Morin exerts cytoprotective effects against oxidative stress in C2C12 myoblasts via the upregulation of Nrf2-dependent HO-1 expression and the activation of the ERK pathway

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Abstract. In the present study, we investigated the cytoprotective efficacy of morin, a natural flavonoid, against oxidative stress and elucidated the underlying mechanisms in C2C12 myoblasts. Our results indicated that morin treatment prior to hydrogen peroxide (H\(_2\)O\(_2\)) exposure significantly increased cell viability and prevented the generation of reactive oxygen species. H\(_2\)O\(_2\)-induced comet-like DNA formation and γH2AX phosphorylation were also markedly suppressed by morin with a parallel inhibition of apoptosis in C2C12 myoblasts, suggesting that morin prevented H\(_2\)O\(_2\)-induced cellular DNA damage. Furthermore, morin markedly enhanced the expression of heme oxygenase-1 (HO-1) associated with the induction and phosphorylation of nuclear factor-erythroid 2-related factor 2 (Nrf2) and the inhibition of Kelch-like ECH-associated protein 1 (Keap1) expression. Notably, these events were eliminated by transient transfection with Nrf2-specific small interfering RNA. Additional experiments demonstrated that the activation of the Nrf2/HO-1 pathway by morin was mediated by the extracellular signal-regulated kinase (ERK) signaling cascade. This phenomenon was confirmed with suppressed Nrf2 phosphorylation and consequently diminished HO-1 expression in cells treated with a pharmacological inhibitor of ERK. Collectively, these results demonstrated that morin augments the cellular antioxidant defense capacity through the activation of Nrf2/HO-1 signaling, which involves the activation of the ERK pathway, thereby protecting C2C12 myoblasts from H\(_2\)O\(_2\)-induced oxidative cytotoxicity.

Introduction

Dysregulation of antioxidant mechanisms along with excessive accumulation of reactive oxygen species (ROS) is causally linked to various health issues, including muscular dystrophy and sarcopenia (1,2). Within the healthy muscle cell, ROS are generated as both a by-product of metabolism and as effectors of signaling cascades (3). However, excessive ROS generation in skeletal muscles can influence their contractile function by causing fatigue and increasing the oxidative damage to cells (4,5). Therefore, increasing the antioxidant capacity of skeletal muscles can be beneficial for muscle performance, disease prevention, and improved quality of life.

Many flavonoids, which are polyphenolic compounds commonly found in a variety of fruits and vegetables and as components of dietary supplements containing herbs, have been shown to protect cells against oxidative stress-induced damage by virtue of their antioxidant properties (6–8). Morin (3,5,7,2,4’-pentahydroxylavone) is a naturally occurring flavonoid that consists of a yellowish pigment found in many fruits and herbs (9,10). It has been reported that this compound may possess strong antioxidant properties that protect cells against oxidative-induced damage (10–13). Morin has also been shown to induce the activity of phase II enzymes, such as quinone reduc-
tase, glutathione S-transferase, and glutathione reductase (14-17). In particular, morin has been reported to protect human lens epithelial cells against oxidative stress through the activation of nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent heme oxygenase-1 (HO-1) expression (18). However, the inhibitory mechanisms of morin vis-à-vis the beneficial effect of morin against oxidative stress have not been fully studied to date.

Under normal conditions, Nrf2, a transcription factor ubiquitously expressed in most tissues, is bound to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm (19,20). Phase II enzyme inducers can disrupt the Nrf2/Keap1 complex, resulting in the release of Nrf2 and its subsequent translocation to the nucleus (20,21). In the nucleus, Nrf2 activates the antioxidant response element (ARE), which transcriptionally activates many antioxidative genes (20,22). It has recently been reported that activation of the Nrf2/HO-1 pathway can be increased by the stabilization and/or phosphorylation of the Nrf2 protein, and several signaling molecules, including phosphatidylinositol-3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs), have been shown to participate in the processes in response to a variety of phase II gene inducers (23,24). Therefore, targeted activation of Nrf2/HO-1 signaling may be considered an important therapeutic strategy for protection against oxidative damage (19,20). In the present study, we investigated the cytoprotective effect of morin against oxidative stress damage in a mouse myoblast C2C12 cell line and the possible protective mechanisms involved.

