THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Up-regulation of Nrf2-mediated heme oxygenase-1 expression by eckol, a phlorotannin compound, through activation of Erk and PI3K/Akt

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Biotechnology

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ABSTRACT

The aim of the present study was to examine the cytoprotective effect of eckol, a phlorotannin found in *Ecklonia cava* and to elucidate underlying mechanisms. Heme oxygenase-1 (HO-1) is an important antioxidant enzyme that plays a role in cytoprotection against oxidative stress. Eckol induced HO-1 expression both at the level of mRNA and protein in Chinese hamster lung fibroblast (V79-4) cells, resulting in increased HO-1 activity. The transcription factor NF-E2-related factor 2 (Nrf2) is a critical regulator of HO-1, achieved by binding to the antioxidant response element (ARE). Eckol treatment resulted in the enhanced level of phosphorylated form, nuclear translocation, ARE binding, and transcriptional activity of Nrf2. Extracellular regulated kinase (Erk) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, Akt) contributed to ARE-driven HO-1 expression. Eckol activated both Erk and Akt, and treatments with U0126 (an Erk kinase inhibitor), LY294002 (a PI3K inhibitor), specific Erk1 siRNA, and Akt siRNA suppressed the eckol induced activation of Nrf2, resulting in a decrease of HO-1 expression. ZnPP (a HO-1 inhibitor), HO-1 siRNA, and Nrf2 siRNA markedly abolished the cytoprotective effect of eckol against hydrogen peroxide-induced cell damage. Likewise, U0126 and LY294002 inhibited the eckol-induced cytoprotective effect against oxidative cell damage. These studies demonstrate that eckol attenuates oxidative stress by activating Nrf2-mediated HO-1 induction via Erk and PI3K/Akt signaling.

Keywords: Eckol; Heme oxygenase-1; NF-E2-related factor 2; Oxidative stress; Cytoprotection; Extracellular regulated kinase; Phosphatidylinositol 3-kinase

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INTRODUCTION

High levels of reactive oxygen species (ROS) cause damage to cells and are involved in various pathological conditions, including aging, cancer, and neurodegenerative diseases [1-7]. Therefore, it has been suggested that the use of antioxidant compounds may help prevent or alleviate diseases, particularly in those for which oxidative stress is the main cause. Eckol, an open-chain trimer of phloroglucinol, is a phlorotannin component of some brown algae such as *Ecklonia cava* (Laminariaceae). Recently, we reported that eckol protected cells from H_2O_2-induced oxidative stress via radical quenching and catalase activation [8] and as well as from ionizing radiation-induced cellular damage [9-11]. Heme oxygenase-1 (HO-1) catalyzes the rate-limiting step in heme catabolism, leading to the formation of biliverdin, free iron, and carbon monoxide. In the presence of biliverdin reductase, biliverdin is further converted to bilirubin [12,13]. Many papers have demonstrated the potent antioxidant and cytoprotective activities of heme-derived metabolites generated by HO-1 catalysis [14-17]. HO-1 is transcriptionally up-regulated by a large variety of stimuli; heme, oxidative stress, signaling protein, and phenolic compounds [18-22]. Therefore, the induction of HO-1 expression represents an adaptive response, increasing a cellular resistance to oxidative injury. A new class of activator protein-1 (AP-1)-related sequences has been demonstrated to mediate the oxidative stress responsiveness of the HO-1 gene. These regions, termed antioxidant responses elements (ARE), are tightly regulated by the redox-sensitive transcription factor, NF-E2-regulated factor 2 (Nrf2) [23]. The ARE is a *cis*-acting enhancer sequence that mediates transcriptional activation of Nrf2 in response to oxidative stress [24]. It is found in the promoter region of genes encoding HO-1 [25] and many other antioxidant enzymes, such as superoxide dismutase and γ-glutamylcysteine synthetase [26-28]. Therefore, genes regulated by the ARE encode proteins that help control of the cellular redox status and protect the cells from oxidative damage [29]. Mitogen activated protein kinase

(MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB, Akt) are important signaling enzymes involved in transduction of various signals from the cell surface to the nucleus. Both of their pathways are associated with the modulation of ARE-driven gene expression via Nrf2 activation [16,30]. In the present study, we investigated the capability of eckol to up-regulate HO-1 expression via activation of the ERK and Akt-Nrf2-ARE pathway in lung fibroblast cell.

