualized using an ECL detection system. C. The cells were stained with annexin V-FITC and PI for flow cytometry analysis, and representative profiles are presented. D. The percentages of apoptotic cells were determined by counting the percentages of annexin V positive cells. E. The cell viability was determined by MTT assay. D and E. The results are expressed as the mean ± SD of three independent experiments; *** $p < 0.001$ compared with the control group, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ compared with the H_2O_2-treated group, $^{\&\&}$ $p < 0.01$ and $^{\&\&\&}$ $p < 0.001$ compared with the PD and H_2O_2-treated group.

2 family members, composed of pro- and anti-apoptotic proteins, play a key role in determining the progression of the intrinsic apoptosis pathway. As a representative pro-apoptotic protein, Bax, located on the outer mitochondrial membrane, induces cytochrome *c* release by promoting mitochondrial permeability transition or weakening the barrier function of the mitochondria outer membrane. In contrast, anti-apoptotic proteins such as Bcl-2 are essential for maintaining mitochondrial permeability and membrane barrier stabilization to inhibit the release of apoptogenic factors (Popgeorgiev et al. 2018; Bock and Tait 2020). Therefore, the balance between the pro-apoptotic Bax member proteins against the anti-apoptotic Bcl-2 member proteins acts as a determinant that induces the activation of the caspase cascade upon initiation of the intrinsic apoptotic pathway. In the present study, the caspase-3 activity and degradation of PARP were obviously increased, and the Bax/Bcl-2 expression ratio was also enhanced in H_2O_2-treated C2C12 cells, both of which findings are consistent with previous studies (Siu et al. 2009; Lee et al. 2016; Choi 2018; Park et al. 2019). However, these changes were all significantly blunted in the presence of platycodin D, indicating that platycodin D can protect C2C12 cell apoptosis by inhibiting the intrinsic apoptosis pathway activated by oxidative stress. Therefore, these results demonstrated that platycodin D antagonism of apoptosis induced by H_2O_2 in C2C12 cells could occur through maintenance of mitochondrial function. Interestingly, Fulle et al. (2013) showed that compared with myogenic precursor cells derived from young muscles, a greater proportion of aged myogenic precursor cells damages the regeneration of skeletal muscle by undergoing apoptosis in response to stress stimuli. Furthermore, because this is closely related to the high ROS levels found in aged muscles (Ji 2015; Di Filippo et al. 2016), I predict that platycodin D, which reduces myoblast death by weakening ROS levels, may contribute to improving muscle differentiation and function in aging as well as other diseases with elevated ROS levels.

Researchers have identified a number of defense mechanisms to protect cells from oxidative stress, and many have focused on controlling the key transcription factors involved in upregulating antioxidants. One such factor is Nrf2, which regulates the production of antioxidant enzymes for defense against oxidative stress disorders (Fetoni et al. 2019; Shaw and Chattopadhyay 2020). Under normal physiological conditions, Nrf2 binds to Keap1 in the cytoplasm and is constitutively ubiquitinated by the Cullin-3-Keap1 E3 ubiquitin ligase complex and degraded *via* the ubiquitin proteasome pathway. When cells are exposed to oxidative stress, Nrf2 escapes from Keap1 and translocates into the nucleus to promote the transcriptional activity of antioxidant response element (ARE) response genes. Of the inducible phase II enzymes regulated at the transcription level by ARE, HO-1 breaks down heme into carbon monoxide, free iron, and biliverdin. Because biliverdin is further broken down into bilirubin, which has antioxidant properties, HO-1 plays a potentially important role in antioxidant defense and iron homeostasis (Bonelli et al. 2012; Yu et al. 2018). These findings suggest that antioxidants that can activate the Nrf2/HO-1 signaling pathway could be a promising therapeutic strategy for preventing and treating oxidative stress-mediated damage.

In addition, various natural products have been reported to have antioxidant properties through the activation of the Nrf2/HO-1 signaling pathway to protect against apoptosis caused by oxidative injury in myoblasts (Kang et al. 2015, 2017; Choi 2016; Han et al. 2017). In present study, platycodin D up-regulated the expression of Nrf2 and p-Nrf2 in the absence of H_2O_2, which is due to antioxidant activity of platycodin D. In addition, I explored the effect of platycodin D on the expression of Nrf2, and found that the expression of p-Nrf2 was markedly upregulated by platycodin D in the presence of H_2O_2. The expression of HO-1 also increased under the same conditions, and Keap1 expression was downregulated, indicating that Nrf2 was activated in platycodin D-treated C2C12 cells under oxidative conditions. Therefore, Nrf2 siRNA was used to elucidate the role of Nrf2 in the antioxidant effects of platycodin D and found that platycodin D increased HO-1 expression in a Nrf2-dependent manner. Consistent with these results, the reduced apoptosis and increased cell viability by pretreatment with platycodin D in the presence of H_2O_2 were significantly reversed in Nrf2 siRNA-transfected cells. These results well support previous findings that activating Nrf2/HO-1 signaling in C2C12 myoblasts could initiate a protective mechanism for initiating an intrinsic apoptosis pathway following oxidative stress-mediated mitochondrial dysfunction and DNA damage (Kang et al. 2015, 2017; Choi 2016; Han et al. 2017).

In conclusion, in the present study, the protective effect of platycodin D against H_2O_2-induced oxidative stress was elucidated and its mechanism of action in C2C12 myoblasts was explored. Platycodin D reversed increased intracellular ROS production and mitochondrial damage caused by H_2O_2, eventually inhibiting DNA damage and apoptosis. In this process, platycodin D activated Nrf2 and promoted the expression of its downstream target protein HO-1, which likely contributed to alleviating oxidative stress and improving oxidant resistance. These results provide evidence for platycodin D's applicability as a therapeutic for maintaining myoblast function during oxidative stress. However, further studies are required to assess how platycodin D can regulate the transcriptional activity of Nrf2 and whether other signaling pathways can intervene in the antioxidant activity of platycodin D.

Conflict of interest. The author has no conflict of interest to declare.

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