The cytoprotective effects of 7,8-dihydroxyflavone against oxidative stress are mediated by the upregulation of Nrf2-dependent HO-1 expression through the activation of the PI3K/Akt and ERK pathways in C2C12 myoblasts

JI SOOK KANG[1*], IL-WHAN CHOI[2*], MIN HO HAN[1,3], GI-YOUNG KIM[4], SU HYUN HONG[3], CHEOL PARK[5], HYE JIN HWANG[1,6], CHEOL MIN KIM[7], BYUNG WOO KIM[1,8] and YUNG HYUN CHOI[1,3]

[1]Blue-Bio Industry RIC and Anti-Aging Research Center, Dongeui University, Busan 614-714; [2]Department of Microbiology, College of Medicine, Inje University, Busan 608-756; [3]Department of Biochemistry, Dongeui University College of Korean Medicine, Busan 614-052; [4]Laboratory of Immunobiology, Department of Marine Life Sciences, Jeju National University, Jeju 690-756; Departments of [5]Molecular Biology and [6]Food and Nutrition, College of Natural Sciences and Human Ecology, Dongeui University, Busan 614-714; [7]Department of Biochemistry, Busan National University College of Medicine, Yangsan, Gyeongsangnam-do 626-870; [8]Department of Life Science and Biotechnology, College of Natural Sciences and Human Ecology, Dongeui University, Busan 614-714, Republic of Korea

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Abstract. Recent studies have demonstrated that 7,8-dihydroxyflavone (7,8-DHF), a newly identified tyrosine kinase receptor B agonist, is a potent antioxidant agent. The present study was designed to confirm the cytoprotective effects of 7,8-DHF against oxidative stress-induced cellular damage and to further elucidate the underlying mechanisms in C2C12 myoblasts. We found that 7,8-DHF attenuated hydrogen peroxide (H_2O_2)-induced growth inhibition and exhibited scavenging activity against intracellular reactive oxygen species (ROS) that were induced by H_2O_2. We also observed that 7,8-DHF significantly attenuated H_2O_2-induced comet tail formation, and decreased the phosphorylation levels of the histone, H2AX, as well as the number of Annexin V-positive cells, suggesting that 7,8-DHF prevents H_2O_2-induced DNA damage and cell apoptosis. Furthermore, 7,8-DHF increased the levels of heme oxygenase-1 (HO-1), which is a potent antioxidant enzyme associated with the induction and phosphorylation of nuclear factor-erythroid 2-related factor 2 (Nrf2), as well as the translocation of Nrf2 from the cytosol to the nucleus. However, the protective effects of 7,8-DHF against H_2O_2-induced ROS generation and growth inhibition were significantly diminished by zinc protoporphyrin IX, an HO-1 competitive inhibitor. Moreover, the potential of 7,8-DHF to mediate HO-1 induction and protect the cells against H_2O_2-mediated growth inhibition was abrogated by transient transfection with Nrf2-specific small interfering RNA (siRNA). In addition, 7,8-DHF induced the activation of Akt, a downstream target of phosphatidylinositol 3-kinase (PI3K), and also that of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK), while specific inhibitors of PI3K and ERK, but not a p38 MAPK inhibitor, abolished the 7,8-DHF induced HO-1 upregulation and Nrf2 induction and phosphorylation. Collectively, these results demonstrate that 7,8-DHF augments the cellular antioxidant defense capacity through activation of the Nrf2/HO-1 pathway, which also involves the activation of the PI3K/Akt and ERK pathways, thereby protecting C2C12 myoblasts from H_2O_2-induced oxidative cytotoxicity.

Correspondence to: Dr Yung Hyun Choi, Department of Biochemistry, Dongeui University College of Korean Medicine, San 45, Yangjung-dong, Busanjin-gu, Busan 614-052, Republic of Korea
E-mail: choiyh@deu.ac.kr

*Contributed equally

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Introduction

Oxidative stress, caused by the increased production of reactive oxygen species (ROS), is the pathological basis of a number of chronic diseases. Under physiological conditions, low levels of ROS are scavenged effectively by the cellular antioxidant defense system. However, the excessive production of ROS causes destructive and irreversible damage to cellular components, including nucleic acids, proteins, lipids and other macromolecules (1,2). Therefore, the induction of antioxidant enzymes plays an important role in the cytoprotection against oxidative stress.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a master cellular sensor for oxidative stress, and it represents the primary response to changes in the redox state of cells (3-5). Under basal conditions, Nrf2 is sequestered in the cytoplasm by the inhibitory partner known as the Kelch-like ECH-associated protein-1 (Keap1). Keap1 functions as an adaptor for the Cullin 3 (Cul3)-based E3 ligase and regulates the proteasomal degradation of Nrf2 (3,4). Upon stimulation, Nrf-2 dissociates from Keap1, and the unbound Nrf-2 translocates to the nucleus (5,6), where it sequentially binds to the antioxidant-response element (ARE), resulting in a cytoprotective response which is characterized by an upregulation of antioxidant enzymes and decreased sensitivity to damage from oxidative stress (3,7,8). The gene families regulated by the ARE include phase II metabolizing/detoxifying enzymes, such as heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1), as well as various antioxidants. Several natural and synthetic compounds are capable of exerting protective effects not only by free radical scavenging, but also by augmenting the expression of cytoprotective and/or antioxidant genes through the Nrf2-driven ARE signaling pathway (7,9). Nrf2 is thus considered a key molecular target of antioxidant enzyme inducers, which convert ROS into less reactive and less damaging forms. This is the primary defense mechanism against ROS.

Flavonoids are natural polyphenolic compounds that are widely distributed in celery and parsley, as well as in a number of other vegetables and herbs (10). There is increasing evidence indicating that flavonoids have diverse biological functions, including antioxidant and anti-inflammatory activities (10), although the underlying mechanisms are not yet fully understood. The flavone derivative 7,8-dihydroxyflavone (7,8-DHF) has been identified as a selective agonist for tyrosine receptor kinase B (TrkB) and activates its downstream signaling cascade (11), thereby exerting potent neuroprotective and neurotrophic effects against Parkinson's disease and other neurological disorders (12). Previous studies have demonstrated that 7,8-DHF is a potent antioxidant and that it protects cells against oxidative stress-induced damage by reducing ROS production (13-16). In our previous studies, we demonstrated that due to its anti-inflammatory properties 7,8-DHF may be used in the treatment of inflammatory and neurodegenerative diseases (17,18). Previous studies have also confirmed that the cytoprotective effects of 7,8-DHF against oxidative stress are associated with the Nrf2-mediated induction of HO-1 expression in lung fibroblasts and keratinocytes (19-21). However, to date, to the best of our knowledge, there is available no information on the beneficial effects of 7,8-DHF against oxidative stress in myoblasts. Therefore, in this study, we aimed to further elucidate the intracellular pathways and determine whether 7,8-DHF has the ability to activate Nrf2 and induce its downstream target genes in murine-derived C2C12 myoblasts.

