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The cytoprotective effect of isorhamnetin against oxidative stress is mediated by the upregulation of the Nrf2-dependent HO-1 expression in C2C12 myoblasts through scavenging reactive oxygen species and ERK inactivation

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Abstract. This study was designed to confirm the protective effects of isorhamnetin against oxidative stress-induced cellular damage. Our results indicated that isorhamnetin inhibited the hydrogen peroxide (H_2O_2)-induced growth inhibition and exhibited scavenging activity against the intracellular reactive oxygen species (ROS) in mouse-derived C2C12 myoblasts. Isorhamnetin also significantly attenuated H_2O_2-induced DNA damage and apoptosis, and increased the levels of the nuclear factor erythroid 2-related factor 2 (Nrf2) and its phosphorylation associated with the induction of heme oxygenase-1 (HO-1). However, the protective effects of isorhamnetin on H_2O_2-induced ROS and growth inhibition were significantly abolished by an HO-1 competitive inhibitor. Moreover, the potential of isorhamnetin to mediate HO-1 induction and protect against H_2O_2-mediated growth inhibition was abrogated by transient transfection with Nrf2-specific small interfering RNA. Additionally, isorhamnetin induced the activation of mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), c-Jun *N*-terminal kinase (JNK), and p38 MAPK. However, the specific inhibitor of ERK, but not JNK and p38 MAPK, was able to abolish the HO-1 upregulation and the Nrf2 phosphorylation. Collectively, these results demonstrate that isorhamnetin augments the cellular antioxidant defense capacity by activating the Nrf2/HO-1 pathway involving the activation of the ERK pathway, thus protecting the C2C12 cells from H_2O_2-induced cytotoxicity.

Key words: Isorhamnetin — ROS — DNA damage — Nrf2/HO-1 — ERK

Introduction

Oxidative stress resulting from the overproduction of reactive oxygen species (ROS) and/or impaired anti-oxidative defense is the pathologic basis of many chronic diseases. In the oxidative stress condition, ROS can induce the oxidative modification of cellular components, such as nucleic acids, proteins, lipids, and other macromolecules (Kregel and Zhang 2007; Finkel 2011). Therefore, the induction of antioxidant enzymes is one of the most important determinants of cytoprotective effects against severe oxidative stress. Heme oxygenase-1 (HO-1) is a rate-limiting antioxidative enzyme that plays a key role in the catabolic process of heme. HO-1 catalyzes the degradation of heme to a few byproducts such as biliverdin, carbon monoxide, and Fe^{2+}, which are considered to be antioxidants that protect cells from undesirable cytotoxic conditions, including excessive production of ROS (Katori et al. 2002; Motterlini and Foresti 2014). Nuclear factor erythroid 2-related factor 2 (Nrf2), which is negatively regulated by Kelch-like ECH-associated protein-1 (Keap1), has been shown to play a critical role in the activation of the HO-1 promoter by the control of antioxidant-response elements (AREs)

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(Ishii et al. 2000; Kobayashi and Yamamoto 2005). Nrf2 is normally sequestered in the cytoplasm by binding to Keap1, which functions as an adaptor for Cul3-based E3 ligase to regulate the proteasomal degradation of Nrf2. Upon stimulation, Nrf2 dissociates from Keap1 and translocates to the nucleus, binds to the ARE, and activates the promoter regions of many gene-encoding phase II metabolizing/detoxifying enzymes, such as HO-1 and NADPH-Quinone oxidoreductase 1 (NQO1), and various antioxidants (Zhang and Gordon 2004; Niture et al. 2014). According to recent studies, the phosphorylation of Nrf2 by diverse kinases enhances its translocation to the nucleus and DNA binding activity (Apopa et al. 2008; Liu et al. 2011). Moreover, many phytochemicals derived from naturally occurring plants have been reported not only to exert protective effects on free radical scavenging but also to augment the expression of controlling the activity of Nrf2 and consequently the expression of HO-1 (Hun Lee et al. 2013; Su et al. 2013; Vriend and Reiter 2014). Therefore, the Nrf2 signaling is considered a key molecular target of antioxidant enzyme inducers in converting high ROS to less reactive and less damaging forms. This conversion is the primary mechanism used to defend against a variety of cellular stresses.

Flavonoids, which are natural polyphenolic compounds widely distributed in herbal drugs and foods, have received considerable attention due to their biological and physiological importance. Isorhamnetin (3-*O*-β-D-glucopyranoside, $C_{16}H_{12}O_7$) is the most abundant flavonol in sea buckthorn (*Hippophae rhamnoides* L.), which has a long history in the treatment of circulatory ischemic heart disease, disorders, liver damage, and cancer (Guliyev et al. 2004; Suryakumar and Gupta 2011). Isorhamnetin is structurally one of the metabolites of quercetin (Morand et al. 1998) and has recently been found to have numerous effects, including scavenging free radicals (Kim et al. 2011; Yang et al. 2014), increased resistance of low-density lipoprotein to oxidation (Janisch et al. 2004), anti-adipogenesis effect (Lee et al. 2009, 2010), and anti-inflammatory (Yang et al. 2013; Chirumbolo 2014) and anti-tumor activity (Shi et al. 2012; Saud et al. 2013). However, the inhibitory mechanisms of isorhamnetin regarding the beneficial effect of isorhamnetin against oxidative stress have not been fully studied to date. Therefore, in this study, we elucidated the intracellular pathways and whether isorhamnetin could activate Nrf2 and induce its mechanisms of action in mouse-derived C2C12 myoblasts.