Materials and methods

Reagents and antibodies. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin antibiotics were purchased from WelGENE Inc. (Daegu, Republic of Korea). Morin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), N-acetyl-L-cysteine (NAC), 4,6-diamidino-2-phenylindole (DAPI), and PD98059 (2'-amino-3'-methoxyflavone) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Abcam, Inc. (Cambridge, MA, USA). All other chemicals were purchased from Sigma-Aldrich Chemical Co. and the American Type Culture Collection (ATCC, Manassas, VA, USA) were used as the secondary antibodies, which were obtained from Santa Cruz Biotechnology, Inc. All other chemicals were purchased from Sigma-Aldrich Chemical Co.

Cell culture and MTT assay. C2C12 myoblasts obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were grown in DMEM supplemented with 10% FBS and 100 µg/ml penicillin/streptomycin antibiotics in a humidified 5% CO2 atmosphere at 37°C. Morin was dissolved in dimethyl sulfoxide (DMSO) and adjusted to final concentrations using complete DMEM prior to use; the final DMSO concentration was <0.1% in all experiments. In order to evaluate the degree to which a single morin treatment affects C2C12 cell viability, the C2C12 cells were seeded at a density of 1x10^4 cells/well in a 96-well plate, incubated at 37°C for 24 h, and treated with morin at different concentrations (100-10,000 µM). Additional cell cultivation occurred for 6 h in media where H2O2 and NAC were simultaneously administered, singularly administered, or were not administered. For the cell viability assay using a colorimetric MTT assay, the medium was discarded, MTT solution was added to each well, and the cells were further incubated for 3 h at 37°C. The medium was discarded and DMSO was added to dissolve the formazan product. The optical density was then read at 450 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR-7000; Dynatech Laboratories, Chantilly, VA, USA). Relative cell cytotoxicity was evaluated according to the quantity of MTT converted to insoluble formazan salt.

Intracellular ROS measurement. In order to monitor ROS generation, the cells were incubated with 10 µM DCF-DA for 20 min at room temperature in the dark. The ROS production in the cells was monitored with a flow cytometer (Becton-Dickinson, San Jose, CA, USA) using CellQuest Pro software (25).

Determination of apoptotic cells by flow cytometry. To quantitatively assess the induced cell apoptosis rate, an Annexin V-FITC staining assay was performed as previously described (26). The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and stained with Annexin V-FITC and propidium iodide (PI) in each sample for 15 min at room temperature in the dark. The degree of apoptosis was quantified as a percentage of Annexin V-positive and PI-negative (Annexin V+/PI- cells) cells using a flow cytometer.

Comet assay. To assess oxidative DNA damage, 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. 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 (Na-EDTA), 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH 10.0) 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 13.0) for 40 min to allow for DNA unwinding and the expression of alkali-labile damage. Next, 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. The cells were washed twice with PBS, and images were then captured using a fluorescence microscope (Carl Zeiss, Jena, Germany) (27).

Protein extraction and western blot analysis. Whole-cell protein extracts from C2C12 myoblasts were prepared with cell lysis buffer (25 mM of Tris-Cl (pH 7.5), 250 mM of NaCl, 5 mM of EDTA, 1% Nonidet P-40, 0.1 mM of sodium orthovanadate, 2 µg/ml of leupeptin, and 100 µg/ml of phenylmethylsulfonyl
flouride] containing protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) for 30 min. In a parallel experiment, nuclear and cytosolic proteins were prepared using nuclear extraction reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's protocol. After cell debris was discarded following centrifugation at 13,000 x g for 15 min, the protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). For western blot analysis, equal amounts of protein extracts were subjected to electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher and Schuell Bioscience, Inc., Keene, NH, USA) by electroblotting. The blots were probed with the desired primary antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibodies, and visualized using an ECL method according to the recommended procedure.

Small interfering RNA (siRNA) transfection. Nrf2 siRNA and control siRNA were purchased from Santa Cruz Biotechnology, Inc. The siRNAs were transfected into cells according to the manufacturer's instructions using Lipofectamine 2000 Transfection Reagent (Life Technologies, Carlsbad, CA, USA). For western blot analysis, equal amounts of protein extracts were subjected to electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher and Schuell Bioscience, Inc., Keene, NH, USA) by electroblotting. The blots were probed with the desired primary antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibodies, and visualized using an ECL method according to the recommended procedure.