Materials and methods

Cell culture and 7,8-DHF treatment. C2C12 myoblasts obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin (both from WelGENE Inc., Daegu, Korea) in a humidified 5% CO_2 atmosphere at 37˚C. 7,8-DHF (purity, ≥98%; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and adjusted to final concentrations using complete DMEM prior to use. In order to investigate the effects of PI3K/Akt and MAPKs pathways on the induction of Nrf2, p-Nrf2 and HO-1 by 7,8-DHF, LY294002 (an inhibitor of Akt), PD98059 (an inhibitor of ERK), SB203580 (an inhibitor of p38) and SP600125 (an inhibitor of JNK) were obtained from Calbiochem (San Diego, CA, USA).

Cell viability assay. As a measure of the overall levels of cell viability, the C2C12 myoblasts were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay. Briefly, the C2C12 myoblasts were seeded in 6-well plates at a density of $1x10^5$ cells/well. Following 24 h of incubation, the cells were treated with the indicated concentrations of 7,8-DHF (2.5, 5, 10, 15 or 20 μM) in the absence or presence of hydrogen peroxide (H_2O_2) and/or zinc protoporphyrin IX (ZnPP, a specific inhibitor of HO-1; Sigma-Aldrich) for the indicated periods of time (24 or 6 h). MTT working solution was then added to the culture plates followed by incubation at 37˚C for 3 h. The culture supernatant was completely removed from the wells, and DMSO was added to dissolve the formazan crystals. The absorbance of each well was then measured at 540 nm using a microplate reader (Molecular Devices, Palo Alto, CA, USA). The protective effects of 7,8-DHF against growth inhibition were assessed as a percentage of cell viability, and the vehicle (0.05 mM DMSO)-treated cells were considered 100% viable.

Comet assay (single cell gel electrophoresis). The cell suspension was mixed with 0.5% low melting agarose (LMA) at 37˚C, and the mixture was spread on a fully frosted microscopic slide precoated with 1% normal melting agarose (NMA). After the solidification of the agarose, the slide was covered with 0.5% LMA and then immersed in a lysis solution [2.5 M NaCl, 100 mM Na-ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10] for 1 h at 4˚C. The slides were then placed in a gel electrophoresis apparatus containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) for 40 min to allow for the unwinding of the DNA and the expression of alkali-labile damage. An electrical field was then used (300 mA, 25 V) for 20 min at 4˚C to draw the negatively charged DNA toward the anode. Following electrophoresis, the slides were washed 3 times for 5 min at 4˚C in neutralizing buffer (0.4 M Tris, pH 7.5), followed by staining with 20 μg/ml propidium iodide (PI; Sigma-Aldrich). The slides were examined under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Protein extraction, electrophoresis and western blot analysis. Whole-cell protein extracts from the C2C12 myoblasts were prepared with cell lysis buffer (20 mM sucrose, 1 mM EDTA, 20 μM Tris-HCl, pH 7.2, 1 mM dithiothreitol, 10 mM KCl, 1.5 mM $MgCl_2$ and 5 μg/ml aprotinin) for 30 min. In a parallel experiment, nuclear proteins were prepared using nuclear extraction reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. The protein extracts were quantified using the Bio-Rad kit (Pierce Biotechnology). For western blot analysis, equal amounts of

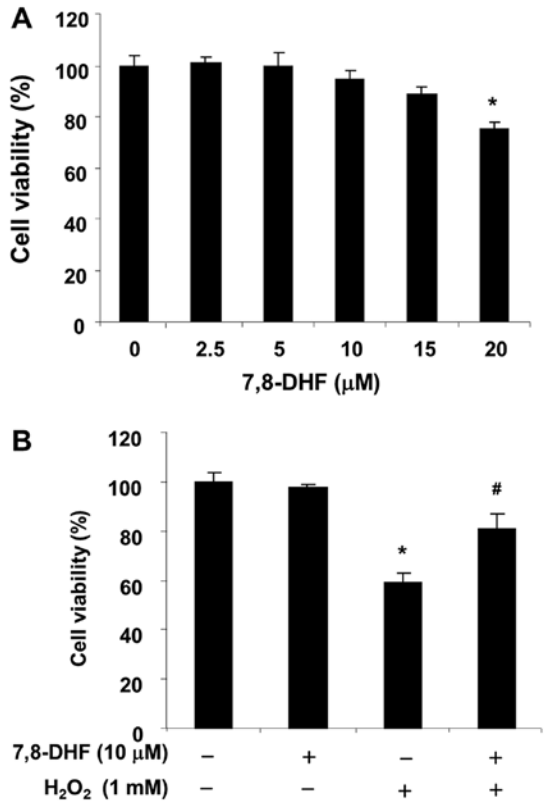

Figure 1. Effects of 7,8-dihydroxyflavone (7,8-DHF) on H_2O_2-induced growth inhibition of C2C12 myoblasts. Cells were treated with various concentrations of 7,8-DHF for 24 h (A) or pre-treated with 10 μM 7,8-DHF for 1 h and then incubated with or without 1 mM H_2O_2 for 6 h (B). Cell viability was assessed by MTT reduction assay. The results are the means ± SD obtained from 3 independent experiments. *P<0.05 compared with the control group; #P<0.05 compared with the H_2O_2-treated group.

protein extracts were separated by denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred electrophoretically onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were then incubated overnight at 4˚C with primary antibodies, probed with enzyme-linked secondary antibodies [mouse IgG, HRP-linked whole antibody (NA931) and rabbit IgG, HRP-linked whole antibody (NA934), Amersham Corp., Arlington Heights, IL, USA)] for 1 h at room temperature, and detected using an enhanced chemiluminescence (ECL) detection system (all from Amersham Co.). The antibodies used were as follows: iNOS (1:500; SC-7271, mouse monoclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), TNF-α (1:500; #3707S, rabbit polyclonal; Cell Signaling Technology, Inc., Danvers, MA, USA), IL-1β (1:500; SC-7884, rabbit polyclonal), NF-κB p65 (1:500; SC-109, rabbit polyclonal), IκBα (1:500; SC-371, rabbit polyclonal), Akt (1:500; SC-8312, rabbit polyclonal), p-Akt (1:500; SC-101629, rabbit polyclonal), ERK (1:1,000; SC-154, rabbit polyclonal; all from Santa Cruz Biotechnology, Inc.), p-ERK (1:500; #9106S, mouse monoclonal; Cell Signaling Technology, Inc.), p38 (1:1,000; SC-535, rabbit polyclonal; Santa Cruz Biotechnology, Inc.), p-p38 (1:500; #9211S, rabbit polyclonal), JNK (1:1,000; #9252S, rabbit polyclonal), p-JNK (1:500; #9255S, mouse monoclonal; all from Cell Signaling Technology, Inc.), Nrf2 (1:500; SC-13032, rabbit polyclonal; Santa Cruz Biotechnology, Inc.), p-Nrf2 (1:500; ab76026, rabbit monoclonal; Abcam, Inc., Cambridge, UK), HO-1 (1:500; SC-136960, mouse monoclonal), Lamin B (1:500; SC-6216, goat polyclonal) and β-actin (1:1,000; sc-1616, goat polyclonal; all from Santa Cruz Biotechnology, Inc.). Actin and poly(ADP ibose) polymerase (PARP) were used as the internal controls of the total cellular and nuclear proteins, respectively.