MATERIALS AND METHODS

Materials

Eckol (Fig. 1A) was provided by professor Nam Ho Lee of Jeju National University (Jeju, Korea). [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) and zinc protoporphyrin (ZnPP) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Primary mouse monoclonal HO-1 antibody was purchased from Stressgen Corporation (Victoria, Canada) and primary rabbit polyclonal ERK, phospho ERK, Nrf2, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary anti-phospho Akt (Ser 473) and Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA, U.S.A). Both U0126 and LY294002 were provided by Calbiochem (San Diego, CA, USA). The ARE-luciferase reporter gene was kindly provided by Professor Young-Joon Surh of Seoul National University (Seoul, Korea). The other chemicals and reagents were of analytical grade.

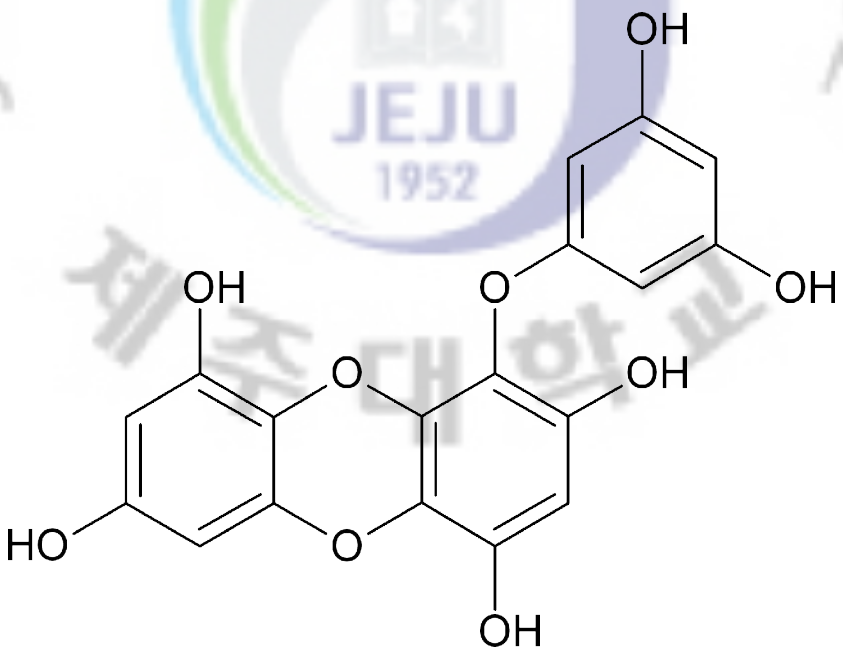

Figure 1A. Chemical structure of *Eckol*

Cell culture

The Chinese hamster lung fibroblast cells (V79-4) were obtained from the American Type Culture Collection. The V79-4 cells were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2 and cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml).

Western blotting analysis

The V79-4 cells were plated at 1×10^5 cells/ml, and sixteen hours after plating, the cells were treated with 0.1, 1, 10 μg/ml of eckol. The cells were harvested at the indicated times, and washed twice with PBS. The harvested cells were then lysed on ice for 30 min in 100 μl of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and centrifuged at 13,000g for 15 min. Supernatants were collected from the lysates and protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed on a 10% SDS-polyacrylamide gel. Blots were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), which were then incubated with primary antibodies. The membranes were further incubated with secondary antibody-horseradish peroxidase conjugates (Pierce, Rockland, IL, USA), and exposed to X-ray film. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

