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Original Article

Ethanol extract of *Prunus mume* fruit attenuates hydrogen peroxide-induced oxidative stress and apoptosis involving Nrf2/HO-1 activation in C2C12 myoblasts

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ABSTRACT

The fruit of the *Prunus mume* (Siebold) Siebold & Zucc., Rosaceae (Korean name: Maesil) has long been used as a health food or valuable medicinal material in traditional herb medicine in Southeast Asian countries. In this study, we determined the potential therapeutic efficacy of the ethanol extract of *P. mume* fruits (EEPM) against H_2O_2-induced oxidative stress and apoptosis in the murine skeletal muscle myoblast cell line C2C12, and sought to understand the associated molecular mechanisms. The results indicated that exposure of C2C12 cells to H_2O_2 caused a reduction in cell viability by increasing the generation of intracellular reactive oxygen species and by disrupting mitochondrial membrane permeability, leading to DNA damage and apoptosis. However, pretreatment of the cells with EEPM before H_2O_2 exposure effectively attenuated these changes, suggesting that EEPM prevented H_2O_2-induced mitochondria-dependent apoptosis. Furthermore, the increased ex-pression and phosphorylation of nuclear factor erythroid 2-related factor 2 (Nrf2) and up-regulation of heme oxygenase-1 (HO-1), a phase II antioxidant enzyme, were detected in EEPM-treated C2C12 cells. We also found that zinc protoporphyrin IX, an HO-1 inhibitor, attenuated the protective effects of EEPM against H_2O_2-induced reactive oxygen species accumulation and cytotoxicity. Therefore, these results indicate that the activation of the Nrf2/HO-1 pathway might be involved in the protection of EEPM against H_2O_2-induced cellular oxidative damage. In conclusion, these results show that EEPM contributes to the prevention of oxidative damage and could be used as a nutritional agent for oxidative stress-related diseases.

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Introduction

Oxidative stress is implicated in numerous diseases caused by the overproduction of reactive oxygen species (ROS). ROS, derivatives of cellular metabolic reactions, modulate the fundamental physiological functions of aerobic life. Excess amounts of ROS damage various cellular molecules, such as proteins, lipids, nucleic acids, and other macromolecular substances, resulting in cellular dysfunction and apoptosis (Wang et al., 2013; Wu et al., 2010). Therefore, supplementation with antioxidants, including synthetic and natural antioxidants, could reduce oxidative stress and ameliorate oxidative stress-related diseases through induction of phase II antioxidant enzymes as well as superoxide dismutase (SOD) and catalase (Wojcik et al., 2010; Guerra-Araiza et al., 2013).

Among phase II antioxidant enzymes, which are regulated by antioxidant responsive elements (AREs) at the transcription level,

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heme oxygenase-1 (HO-1) is a rate-limiting enzyme involved in the conversion of heme to biliverdin and carbon monoxide, among other cellular reactions (Martin et al., 2004; Chen et al., 2003). Bilirubin functions as a potent antioxidant and carbon monoxide has been reported to mediate the anti-apoptotic effects of HO-1 in response to inflammatory cytokine stimulation (Surh et al., 2008; Son et al., 2013). Nuclear factor erythroid 2-related factor 2 (Nrf2), a leucine zipper redox-sensitive transcription factor, is a key regulator of antioxidant and detoxification gene expression. Under normal conditions, Nrf2 binds to kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and is later subjected to proteasomal degradation. However, Nrf2 translocates from the cytoplasm to the nucleus following a variety of stimuli, and subsequently binds to ARE present within the promoter region of genes encoding for phase II enzymes, resulting in the up-regulation of their transcription (Terazawa et al., 2013; Niture et al., 2014). Moreover, recent evidence indicates that Nrf2 promotes cell survival by preventing an increase in ROS in various conditions of oxidative stress (Hybertson et al., 2011; Niture et al., 2014). Therefore, the Nrf2-ARE pathway is currently the most important endogenous antioxidant signaling pathway.