Assessment of apoptosis by flow cytometry. To quantitatively assess the cell apoptotic rate, a fluorescein-conjugated Annexin V (Annexin V-FITC) staining assay was performed according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Briefly, the cells were stained with 5 μl Annexin V-FITC and 5 μl PI. Following incubation for 15 min at room temperature in the dark, the degree of apoptosis was quantified as a percentage of the Annexin V-positive and PI-negative cells by flow cytometry, as previously described (22).

Measurement of ROS production. The intracellular accumulation of ROS was determined using the fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; obtained from Molecular Probes, Eugene, OR, USA). In order to monitor the generation of ROS, the cells were treated with 5 mM N-acetyl-L-cysteine (NAC; Sigma-Aldrich) for 30 min and then treated with 7,8-DHF for 6 h. After the addition of 10 μM H2DCFDA for 20 min at room temperature in the dark, ROS production in the cells was monitored using a flow cytometer (BD Biosciences) with CellQuest Pro software, as previously described (23).

Transfection with small interfering RNA (siRNA). Nrf2 siRNA and control siRNA were purchased from Santa Cruz Biotechnology, Inc. The cells were transfected with the siRNAs according to the manufacturer's instructions using Lipofectamine 2000 Transfection Reagent (Life Technologies, Carlsbad, CA, USA). For transfection, the cells were seeded in 6-well culture plates and incubated with the control siRNA or Nrf2 siRNA at 50 nM for 6 h in serum-free OPTI-MEM medium. Following incubation, the transfected cells were subjected to treatment as described in the figure legends and as previously described (24).

Statistical analysis. Data are expressed as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparisons in the experiments with multiple time points and concentrations. When ANOVA indicated statistical significance, Duncan's multiple range test was used to determine which means were significantly different. A probability value of P<0.05 was used as the criterion for statistical significance.

Results

Protective effects of 7,8-DHF against H_2O_2-induced cytotoxicity to C2C12 myoblasts. We first determined the effects of 7,8-DHF on the viability of C2C12 myoblasts by MTT assay. The cells were treated with a range of 7,8-DHF concentrations, from 2.5 to 20 μM for 24 h (Fig. 1A). Treatment of the C2C12 myoblasts with up to 10 μM 7,8-DHF did not result in any cytotoxic effects, whereas cell viability decreased in a dose-dependant manner following treatment with 7,8-DHF at concentrations >10 μM (Fig. 1A). Therefore, the dose of 10 μM 7,8-DHF was selected as the optimal dose for examining the

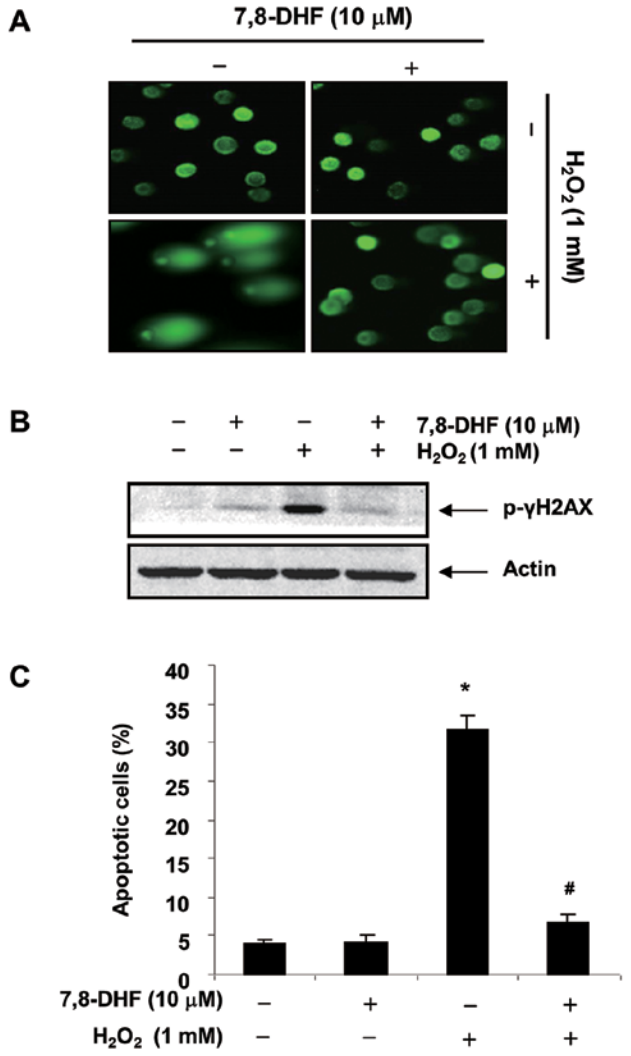

Figure 2. 7,8-Dihydroxyflavone (7,8-DHF) protects against H_2O_2-induced DNA damage and apoptosis in C2C12 myoblasts. C2C12 myoblasts were pre-treated with 10 μM 7,8-DHF for 1 h and then incubated with or without 1 mM H_2O_2 for 6 h. (A) To detect cellular DNA damage, a comet assay was performed and representative images of the comets were acquired using a fluorescence microscope (original magnification, x200). (B) The cells were lysed, and then equal amounts of cell lysates were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with specific antibodies against phosphorylated-histone H2AX (γH2AX) and actin, as an internal control, and the proteins were visualized using an enhanced chemiluminescence (ECL) detection system. A representative blot from 3 independent experiments is shown. (C) The cells were also stained with Annexin V-FITC and propidium iodide (PI), and the percentages of apoptotic cells (Annexin V$^+$ cells) were then analyzed by flow cytometry. The results are the means ± SD obtained from 3 independent experiments. *P<0.05 compared with the control group; $^\#$P<0.05 compared with the H_2O_2-treated group.

cytoprotective effects of 7,8-DHF against H_2O_2-induced cell damage. To examine the protective effects of 7,8-DHF against H_2O_2-induced cytotoxicity, we treated the C2C12 myoblasts with 10 μM 7,8-DHF 1 h prior to exposure to H_2O_2, and cell viability was then measured by MTT assay. Following exposure to 1 mM H_2O_2 alone, cell viability was reduced to approximately 60% at 6 h, whereas the H_2O_2-induced decrease in of cell viability was significantly attenuated by pre-treatment with 7,8-DHF (Fig. 1B). These results clearly indicate that the exposure of C2C12 myoblasts to 7,8-DHF confers a significant protective effect against oxidative stress.