Reverse transcriptase polymerase chain reaction

Total RNA was isolated from cells using Trizol (GibcoBRL, Grand Island, NY, USA). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described previously [31]. PCR conditions for HO-1 and for the housekeeping gene, GAPDH, were as follows: 27 cycles of 94 °C for 45 sec; 53 °C for 45 sec; and 72 °C for 60 sec. The primer

pairs (Bionics, Seoul, Korea) were follows (forward and reverse, respectively): HO-1, 5'-GAGAATGCTGAGTTCATG-3' and 5'-ATGTTGAGCAGGAAGGC-3'; and GAPDH, 5'-GTGGGCCGCCCTAGGCACCA GG-3'; and 5'-GGAGGAAGAGGATGCGGCAGTG-3'. Amplified products were resolved on 1% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light.

Heme oxygenase-1 activity assay

HO-1 enzyme activity was measured as described previously [32]. Cells were homogenized in 0.5 ml ice-cold 0.25 M sucrose solution containing 50 mM potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 200g for 10 min. The supernatants were centrifuged at 9000g for 20 min, and further centrifuged at 15,000g for 60 min. The pellet was then resuspended in 50 mM potassium phosphate buffer (pH 7.4) and the amount of protein was determined. The reaction mixture (200 μl) containing 0.2 mM of the substrate hemin, 500 μg/ml of cell lysate, 0.5 mg/ml rat liver cytosol as a source of biliveridin reductase, 0.2 mM $MgCl_2$, 2 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, 1 mM NADPH, and 50 mM potassium phosphate buffer (pH 7.4) was incubated at 37 °C for 1 h. The reaction was stopped with 0.5 ml of chloroform, and after extraction the chloroform layer was measured spectrophotometrically. Bilirubin formation was calculated from the difference in absorption between 464 and 530 nm.

Immunocytochemistry

Cells plated on coverslips were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 2.5 min. Cells were treated with blocking medium (3% bovine serum albumin in PBS) for 1 h and incubated with Nrf2 antibody diluted in blocking medium for 2 h. Immuno-reacted primary Nrf2 antibody was detected by a 1:500 dilution of FITC-conjugated secondary antibody (Jackson ImmunoResearch

Laboratories, West Grove, PA, USA) for 1 h. After washing with PBS, stained cells were mounted onto microscope slides in mounting medium with DAPI (Vector, Burlingame, CA, USA). Images were collected using the LSM 510 program on a Zeiss confocal microscope.

Nuclear extract preparation and electrophoretic mobility shift assay

Cells were harvested at the indicated times, and were then lysed on ice with 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, and 1% NP-40) for 4 min. After 10 min of centrifugation at 3,000g, the pellets were re-suspended in 50 μl of extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 300 mM NaCl, 0.2 mM EDTA, 1 mM DTT, and 1 mM PMSF), incubated on ice for 30 min, and centrifuged at 13,000g for 5 min. The supernatant was then harvested as nuclear protein extracts and stored at -70 ºC after determination of protein concentration. Synthetic double strand oligonucleotides containing the Nrf2-binding domain (ARE) were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase, and subsequently used as probes. The double stranded sequence of oligonucleotides was 5'-TTT TCT GCT GAG TCA AGG GTC CG-3' and 3'-AAA AGA CGA CTC AGT TCC AGG C-5'. The probes (50,000 cpm) were incubated with 6 μg of the nuclear extracts at 4 ^{o}C for 30 min in a final volume of 20 μl containing 12.5% glycerol, 12.5 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM DTT with 1 μg of poly (dI-dC). Binding products were resolved on a 5% polyacrylamide gel and the bands were visualized by autoradiography [33].

Immunoprecipitation and Western blot for detection of phosphorylated Nrf2

Nrf2 was immunopreciptated from the nuclear extracts using Nrf2 antibody. Immune complexes were collected with protein G beads and washed with immunoprecipitation buffer. Equal amounts precipitates were run on a SDSpolyacrylamide gel, and Western blotting was performed with antibody specific to phosphoserine/phosphothreonine (Amos et al., 2005).