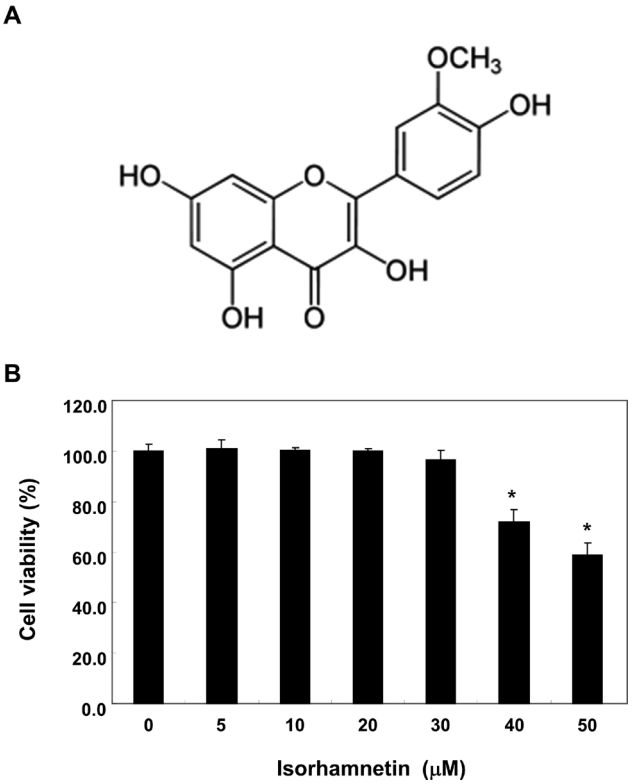

Figure 1. Effects of isorhamnetin on the growth of C2C12 cells. **A.** Chemical structure of isorhamnetin. **B.** Cells were treated with various concentrations of isorhamnetin for 24 h, and then cell viability was assessed using an MTT reduction assay. The results were the mean ± SD values obtained from three independent experiments (* $p < 0.05$ compared with control group).

Materials and Methods

Cell culture and treatment with isorhamnetin

C2C12 myoblasts obtained from American Type Culture Collection (Manassa, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc., Daegu, Republic of Korea) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin antibiotics (WelGENE Inc.) in a humidified 5% CO_2 atmosphere at 37°C. Isorhamnetin (Figure 1A, Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and adjusted to the final concentrations using complete DMEM prior to use; the final DMSO concentration was <0.1% in all experiments.

Cell viability assay

Cell viability was measured on the basis of the formation of blue formazan that was metabolized from colorless 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) by mitochondrial dehydrogenases, which are active only in live cells. In brief, C2C12 cells (1×10^5 cells) were seeded in a 6-well plate. After 24 h incubation, the cells were treated with the indicated concentrations of isorhamnetin in the absence and presence of H_2O_2

and/or zinc protoporphyrin IX (ZnPP, Sigma-Aldrich). After incubation for the indicated times, the cultured medium was replaced by fresh medium, and the cells were incubated with 0.5 mg/ml of MTT solution for 3 h. Then, the supernatant was discarded, and formazan blue, which was formed in the cells, was dissolved with DMSO. The optical density was measured at 540 nm with an enzyme-linked immunosorbent assay plate reader (Dynatech Laboratories, Chantilly, VA, USA).

Flow cytometric detection of apoptosis

A combination of annexin-V and propidium iodide (PI) staining is used to distinguish between early (annexin-V^+/PI^-) and late (annexin-V^+/PI^-) apoptotic, necrotic, and live cells using a fluorescein-conjugated annexin V (annexin V-FITC) staining assay kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol (BD Biosciences). Briefly, cells were washed with phosphate buffered saline and resuspended in 100 μl binding buffer containing 5 μl annexin-V-FITC and 5 μl PI for 15 min at room temperature in the dark. The cells were immediately analyzed by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA, USA). The percentages of apoptotic cells (annexin-V^+ cells) were presented as the mean ± standard deviation (SD) as described previously (Kim et al. 2014).

Measurement of intracellular ROS

The ROS production was monitored using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H2D-CFDA, Molecular Probes, Eugene, OR, USA). To monitor the ROS generation, the cells were incubated with 10 μM H2DCFDA for 20 min at room temperature in the dark. The ROS production in the cells was monitored with a flow cytometer using the Cell-Quest pro software (Becton Dickinson) (Song et al. 2014).

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 pre-coated with 1% normal melting agarose. After the agarose solidified, 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% Trion 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 DNA unwinding and the expression of alkali-labile damage. An electrical field was applied (300 mA, 25 V) for 20 min at 4°C to draw the negatively charged DNA toward the anode. After electrophoresis, the slides were washed three times for 5 min at 4°C in a neutralizing buffer (0.4 M Tris, pH 7.5), followed by staining with 20 μg/ml PI (Sigma-Aldrich). The slides were examined under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) (Jeong et al. 2014). Standard of assessing DNA single strand breaks was based on the percentage of cells with tail by visual estimation.