Prunus mume (Siebold) Siebold & Zucc. (Korean name: Maesil) is a deciduous tree of the Family Rosaceae, which is now widely cultivated in Southeast Asia countries, including Korea, China, and Japan (Matsuda et al., 2003; Wen and Shi, 2012). The various parts of this plant have been used as health foods or medicinal materials in traditional medicine for generations (Wen and Shi, 2012; Yan et al., 2014). In particular, its fruit has been eaten since ancient times in Asian countries as a traditional herbal medicine for relief of fatigue, diarrhea, fever, dyspepsia, and intestinal and skin disorders for thousands of years (Yan et al., 2014; Wen and Shi, 2012; Zhang et al., 2011; Jeong et al., 2006). The fruit of the *P. mume* contains abundant phenolic compounds, such as phenolic acids and flavonoids (Jeong et al., 2006; Kita et al., 2007; Mitani et al., 2013), which may be involved in the biological effects of anti-viral, anti-inflammatory, immunoenhancing, and antineoplastic activities (Zhang et al., 2011; Yingsakmongkon et al., 2008; Park et al., 2011; Enomoto et al., 2010; Tsuji et al., 2011; Jung et al., 2010; Tada et al., 2012; Lee et al., 2013; Jeong et al., 2006). Although there are several reports on the antioxidant activity and free radical scavenging activities of *P. mume* (Yan et al., 2014; Sang et al., 2002; Lee et al., 2013), the exact molecular mechanism(s) of actions of *P. mume* extract against oxidative stress involved in the Nrf2/HO-1 signaling pathway are yet to be described. Therefore, in the present study, we examined the ability of the ethanol extract of *P. mume* fruits (EEPM) to protect cells from hydrogen peroxide (H_2O_2)-induced cell damage and elucidated the mechanism underlying the protective action in a mouse-derived C2C12 myoblast model.

Materials and methods

Preparation of EEPM

Dried fruits of *Prunus mume* (Siebold) Siebold & Zucc., Rosaceae, were obtained from Institute of Hukyong Food (Busan, Republic of Korea), which were authenticated by Professor Su Hyun Hong, Department of Biochemistry, Dongeui University College of Korean Medicine (Busan, Republic of Korea). For the preparation of EEPM, freeze-dried fruits of *P. mume* were extracted with ethanol (100 g per 1 l) at 60 °C for three days using a blender. The extract was centrifuged at 10,000 × *g* for 20 min, and the supernatants were then collected and immediately filtered through a Whatman filter (pore size, 0.22 μm). The filtrate was lyophilized and stored at −70 °C. The yield (w/w) of the extract was ∼5.0%. The powder was dissolved to a 100 mg/ml concentration with dimethylsulfoxide (DMSO, Sigma–Aldrich Chemical Co., St. Paul, MN, USA). The voucher specimens (accession number DEU-36) have been deposited at a publicly available Natural Resource Bank of Dongeui University College of Oriental Medicine.

Cell culture and EEPM treatment

The C2C12 myoblast cell line was purchased from the American Type Culture Collection (Manassa, VA, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc., Daegu, Republic of Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS, WelGENE Inc.) and 100 μg/ml penicillin/streptomycin antibiotics (WelGENE Inc.) in a humidified 5% CO_2 atmosphere at 37 °C. The stock solution of EEPM was diluted with DMEM before every experiment.

Measurement of cell viability

To investigate the cytotoxicity, cells were seeded into 6-well plates and exposed to various concentrations of EEPM in the absence or presence of H_2O_2 and/or zinc protoporphyrin IX, a HO-1 inhibitor (ZnPP, Sigma–Aldrich) for the indicated times. After completion of the treatments, 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich) was added to each well continuously at 37 °C. After 3 h incubation, the culture supernatant was removed from the wells and the formazan complex was dissolved in DMSO. The absorbance of each well was detected at 540 nm with a microplate reader (Molecular Devices, Palo Alto, CA, USA). Cell viability is expressed as a percentage of untreated cells.

Measurement of intracellular ROS levels

The production of intracellular ROS was quantified using the oxidation-sensitive fluorescent probes 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes, Eugene, OR, USA). After treatment, the cells were harvested, suspended in phosphate-buffered saline (PBS), and then incubated with 10 μM H2DCFDA for 20 min at room temperature in the dark. The fluorescence intensity was measured by a flow cytometer (Becton Dickinson, San Jose, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm (Kim et al., 2014).

Measurement of mitochondrial membrane potential (MMP)

The mitochondrial transmembrane electrochemical gradient was measured using the mitochondrial potential sensor 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1, Sigma–Aldrich), a cell permeable, cationic, and lipophilic dye, which is internalized and concentrated by respiring mitochondria, reflecting changes in MMP in live cells. Briefly, cells were collected, resuspended in PBS, and then incubated with 10 μM JC-1 under dark conditions for 30 min at 37 °C. After the JC-1 was removed, the cells were washed with PBS to remove unbound dye. The cellular fluorescence intensity was quantified using a flow cytometry at an excitation wavelength of 480 nm and an emission wavelength of 530 nm (Seo et al., 2014).