7,8-DHF prevents H_2O_2-induced DNA damage and apoptosis in C2C12 cells. As DNA strand breakage is considered one of the most frequent types of damage that can be induced by oxidative stress (25), we examined the effects of 7,8-DHF on H_2O_2-mediated damage to C2C12 cell DNA using single-cell gel electrophoresis (comet assay) and western blot analysis. Treatment with H_2O_2 alone induced significant DNA damage in the C2C12 myoblasts; however, this adverse effect was markedly reduced by pre-treatment with 7,8-DHF (Fig. 2A). In addition, our results revealed that the exposure of C2C12 myoblasts to H_2O_2 resulted in an upregulation in the levels of the phosphorylated histone variant H2A.X (p-γH2AX) at serine 139, a sensitive marker of DNA double-strand breaks (25); however, pre-treatment with 7,8-DHF resulted in a significant decrease in p-γH2AX expression (Fig. 2B). In order to investigate the protective effects of 7,8-DHF against H_2O_2-induced apoptosis, the frequency of apoptotic cells was detected by flow cytometry, and the results revealed that the treatment of the cells with 7,8-DHF prior to exposure to H_2O_2 protected the C2C12 myoblasts against apoptosis.

7,8-DHF modulates the H_2O_2-induced ROS generation in C2C12 cells. Using H2DCFDA assay, we then investigated whether 7,8-DHF affects intracellular ROS generation induced by exposure of the cells to H_2O_2. As expected, significantly increased levels of ROS were detected following the epxosure of the cells to H_2O_2 compared with the levels observed in the untreated cells; however, this increase in ROS levels was significantly inhibited by treatment with 7,8-DHF (Fig. 3A). Moreover, no fluorescence was detected in the cells treated with 7,8-DHF alone (data not shown), indicating that 7,8-DHF itself does not contribute to ROS generation. As a positive control, the ROS scavenger, NAC, was also used. Treatment with NAC attenuated the H_2O_2-induced ROS generation and decreased the apoptotic rate; it also reversed the decrease in cell viability induced by exposure to H_2O_2; these effects were similar to those of 7,8-DHF (Fig. 3B and C). The results indicate that the H_2O_2-induced induction of apoptosis and the reduction in cell viability are mediated by the generation of ROS, and that 7,8-DHF exerts a potent ROS-scavenging effect, protecting the C2C12 myoblasts against H_2O_2-damage.

7,8-DHF enhances the expression of HO-1, as well as Nrf2 phosphorylation and nuclear accumulation in C2C12 cells. It has been well documented that antioxidant enzymes play an important role against oxidative stress (1,2); thus, we hypothesized that the effects of 7,8-DHF may be mediated by the induction of antioxidant enzymes. Treatment of the C2C12 myoblasts with 7,8-DHF induced the protein expression of HO-1 in a time-dependent manner; however, the levels of the other antioxidant enzymes, NQO-1 and thioredoxin reductase 1 (TrxR1), were unaffected (Fig. 4A). Previous studies have demonstrated that, under normal conditions, Nrf-2 is inactive and bound in the cytosol by Keap1, and that the translocation of Nrf2 into the nucleus is essential for the transactivation of various target genes, such as HO-1. Moreover, the phosphorylation of Nrf2 at Ser40 by several kinases is also a critical process in its stabilization and nuclear translocation (6,7). Therefore, in this study, we examined the phosphorylation and subcellular localization of Nrf2 following treatment with

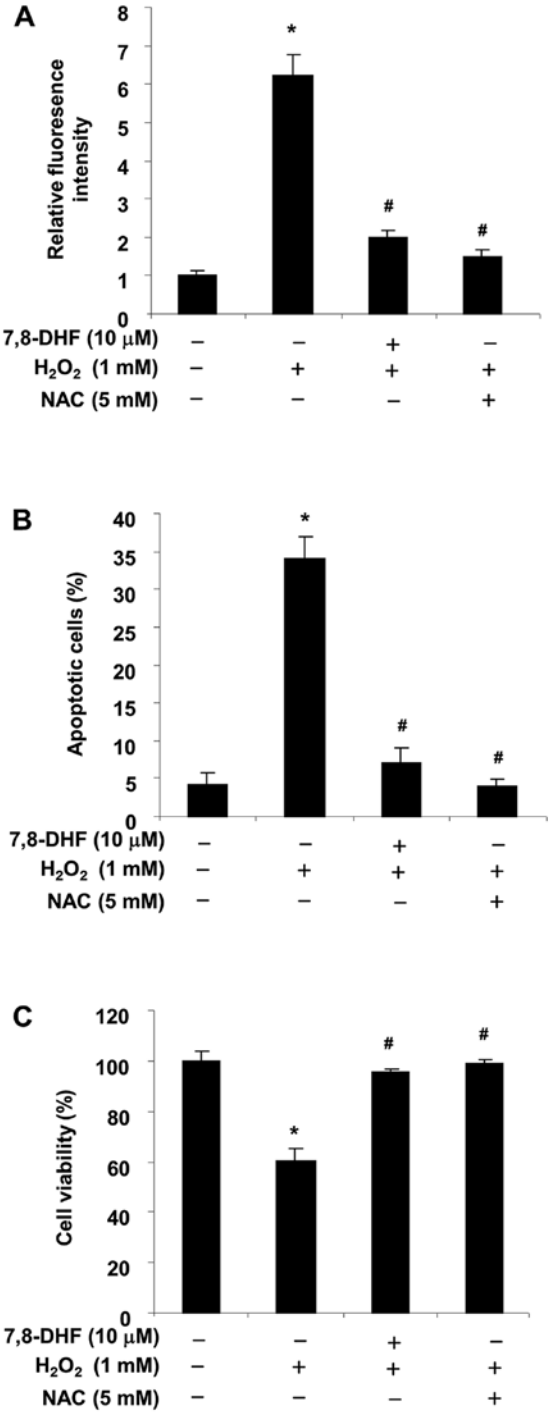

Figure 3. 7,8-Dihydroxyflavone (7,8-DHF) attenuates H_2O_2-induced reactive oxygen species (ROS) generation in C2C12 myoblasts. C2C12 myoblasts were pre-treated with 10 μM 7,8-DHF or 5 mM N-acetyl-L-cysteine (NAC) for 1 h and then stimulated with or without 1 mM H_2O_2 for 6 h. (A) In order to monitor ROS production, the cells were incubated at 37˚C in the dark for 20 min with new culture medium containing 10 μM H2DCFDA. ROS generation was measured using a flow cytometer. The degree of (B) apoptosis and (C) cell viability were also assessed using a flow cytometer and MTT reduction assay, respectively. The results are the means ± SD values obtained from 3 independent experiments. *P<0.05 compared with the control group; #P<0.05 compared with the H_2O_2-treated group.