Transient transfection of ARE promoter and luciferase assay

A day before transfection, cells were sub-cultured at a density of 1×10^6 cells in 60 mm dish to reach approximately 60-80% confluence. The cells were transiently transfected with a plasmid harboring the ARE-promoter using the transfection reagent DOTAP, according to the manufacturer's instructions (Roche, Mannheim, Germany). After overnight transfection, cells were treated with 10 μg/ml of eckol. Cell were then washed twice with PBS and lysed with reporter lysis buffer. After vortex-mixing and centrifugation at 12,000g for 1 min at 4 °C, the supernatant was stored at -70 °C for the luciferase assay. After 20 μl of the cell extract was mixed with 100 μl of the luciferase assay reagent at room temperature, the mixture was placed in a luminometer in order to measure the light produced.

Transient transfection of small RNA interference (siRNA)

V79-4 cells were seeded at 1.5 x 10^5 cells/well in 24 well plate and allowed to reach approximately 50% confluence on the day of transfection. The siRNA construct used were obtained as mismatched siRNA control (siControl, Santa Cruz Biotechnology, Santa Cruz, CA, USA), siRNA against Nrf2 (siNrf2, Santa Cruz Biotechnology, Santa Cruz, CA, USA), siRNA against Akt (siAkt, Dharmacon, Lafayette, CO, USA), siRNA against HO-1 (siHO-1, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and siRNA against Erk1 (siErk1, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were transfected with 10-50 nM siRNA using lipofectamineTM 2000 (Invitrogen, Carlsbad, CA) based on the manufacturer's instruction. At 24 h after transfection, the cells were treated with 10 μg/ml of eckol for 24 h and examined by either Western blot analysis or MTT assay.

Cell viability

The effect of eckol on the viability of the V79-4 cells was determined by the MTT assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in

viable cells [34]. The cells were seeded in a 96 well plate at a density of 1×10^5 cells/ml and treated with 10 μg/ml of eckol, 10 μM ZnPP (an inhibitor of HO-1), 10 nM U0126 (an inhibitor of ERK kinase), 5 μM LY294002 (an inhibitor of PI3K), followed 2 h later by 1 mM of H_2O_2. After incubating for 48 h at 37 °C, 50 μl of the MTT stock solution (2 mg/ml) was then added to each well to attain a total reaction volume of 250 μl. After incubation for 2.5 h, the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl DMSO, and the ABS_{540} was read on a scanning multi-well spectrophotometer.

Statistical analysis

All the measurements were made in triplicate and all values are represented as mean ± standard error of the mean (SEM). The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference. A value of $p<0.05$ was considered significant.

RESULTS

Eckol induces HO-1 expression and activity in a concentration- and time-dependent manner

Treatment of lung fibroblast V79-4 cells with eckol for 12 hours induced a concentration-dependent enhancement in HO-1 protein expression. Additionally, eckol treatment at 10 μg/mL increased HO-1 protein expression in a time-dependent manner (Fig. 1B). The increased HO-1 expression correlated with *HO-1* mRNA levels in both concentration- and time-dependent fashions in eckol-treated cells (Fig. 1C). Given that HO-1 requires posttranslational alteration for its activity (Salinas et al., 2004), its activity was also assessed. A significant increase in HO-1 activity was observed at 1 and 10 μg/mL of eckol, and eckol at 10 μg/mL increased the enzyme activity after 12 hour (Fig. 1D). This result suggests that the increase in HO-1 mRNA and protein is accompanied by enhanced HO-1 activity.

