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

MOON HEE LEE[1,2], MIN HO HAN[3], DAE-SUNG LEE[3], CHEOL PARK[4], SU-HYUN HONG[1], GI-YOUNG KIM[5], SANG HOON HONG[6], KYOUNG SEOB SONG[7], IL-WHAN CHOI[8], HEE-JAE CHA[9] and YUNG HYUN CHOI[1,2]

[1]Department of Biochemistry, Dongeui University College of Korean Medicine, Busan 614-052; [2]Anti-Aging Research Center, Dongeui University, Busan 614-714; [3]Marine Biodiversity Institute of Korea, Seocheon 325-902; [4]Department of Molecular Biology, College of Natural Sciences and Human Ecology, Dongeui University, Busan 614-714; [5]Laboratory of Immunobiology, Department of Marine Life Sciences, Jeju National University, Jeju 690-756; [6]Department of Internal Medicine, Dongeui University College of Korean Medicine, Busan 614-052; [7]Department of Physiology, Kosin University College of Medicine, Busan 602-072; [8]Department of Microbiology, College of Medicine, Inje University, Busan 608-737; [9]Department of Parasitology and Genetics, Kosin University College of Medicine, Busan 602-072, Republic of Korea

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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_2O_2) exposure significantly increased cell viability and prevented the generation of reactive oxygen species. H_2O_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_2O_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_2O_2-induced oxidative cytotoxicity.

Correspondence to: Dr Hee-Jae Cha, Department of Parasitology and Genetics, Kosin University College of Medicine, Gamcheollo 262, Seo-gu, Busan 602-072, Republic of Korea
E-mail: hcha@kosin.ac.kr

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

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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'-pentahydroxyflavone) 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-phenyllindile (DAPI), and PD98059 (2'-amino-3'-methoxyflavone) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCF-DA) and a fluorescein-conjugated Annexin V (Annexin V-FITC) staining assay kit were purchased from Molecular Probes (Eugene, OR, USA) and BD Biosciences (San Jose, CA, USA), respectively. An enhanced chemiluminescence (ECL) detection kit and FITC-conjugated donkey anti-rabbit IgG were purchased from Amersham Co. (Arlington Heights, IL, USA) and Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), respectively. Various primary antibodies (Table I) for western blot analysis were obtained from Cell Signaling Technology, Inc. (Boston, MA, USA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Abcam, Inc. (Cambridge, MA, USA). Horseradish peroxidase-conjugated anti-rabbit (SC-2004), anti-mouse (SC-2005) and anti-goat (SC-2350) antibodies 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% CO_2 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 H_2O_2 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

Table I. Antibodies used in the present study.

Antibody	Origin	Company	Catalogue no.
Actin	Mouse monoclonal	Santa Cruz Biotechnology, Inc.	SC-47778
p-γH2AX	Rabbit monoclonal	Cell Signaling Technology, Inc.	9718
γH2AX	Rabbit monoclonal	Cell Signaling Technology, Inc.	7631
p-Nrf2	Rabbit monoclonal	Abcam, Inc.	ab76026
Nrf2	Rabbit polyclonal	Santa Cruz Biotechnology, Inc.	SC-13032
Keap1	Goat polyclonal	Santa Cruz Biotechnology, Inc.	SC-15246
HO-1	Rabbit polyclonal	Santa Cruz Biotechnology, Inc.	SC-10789
NQO1	Goat polyclonal	Santa Cruz Biotechnology, Inc.	SC-16464
TrxR1	Mouse monoclonal	Santa Cruz Biotechnology, Inc.	SC-28321
Lamin B	Goat polyclonal	Santa Cruz Biotechnology, Inc.	SC-6216
p-PI3K	Rabbit polyclonal	Cell Signaling Technology, Inc.	4228
PI3K	Rabbit polyclonal	Cell Signaling Technology, Inc.	4249
p-Akt	Rabbit polyclonal	Santa Cruz Biotechnology, Inc.	SC-101629
Akt	Rabbit polyclonal	Santa Cruz Biotechnology, Inc.	SC-8312
p-ERK	Mouse monoclonal	Cell Signaling Technology, Inc.	9106
ERK	Rabbit polyclonal	Santa Cruz Biotechnology, Inc.	SC-154
p-JNK	Mouse monoclonal	Cell Signaling Technology, Inc.	9255
JNK	Rabbit monoclonal	Cell Signaling Technology, Inc.	9252
p-p38 MAPK	Rabbit monoclonal	Cell Signaling Technology, Inc.	9211
p38 MAPK	Rabbit polyclonal	Santa Cruz Biotechnology, Inc.	SC-535

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 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 were subjected to treatment, as previously described (28).