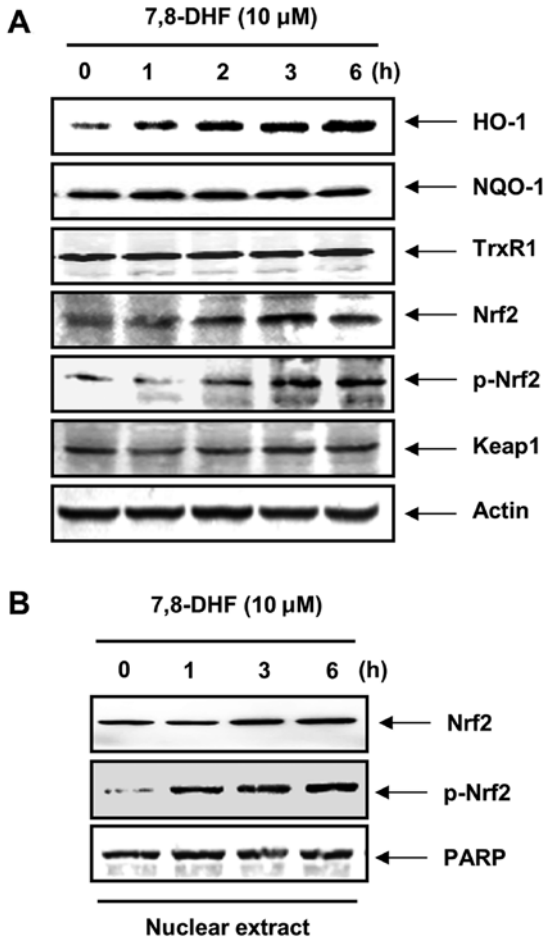

Figure 4. Induction of nuclear factor-erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) expression by 7,8-DHF in C2C12 myoblasts. Cells were incubated with 10 μM 7,8-DHF for the indicated periods of time. (A) Total cellular or (B) nuclear proteins were separated on SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were probed with the specific antibodies against HO-1, NQO-1, thioredoxin reductase 1 (TrxR1), Nrf2, p-Nrf2 and Kelch-like ECH-associated protein-1 (Keap1). Proteins were visualized using an enhanced chemiluminescence (ECL) detection system. Actin and poly(ADP-ribose) polymerase (PARP) were used as the internal controls of the total cellular and nuclear proteins, respectively.

7,8-DHF in order to confirm the Nrf2-activating properties of 7,8-DHF. We observed that 7,8-DHF increased the expression levels of total and phosphorylated Nrf2 in a time-dependent manner (Fig. 4A). Furthermore, western blot analysis of the nuclear fraction revealed a significant augmentation of Nrf2 phosphorylation and nuclear accumulation following treatment with 7,8-DHF, in a time-dependent manner (Fig. 4B).

7,8-DHF upregulates HO-1 expression through the activation of Nrf2 in C2C12 cells. We developed a Nrf2 gene knockout model using siRNA transfection in order to demonstrate the importance of Nrf2 upregulation. The results of western blot analysis revealed that the silencing of Nrf2 using specific siRNA abolished the 7,8-DHF-induced increase in Nrf2 expression and HO-1 upregulation (Fig. 5A), which is evidence that the augmentation of HO-1 is mediated by Nrf2. To further confirm the involvement of Nrf2, the protective effects of 7,8-DHF against the H_2O_2-induced decrease in cell viability were determined in cells in which Nrf2 had been knocked down. As shown in Fig. 5B, siNrf2 transfection abrogated the cytoprotective effects of 7,8-DHF compared to the control siRNA-transfected cells, thus proving that 7,8-DHF diminishes