DAPI nuclear staining

For the assessment of apoptosis, morphological changes of nuclei were visualized following DNA staining by t4,6-diamidino-2-phenyllindile (DAPI, Sigma–Aldrich), a fluorescent dye. In brief, the cells were fixed with 3.7% paraformaldehyde (Sigma–Aldrich) in PBS for 10 min at room temperature, and then stained with

2.5 μg/ml DAPI solution for 10 min at room temperature. The cells were washed twice with PBS, and stained nuclei were analyzed using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Determination of apoptotic cells by flow cytometry

To assess the induced cell apoptosis rate quantitatively, a fluorescein-conjugated annexin V (annexin V-FITC) staining assay was performed according to the manufacturer's protocol (BD Biosciences, San Jose, CA, USA). The cells were washed twice with ice-cold PBS and stained with annexin V-FITC and propidium iodide (PI) for 15 min at room temperature in the dark. The degree of apoptosis was quantified as a percentage of the annexin V-positive and PI-negative (annexin V^+/PI^- cells) cells by flow cytometry.

Comet assay (single-cell gel electrophoresis)

The degree of oxidative DNA damage was assessed in a comet assay. The cell suspension was mixed with 0.5% low melting agarose (LMA) at 37 °C, and the mixture was spread on a fully frosted microscopic slide precoated with 1% normal melting agarose (NMA). After the solidification of the agarose, the slide was covered with 0.5% LMA and then immersed in a lysis solution [2.5 M NaCl, 100 mM Na-ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 1% 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 then applied (300 mA, 25 V) for 20 min at 25 °C to draw the negatively charged DNA toward the anode. The slides were washed three times for 5 min at 25 °C in a neutralizing buffer (0.4 M Tris, pH 7.5), followed by staining with 20 μg/ml propidium iodide (PI, Sigma–Aldrich). The slides were examined under a fluorescence microscope.

Western blot analysis

The total cellular protein was extracted with lysis buffer (20 mM of sucrose, 1 mM of EDTA, 20 μM of Tris–HCl, pH 7.2, 1 mM of dithiothreitol, 10 mM of KCl, 1.5 mM of $MgCl_2$ and 5 μg/ml of aprotinin) for 30 min. The protein concentration was measured using a Bio-Rad protein assay (Bio-Rad Lab., Hercules, CA, USA) according to the manufacturer's instructions. Aliquots of each sample were loaded into dedicated wells of SDS-polyacrylamide gels, separated by electrophoresis, and then transferred to polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH, USA). After blocking nonspecific binding with 5% nonfat dry milk in TBST (Tris-buffered saline-Tween), the membranes were then probed with the desired primary antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Danvers, MA, USA), and incubated overnight at 4 °C. The membranes were washed with Tris-buffered saline-0.01% (v/v) containing Tween-20 at room temperature for 15 min and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Co., Arlington Heights, IL, USA) in TBST for 2 h at room temperature. Proteins were visualized by using an enhanced chemiluminescence (ECL, Amersham Co.) detection method followed by film exposure.