Protein extraction, electrophoresis, and Western blot analysis

For total protein extraction, the cells were lysed using a lysis buffer (25 mM Tris-Cl (pH 7.5), 250 mM of NaCl, 5 mM of EDTA, 1% nonidet-P40, 1 mM of pheny-methylsulfonyl fluoride, and 5 mM of dithiothreitol) for 1 h. Insoluble materials were discarded by centrifugation at 14,000 rpm for 20 min at 4°C. The protein concentration in the cell lysate was determined using a detergent-compatible protein assay from Bio-Rad (Hercules, CA, USA). Equal amounts of protein were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The separated protein was transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA) and subsequently blocked with Tris-buffered saline (10 mM of Tris-Cl, pH 7.4) containing 0.5% Tween 20 and 5% nonfat dry milk for 1 h at room temperature. The proteins were probed with primary antibodies overnight at 4°C. After probing with primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG as secondary antibodies purchased from Amersham Corp. (Arlington Heights, IL, USA). Using an enhanced chemiluminescence (ECL) detection system (Amersham Corp.), immunoreactive bands were detected and exposed to X-ray film. The antibodies used were as follows: Nrf2 (1:500; SC-13032, rabbit polyclonal), NQO-1 (1:500: SC-16464, goat polyclonal), HO-1 (1:500; SC-136960, mouse monoclonal), actin (1:1,000; sc-1616, goat polyclonal), Akt (1:500; SC-8312, rabbit polyclonal), p-Akt (1:500; SC-101629, rabbit polyclonal), p38 (1:1,000; SC-535, rabbit polyclonal), ERK (1:1,000; SC-154, rabbit polyclonal; all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), γH2A.X (1:1000; #7631S, rabbit monoclonal), p-γH2A.X (1:500; #9718S, rabbit monoclonal), p-ERK (1:500; #9106S, mouse monoclonal), 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., Danvers, MA, USA), p-Nrf2 (1:500; #2073-1, rabbit monoclonal; Abcam, Inc., Cambridge, UK),

Small interfering RNA (siRNA) transfection

Nrf2 siRNA and control siRNA were purchased from Santa Cruz Biotechnology. The siRNAs were transfected into cells according to the manufacturer's instruction using the Lipofectamine 2000 Transfection Reagent (Life Technologies,

Carlsbad, CA, USA). In the 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 media (Life Technologies). After incubation, the transfected cells underwent treatment as described in the figure legends (Jiang et al. 2014).

Data analysis

Results were expressed as means ± SD. The differences in the mean values between groups were analyzed by a one-way analysis of variance followed by the Dunnett's test. The differences were considered significant when the p values were less than 0.05.

Results

Isorhamnetin protects C2C12 cell against H2O2-induced cytotoxicity

The effect of isorhamnetin on the viability of C2C12 cells was first determined using the MTT assay. The treatment of C2C12 cells with isorhamnetin did not result in any cytotoxic effect up to the concentration of 30 μM, and cell viability was dose-dependently decreased at concentrations higher than 40 μM (Figure 1B). Then, we treated the C2C12 cells with 20 μM and 30 μM isorhamnetin 1 h prior to H_2O_2 treatment. Cell viability was measured using the MTT assay to examine the protective effect of isorhamnetin on H_2O_2-induced cytotoxicity. The results indicated that cell viability after treatment with 1 mM H_2O_2 alone was reduced by approximately 60% at 6 h and the H_2O_2-induced reduction of cell viability was significantly protected by isorhamnetin pre-treatment in a concentration-dependent manner (Figure 2A). To investigate the protective effect of isorhamnetin on H_2O_2-induced apoptosis, the frequency of apoptotic cells was further detected by flow cytometry. We observed that the treatment of cells with isorhamnetin prior to the H_2O_2 exposure strongly protected the C2C12 cells against apoptosis (Figure 2B).

Isorhamnetin modulates the H_2O_2-induced ROS generation in C2C12 cells

We also investigated whether isorhamnetin affected the intracellular ROS generation by H_2O_2 treatment using the H2DCFDA assay. As shown in Figure 2C, a significantly increased ROS level was detected after H_2O_2 treatment unlike in untreated cells; however, this increase was significantly