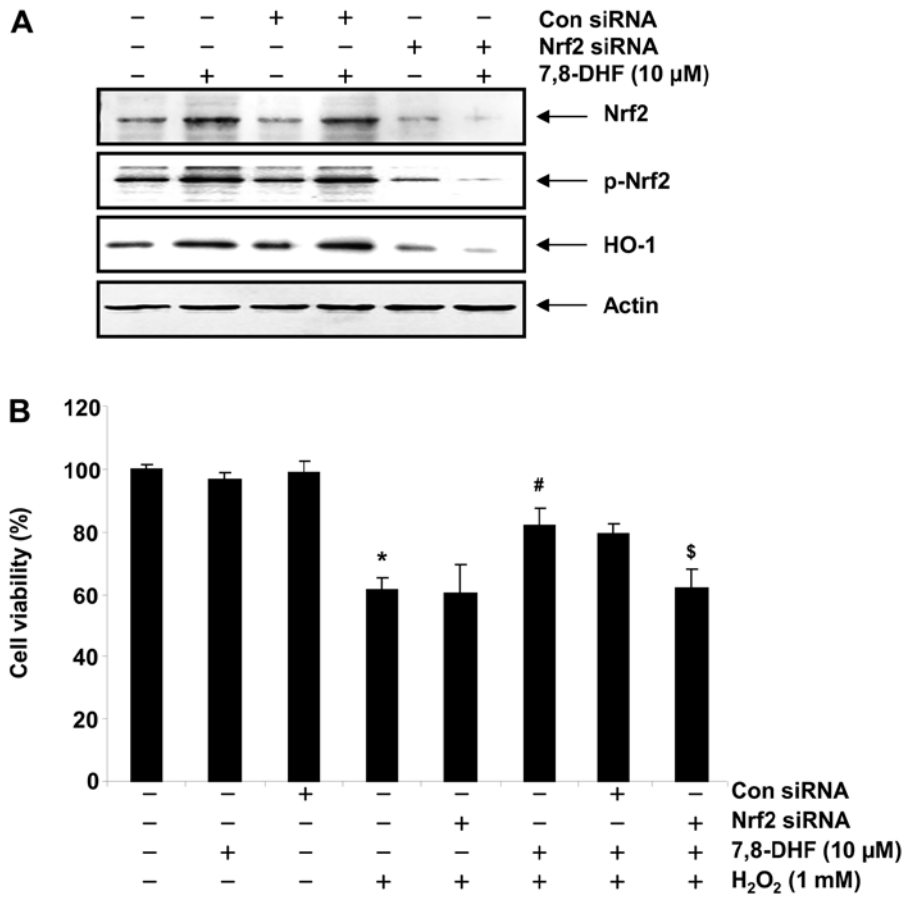

Figure 5. Nuclear factor-erythroid 2-related factor 2 (Nrf2)-mediated induction of heme oxygenase-1 (HO-1) expression by 7,8-dihydroxyflavone (7,8-DHF) in C2C12 myoblasts. Cells were transfected with control (Con siRNA, as a negative control for RNA interference) and Nrf2 siRNA. After 24 h, the cells were treated with 10 μM 7,8-DHF for 6 h (A) or pre-treated with 10 μM 7,8-DHF for 1 h and then stimulated with or without 1 mM H_2O_2 for 6 h (B). Cellular proteins were separated on SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes (A). The membranes were probed with the specific antibodies against Nrf2, p-Nrf2 and HO-1. Proteins were visualized using an enhanced chemiluminescence (ECL) detection system. Actin was used as a loading control. (B) Cell viability was estimated by MTT assay. The results are the means ± SD values obtained from 3 independent experiments *P<0.05 compared with the control group; $^{\#}$P<0.05 compared with the H_2O_2-treated group; $^{\$}$P<0.05 compared with the H_2O_2 and 7,8-DHF-treated group.

the H_2O_2-induced decrease in cell viability through the activation of the Nrf2/HO-1 signaling pathway.

7,8-DHF exerts protective effects against H_2O_2-induced damage in C2C12 cells through the Nrf2/HO-1 pathway. To further determine whether the 7,8-DHF-induced antioxidant and cytoprotective activities against oxidative stress in C2C12 cellsmyoblasts are mediated through the activation of the Nrf2/HO-1 pathway, the C2C12 myoblasts were pre-incubated with or without a specific inhibitor of HO-1, ZnPP, and the levels of ROS and cell viability were then assessed. ZnPP abrogated the protective effects of 7,8-DHF against the H_2O_2-induced production of ROS and the decrease in cell viability (Fig. 6). These results indicate that 7,8-DHF exerts its protective effects by inducing the cellular defense mechanism against oxidative stress through the Nrf2-related cytoprotective pathway, and that HO-1 plays a crucial role in this protection of C2C12 myoblasts.

The phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK) signaling pathways are associated with the Nrf2-mediated induction of HO-1 by 7,8-DHF in C2C12 cells. A number of studies have noted that multiple phosphorylation cascades participate in the regulation of the translocation of Nrf2 and Nrf2-mediated HO-1 gene expression (26-28). Thus, to identify the upstream signaling events involved in the 7,8-DHF-mediated activation of Nrf2 and the induction of HO-1, the potential involvement of PI3K/Akt and mitogen-activated protein kinases (MAPKs) were explored. Although the total levels of Akt, a downstream target of PI3K, did not show a notable change, the Akt phosphorylation levels markedly increased following treatment with 7,8-DHF within 30 min (Fig. 7A). However, treatment with LY294002, a pharmacological inhibitor of PI3K, prevented the increase in the phosphorylation levels of Nrf2 and resulted in a blockade of Nrf2 and HO-1 induction which was by 7,8-DHF (Fig. 7B), suggesting that the 7,8-DHF-induced activation of the Nrf2/HO-1 pathway may be a process necessary to the PI3K cascade.

Subsequently, we investigated the effects of 7,8-DHF on the activation of MAPKs in C2C12 myoblasts. The increase in the phosphorylation levels of ERK and p38 MAPK were observed 30 min following treatment with 7,8-DHF and this increase was sustained for up to 2 h following treatment with 7,8-DHF. However, there were no notable changes observed in the phosphorylation levels of c-Jun N-terminal kinase (JNK) (Fig. 8A). When a selec-

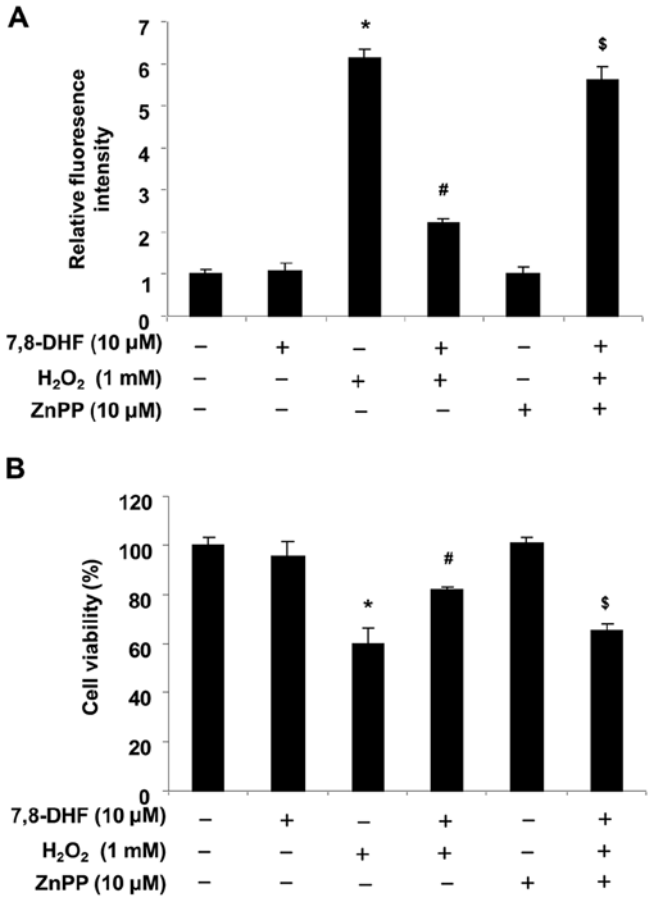

Figure 6. Effects of the inhibitor of heme oxygenase-1 (HO-1), zinc protoporphyrin IX (ZnPP), on the 7,8-dihydroxyflavone (7,8-DHF)-mediated attenuation of reactive oxygen species (ROS) formation and growth inhibition by H_2O_2 in C2C12 myoblasts. Cells were pre-treated for 1 h with 10 μM 7,8-DHF and then treated for 6 h with or without 1 mM H_2O_2 in the absence or presence of 10 μM ZnPP. (A) ROS generation and (B) cell viabilit were then estimated. The results are the means ± SD values obtained from 3 independent experiments. *P<0.05 compared with the control group; $^{\#}$P<0.05 compared with the H_2O_2-treated group; $^{\$}$P<0.05 compared with the H_2O_2 and 7,8-DHF-treated group.

