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The cytoprotective effects of ethanol extract of *Ecklonia cava* against oxidative stress are associated with upregulation of Nrf2-mediated HO-1 and NQO-1 expression through activation of the MAPK pathway

Yung Hyun Choi[1,2]

[1] *Department of Biochemistry, Dongeui University College of Korean Medicine, 52-57, Yangjeong-ro, Busanjin, Busan 614-052, Republic of Korea*
[2] *Anti-Aging Research Center and Blue-Bio Industry RIC, Dongeui University, 176 Eomgwangno Busanjin-gu, Busan 614-714, Republic of Korea*

Abstract. The aim of the present study was to examine the cytoprotective effect of *Ecklonia cava* against oxidative stress in C2C12 myoblasts. The ethanol extract of *E. cava* (EEEC) prevented hydrogen peroxide (H_2O_2)-induced inhibition of the growth of C2C12 myoblasts and exhibited scavenging activity against intracellular reactive oxygen species (ROS) induced by H_2O_2. EEEC treatment attenuated H_2O_2-induced comet tail formation and phospho-histone γH2A.X expression. Furthermore, EEEC treatment enhanced the level of the phosphorylated form of nuclear factor erythroid 2-related factor 2 (Nrf2) and its nuclear translocation, which was associated with the induction of heme oxygenase-1 (HO-1) and NADPH-quinone oxidoreductase 1 (NQO-1). Zinc protoporphyrin IX, a HO-1 competitive inhibitor, significantly abolished the protective effects of EEEC against H_2O_2-induced ROS generation and growth inhibition in C2C12 myoblasts. Transient transfection with Nrf2-specific small interfering RNA restored the elevated HO-1 and NQO-1 expression and the phosphorylation of Nrf2 to near normal levels. The EEEC treatment also induced the activation of mitogen-activated protein kinases (MAPKs), and specific inhibitors of MAPKs abolished upregulated HO-1 and NQO-1, as well as the phosphorylation of Nrf2. Taken together, these data suggest that EEEC attenuates oxidative stress by activating Nrf2-mediated HO-1 and inducing NQO-1 *via* the activation of MAPK signaling pathways.

Key words: *Ecklonia cava* — ROS — Nrf2 — MAPKs

Introduction

A balance of intracellular oxidation and reduction is essential for maintaining cellular functions. Reactive oxygen species (ROS), which are a natural byproduct of the normal metabolism of oxygen, are chemically reactive molecules containing oxygen. Under physiological conditions, ROS have important roles in cell signaling and homeostasis. However, excessive production of ROS, known as oxidative stress, causes destructive and irreversible damage to cellular components, such as proteins, lipids, nucleic acids, 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 oxidative stress.

The nuclear factor erythroid 2-related factor 2 (Nrf2) acts as a master cellular sensor, regulating the cellular adaptive response to oxidative stress through the cis-regulating antioxidant response element (ARE). In the absence of stimuli, Nrf2 is primarily sequestered in the cytoplasm and is controlled mainly by an inhibitory partner called Kelch-like ECH-associated protein 1 (Keap-1) through ubiquitination and proteasomal degradation (Venugopal and Jaiswal 1996; Zhang and Gordon 2004). Oxidation of redox-sensitive cysteines in Keap-1 releases Nrf2, and the unbound Nrf2

Correspondence to: Yung Hyun Choi, Department of Biochemistry, Dongeui University College of Korean Medicine, 52-57, Yangjeong-ro, Busanjin, Busan 614-052, Republic of Korea
E-mail: choiyh@deu.ac.kr

then translocates from the cytosol to the nucleus. In the nucleus, Nrf2 increases gene expression by binding to the ARE of phase II metabolizing/detoxifying genes, such as heme oxygenase-1 (HO-1) and NADPH-quinone oxidoreductase 1 (NQO-1) (Venugopal and Jaiswal 1996; Kweon et al. 2006; Surh et al. 2008). Nrf2 is considered a key molecular target of antioxidant enzyme inducers to convert highly ROS to less reactive and less damaging forms, which is the primary mechanism used to defend against oxidative stress.