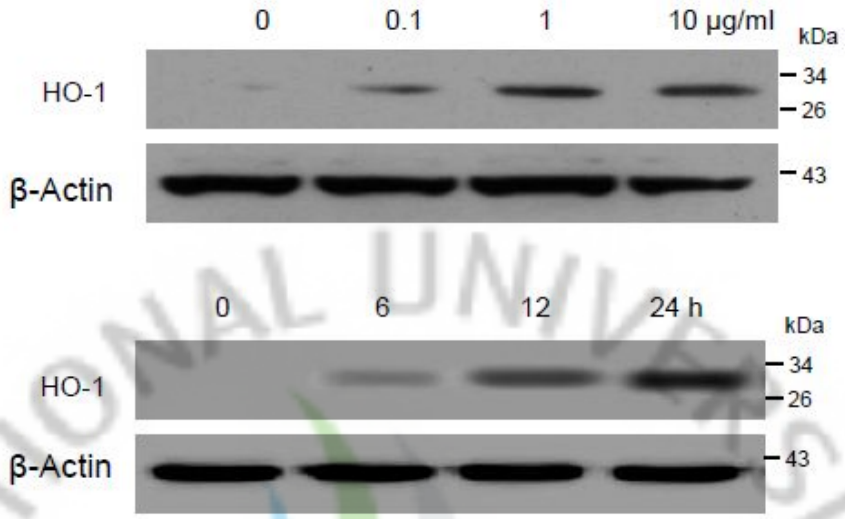

Figure 1B. Cell lysates treated by various concentration of eckol at 12 h, and by a variety of time courses of eckol at 10 μg/ml were electrophoresed and the expression of HO-1 protein was detected using a HO-1 specific antibody.

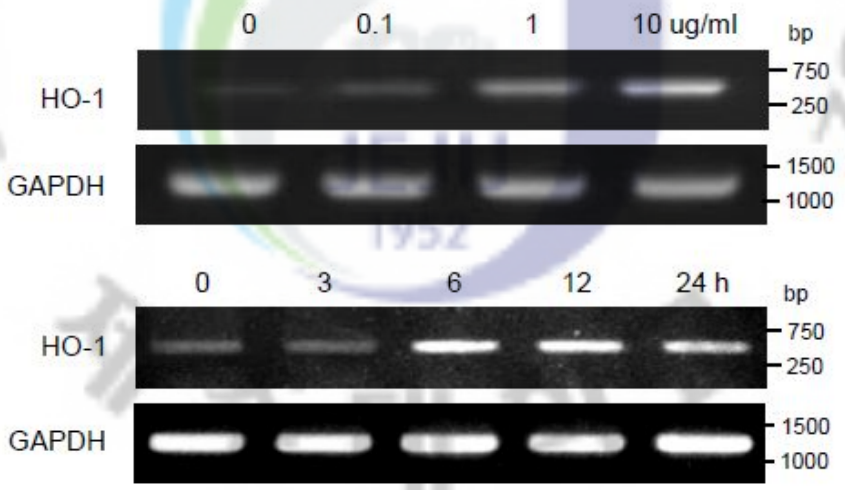

Figure 1C. Cells were treated with eckol, total RNA was extracted, and HO-1 mRNA expression was analyzed by RT-PCR. The GAPDH band is shown to confirm integrity and equal loading of RNA.