Immunofluorescent staining for Nrf2. C2C12 cells were seeded on glass coverslips in 6-well plates for 24 h, and the cells were treated with morin for 6 h. Next, the cells were rinsed twice with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at 4˚C. 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.

Results

Effects of morin on H_2O_2-induced cytotoxicity in C2C12 myoblasts. To evaluate the protective effect of morin on H_2O_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 viability of the C2C12 cells when exposed to H_2O_2 at the concentration of 1 mM for 6 h was significantly decreased as compared with the control group, and the survival rate was ~60% that of the control group. When the C2C12 cells were pretreated

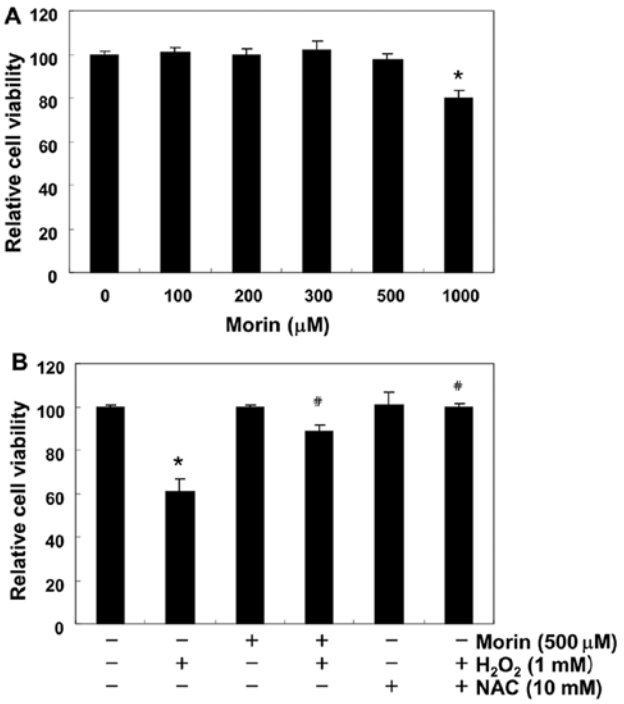

Figure 1. Morin attenuates H_2O_2-induced growth inhibition in C2C12 myoblasts. Cells were (A) treated with various concentrations of morin for 24 h or (B) pretreated with 500 μM morin or 10 mM *N*-acetyl-L-cysteine (NAC) for 1 h and then stimulated with or without 1 mM H_2O_2 for 6 h. The cell viability 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_2O_2-treated group).

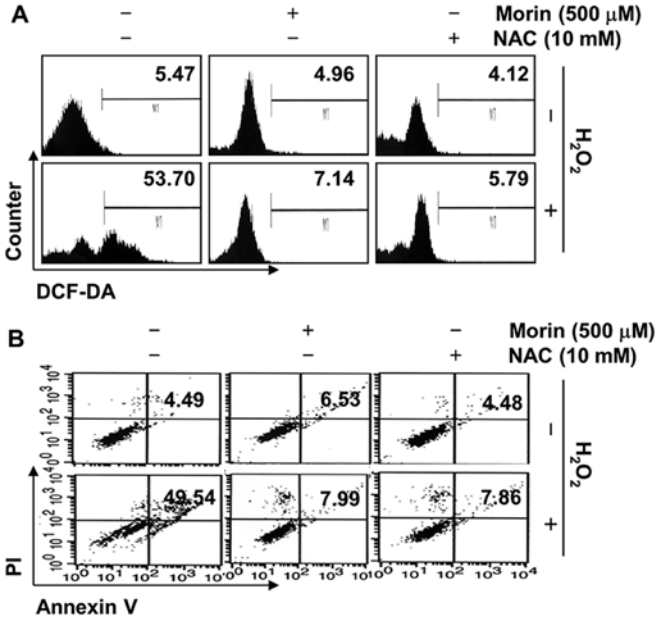

Figure 2. Morin blocks H_2O_2-induced reactive oxygen species (ROS) generation and apoptosis in C2C12 myoblasts. Cells were pretreated with 500 μM morin or 10 mM *N*-acetyl-L-cysteine (NAC) for 1 h and then stimulated with and without 1 mM H_2O_2 for 6 h. (A) In order to monitor ROS production, the cells were incubated at 37˚C in the dark for 20 min with culture medium containing 10 μM DCF-DA. ROS generation was evaluated using a flow cytometer. (B) The cells were stained with Annexin V-FITC and propidium iodide (PI), and the percentages of apoptotic cells (Annexin V^+/PI^- cells) were then analyzed using a flow cytometer. Data are the mean of two different experiments.