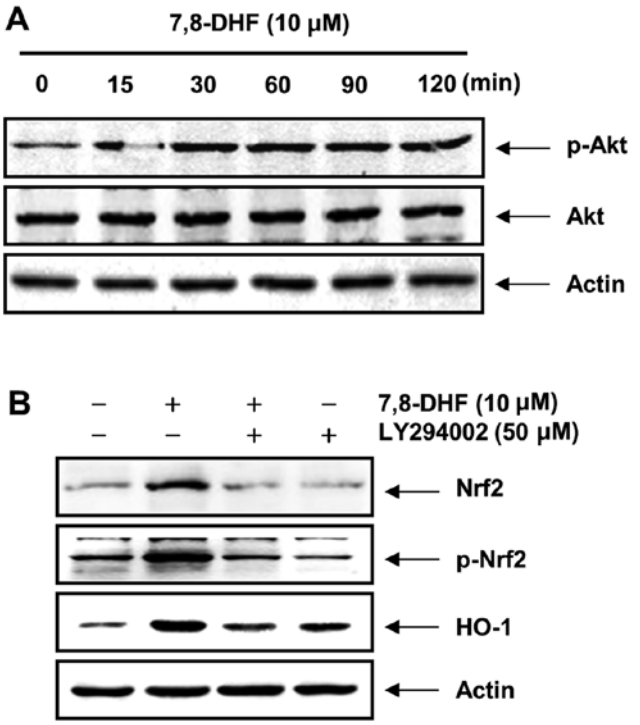

Figure 7. Involvement of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in the nuclear factor-erythroid 2-related factor 2 (Nrf2)-mediated induction of heme oxygenase-1 (HO-1) by 7,8-dihydroxyflavone (7,8-DHF) in C2C12 myoblasts. Cells were treated with 10 μM 7,8-DHF for the indicated periods of time (A) or pre-treated for 1 h with or without LY294002, an inhibitor of PI3K, and then treated with 10 μM 7,8-DHF for an additional 6 h (B). The cells were lysed and then equal amounts of cell lysates were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies and the proteins were visualized using an enhanced chemiluminescence (ECL) detection system. Actin was used as an internal control.

tive inhibitor of ERK (PD98059) was utilized, the induction and phosphorylation of Nrf2 were blocked and, accordingly, HO-1 induction was diminished (Fig. 8B). By contrast, inhibitors of p38 MAPK (SB203580) and JNK (SP600125) did not reduce the 7,8-DHF-induced HO-1 and Nrf2 expression or Nrf2 phosphorylation. These data indicate that the 7,8-DHF-mediated activation of the Nrf2/HO-1 pathway involves the ERK pathway, but not the p38 MAPK and JNK pathways. Taken together, these observations support the hypothesis that 7,8-DHF activates the PI3K/Akt and ERK pathways, which subsequently induces Nrf2/HO-1 activation in C2C12 myoblasts.

Discussion

Previous studies have demonstrated that 7,8-DHF, a small-molecule TrkB agonist, has potent antioxidant potential (4,13,14) as a direct free radical scavenger due to two adjacent hydroxyl groups which act as electron donors (29,30). For example, it has been reported that this compound provides neuroprotection against glutamate-induced toxicity by increasing the cellular glutathione levels and reducing ROS production caused by glutamate in hippocampal HT-22 cells (14). Zhang *et al* (15) demonstrated that 7,8-DHF exerts cytoprotective effects against oxidative stress by scavenging intracellular ROS and enhancing PI3K/Akt signaling in lung fibroblasts. Han *et al* (16) also indicated that 7,8-DHF prevents hydroxydopamine-induced DNA damage and apoptosis by directly scavenging intracellular ROS in PC12 pheochromocytoma cells. Recently, 7,8-DHF has been shown to activate the ERK- and Akt-Nrf2 signaling cascades in cultured human HaCaT keratinocytes, leading to the upregulation of HO-1 and cytoprotection against oxidative stress (19). 7,8-DHF has also been shown to protect cells from oxidative stress through the activation of the ERK/Nrf2/HO-1 signaling pathway in lung fibroblasts (20).

Oxidative stress which results from the overproduction of ROS and/or impaired antioxidan defense is a major cause of cell death in a number of pathological conditions (1,2). H_2O_2, one of the main ROS, may be involved in the formation of hydroxyl radicals, which are highly reactive and destructive substances that cause DNA damage in cells, and this in turn results in cell death (31,32). In the present study, C2C12 myoblasts exposed to H_2O_2 exhibited a significant decrease in cell viability and an increased apoptosis; however, treatment with 7,8-DHF increased cell viability by inhibiting H_2O_2-induced apop-

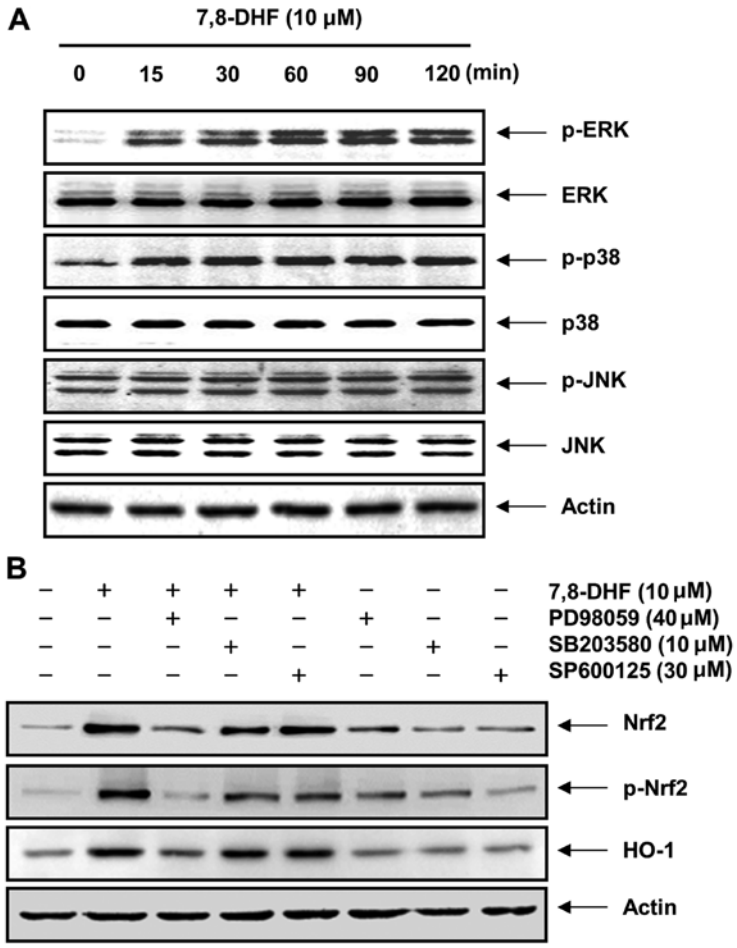

Figure 8. Involvement of the extracellular signal-regulated kinase (ERK) signaling pathway in the nuclear factor-erythroid 2-related factor 2 (Nrf2)-mediated induction of heme oxygenase-1 (HO-1) by 7,8-dihydroxyflavone (7,8-DHF) in C2C12 myoblasts. Cells were treated with 10 μM 7,8-DHF for the indicated periods of time (A) or pre-treated for 1 h with or without the indicated inhibitors and then treated with 10 μM 7,8-DHF for an additional 6 h (B). The cells were lysed and equal amounts of cell lysates were then separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized using an enhanced chemiluminescence (ECL) detection system. Actin was used as an internal control.