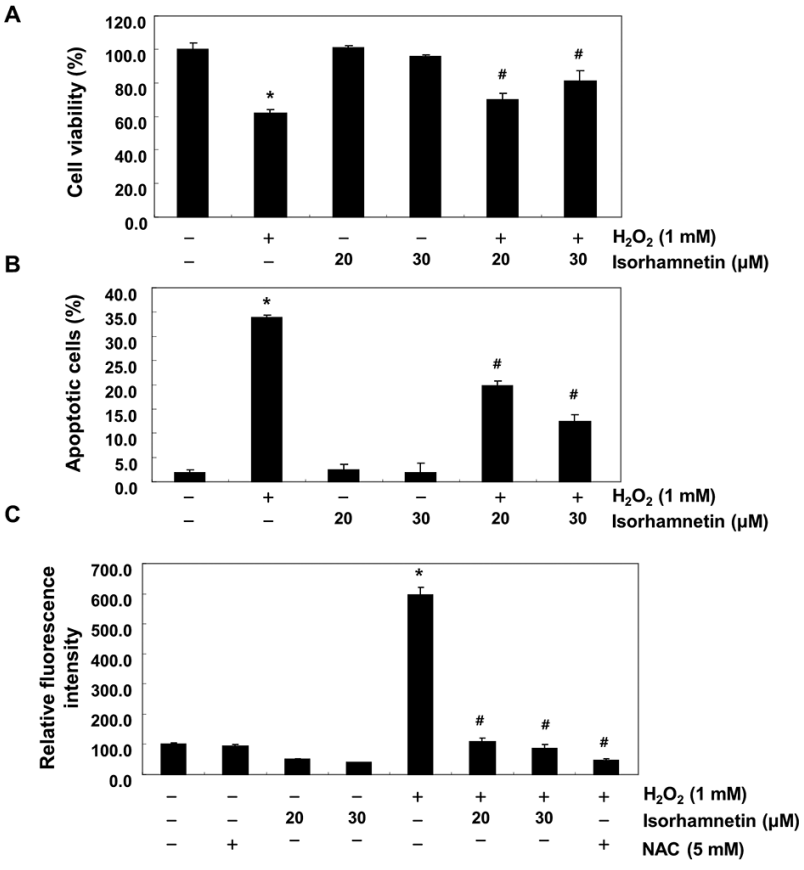

Figure 2. Attenuation of H_2O_2-induced ROS generation and apoptosis by isorhamnetin in C2C12 cells. C2C12 cells were pre-treated with the indicated concentrations of isorhamnetin or 5 mM NAC for 1 h and then stimulated with and without 1 mM H_2O_2 for 6 h. **A.** Cell viability was assessed using an MTT reduction assay. **B.** The cells were stained with annexin V-FITC and PI, and the percentages of apoptotic cells (annexin V^+ cells) were then analyzed using flow cytometric analysis. **C.** To monitor the ROS production, the cells were incubated at 37°C in the dark for 20 min with a new culture medium containing 10 μM H2DCFDA. The ROS generation was measured using a flow cytometer. The results were the mean ± SD values obtained from three independent experiments (* $p < 0.05$ compared with control group; # $p < 0.05$ compared with H_2O_2-treated group).

reduced in the presence of isorhamnetin (Figure 2C). As a positive control, the ROS scavenger N-acetyl-L-cysteine (NAC) also significantly attenuated the H_2O_2-induced ROS generation, and isorhamnetin itself did not contribute to the ROS generation.

Isorhamnetin prevents H_2O_2-induced DNA damage in C2C12 cells

Therefore, we examined the effects of isorhamnetin on H_2O_2-mediated damage to C2C12 cell DNA, as DNA strand breakage is considered one of the most frequent damages induced by oxidative stress. The single-cell gel electrophoresis (comet assay) results indicated that treatment with H_2O_2 alone significantly induced the DNA damage in C2C12 cells; however, this adverse effect was markedly reduced by isorhamnetin pre-treatment (Figure 3A). In addition, Western blotting results showed that treating C2C12 cells with H_2O_2 resulted in the up-regulation of the level of phosphorylated histone variant γH2A.X in serine 139 (p-γH2A.X), a sensitive marker for DNA double-strand breaks (Rogakou et al. 1998) (Figure 3B). However, pre-treatment with isorhamnetin resulted in a marked decreased in p-γH2A.X expression.

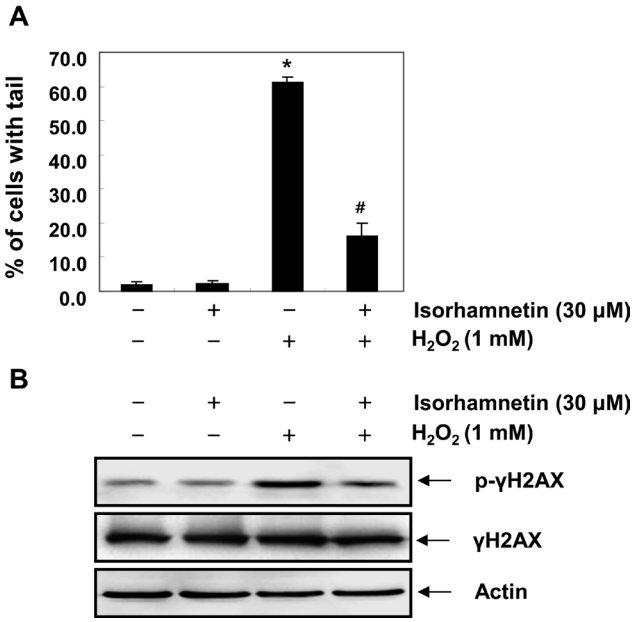

Figure 3. Protection of H_2O_2-induced DNA damage by isorhamnetin in C2C12 cells. C2C12 cells were pre-treated with 30 μM isorhamnetin for 1 h and then incubated with and without 1 mM H_2O_2 for 6 h. **A.** DNA damage was assessed in an alkaline comet assay and characterized by the percentage of cells with comet tail/100 cells (%) in C2C12 cells (* $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the H_2O_2-treated group). **B.** The cells were lysed, and equal amounts of cell lysates were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the specific antibodies against p-γH2A.X, γH2A.X, and actin as an internal control, and the proteins were visualized using an ECL detection system. A representative blot from three independent experiments is shown.