Several epidemiological studies have confirmed that an intake of marine algae is linked with a reduced risk of various chronic diseases (Gomez-Gutierrez et al. 2011; Vo et al. 2012; Lee et al. 2013). The positive properties of marine algae products have been partly ascribed to the components that possess antioxidant properties (Thomas and Kim 2011; Balboa et al. 2013; Park and Pezzuto 2013). *Ecklonia cava* is an edible marine brown alga belonging to the *Laminariaceae* family of the order Laminariales. It is abundant in the subtidal regions of Jeju Island, Korea and is popular in Asian countries, such as Korea and Japan, as a food ingredient. *E. cava* is also used in foodstuffs and as a herbal remedy. Recently, the potential health benefits of extracts and components of *E. cava* have been extensively studied, including their antioxidant (Lee et al. 2012; Kim et al. 2014a, 2014b), antiviral and antibacterial (Choi et al. 2010; Ryu et al. 2011; Kwon et al. 2013), anti-inflammatory (Jung et al. 2009; Kim and Bae 2010), antidiabetic (Kang et al. 2010; Yokogawa et al. 2011), antiallergic (Kim et al. 2008; Shim et al. 2009), and anticancer (Kong et al. 2009; Ahn et al. 2015) properties. Although there are many reports on the antioxidant activities of *E. cava*, only a few studies have focused on the Nrf2/HO-1 signaling pathway (Kang et al. 2007; Kim et al. 2010; Jun et al. 2014), and the molecular mechanisms remain poorly understood. Thus, in the present study, we investigated the protective effect of ethanol extract of *E. cava* (EEEC) on cell damage induced by oxidative stress and its mechanism of action in mouse-derived C2C12 myoblasts.

Materials and Methods

Cell culture and treatment with EEEC

C2C12 myoblasts obtained from the American Type Culture Collection (Manassa, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc., Daegu, Republic of Korea) containing 10% heat-inactivated fetal bovine serum and 100 μg/ml of penicillin/streptomycin antibiotics (WelGENE Inc.) in a humidified 5% CO_2 atmosphere at 37°C. For the preparation of EEEC, *E. cava* was collected along coast of Jeju Island, Korea between February and May 2012. The samples were washed three times in tap water to remove any attached salt, epiphytes, and sand, and then rinsed carefully with fresh distilled water and chopped before storage at –20°C. The frozen samples were lyophilized and homogenized using a grinder prior to extraction. The dried powder was extracted with 80% methanol (EEEC) and evaporated *in vacuum*. The PBME was dissolved with a dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical Co., St Paul, MN, USA) stock solution at a concentration of 50 mg/ml and then diluted with medium to the desired concentration prior to use. In order to measure the degree to which a single EEEC treatment affected the cell viability of C2C12, different concentrations of EEEC (25~300 μg/ml) were treated for 24 h, and then an MTT assay was conducted. Additional cell cultivation occurred for 6 h in media where H_2O_2 and zinc protoporphyrin IX (ZnPP, Sigma-Aldrich) were simultaneously treated, singularly treated, or were not treated. Then an MMT assay was carried out. In order to examine the effects of EEEC on DNA damage and on the decrease of cell viability due to H_2O_2 treatment, EEEC (200 μg/ml) was treated for one hour. In order to examine the effects of EEEC on the generation of ROS according to H_2O_2 treatment, EEEC (200 μg/ml) was treated for one hour. Then in media where H_2O_2 and ZnPP were simultaneously treated, singularly treated, or were not treated, additional cell cultivation occurred for six hours, and then the amount of ROS generation was compared.

Cell viability assay

The effect of EEEC on the viability of the C2C12 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) assay, which depends on the conversion of yellow tetrazolium bromide to its purple formazan derivative *via* mitochondrial succinate dehydrogenase in viable cells. For the MTT assay, MTT working solution was then added to the culture plates and incubated continuously at 37°C. Three hours later, the plates were centrifuged for 5 min at 800 × *g*, and the supernatants were aspirated. The formazan crystals in each well were dissolved in DMSO, and the absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR-7000; Dynatech Laboratories, Chantilly, VA, USA) at a wavelength of 540 nm. Relative cell cytotoxicity was evaluated according to the quantity of MTT converted to insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The data were expressed as the mean percentage of the viable cells *versus* the respective control.

Comet assay

The cell suspension was mixed with 0.5% melting point agarose (LMPA) at 37°C, and the mixture was spread on

a fully frosted microscopic slide that was precoated with 1% normal melting point agarose. After the solidification of the agarose, the slide was covered with 0.5% LMPA and subsequently 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 buffer containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) for 20 min to allow DNA unwinding and to measure the alkali labile damage. An electrical field was applied (300 mA, 25 V) for 20 min at 4°C to draw 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 of propidium iodide (Sigma-Aldrich). The slides were examined under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany), and the resulting images were analyzed.