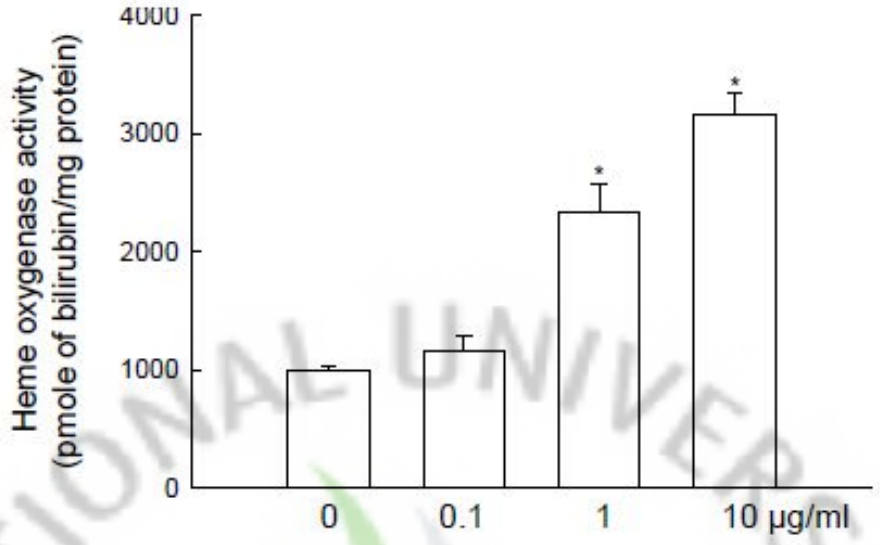

Figure 1D. HO-1 activity was measured with various concentration of eckol at 12 h. Each bar represents the mean ± SEM in triplicate experiments. *Significantly different from control ($p<0.05$).

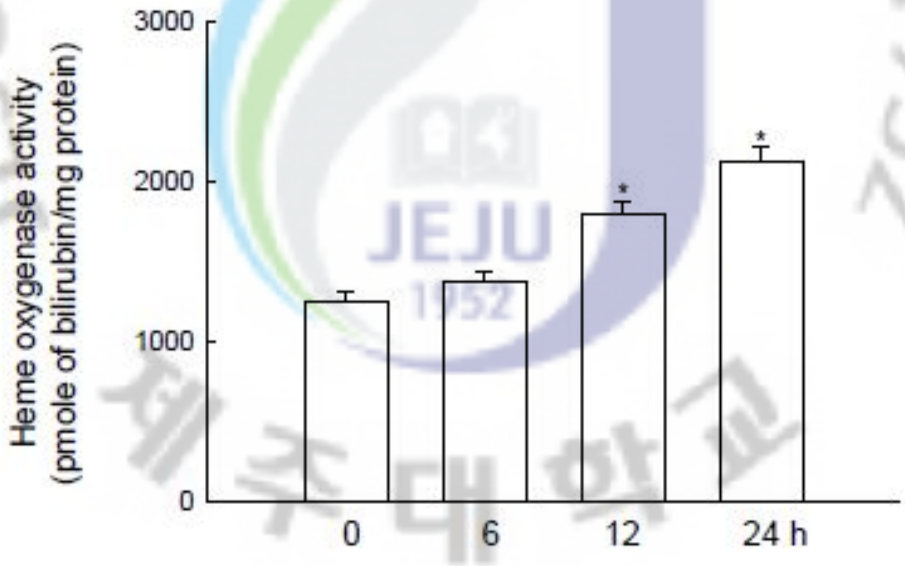

Figure 1D. HO-1 activity was measured at a variety of time courses of eckol with10 μg/ml. Each bar represents the mean ± SEM in triplicate experiments. *Significantly different from control ($p<0.05$).

Eckol increases the levels of Nrf2 transcription factor

Most of the genes encoding phase II detoxifying and antioxidant enzymes have an ARE sequence in their promoter region. Nrf2 is an important transcription factor that regulates ARE-driven HO-1 gene expression. We examined whether eckol is able to activate Nrf2 in association with HO-1 up-regulation. Eckol treatment increased Nrf2 protein expression (Fig. 2A). To detect Nrf2 phosphorylation, the nuclear lysates were immunoprecipitated with anti Nrf2 antibody and immunoblotted with phosphoserine/phosphothreonine antibody. The phosphorylated Nrf2 was clearly increased in the eluted fraction of cells treated with eckol (Fig. 2B), and resulted in a translocalization of Nrf2 protein from the cytosol to the nucleus (Fig. 2C). Moreover, eckol treated cells exhibited significantly elevated Nrf2 binding to the ARE sequence as assessed by the gel shift assay (Fig. 2D). To verify the functional relevance of Nrf2 binding to the ARE sequence of HO-1, an ARE promoter construct containing the Nrf2 binding DNA consensus site linked to a luciferase reporter gene was used. As illustrated in Fig. 2E, eckol increased the transcriptional activity of Nrf2. These results further suggest that Nrf2 mediates the eckol-induced activation of the HO-1 promoter. To confirm the requirement of Nrf2 for eckol-induced HO-1 expression, cells were transfected with siNrf2 for 24 h prior to eckol treatment. As shown in Fig. 2F, the both constitutive and eckol-induced expression of HO-1 was markedly inhibited by siRNA knock down of Nrf2 gene.