tosis and reducing ROS production generated by exposure to H_2O_2 (Figs. 1-3). It is well-known that ROS generation is associated with mitochondrial dysfunction, and thus we presumed that 7,8-DHF would improve mitochondrial function by eliminating the overproduction of ROS induced by H_2O_2, and thereby reducing H_2O_2-induced apoptosis. To assess the oxidative damage induced by H_2O_2, we measured both the tail length of DNA using comet assay and the levels of p-γH2AX by western blot analyses, which are widely used markers for the detection of DNA damage (25). Our data indicated that exposure to H_2O_2 increased the tail length (DNA migration) and the expression of p-γH2AX; however, these effects were mitigated in the C2C12 myoblasts by treatment with 7,8-DHF prior to H_2O_2 exposure (Fig. 2A and B). Therefore, the attenuation of apoptosis and DNA damage by 7,8-DHF may be associated with its potent antioxidant effects.

Accumulating evidence has demonstrated that HO-1 plays a central role in cellular antioxidant defense in cells (5,7,9). The transcriptional regulation of HO-1 is linked to the transcription factor, Nrf2, which is a key component in protection against oxidative stress. Under normal conditions, Nrf2 is anchored in the cytoplasm by binding to Keap1, which facilitates the ubiquitination and subsequent proteolysis of Nrf2. However, upon exposure to various types of stress, the Nrf2/Keap1 complex is disrupted, leading to the phosphorylation of Nrf2; this is a critical process in the nuclear translocation of Nrf2, as well as its transcriptional activity (5-7). In the present study, 7,8-DHF induced HO-1 expression, as well as the induction of Nrf2 expression and phosphorylation, and the accumulation of Nrf2 in the nucleus (Fig. 4); these results are consistent with those of previous studies showing treatment with other Nrf2 activators, such as berberine (33), α-lipoic acid (34), 4-ketopinoresinol (35), genipin (36) and phenethyl isothiocyanate (37). However, in our study, the knockdown of Nrf2 by Nrf2-targeted siRNA markedly abrogated the 7,8-DHF-induced increase in HO-1 expression (Fig. 5A), suggesting that Nrf2 is a critical upstream regulator of the 7,8-DHF-mediated induction of HO-1 in C2C12 myoblasts. The silencing of Nrf2 abolished the 7,8-DHF-induced restoration of the H_2O_2-mediated growth inhibition of C2C12 myoblasts (Fig. 5B). Pre-treatment with ZnPP, a HO-1 inhibitor, also markedly abrogated the protective effects of 7,8-DHF against the H_2O_2-induced ROS generation and growth inhibition (Fig. 6). These results suggest that the Nrf2-dependent induction of HO-1 by 7,8-DHF may, at least in part, participate in the protection against oxidative stress, which is in agreement with the results of other studies (15,38).

A number of studies have suggested that diverse protein kinases are involved in the signals that trigger Nrf2-Keap1 dissociation and the phosphorylation of Nrf2, as well as the antioxidant-induced activation of the Nrf2/HO-1 signaling pathway. For example, the phenolic glucoside gastrodin stimulated HO-1 expression through the activation of the p38 MAPK/Nrf2 signaling pathway (39), the flavonoid sulfuretin upregulation of the activity of HO-1 through the JNK/ERK/Nrf2 signaling pathway (40), the eckol induction of Nrf2-dependent HO-1 expression through the JNK and PI3K/Akt signaling pathways (41), and the induction of expression of HO-1 by prenylated flavone cudraflavone B mediated through the PI3K/Akt pathway (42). These findings prove that the role of each pathway in the activation of Nrf2/HO-1 signaling, and their molecular targets, may be specific to the stimulus and cell type. In the present study, the PI3K/Akt signaling pathway was rapidly activated following treatment with 7,8-DHF (Fig. 7A). ERK and p38 MAPK, but not JNK, were also markedly activated. To identify which signaling cascade controlsl the activation of Nrf2 by 7,8-DHF, we examined the effects of specific inhibitors of PI3K and MAPKs on the induction of HO-1 and Nrf2, and the phosphorylation of Nrf2. The results demonstrated that p38 MAPK was not involved in the 7,8-DHF-induced activation of Nrf2/HO-1 signaling, as its inhibitor (SB203580) had no effect on the 7,8-DHF-induced HO-1 and Nrf2 expression and Nrf2 phosphorylation (Fig. 7B). However, both the PI3K inhibitor, LY294002, and the ERK inhibitor, PD98059, suppressed the 7,8-DHF-induced changes in the levels of HO and Nrf2 (Figs. 7B and 8B), a finding which is consistent with the results of the study by Ryu *et al* (19). Akt can be activated in a PI3K-dependent manner by various growth and survival factors, and it plays a key role in cell survival and metabolism by binding and regulating a number of downstream effectors. Moreover, previous studies have suggested that Akt kinase phosphorylates specific serine or threonine residues present in Nrf2, thus facilitating the nuclear localization of Nrf2 and thereby inducing HO-1 (6). Recently, it was shown that PI3K inhibitor partially blocked the protective effects of 7,8-DHF, indicating that the PI3K/Akt pathway plays a crucial role in 7,8-DHF-mediated protection (16). On the other hand, among the MAPKs, the activation of the p38 MAPK and JNK pathways has been shwon to lead to the induction of apoptosis through the phosphorylation of a variety of pro-apoptotic downstream effectors, whereas the ERK pathway is more often associated with cell survival (43). Certain studies have also reported that 7,8-DHF prevents oxidative stress-induced cytotoxicity by enhancing ERK signaling (13,15,19,20). Therefore, these observations suggest that PI3K/Akt and ERK play a crucial role in the Nrf2-dependent induction of HO-1 and suggest that Nrf2 is a downstream effector of Akt and ERK, which are activated in response to 7,8-DHF treatment and oxidative stress. Therefore, 7,8-DHF treatment prevents cell injury induced by oxidative damage.

In conclusion, the present study demonstrates that 7,8-DHF induces Nrf2-mediated HO-1 expression, which, at least in part, contributes to a cellular defense mechanism against oxidative stress-induced genotoxic events. We suggest that the cytoprotective effects induced by 7,8-DHF may come directly from scavenging ROS and the activation of the PI3K/Akt and ERK signaling pathways. These findings suggest that 7,8-DHF may have potential for use as a therapeutic agent, as it acts as an antioxidant.

Acknowledgements

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