Protein extraction, electrophoresis, and Western blot analysis

The harvested cells were lysed on ice for 30 min in lysis buffer (20 mM sucrose, 1 mM EDTA, 20 μM Tris-HCl, pH 7.2, 1 mM dithiothreitol, 10 mM KCl, 1.5 mM $MgCl_2$, and 5 μg/ml aprotinin) and centrifuged at 13,000 × *g* for 15 min. Supernatants were collected from the lysates. 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. The protein extracts were quantified using a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA, USA). For Western blotting, aliquots of the lysates were separated by SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA), which were then incubated overnight at 4°C with desired primary antibodies. The membranes were further incubated with enzyme-linked secondary antibodies (Amersham Co., Arlington Heights, IL, USA) for 1 h at room temperature and the specific proteins were detected using an enhanced chemiluminescence (ECL) detection system (Amersham Co.). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Danvers, MA, USA).

Measurement of intracellular ROS

Intracellular accumulation of ROS was determined using fluorescent probes 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes, Eugene, OR, USA). For the detection of ROS, the cells were incubated with 10 μM of H2DCFDA for 20 min at room temperature in the dark. The ROS production in the cells was monitored with flow cytometer (Becton Dickinson, San Jose, CA, USA) using Cell-Quest pro software (Song et al. 2014).

Small interfering RNA (siRNA) transfection

Nrf2 siRNA and control siRNA were purchased from Santa Cruz Biotechnology. The siRNA was transfected into cells according to the manufacturer's instruction using the Lipofectamine 2000 Transfection Reagent (Life Technologies, Carlsbad, CA, USA). For transfection, the cells were seeded in 6-well culture plates and incubated with 50 nM of control siRNA or Nrf2 siRNA for 6 h in serum-free OPTI-MEM media. After incubation, the transfected cells were subjected to the treatment described in Jiang et al. (2014a).

Statistical analysis

All the data were expressed as the mean ± standard deviation (SD) of three determinations. Statistical comparison was performed *via* a one-way analysis of variance, followed by Duncan's' multiple-range test. A probability value of $p < 0.05$ was used as the criterion for statistical significance.

Results

EEEC provided protection against H_2O_2-induced C2C12 cell cytotoxicity

To examine the protective effect of EEEC on H_2O_2-induced cytotoxicity, the effect of EEEC on the viability of C2C12 cells was first determined using an MTT assay. The EEEC treatment did not result in any cytotoxic effects up to a concentration of 200 μg/ml, whereas cell viability was significantly decreased at a concentration of 300 μg/ml (Figure 1). Therefore, 200 μg/ml of EEEC was chosen as the optimal

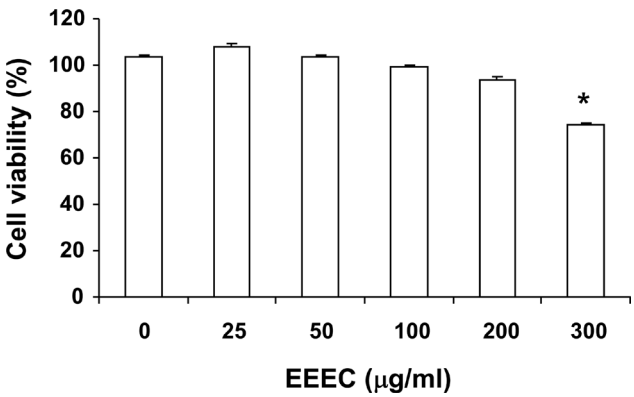

Figure 1. Effects of EEEC on the inhibition of the C2C12 cells growth. The cells were treated with various concentrations of EEEC for 24 h. The cell viability was assessed using an MTT reduction assay. The results were the mean ± SD values obtained in three independent experiments (* $p < 0.05$ compared with the control group).

dose for studying the cytoprotective effect of EEEC against H_2O_2-induced cell damage. Cell viability after the treatment with 1 mM H_2O_2 alone was reduced by approximately 60% at 6 h, whereas the H_2O_2-induced reduction of cell viability was significantly protected by pretreatment with EEEC (200 μg/ml; Figure 2A).

EEEC prevented H_2O_2-induced DNA damage and ROS generation in the C2C12 cells

We next examined the effects of EEEC on H_2O_2-mediated damage to C2C12 cell DNA using a comet assay and Western blotting analysis. As shown in Figure 2B, exposure of cells to H_2O_2 alone increased the number of DNA breaks, enhancing the fluorescence intensity in the tails of the comet-like structures. However, pretreatment with EEEC markedly reduced this adverse effect. Treating the C2C12 cells with H_2O_2 upregulated the level of the phosphorylated histone variant H2A.X at serine 139 (p-γH2A.X), a sensitive marker of DNA double-strand breaks (Rogakou et al. 1988) (Figure 2C). However, pretreatment with EEEC significantly decreased p-γH2A.X expression. The levels of ROS significantly increased in the H_2O_2-treated cells compared to those of the untreated cells. However, these were significantly inhibited in