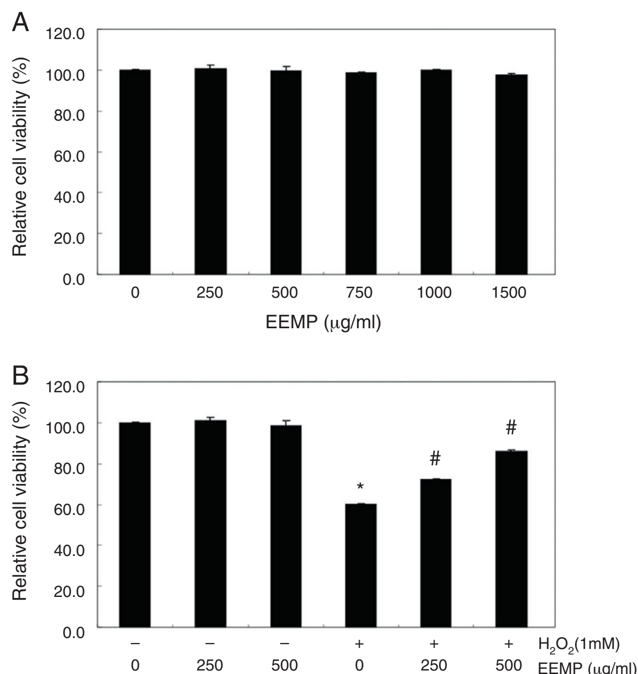

Fig. 1. Effects of EEPM on H_2O_2-induced growth inhibition in C2C12 cells. Cells were treated with various concentrations of EEPM for 24 h (A) or pretreated with 250 μg/ml and 500 μg/ml of EEPM for 1 h and then incubated with or without 1 mM H_2O_2 for 6 h (B). Cell viability was assessed using an MTT reduction assay. The results are the mean ± SD values obtained in three independent experiments (*$p < 0.05$ compared with control group; #$p < 0.05$ compared with H_2O_2-treated group).

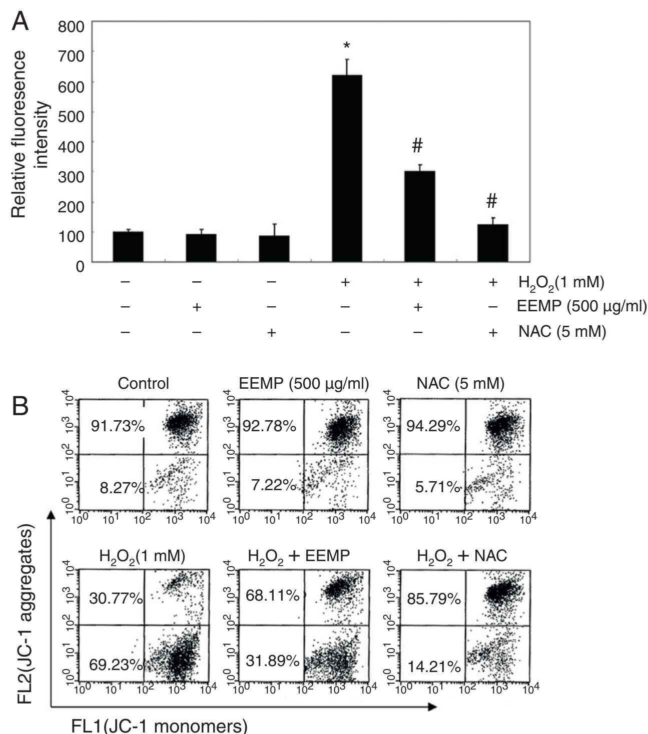

Fig. 2. Attenuation of H_2O_2-induced ROS generation and mitochondrial dysfunction by EEPM in C2C12 cells. C2C12 cells were pretreated with 500 μg/ml EEPM or 5 mM NAC for 1 h and then stimulated with or without 1 mM H_2O_2 for 6 h. (A) In order to monitor ROS production, the cells were incubated at 37 °C in the dark for 20 min with new culture medium containing 10 μM H2DCFDA. ROS generation was measured using a flow cytometer. The results are the mean ± SD values obtained in three independent experiments (*$p < 0.05$ compared with control group; #$p < 0.05$ compared with H_2O_2-treated group). (B) The MMPs of cells treated under the same conditions were evaluated using a flow cytometer. The data represent the average of two independent experiments.

Statistical analysis

All measurements were made in triplicate and all values are presented as mean ± standard deviation (SD). The results were subjected to an analysis of variance (ANOVA) using the Tukey test to analyze the difference. $p < 0.05$ was considered to be statistically significant.

Results

EEPM protects C2C12 cells against H_2O_2-induced cytotoxicity

To examine the protective effect of EEPM on H_2O_2-induced cytotoxicity, the effect of EEPM on the viability of C2C12 cells was first performed for primary dose selection. As shown in Fig. 1A, the EEPM treatment did not result in any cytotoxic effects up to the concentration of 1500 μg/ml. Therefore, 250 and 500 μg/ml EEPM was chosen as the optimal doses for studying the cytoprotective effect of EEPM against H_2O_2-induced cytotoxicity. In order to determine the effects of EEPM, the cells were pre-treated with the EEPM for 1 h prior to H_2O_2 administration. As estimated by an MTT assay, cell viability was markedly decreased, to approximately 60%, after a 6 h exposure to 1 mM H_2O_2. However, when cells were pre-incubated with EEPM, the reduction of cell viability was significantly attenuated in a dose-dependent manner (Fig. 2B).