**Results**

**Effects of morin on H$_2$O$_2$-induced cytotoxicity in C2C12 myoblasts.** To evaluate the protective effect of morin on H$_2$O$_2$-induced cytotoxicity, the cells were treated with various concentrations of morin for 24 h, and the cell viability was evaluated for primary dose selection by determining the percentage of MTT reduction. Morin alone at 100-500 µM showed no cytotoxic effects, but significant cytotoxicity was noted at 1,000 µM morin (Fig. 1A). Thus, 500 µM morin was chosen as the optimal dose for studying the cytoprotective effect of this flavonoid. The cells were incubated with 0.4% Triton X-100 for 10 min and then blocked with 5% bovine serum albumin for 1 h, followed by probing with the anti-Nrf2 antibody overnight at 4°C and incubation with FITC-conjugated donkey anti-rabbit IgG for 2 h at room temperature. After washing with PBS, nuclei were counterstained with DAPI solution (1 mg/ml) for 15 min in the dark. Images were collected using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

**Statistical analysis.** Data are expressed as mean ± standard deviation (SD) from at least three independent experiments. Statistical comparisons between different groups were performed using one-way ANOVA, followed by Student's t-tests after comparing each treated group to the negative control. Probability values P<0.01 were considered to indicate statistically significant differences.

**Table I. Antibodies used in the present study.**

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Figure 1. Morin attenuates H$_2$O$_2$-induced growth inhibition in C2C12 myoblasts. Cells were (A) treated with various concentrations of morin for 24 h or (B) pretreated with 500 µM morin or 10 mM N-acetyl-L-cysteine (NAC) for 1 h and then stimulated with or without 1 mM H$_2$O$_2$ for 6 h. The cell viability was assessed using an MTT reduction assay. The results are the mean ± SD values obtained in three independent experiments (*P<0.05 compared with the control group; †P<0.05 compared with the H$_2$O$_2$-treated group).

with 500 µM morin or 10 mM NAC, an ROS scavenger that was used as positive control, for 1 h and exposed to H$_2$O$_2$ for an additional 6 h, the cell viability was significantly increased compared to the H$_2$O$_2$ group, and the survival rate was 81.00 and 99.08% that of the control group, respectively (Fig. 1B).

Inhibition of H$_2$O$_2$-induced ROS production by morin in C2C12 myoblasts. As the cytotoxicity of H$_2$O$_2$ is mainly mediated by oxidative stress, we investigated the effect of morin on H$_2$O$_2$-induced ROS accumulation using DCF-DA reagent. Compared with the non-treated control cells, the level of intracellular ROS was markedly increased in the C2C12 cells treated with 1 mM H$_2$O$_2$ for 6 h, as indicated by the increase in the DCF-liberated fluorescent signal (Fig. 2A). However, when the C2C12 cells were pretreated with morin or NAC, the ROS formation was significantly decreased, suggesting that morin pretreatment induced a cellular antioxidative response.

Protection against H$_2$O$_2$-induced C2C12 cell apoptosis by morin. The effects of morin on C2C12 cell apoptosis induced by H$_2$O$_2$ were observed using an Annexin V-FITC/PI assay. As illustrated in Fig. 2B, the percentage of apoptotic cells treated with 1 mM H$_2$O$_2$ was ~49.54%; however, morin or NAC pretreatment effectively decreased cell apoptosis induced by H$_2$O$_2$ to 7.99 and 7.86%, respectively. The results indicate that the H$_2$O$_2$-induced apoptosis was mediated by ROS generation and that morin exerted a potent ROS scavenging effect, preventing H$_2$O$_2$-induced apoptosis.

Prevention of H$_2$O$_2$-induced DNA damage by morin in C2C12 myoblasts. We next examined the effects of morin on H$_2$O$_2$-mediated DNA damage in the C2C12 cells using the comet assay (single cell gel electrophoresis) and western blot analysis. Exposure to H$_2$O$_2$ alone induced significant DNA breaks, resulting in an increase in fluorescence intensity in the tails of the comet-like structures (Fig. 3A); however, this adverse effect was markedly reduced by pretreatment with morin or NAC. In addition, the exposure of the C2C12 cells to H$_2$O$_2$ resulted in upregulation in the level of the phosphorylated histone variant H2AX (p-γH2AX) at serine 139, a sensitive marker of DNA double strand breaks (29); however, pretreatment with morin or NAC resulted in a significant decrease in p-γH2AX expression.