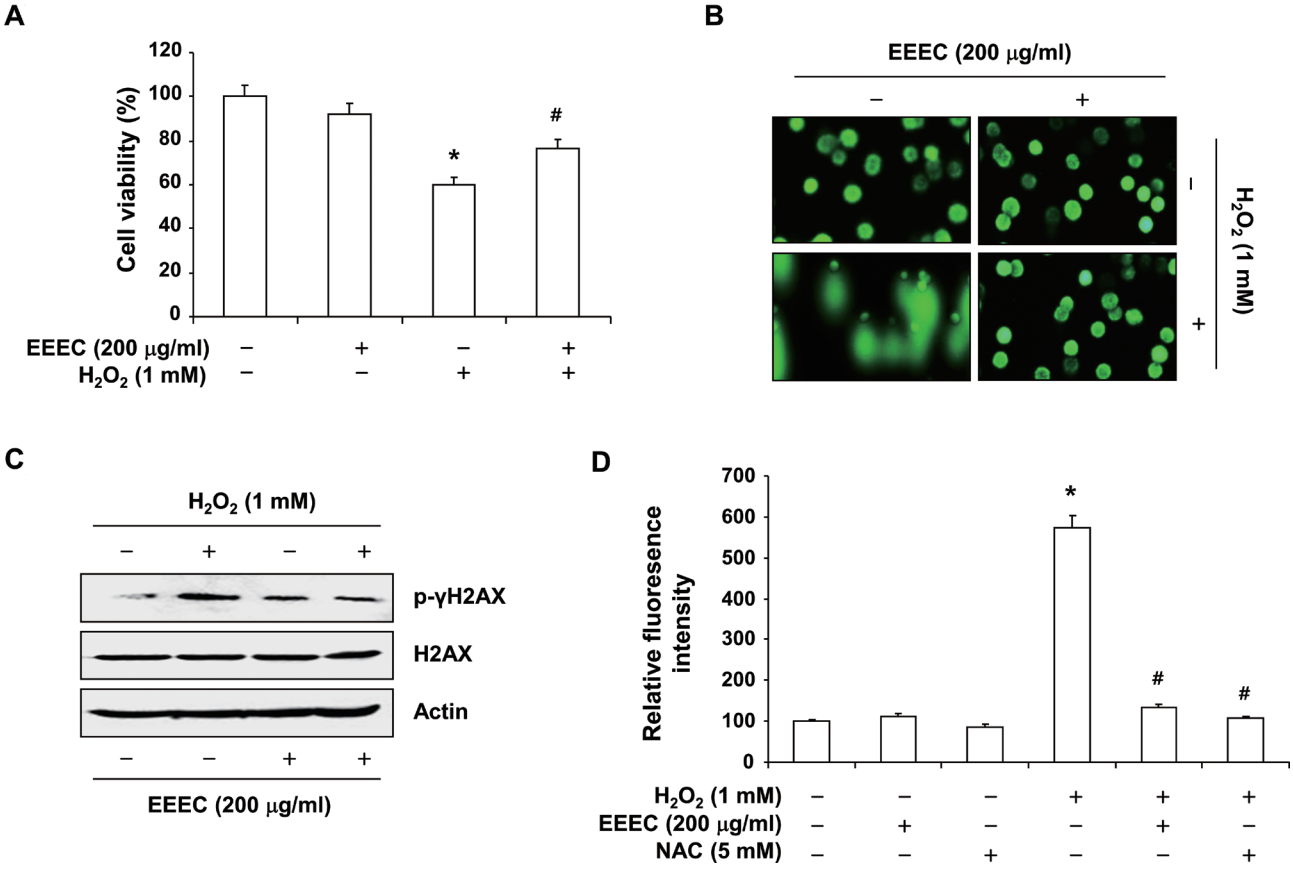

Figure 2. EEEC-induced protection against H_2O_2-induced ROS generation and DNA damage in the C2C12 cells. The C2C12 cells were pretreated with 200 μg/ml of EEEC for 1 h. Then cells were stimulated with or without 1 mM H_2O_2 in the presence of EEEC for further 6 h. **A.** Cell viability was assessed using an MTT reduction assay. The results were the mean ± SD values obtained in three independent experiments. **B.** To detect cellular DNA damage, a comet assay was performed, and representative pictures of the comets were taken using a fluorescence microscope (200× original magnification). **C.** The cells were lysed, and equal amounts of cell lysates were then separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with specific antibodies against phospho-γH2A.X, γH2A.X, and actin as an internal control, and the proteins were visualized using an ECL detection system. **D.** In order to investigate the effects of EEEC on the generation of ROS increased by H_2O_2 treatment, EEC or NAC was treated for 1 h in C2C12 cells, and additional cultivation occurred for 6 h in media that were either treated or not treated with H_2O_2. To monitor ROS production, the cells were incubated at 37°C in the dark for 20 min with new culture medium containing 10 μM H2DCFDA. ROS generation was measured using a flow cytometer. The 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).

the presence of EEEC, as well as those of the ROS scavenger N-acetyl-L-cysteine (NAC) (Figure 2D).