Isorhamnetin enhances the expression of HO-1 and Nrf2 and the phosphorylation of Nrf2 in C2C12 cells

We then investigated whether the inhibitory effect of isorhamnetin against oxidative stress could be mediated by the induction of genes encoding antioxidant enzymes have been well documented to play an important role against oxidative stress. As shown in Figure 4, the treatment of C2C12 cells with isorhamnetin induced the expression of HO-1 protein in a time-dependent manner, but the other antioxidant enzyme, NQO-1, was unaffected by the isorhamnetin treatment. We also observed that isorhamnetin time-dependently increased the total levels of Nrf2 and rapidly induced the phosphorylation of Nrf2 in Ser40 within 1 h.

Nrf2/HO-1 pathway is involved in the isorhamnetin protection against H_2O_2 treatment in C2C12 cells

To determine whether the isorhamnetin-induced antioxidant and the cytoprotective activities against oxidative stress in C2C12 cells were mediated through the activation of the Nrf2/HO-1 pathway, C2C12 cells were pre-incubated with and without a specific inhibitor of HO-1, ZnPP. The levels

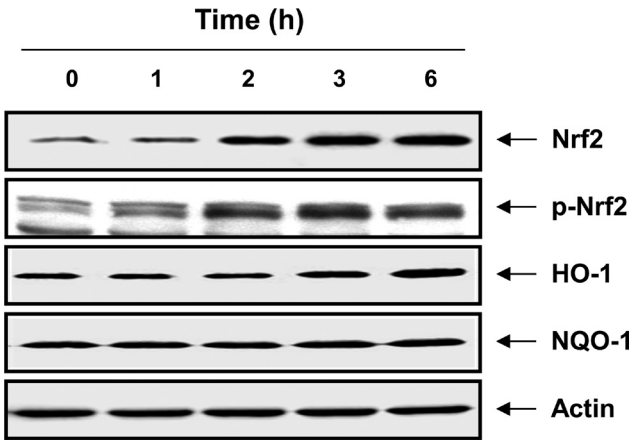

Figure 4. Induction of Nrf2 and HO-1 expression by isorhamnetin in C2C12 cells. Cells were incubated with 30 μM isorhamnetin for the indicated periods. The proteins were separated on SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were probed with the specific antibodies against Nrf2, p-Nrf2, HO-1, and NQO-1. Proteins were visualized using an ECL detection system. Actin was used as an internal control.

of ROS and cell viability were also assessed. As shown in Figure 5, ZnPP abrogated the protective effect of isorhamnetin on the H_2O_2-induced production of ROS and the reduction of cell viability.

Isorhamnetin upregulates the HO-1 expression via the Nrf2 activation in C2C12 cells

We next developed an Nrf2 gene knockdown model using siRNA transfection to demonstrate the importance of the Nrf2 upregulation. Western blot analysis indicated that silencing Nrf2 through a specific siRNA eliminated the isorhamnetin-induced expression and phosphorylation of Nrf2 and the HO-1 upregulation (Figure 6A). To further confirm the involvement of Nrf2, the protective effect of isorhamnetin on the H_2O_2-induced reduction of cell viability was measured in Nrf2 knockdown cells. As indicated in Figure 6B, the siNrf2 transfection abrogated the cytoprotective effect of isorhamnetin unlike in the control siRNA-transfected cells.

ERK signaling pathway is associated with the Nrf2-mediated HO-1 induction by isorhamnetin in C2C12 cells

To identify the upstream signaling events involved in the isorhamnetin-mediated Nrf2 activation and HO-1 induction, the potential involvement of several phosphorylation cascades, such as phosphatidyl-inositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs), was explored. As shown in Figure 7, the total and phosphorylated levels of Akt, a downstream target of PI3K, did not show a notable change, and treatment with LY294002, a pharmacological inhibitor of PI3K, did not prevent the isorhamnetin-induced Nrf2 phosphorylation and expressions of HO-1 and Nrf2. Then, we investigated the effect of isorhamnetin on the activation of MAPKs, which are classified into three major subgroups, namely, extracellular signal-regulated kinase (ERK), c-Jun *N*-terminal kinase (JNK), and p38 MAPK, in C2C12 cells. As illustrated in Figure 8A, the isorhamnetin treatment had a notable inducing effect on the phosphorylation of ERK and p38 MAPK in a time-dependent manner, and JNK phosphorylation acutely increased after the treatment with isorhamnetin within 2 h and then declined. When a selective inhibitor of ERK, PD98059, was incubated, the levels of the isorhamnetin-induced phosphorylation of Nrf2 were blocked, thus decreasing the HO-1 induction (Figure 8B). On the contrary, the inhibitors of p38 MAPK, SB203580, and JNK, SP600125, could not reduce the isorhamnetin-induced HO-1 expression and the Nrf2 phosphorylation.