EEPM decreases H_2O_2-induced ROS production in C2C12 cells

To investigate whether EEPM could prevent H_2O_2-induced ROS generation and the resulting oxidative stress, we next measured the ROS production in cells by using H2DCFDA reagent, a fluorescent dye that visualizes ROS. As shown in Fig. 2A, the intensity of the DCF-liberated fluorescent signal from the H_2O_2-treated cells was significantly increased, and the signal was markedly reduced in the presence of EEEC as well as the ROS scavenger N-acetyl-L-cysteine (NAC), which was used as a positive control.

EEPM attenuates H_2O_2-induced loss of MMP in C2C12 cells

Since MMP regulates mitochondrial permeability, which may be critical for inducing or arresting the oxidative stress-induced apoptotic pathway, we evaluated the effect of EEPM on the MMP of C2C12 cells using flow cytometry. After incubation with 1 mM H_2O_2 for 6 h, the MMP level was markedly reduced as compared to the untreated control (Fig. 2B), indicating mitochondrial damage and dysfunction. In contrast, pretreatment with EEPM or NAC effectively prevented the loss of MMP induced by H_2O_2. The data indicated that EEPM protected the cells against the H_2O_2-induced lowering of ROS generation by blocking mitochondrial dysfunction in C2C12 cells.

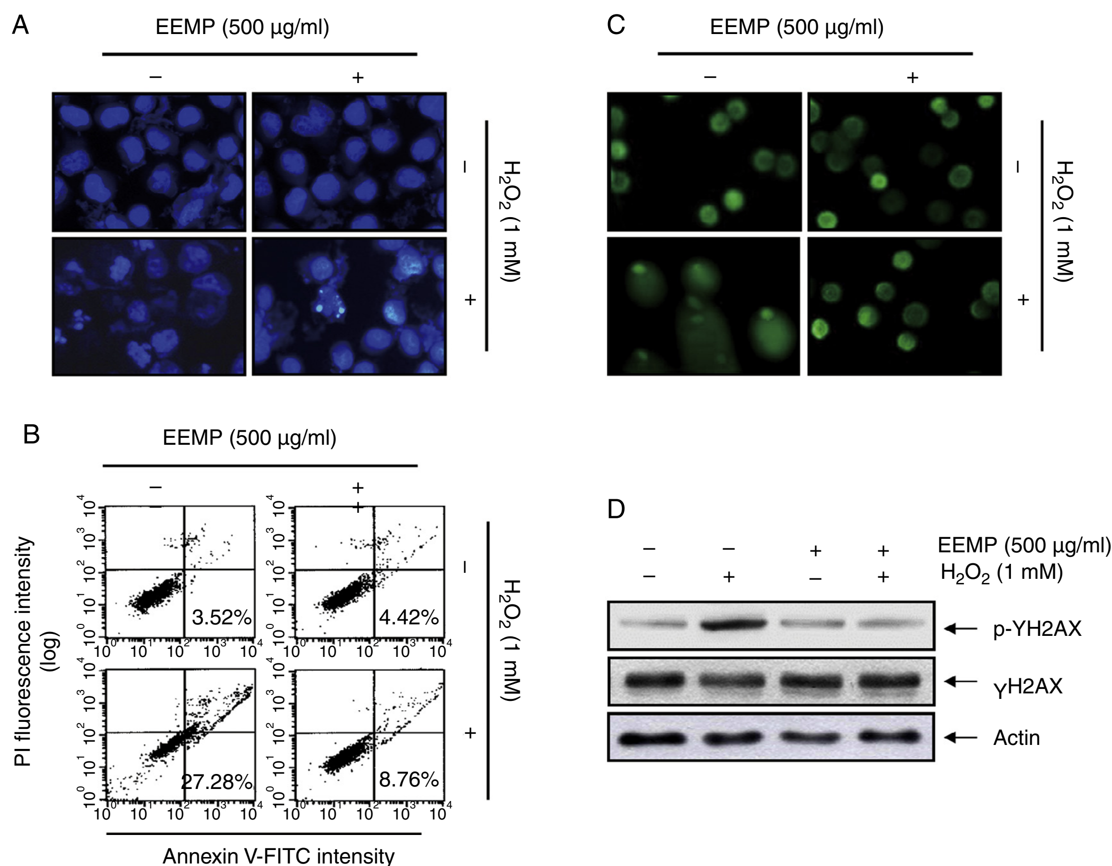

Fig. 3. Protection of H_2O_2-induced apoptosis and DNA damage by EEPM in C2C12 cells. C2C12 cells were pretreated with 500 μg/ml EEPM for 1 h and then incubated with or without 1 mM H_2O_2 for 6 h. (A) The cells were fixed and stained with DAPI solution. After 10 min incubation at room temperature, stained nuclei were observed using a fluorescent microscope (original magnification, 400×). (B) The cells were stained with FITC-conjugated Annexin-V and PI for flow cytometry analysis. The percentages of apoptotic cells were determined by counting the percentage of Annexin V-positive cells. Each point represents the mean of two independent experiments. (C) To detect cellular DNA damage, the comet assay was performed and representative pictures of the comets were taken using a fluorescence microscope (original magnification, 200×). (D) 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 specific antibodies against γH2A.X, p-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.

EEPM protects H_2O_2-induced C2C12 cell apoptosis

We also examined the ability of EEPM to protect against H_2O_2-triggered C2C12 cell apoptosis using the DAPI and Annexin/PI-staining assays. As illustrated in Fig. 3A, C2C12 cells exposed to 1 mM H_2O_2 alone demonstrated characteristic apoptotic features, including cell shrinkage and chromatin condensation and fragmentation in the nucleus detected by DAPI staining. However, the pre-treatment with EEPM on H_2O_2-treated C2C12 cells significantly reduced these morphological changes. In addition, the percentage of apoptotic cells treated with 1 mM H_2O_2 was approximately 24.60% by the flow cytometry assay (Fig. 3B); however, 500 μg/ml EEPM effectively decreased the cell apoptosis induced by H_2O_2 to 7.70%. These results show that preconditioning by EEPM protects against oxidative stress-induced C2C12 cell death.