EEEC induced the phosphorylation and nuclear accumulation of Nrf2 and the expression of HO-1 and NQO-1 in the C2C12 cells

As Nrf2 is an important transcription factor that regulates ARE-driven phase II antioxidant enzymes, we investigated whether EEEC upregulated phase II antioxidant enzymes by activating Nrf2. As shown in Figure 3A, the treatment of the C2C12 cells with EEEC induced the expression of HO-1 and the NQO-1 protein in a time-dependent manner, which was associated with increased expression of Nrf2, as well as its phosphorylation. We also examined the phosphorylation and subcellular localization of Nrf2 following EEEC treatment to confirm the Nrf2-activating property of EEEC. As illustrated in Figure 3B, Western blot analyses of the cytosolic and nuclear fractions demonstrated that the EEEC treatment restored Nrf2 protein expression, resulting in the translocation of phosphorylated Nrf2 from the cytosol to the nucleus.

The Nrf2/HO-1 pathway was involved in the protective effects of EEEC in the H_2O_2 -treated C2C12 cells

To determine whether the EEEC-induced antioxidant and cytoprotective activities against oxidative stress were mediated through the activation of the Nrf2/HO-1 pathway, the C2C12 cells were preincubated with or without a specific inhibitor of HO-1, ZnPP, and the levels of ROS and cell viability were assessed. As shown in Figure 4, ZnPP abrogated the protective effect of EEEC on the H_2O_2-induced production of ROS and the reduction of cell viability.

EEEC up-regulated HO-1 and NQO-1 expression via Nrf2 activation in the C2C12 cells

We next developed an Nrf2 gene knockdown model using siRNA transfection to demonstrate the importance of Nrf2 activation. The results indicated that silencing Nrf2 by specific siRNA abolished EEEC-induced expression and phosphorylation of Nrf2, as well as the upregulation of HO-1 and NQO-1 (Figure 5A). To further confirm the involvement of Nrf2, the protective effect of EEEC against the H_2O_2-induced reduction of cell viability was measured in Nrf2 knock-down cells. As indicated in Figure 5B, siNrf2 transfection abrogated the cytoprotective effect of EEEC compared to the control siRNA-transfected cells.

The MAPK signaling pathway was involved in the Nrf2-mediated induction of HO-1 and NQO-1 by EEEC in the C2C12 cells

Furthermore, the potential involvement of MAPKS in upstream signaling events involved in EEEC-mediated Nrf2