Discussion

Oxidative stress is the most common cause of cellular dysfunctions. Many studies have been conducted to determine the antioxidant capacities of natural products (Ševčovičová et al. 2015). Consequently, a large number of natural compounds have been reported to possess strong antioxidant properties. Several previous works showed that isorham-

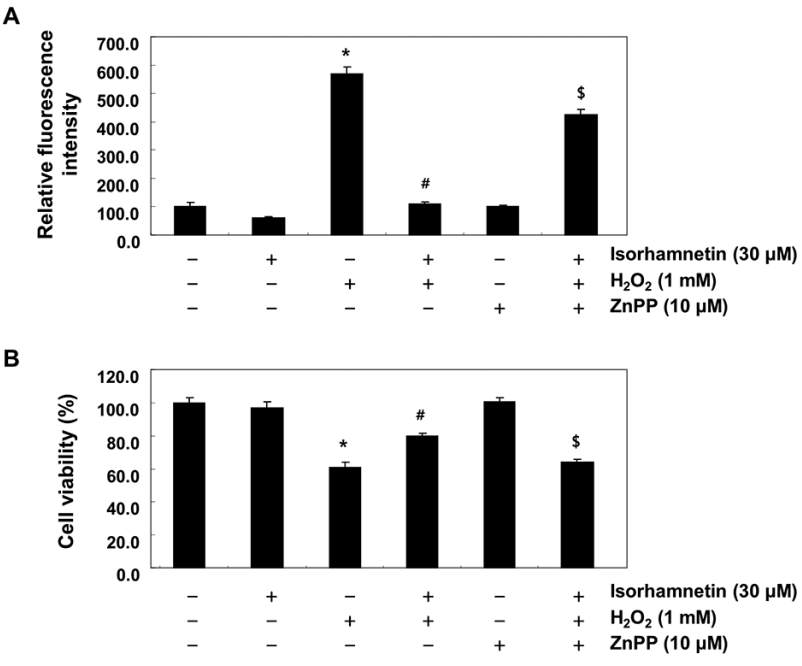

Figure 5. Effects of an inhibitor of HO-1 on the isorhamnetin-mediated attenuation of ROS formation and cytotoxicity induced by H_2O_2 in C2C12 cells. Cells were pre-treated for 1 h with 30 μM isorhamnetin and then treated for 6 h with and without 1 mM H_2O_2 in the absence and presence of 10 μM ZnPP. ROS generation (**A**) and cell viability (**B**) were estimated, respectively. The results were the mean ± SD values obtained in three 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 isorhamnetin-treated group).

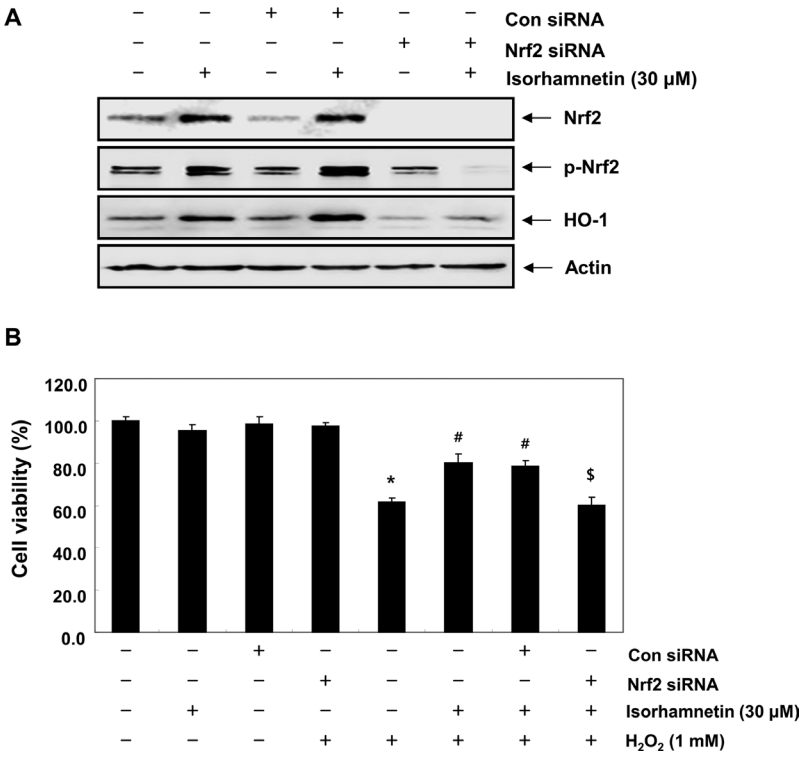

Figure 6. Nrf2-mediated induction of the HO-1 expression by isorhamnetin in C2C12 cells. Cells were transfected with control (Con siRNA, as a negative control for the RNA interference) and Nrf2 siRNA. After 24 h, the cells were treated with 30 μM isorhamnetin for 6 h (**A**) or pre-treated with 30 μM isorhamnetin for 1 h and then stimulated with and without 1 mM H_2O_2 for 6 h (**B**). A. Cellular proteins were separated on SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were probed with the specific antibodies against Nrf2, p-Nrf2, and HO-1. Proteins were visualized using an ECL detection system. Actin was used as a loading control. B. Cell viability was estimated by the MTT assay. The results were the mean ± SD values obtained from three 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 isorhamnetin-treated group).