Effects of morin on the expression of Nrf2 and HO-1 in C2C12 myoblasts. As Nrf2 signaling regulates cellular antioxidant response (20,22), we examined whether morin protects cells from oxidative stress by activating the Nrf2 signaling pathway. Our immunoblotting results indicated that treatment of C2C12 cells with morin induced the expression of HO-1 protein in a time-dependent manner, but other antioxidant enzymes, NADPH-quinone oxidoreductase 1 (NQO1) and thioredoxin reductase 1 (TrxR1), were unaffected by morin treatment, which was associated with the upregulation of Nrf2 expression and the downregulation of Keap1 (Fig. 4A).

Since the phosphorylation of Nrf2 at Ser40 by several kinases is also a critical process in its stabilization and nuclear translocation (20,23,24), we examined the phosphorylation of Nrf2 under morin treatment to further confirm the Nrf2 activating property.
of morin and observed that the treatment of cells with morin time-dependently increased the expression levels of phosphorylated Nrf2 (Fig. 4A). Additionally, western blot analysis was carried out using the nuclear and cytosolic fractions of the C2C12 cells. The results shown in Fig. 5A and B indicate that the amounts of total and phosphorylated Nrf2 proteins in the nucleus were markedly increased following treatment with morin. The immunofluorescence images also revealed that the nuclear localization and accumulation of Nrf2 in the C2C12 cells was significantly increased after stimulation with morin (Fig. 5C).

**Nrf2-dependent induction of HO-1 by morin in C2C12 myoblasts.** In order to provide evidence for the involvement of Nrf2 in the induction of HO-1 by morin, we transiently transfected C2C12 cells with Nrf2 siRNA. Western blot analysis results revealed that the silencing of Nrf2 using specific siRNA abrogated the morin-induced increase and phosphorylation in Nrf2 expression (Fig. 4B). Therefore, we investigated whether Nrf2 siRNA transfection attenuated morin-induced upregulation of HO-1 and downregulation of Keap1 and found that Nrf2 siRNA reversed these effects, which is evidence that the augmentation of HO-1 by morin was mediated by Nrf2.

**Effect of extracellular signal-regulated kinase (ERK) signaling pathway on Nrf2-mediated HO-1 induction by morin in C2C12 myoblasts.** To identify the upstream signaling events involved in morin-mediated Nrf2-dependent HO-1 induction by morin in C2C12 myoblasts, we investigated the potential involvement of the PI3K/Akt and MAPK signaling pathways. The total and phosphorylated levels of PI3K and Akt, a downstream target of PI3K, did not show a notable change in the morin-treated C2C12 cells as compared with levels in the untreated control cells (Fig. 6). Subsequently, although there were no notable changes observed in the phosphorylated levels of c-Jun N-terminal kinase (JNK) and p38 MAPK, the activation of c-Jun N-terminal kinase (JNK) and p38 MAPK, the activation of...
extracellular signal-regulated kinase (ERK) was noted as early as 30 min after morin treatment, and it lasted at least for 6 h. However, treatment with a selective inhibitor of ERK, PD98059, blocked the morin-triggered phosphorylation of Nrf2, and HO-1 induction was accordingly diminished without the blockade of Nrf2 induction (Fig. 7). By contrast, when an inhibitor of ERK...
was utilized, the induction of the total protein level of Nrf2 in the morin-treated C2C12 cells was not attenuated.

Discussion

Although there have been few studies on how oxidative stress may quantitatively affect the load-carrying capacity of muscle cells, oxidative stress on myoblasts should accompany the dysfunction of muscles (3,4). In this investigation, as part of the screening program for therapeutic antioxidative agents from phytochemicals, we attempted to determine whether morin offers protection from oxidative stress-induced cytotoxicity using a C2C12 myoblast cell model. We first observed that when C2C12 myoblasts were treated with morin in the presence of H$_2$O$_2$, cell viability was significantly recovered by inhibiting H$_2$O$_2$-induced ROS generation, compared with exposure to H$_2$O$_2$ alone. Our data also showed that H$_2$O$_2$ exposure increased cell death and the expression of p-$\gamma$H2AX, whereas each event was mitigated in C2C12 cells that had been treated with morin prior to H$_2$O$_2$ exposure. As a result, these findings suggest that morin may be useful for the prevention of H$_2$O$_2$-induced cytotoxicity due to its strong antioxidative effect.