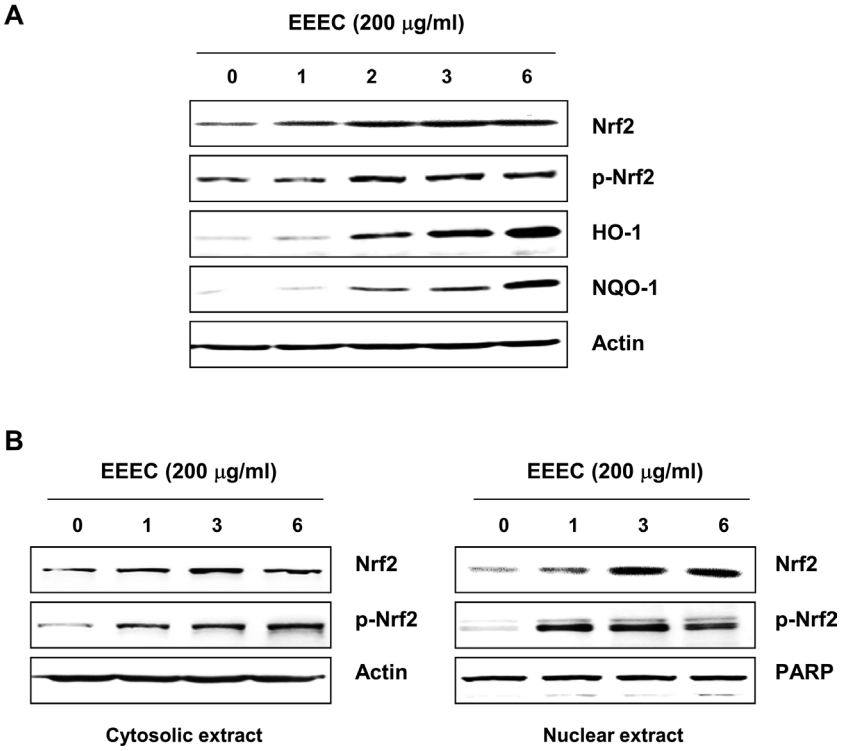

Figure 3. Effects of EEEC on the expression of Nrf2, HO-1, and NQO-1 in the C2C12 cells. The cells were incubated with 200 μg/ml of EEEC for the indicated periods. Total cellular (**A**) and nuclear or cytosolic proteins (**B**) were separated on SDS-polyacrylamide gels and then transferred onto nitrocellulose 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 and poly(ADP-ribose) polymerase (PARP) were used as the internal controls of total cellular and nuclear proteins, respectively.

activation was examined. As shown in Figure 6A, although the total levels of three MAPKs, extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK, did not show notable changes, the EEC treatment increased their phosphorylated levels within 2 h. However, treatment with specific pharmacological inhibitors of ERK (PD98059), JNK (SP600125), and p38 MAPK (SB203580) markedly reduced the phosphorylation of Nrf2, with a resulting drop in the induction of HO-1 and NQO-1, suggesting that EEEC-induced activation of the Nrf2/HO-1 pathway may be required in the MAPK cascade.

Discussion

In the present study, we investigated the protective effect of EEEC against H_2O_2-induced oxidative stress and its underlying mechanism in cultured mouse C2C12 cells. Our data demonstrated that EEEC provided protection against

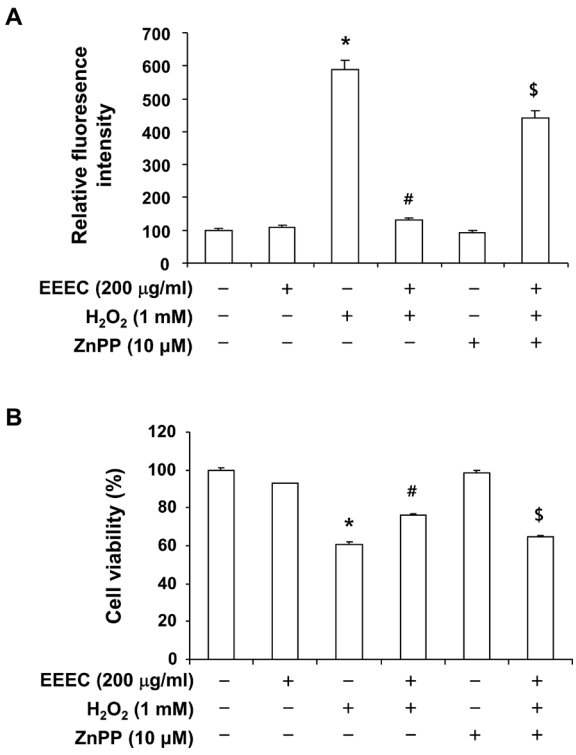

Figure 4. Effects of an HO-1 inhibitor on EEEC-mediated attenuation of ROS formation and growth inhibition by H_2O_2 in the C2C12 cells. The cells were pretreated for 1 h with 200 μg/ml of EEEC and then stimulated with or without 1 mM H_2O_2 for further 6 h in the absence or presence of 10 μM of ZnPP. ROS generation (**A**) and cell viability (**B**) were then estimated. 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 EEEC-treated group).