netin, the most abundant flavonoid in herbs and plants used as traditional medicines including sea buckthorn, has a strong antioxidant activity with complex functions in many pathological processes (Kim et al. 2011; Yang et al. 2014). In the current study, we evaluated the protective effects of isorhamnetin against oxidative stress-induced cytotoxicity and explored the underlying mechanisms using the C2C12 myoblast cell line. The results of this study demonstrated that isorhamnetin pre-treatment effectively antagonized H_2O_2-induced growth inhibition by inhibiting H_2O_2-induced apoptosis and reduced ROS accumulation generated by the H_2O_2 treatment in C2C12 cells (Figure 2). As ROS generation is generally associated with mitochondrial dysfunction, we presumed that isorhamnetin could improve the mitochondrial function by eliminating the overproduction of ROS induced by H_2O_2, thus reducing the apoptosis. Our data indicated that H_2O_2 treatment increased the tail length (DNA migration) and the expression of p-γH2A.X, which are widely used markers for the detection of DNA damage (Rogakou et al. 1998). Each event was mitigated by the isorhamnetin treatment prior to H_2O_2 exposure (Figure 3). These results directly reveal that the protective effects of isorhamnetin contribute to its role in the ROS scavenging effect and the antioxidant defense activity against H_2O_2 treatment in C2C12 cells.

Accumulating evidence indicates that the transcription factor Nrf2 is crucial in the detoxification mechanisms

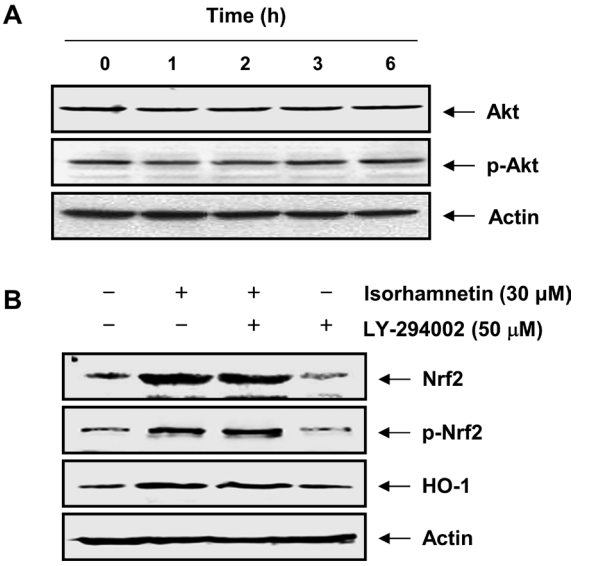

Figure 7. Effects of the PI3K/Akt signaling pathway on the Nrf2-medicated HO-1 induction by isorhamnetin in C2C12 cells. Cells were treated with 30 μM isorhamnetin for the indicated times (**A**) or pre-treated for 1 h with and without LY294002, an inhibitor of PI3K, and then treated with 30 μM isorhamnetin 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 to the nitrocellulose membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized using an ECL detection system. Actin was used as an internal control.

for scavenging reactive metabolites and ROS (Ishii et al. 2000; Kobayashi and Yamamoto 2005; Surh et al. 2008; Niture et al. 2014). Moreover, the phosphorylation and the translocation of Nrf2 into the nucleus are critical for cytoprotection against various oxidative stresses (Zhang and Gordon 2004; Apopa et al. 2008; Niture et al. 2014). In the present study, isorhamnetin was shown to effectively induce the Nrf2 gene expression in a time-dependent manner. The phosphorylation of Nrf2 was also observed following the isorhamnetin treatment (Figure 4). Therefore, the effects of isorhamnetin on the expression of genes encoding antioxidant enzymes, e.g. HO-1 and NQO1 were determined. Western blotting data indicated found that isorhamnetin significantly induced HO-1 expression in a time-dependent manner but not NQO1 expression. Although several transcriptional factors and signaling cascades are involved in HO-1 regulation, the most important factor seems to be Nrf2, as it plays a central role in cellular antioxidant defense (Alam et al. 1999; Vriend and Reiter 2014). Therefore, we further examined the potential role of the Nrf2/HO1 pathway in H_2O_2-induced C2C12 cell damage and isorhamnetin-mediated cytoprotection. The data indicated that the inhibition of the HO-1 function using an HO-1 inhibitor, ZnPP, effectively reduced the protective effect of isorhamnetin against H_2O_2-induced ROS generation and cytoprotection (Figure 5). In addition, the knockdown of Nrf2 by Nrf2-targeted siRNA markedly abrogated the isorhamnetin-induced HO-1 expression, suggesting that Nrf2 is a critical upstream regulator of the isorhamnetin-mediated induction of HO-1 in C2C12 cells. As previously indicated, the silencing of Nrf2 abolished the isorhamnetin-induced restoration of the H_2O_2-mediated growth inhibition of C2C12 cells (Figure 5B). The present results clearly demonstrate that the HO-1 induction by isorhamnetin is responsible for protecting C2C12 cells against H_2O_2-induced oxidative stress. Moreover, the findings suggest that the isorhamnetin-induced cytoprotection of C2C12 cells against oxidative stress is critically dependent on the activation of the Nrf2/HO1 pathway, consistent with previous results (Seo et al. 2014; Yang et al. 2014).