EEPM prevents H_2O_2-induced DNA damage in C2C12 cells

We next examined the effects of EEPM on H_2O_2-mediated damage to C2C12 cell DNA using the comet assay and Western blotting analysis. As shown in Fig. 3C, exposure of cells to H_2O_2 alone increased the number of DNA breaks, resulting in an increase in fluorescence intensity in the tails of the comet-like structures; however, this adverse effect was markedly reduced by EEPM pretreatment. Our results also showed that treating C2C12 cells with H_2O_2 resulted in the up-regulation of the level of the phosphorylated histone variant H2A.X at serine 139 (p-γH2A.X), a sensitive marker for DNA double-strand breaks (Rogakou et al., 1998) (Fig. 2C); however, pretreatment with EEPM resulted in a significant decreased p-γH2A.X expression.

EEPM promotes the expression of Nrf2 and HO-1 in C2C12 cells

To examine whether or not Nrf2 activation is associated with EEPM-mediated cytoprotection, we monitored the levels of the HO-1 protein. As shown in Fig. 4, treatment of C2C12 cells with EEPM induced the expression of the HO-1 protein in a dose- and time-dependent manner, but that other antioxidant enzymes, NADPH-quinone oxidoreductase 1 (NQO1) and thioredoxin reductase 1 (TrxR1), were unaffected by EEPM treatment, which was associated with the induction of Nrf2. Since the phosphorylation of Nrf2 at Ser40 by several kinases is also a critical process in its stabilization and nuclear translocation (Surh et al., 2008; Niture et al., 2014), we examined the phosphorylation of Nrf2 under EEPM treatment to further confirm the Nrf2-activating property of EEPM and observed that treatment of cells with EEPM caused dose- and time-dependent increases in the levels of phosphorylated Nrf2 expression.

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

In order to provide evidence for the involvement of Nrf2-mediated HO-1 induction in EEPM-mediated antioxidant and cytoprotective activities against oxidative stress, we inhibited HO-1 activity by using ZnPP, a specific inhibitor of HO-1. As shown in Fig. 5, in the presence of ZnPP, the protective effect of EEPM on H_2O_2-induced production of ROS and the reduction of cell viability were significantly attenuated. Taken together, these results suggest that EEPM exerts its protective effects by inducing the cellular defense mechanism against oxidative stress through HO-1 induction *via* Nrf2 activation, and that HO-1 plays a crucial role in this protection in C2C12 cells.