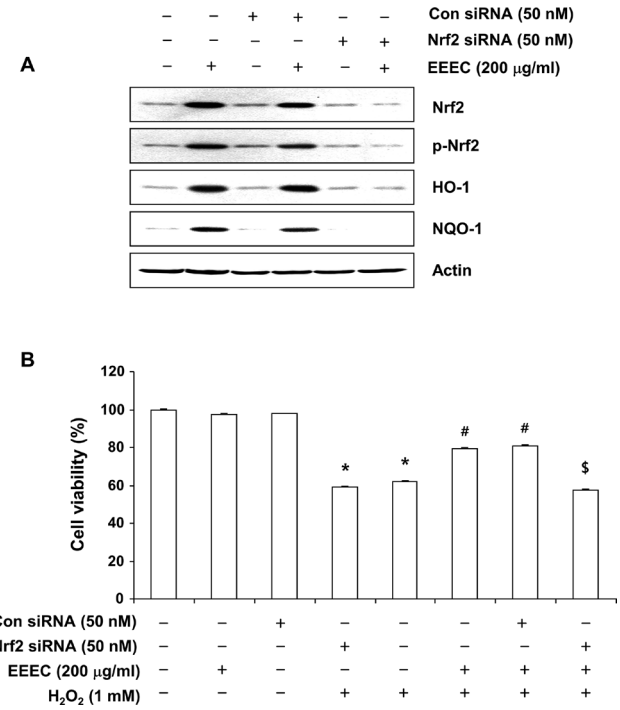

Figure 5. Nrf2-mediated induction of HO-1 and NQO-1 expression by EEEC in the C2C12 cells. The cells were transfected with control (Con siRNA, as a negative control for RNA interference) and Nrf2 siRNA. After 24 h, the cells were treated with 200 μg/ml of EEEC for 6 h (**A**) or pretreated with 200 μg/ml of EEEC for 1 h and then stimulated with or without 1 mM H_2O_2 for further 6 h (**B**). A. Cellular proteins were separated on SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were probed with specific antibodies against Nrf2, p-Nrf2, HO-1, and NQO-1. Proteins were visualized using an ECL detection system. Actin was used as a loading control. (B) Cell viability was estimated with an MTT assay. The results were the mean ± SD values obtained in three independent experiments (* $p < 0.05$ compared with a control group; # $p < 0.05$ compared with the H_2O_2-treated group; $ $p < 0.05$ compared with the H_2O_2- and EEEC-treated group).

H_2O_2-mediated cell toxicity and DNA damage through inhibition of intracellular ROS generation. Moreover, our results indicated that the mechanism by which EEEC-induced cytoprotection could include the Nrf2/ARE and MAPK pathways.

Oxidative stress is caused by an imbalance between the production of ROS and the ability of the antioxidative defense system to readily detoxify the reactive intermediates or easily repair the resulting damage (Chen and Keaney 2004; Chapple et al. 2012). The present study demonstrated that C2C12 cells treated with H_2O_2 showed a marked loss of viability and that pretreatment with EEEC significantly decreased the loss of cell viability (Figure 2A). We also observed much lower levels of H_2O_2-induced intracellular

accumulation of ROS in the EEEC-treated cells (Figure 2D). Moreover, our data indicated that the H_2O_2 treatment increased tail length (DNA migration) and the expression of p-γH2A.X, both of which are widely used markers for the detection of DNA damage (Rogakou et al. 1988). The treatment with EEEC prior to H_2O_2 exposure mitigated each of these events in the C2C12 cells (Figure 2B and C). These findings support the idea that EEEC protects C2C12 cells from H_2O_2-induced cell damage *via* its antioxidative properties.

Recent studies have characterized the Nrf2/ARE pathway as an important endogenous mechanism, which contributes significantly to cellular protection against redox cycling and oxidative stress (Venugopal and Jaiswal 1996; Zhang and Gordon 2004). Various natural products and compounds have been identified as activators of the Nrf2/ARE pathway. Under normal conditions, Nrf2 is anchored in the cytoplasm by binding to Keap1, which facilitates the ubiquitination and subsequent proteolysis of Nrf2. Upon stimulation by inducers, Nrf2 dissociates from Keap1, leading to phosphorylation of Nrf2. The latter is a critical process in the nuclear translocation of Nrf2. In the nucleus, Nrf2 dimerizes with specific cofactors and binds to ARE in a regulatory sequence involving the coordinated transcriptional activation of genes, which code for a number of antioxidant enzymes, including HO-1 and NQO-1 (Venugopal and Jaiswal 1996; Kweon et al. 2006; Surh et al. 2008). HO-1 is an enzyme that catalyzes the degradation of heme, resulting in the formation of the antioxidant bilirubin when biliverdin reductase is present (Mancuso and Barone 2009; Wegiel et al. 2014). NQO-1 is a cytosolic flavoprotein, which facilitates the detoxification and excretion of endogenous and exogenous chemicals through a reduction reaction from quinones to hydroquinones, limiting the subsequent generation of ROS (Baulig et al. 2003; Piao et al. 2011). As shown in our results, EEEC effectively induced and phosphorylated Nrf2. Consequently, the expression of HO-1 and NQO-1 was induced in a time-dependent manner. We also found that EEEC promoted the nuclear translocation of phosphorylated Nrf2 at a very early stage (Figure 3). However, pretreatment with ZnPP, an inhibitor of HO-1, markedly abrogated the protective effects of EEEC against H_2O_2-induced ROS generation and inhibition of the growth of the C2C12 cells (Figure 4). In addition, contrary to the control siRNA-treated cells, Nrf2 siRNA successfully blocked Nrf2 protein expression and phosphorylation, and EEEC-induced HO-1 and NQO-1 expression (Figure 5A). Again, silencing of Nrf2 abolished the EEEC-induced restoration of H_2O_2-mediated growth inhibition of the C2C12 cells (Figure 5B), suggesting that Nrf2 is a critical upstream regulator of the EEEC-mediated induction of HO-1 and NQO-1 in C2C12 cells.