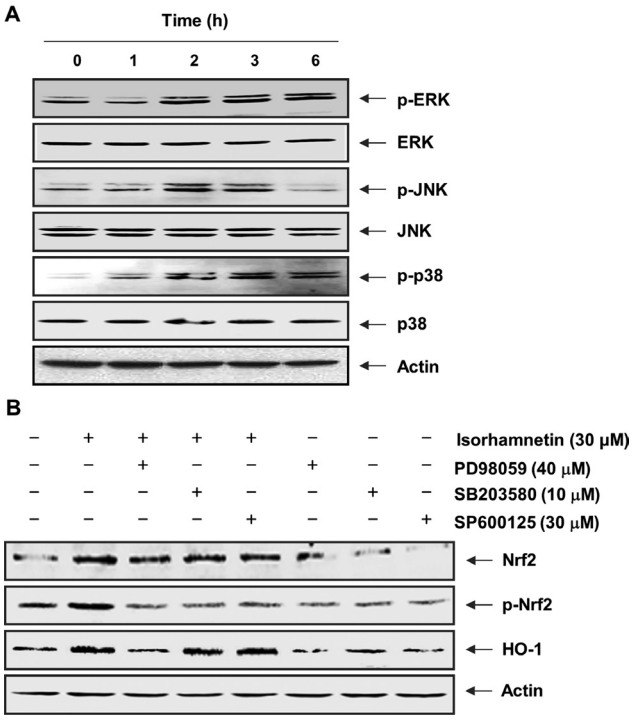

Figure 8. Involvement of the ERK signaling pathway in the Nrf2-medicated HO-1 induction by isorhamnetin in C2C12 cells. Cells were treated with 30 μM isorhamnetin for the indicated times (**A**) or pre-treated for 1 h with and without the indicated inhibitors and then treated with 30 μM isorhamnetin 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 to nitrocellulose membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized using an ECL detection system. Actin was used as an internal control.

A number of reports have implicated multiple protein kinases in the signals that trigger the Nrf2-Keap1 dissociation, the phosphorylation of Nrf2, and the antioxidant-induced activation of the Nrf2/HO-1 signaling pathway. Moreover, recent findings show that the role of each pathway in the activation of Nrf2/HO-1 signaling, as well as its molecular target, may be specific to the stimulus and cell type (Jiang et al. 2014; Jun et al. 2014; Lee et al. 2014). In the present study, the PI3K/Akt signaling pathway was not activated after the isorhamnetin treatment (Figure 7A). In a similar time zone, three MAPKs, namely, ERK, JNK, and p38 MAPK, were effectively activated. Therefore, we examined the effects of the specific inhibitors of PI3K and MAPKs on the induction of HO-1 and the phosphorylation of Nrf2 to identify which signal cascade controlled the activation of Nrf2 by isorhamnetin. The present results demonstrated that PI3K, JNK, and p38 MAPK were not involved in the isorhamnetin-induced activation of the Nrf2/HO-1 signaling because their inhibitors had no effect on the isorhamnetin-induced changes in HO and Nrf2. However, the ERK inhibitor, PD98059, suppressed the isorhamnetin-induced HO-1 and the Nrf2 phosphorylation (Figure 8B). Recently, Sun et al. (2012) demonstrated that the activation of the ERK signaling is involved in the H_2O_2-induced apoptosis in cardiomyocytes and that the anti-apoptotic effects associated with isorhamnetin are attributable to the suppression of the H_2O_2-induced ERK activation. In our study, a potent ROS scavenger, NAC, attenuated the H_2O_2-induced apoptosis, but PD98059 did not effectively antagonize the ROS generation (data not shown). Although more studies are required to understand the reason for these results, we suggest that ROS may be an upstream regulator of the ERK signaling that

contributes to the cytoprotective activity of isorhamnetin in C2C12 cells.

In conclusion, the results indicate that isorhamnetin exhibits potent cytoprotective effects against cell toxicity resulting from exposure to H_2O_2 *via* scavenging ROS and induces Nrf2-mediated HO-1 expression. We also show that the cytoprotective effects induced by isorhamnetin may come from the activation of the ERK signaling axis. Although further investigations including animal studies are needed, these findings suggest that isorhamnetin is a promising agent for the treatment of oxidative stress-induced diverse injuries and may have implications for other diseases associated with ROS overproduction.

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

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