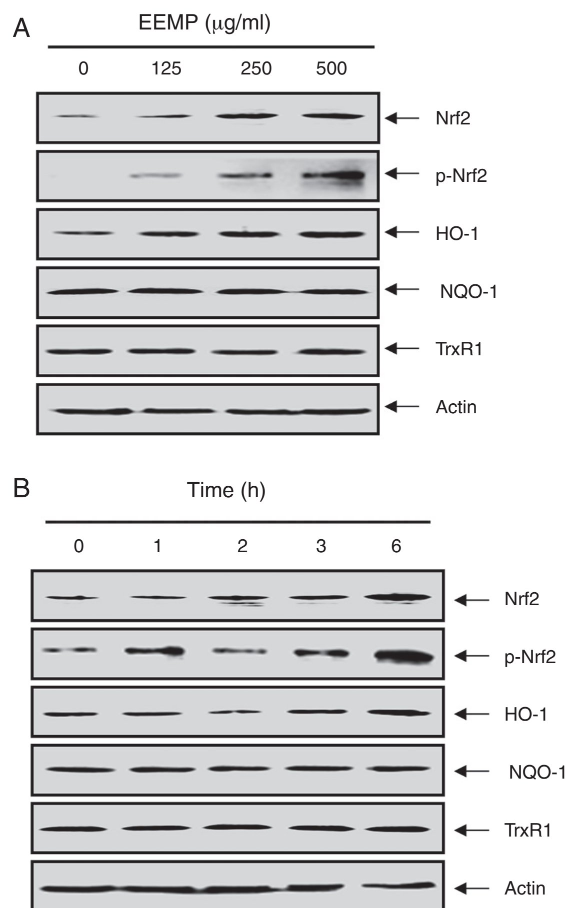

Fig. 4. Induction of Nrf2 and HO-1 expression by EEPM in C2C12 cells. Cells were incubated with the indicated concentrations of EEPM for 6 h (A) or 500 μg/ml EEPM for the indicated time periods (B). Total cellular proteins were separated on SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were probed with the specific antibodies against Nrf2, p-Nrf2, HO-1, NQO-1, and TrxR1. Proteins were visualized using an ECL detection system. Actin was used as an internal control.

Discussion

The mitochondrial electron transport system in the mitochondrial membrane is one of the major sources of intracellular ROS generation (Fleury et al., 2002), whereby the mitochondria play a pivotal role in the ROS-mediated cell death process. Excessive production of ROS by mitochondrial dysfunction following oxidative DNA damage and exposure to other genotoxic factors ultimately leads to the activation of the mitochondrial apoptotic pathway (also termed the intrinsic apoptotic pathway) rather than necrosis (Wang et al., 2013; Wu et al., 2010). Moreover, H_2O_2 directly induces mitochondrial dysfunction followed by a rapid efflux of intracellular ROS, which increases the permeabilization and depolarization of the mitochondrial membrane. This event could facilitate a rapid disruption of MMP and the release of apoptosis-inducing factors, which activate the caspase-dependent signaling cascades (Kim et al., 2006; Fleury et al., 2002). In the present study, treatment of C2C12 cells with H_2O_2 caused a marked decrease in cell survival, intracellular accumulation of ROS, and further induced the loss of MMP, leading to apoptosis. However, when C2C12 cells were pretreated with EEPM, the H_2O_2-induced reduction of cell viability, accumulation of ROS, loss of MMP, and apoptosis were significantly attenuated.

Literature reports indicate that DNA is sensitive to ROS-induced oxidative injury and that DNA is the most frequent target of