There is mounting evidence that oxidative stress is implicated in the pathogenesis of muscle dysfunction (4,5). Oxidative stress occurs when the production of ROS exceeds its catabolism. ROS, including superoxide radical anions, hydroxyl radicals, singlet oxygen and hydrogen peroxide, are a class of endogenous signaling molecules with specific functions depending on their subcellular localization, local concentration and duration of production. Although a moderate level of ROS transiently oxidizes the cysteine sulfhydryl that contributes to the active sites of most proteins, when ROS accumulate to a certain level, the active effects may convert to inhibitory effects that damage cellular functions (30,31). Excessive ROS generation in skeletal muscles can influence the contractile function of skeletal muscles by causing fatigue and increasing the oxidative damage to cells, which is implicated in the development of multiple diseases, including muscular dystrophy and sarcopenia (4,5). Therefore, the discovery of an efficient agent that is able to reduce ROS is valuable in the treatment of muscle-related diseases. As expected, our results showed that ROS generation in C2C12 myoblasts was significantly increased by H$_2$O$_2$ challenge and that morin markedly reduced ROS production by attenuating DNA damage.

Nrf2, a transcription factor that is part of the redox homeostatic gene regulatory network, and its repressor, Keap1, play indispensable roles in protecting a variety of tissues from a wide array of toxic insults, including oxidative stress. As yet, the role of Nrf2 signaling has not been well characterized in skeletal muscle. However, coordinated regulation of Nrf2 redox signaling is believed to preserve the redox state and to protect skeletal muscle structure and function (2,32). Previous studies have shown that active skeletal muscle exhibit increased nuclear Nrf2 levels and subsequent activation of its major target antioxidant enzymes, including HO-1 (33). Contrary to this, the loss of Nrf2 is strongly coupled with the dysregulation of antioxidant pathways and the progression of muscle dysfunction (34,35). Nrf2 is sequestered in the cytosol as an inactive complex with its repressor Keap1. Dissociation of Nrf2 from Keap1 is a prerequisite for nuclear translocation and subsequent DNA binding of Nrf2 to regulate the inducible expression of cytoprotective genes, such as that of HO-1 (21,22). HO-1 is an inducible rate-limiting enzyme initially identified as a phase II detoxifying enzyme that facilitates the degradation of heme into bilirubin, free iron, and carbon monoxide. The final products of heme catabolism exert certain antioxidant effects by neutralizing intracellular ROS (36,37). Therefore, Nrf2/HO-1 signaling may represent a potential therapeutic target in the management of oxidative stress-related diseases. In the present study, we observed a significant increase in total Nrf2 expression in morin-treated C2C12 myoblasts that was correlated with a concomitant reduction in Keap1 expression. Subsequently, we found that morin triggered the phosphorylation of Nrf2 and led to HO-1 induction along with a concomitant translocation of Nrf2 into the nuclei. However, Nrf2-specific siRNA markedly suppressed morin-enhanced HO-1 protein levels, suggesting that Nrf2 is a critical upstream regulator of the morin-mediated induction of HO-1 in C2C12 myoblasts.

It has been proposed that the induction of phosphorylation plays a key role in the regulation of the transcriptional activity of Nrf2 (23,24). In addition, a number of protein kinases, such as PI3K/Akt and MAPKs, have been implicated as upstream signals in the regulation of Nrf2 activity by facilitating the translocation of Nrf2 into the nucleus, whereupon it binds to ARE in the promoter regions (38-42). Therefore, to investigate whether any protein kinases may be involved in the activation of Nrf2, the phosphorylated level of different protein kinases following morin treatment was analyzed. Our results revealed that morin treatment only had a notable inducing effect on ERK phosphorylation within 30 min. In contrast, phosphorylation of PI3K, Akt, JNK and p38 MAPK was not observed at any time point. Furthermore, our data indicated that morin-induced phosphorylation of Nrf2 and induction of HO-1 were effectively inhibited by the ERK kinase inhibitor, whereas the increased total protein levels of Nrf2 were not affected. These observations suggest that morin may activate ERK by inducing its phosphorylation and in turn post-translationally phosphorylate Nrf2 as a downstream signal, which is required for Nrf2 accumulation in the nucleus leading to HO-1 expression.

In conclusion, our results showed that in C2C12 myoblasts, morin exhibited protective ability against H$_2$O$_2$-induced cytotoxicity and DNA damage through the suppression of intracellular ROS generation. In addition, our overall results imply that morin may activate Nrf2 by activating ERK to contribute to the induction of phase II antioxidant HO-1 in C2C12 myoblasts, which, at least in part, contributes to a cellular defense mechanism against oxidative stress-induced genotoxic events. In future studies, this molecular mechanism must be validated in vivo. A positive result, if confirmed, would be invaluable to the development of new approaches for effective stress-responsive antioxidants against assaults triggered by ROS.

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