with 500 μM morin or 10 mM NAC, an ROS scavenger that was used as positive control, for 1 h and exposed to H_2O_2 for an additional 6 h, the cell viability was significantly increased compared to the H_2O_2 group, and the survival rate was 81.00 and 99.08% that of the control group, respectively (Fig. 1B).

Inhibition of H_2O_2-induced ROS production by morin in C2C12 myoblasts. As the cytotoxicity of H_2O_2 is mainly mediated by oxidative stress, we investigated the effect of morin on H_2O_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_2O_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_2O_2-induced C2C12 cell apoptosis by morin. The effects of morin on C2C12 cell apoptosis induced by H_2O_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_2O_2 was ~49.54%; however, morin or NAC pretreatment effectively decreased cell apoptosis induced by H_2O_2 to 7.99 and 7.86%, respectively. The results indicate that the H_2O_2-induced apoptosis was mediated by ROS generation and that morin exerted a potent ROS scavenging effect, preventing H_2O_2-induced apoptosis.

Prevention of H_2O_2-induced DNA damage by morin in C2C12 myoblasts. We next examined the effects of morin on H_2O_2-mediated DNA damage in the C2C12 cells using the comet assay (single cell gel electrophoresis) and western blot analysis. Exposure to H_2O_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_2O_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

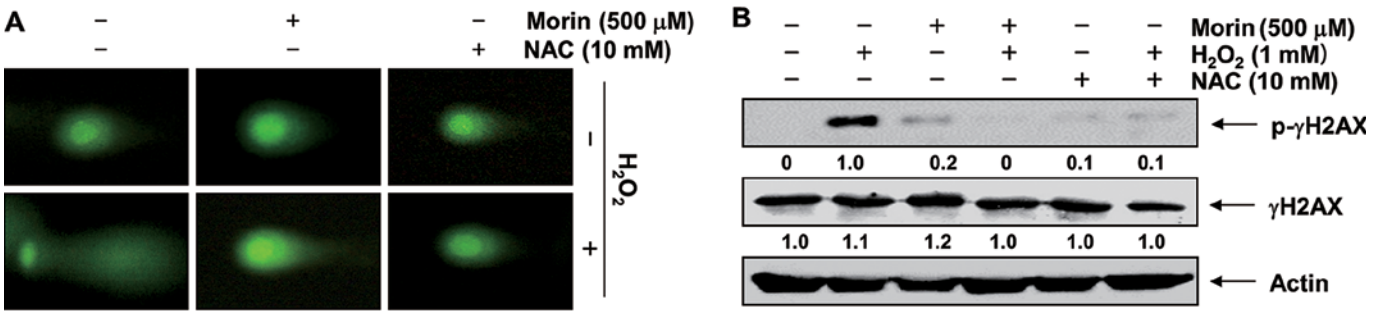

Figure 3. Morin protects against H_2O_2-induced DNA damage in C2C12 myoblasts. Cells were pretreated with 500 μM morin or 10 mM *N*-acetyl-L-cysteine (NAC) for 1 h and then stimulated with and without 1 mM H_2O_2 for 6 h. (A) To detect cellular DNA damage, the comet assay was performed, and representative images of the comets were captured using a fluorescence microscope (x400 original magnification). (B) The cells were lysed and then equal amounts of cell lysates were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with specific antibodies against p-γH2AX, γH2AX, and actin as an internal control, and the proteins were visualized using an enhanced chemiluminescence (ECL) detection system. The relative ratios of expression in the western blotting results are shown at the bottom of each of the results as relative values to actin expression.