Many previous reports have implicated diverse protein kinases in the signals that trigger Nrf2-Keap1 dissociation and phosphorylation of Nrf2, as well as antioxidant-induced activation of the Nrf2/ARE signaling pathway (Kweon et al. 2006; Surh et al. 2008). Among several kinase cascades, we focused on the MAPK signaling pathway because it has a key role in regulating many important proteins involved in cell survival and death (Kong et al. 2001; Yang et al. 2013). In the present study, EEEC time-dependently increased the phosphorylation of ERK and p38 MAPK. The EEEC treatment also markedly activated JNK in a time-dependent manner, with levels declining after 3 h of exposure (Figure 6A). To identify which signal cascade controlled the EEEC-induced activation of Nrf2, we further examined the effects of specific inhibitors of MAPKs on the phosphorylation of Nrf2 as well as on the increased levels of HO-1 and NQO-1. The results demonstrated that each specific inhibitor of three MAPKs

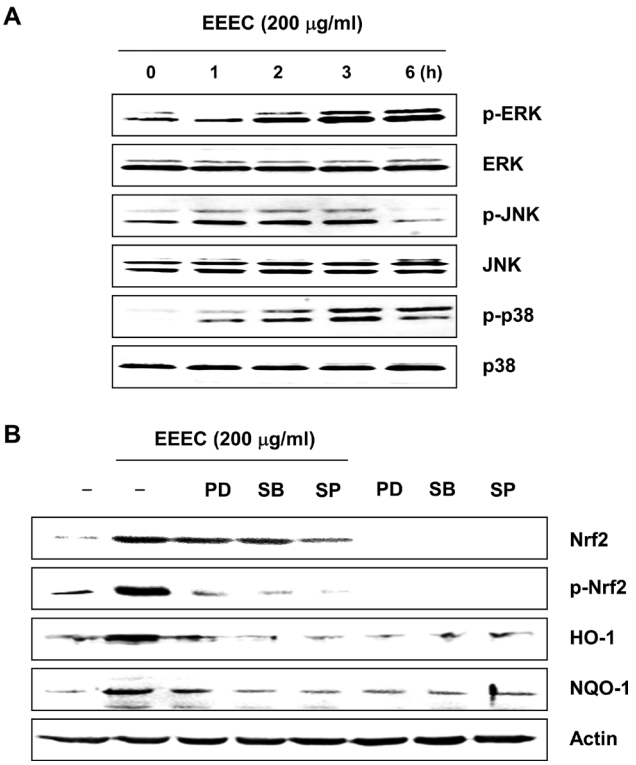

Figure 6. Involvement of the MAPK signaling pathway in Nrf2-medicated HO-1 induction by EEEC in the C2C12 cells. The cells were treated with 200 μg/ml of EEEC for the indicated times (**A**) or pretreated for 1 h with or without the indicated inhibitors [PD (PD98059), a specific ERK inhibitor; SB (SB203580), a specific p38 MAPK inhibitor; SP (SP600125), a specific JNK inhibitor, SP600125] and then stimulated with or without 200 μg/ml of EEEC for an additional 6 h (**B**). The cells were lysed, and equal amounts of cell lysates were then separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized with an ECL detection system. Actin was used as an internal control.

suppressed the EEEC-induced HO-1 and NQO-1 expression and Nrf2 phosphorylation (Figure 6B). These observations suggest that EEEC enhanced the phase II antioxidant enzyme system and inhibited cell damage induced by H_2O_2 through activation of the MAPK signaling pathway.

Taken together, the results of the present study provide strong evidence that the EEEC treatment attenuated H_2O_2-induced oxidative stress in C2C12 cells by preventing ROS generation. As shown in our results, the phosphorylation and nuclear localization of Nrf2 and the activation of MAPK signaling by following EEEC-induced activation of HO-1 and NQO-1 are critical for the protection against H_2O_2-induced oxidative stress. Although further research and clinical trials are needed to further elucidate the molecular mechanisms detected herein, these findings suggest that EEEC may be a desirable food supplement to treat a variety of oxidative stress conditions.

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

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