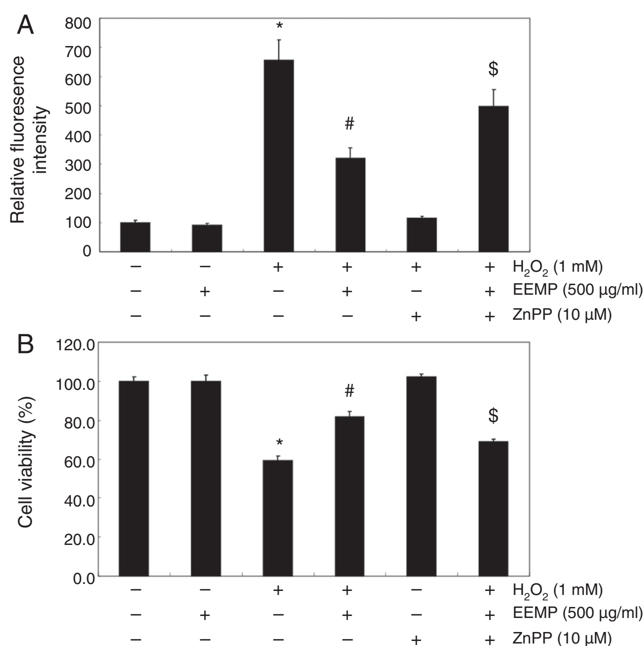

Fig. 5. Effects of an inhibitor of HO-1 on EEPM-mediated attenuation of ROS formation and growth inhibition by H_2O_2 in C2C12 cells. Cells were pretreated for 1 h with 1500 µg/ml EEPM and then treated for 6 h with or without 1 mM H_2O_2 in the absence or presence of 10 µM ZnPP. Then, ROS generation (A) and cell viability (B) were estimated. The results are the mean ± SD values obtained in three independent experiments ($^*p < 0.05$ compared with control group; $^\#p < 0.05$ compared with H_2O_2-treated group; $^\$p < 0.05$ compared with H_2O_2 and EEPM-treated group).

oxidative stress stemming from ROS production (Ayala-Peña, 2013; Maynard et al., 2009), suggesting a direct link between ROS signaling and oxidative DNA damage. Severe ROS-induced oxidative stress leads to the induction of mitochondria-medicated apoptosis in affected cells due to high levels of DNA damage (Deavall et al., 2012; Chistiakov et al., 2014). Thus, we presumed that EEPM might improve mitochondrial function through eliminating the overproduction of ROS induced by H_2O_2, thereby reducing the H_2O_2-induced apoptosis. Our study showed that H_2O_2 treatment increased DNA tail length in the comet assay, as well as the expression of phospho-H2A.X, which are widely-used markers for the detection of DNA damage (Rogakou et al., 1998). Both were attenuated by EEPM in the present study. These results indicated that EEPM protected against the H_2O_2-induced apoptosis of C2C12 cells by reducing DNA damage from the destructive impact of oxidative stress in C2C12 cells.

Nrf2, a transcription factor that is part of the redox homeostatic gene regulatory network, is a key regulator of the expression of an expansive set of ARE-mediated genes which remove ROS by inducing the actions of detoxifying enzymes (Hybertson et al., 2011; Niture et al., 2014). Increasing evidence suggests that the elevation of Nrf2-mediated target genes, including HO-1, also promotes cell survival in oxidizing environments *via* enhancement of free radical metabolism, inhibition of cytokine-mediated inflammation, and recognition of damaged DNA (Surh et al., 2008; Son et al., 2013). Many antioxidant agents have been reported to reduce ROS production by the activation of Nrf2/HO-1 pathway through a variety of signaling pathways (Terazawa et al., 2013; Niture et al., 2014). Moreover, it has been reported that Nrf2 phosphorylation by several protein kinases such as phosphatidylinositol-3 kinase/Akt, mitogen-activated protein kinases, and protein kinase C facilitates its translocation into the nucleus, whereupon it binds to ARE in the promoter regions (Surh et al., 2008; Niture et al., 2014; Lee et al., 2014). Therefore, we determined the potential role of Nrf2 and HO-1 in H_2O_2-induced C2C12 cell damage and EEPM-mediated cytoprotection. The data from the present study indicated that treatment of C2C12 cells with EEPM resulted in an increase of Nrf2 expression as well as its phosphorylated form with a concomitant increase in HO-1 expression. Furthermore, treatment with an HO-1 inhibitor, ZnPP, significantly abrogated the protective effects of EEPM on H_2O_2-induced growth inhibition and ROS generation, providing evidence for the involvement of HO-1 induction and Nrf2 activation in EEPM-mediated antioxidant potential.

In summary, our results clearly indicated that EEPM exhibited a protective ability against H_2O_2-induced cytotoxicity and apoptosis in C2C12 myoblasts. EEPM also successfully suppressed the formation of intracellular ROS, leading to a substantial regaining of MMP through, at least in part, activation of Nrf2 signaling and induction of phase II antioxidant enzymes, such as HO-1. In future studies, this molecular mechanism needs to be validated *in vivo* as a positive result, if confirmed, would provide essential information toward the development of new approaches for effective stress-responsive antioxidants against assaults triggered by ROS.

Authors' contributions

JSK, DJK, GYK and CP contributed in running the laboratory work, analysis of the data and drafted the paper. HJC, SK and HSK contributed to critical reading of the manuscript. HJK, BWK, CMK and YHC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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