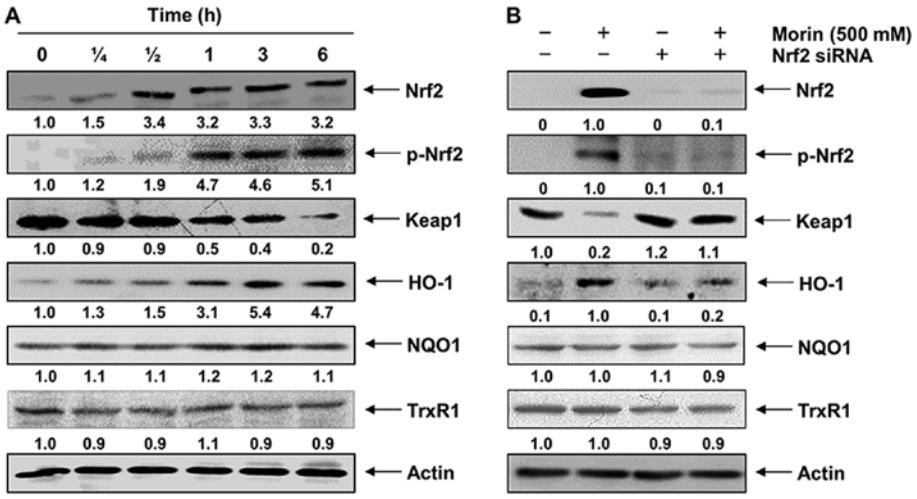

Figure 4. Morin induces nuclear factor-erythroid 2-related factor 2 (Nrf2)-dependent heme oxygenase-1 (HO-1) expression in C2C12 myoblasts. (A) Cells were incubated with 500 μM morin for the indicated time periods. The cells were lysed and then equal amounts of cell lysates were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. (B) Cells were transfected with Nrf2 siRNA. After 24 h, the cells were treated with or without 500 μM morin for 6 h. 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, Keap1 and HO-1. Proteins were visualized using an enhanced chemiluminescence (ECL) detection system. Actin was used as an internal control. The relative ratios of expression in the western blotting results are shown at the bottom of each of the results as relative values to actin expression.

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, the potential involvement of the PI3K/Akt and MAPK signaling pathways was investigated. 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

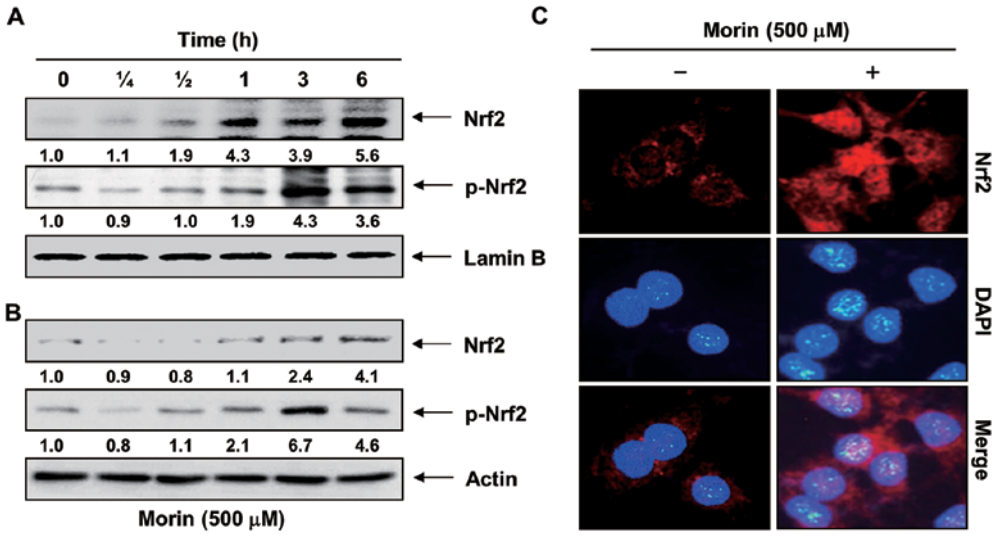

Figure 5. Morin enhances nuclear translocation of nuclear factor-erythroid 2-related factor 2 (Nrf2) in C2C12 myoblasts. (A and B) Cells were incubated with 500 μM morin for the indicated time periods. (A) Nuclear or (B) cytosolic proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were probed with anti-Nrf2 and anti-p-Nrf2 antibodies. Proteins were visualized using an enhanced chemiluminescence (ECL) detection system. Lamin B and actin were used as the internal controls of nuclear and cytosolic proteins, respectively. The relative ratios of expression in the western blotting results are shown at the bottom of each of the results as relative values to lamin B (A) or actin (B) expression. (C) Cells were treated with 500 μM morin for 6 h and then localization of Nrf2 was visualized with a fluorescence microscope after immunofluorescence staining with anti-Nrf2 antibody and an FITC-labeled anti-rabbit IgG antibody (red). Nuclei of the corresponding cells were visualized with DAPI (blue).

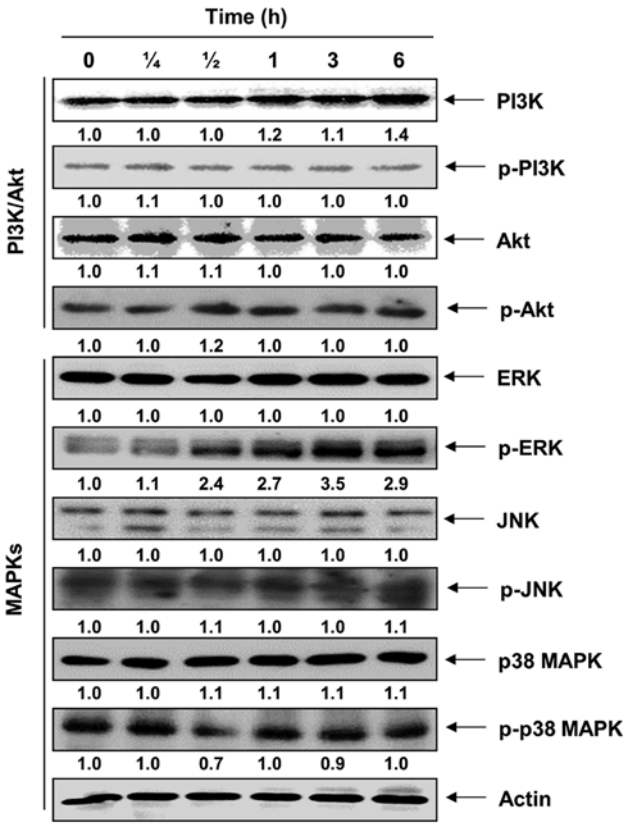

Figure 6. Morin induces phosphorylation of extracellular signal-regulated kinase (ERK) in C2C12 myoblasts. Cells were incubated with 500 μM morin for the indicated time periods. The cells were lysed and then equal amounts of cell lysates were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies and then the proteins were visualized using an enhanced chemiluminescence (ECL) detection system. Actin was used as an internal control. The relative ratios of expression in the western blotting results are shown at the bottom of each of the results as relative values to actin expression.

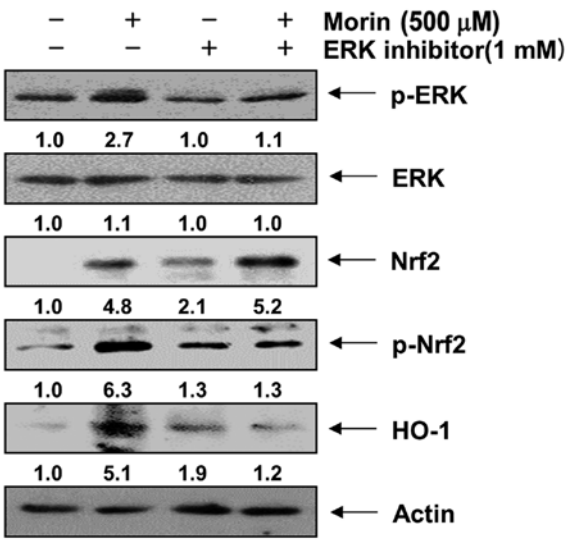

Figure 7. Morin phosphorylates nuclear factor-erythroid 2-related factor 2 (Nrf2) through extracellular signal-regulated kinase (ERK) activation in C2C12 myoblasts. Cells were pretreated for 1 h with or without PD98059, an inhibitor of ERK, and then treated with 500 μM morin for an additional 6 h. The cells were lysed and then equal amounts of cell lysates were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to 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. The relative ratios of expression in the western blotting results are shown at the bottom of each of the results as relative values to actin expression.

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_2O_2, cell viability was significantly recovered by inhibiting H_2O_2-induced ROS generation, compared with exposure to H_2O_2 alone. Our data also showed that H_2O_2 exposure increased tail length and the expression of p-γH2AX, whereas each event was mitigated in C2C12 cells that had been treated with morin prior to H_2O_2 exposure. As a result, these findings suggest that morin may be useful for the prevention of H_2O_2-induced cytotoxicity due to its strong antioxidant 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_2O_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. Howevere, 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_2O_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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