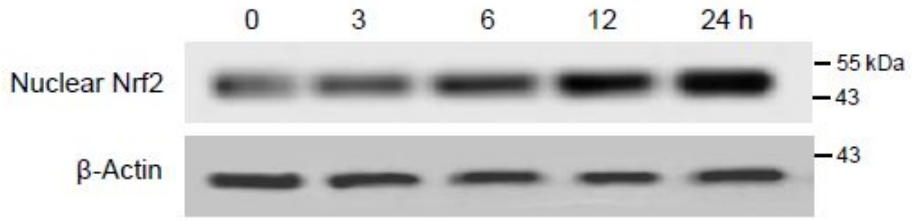

Figure 2A. Nuclear extracts from V79-4 cells were prepared after treatment with 10 μg/ml of eckol for the indicated times. Western blot for nuclear lysates were detected with a Nrf2-specific antibody.

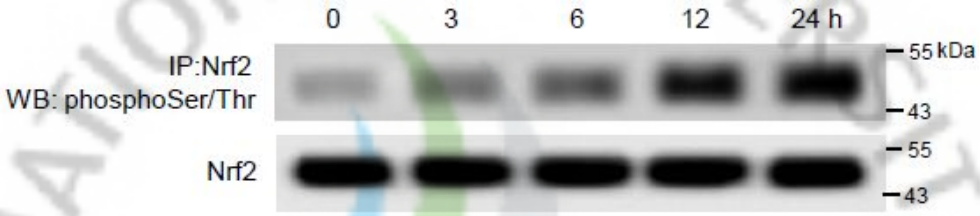

Figure 2B. The cell lysates were immunoprecipitated with Nrf2 antibody, and precipitates were Western blotted with antibodies against phosphoserine/phosphothreonine and Nrf2

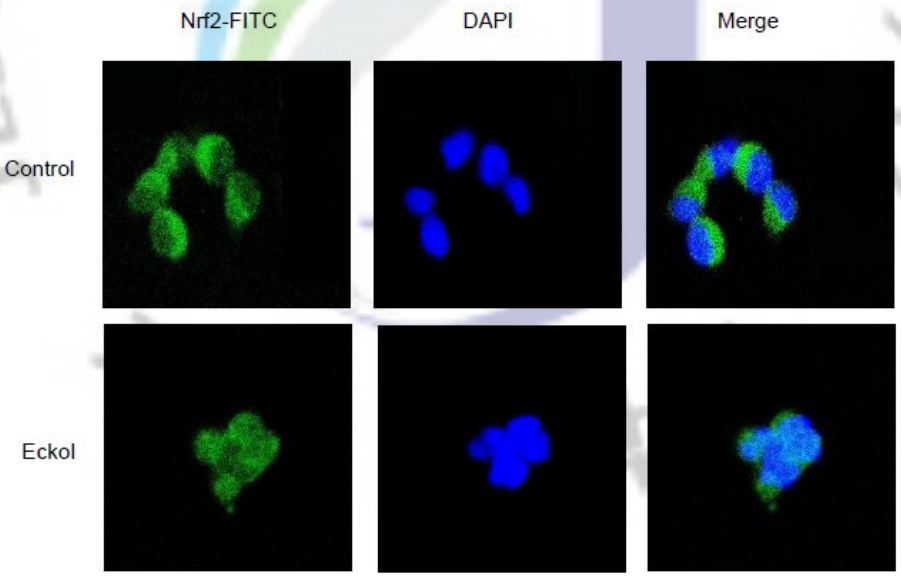

Figure 2C. Confocal imaging using FITC-conjugated secondary antibody staining indicates the location of Nrf2 (green) by anti- Nrf2 antibody, DAPI staining indicates the location of the nucleus (blue), and the merged image in eckol-treated cells indicates the nuclear location of Nrf2 protein.

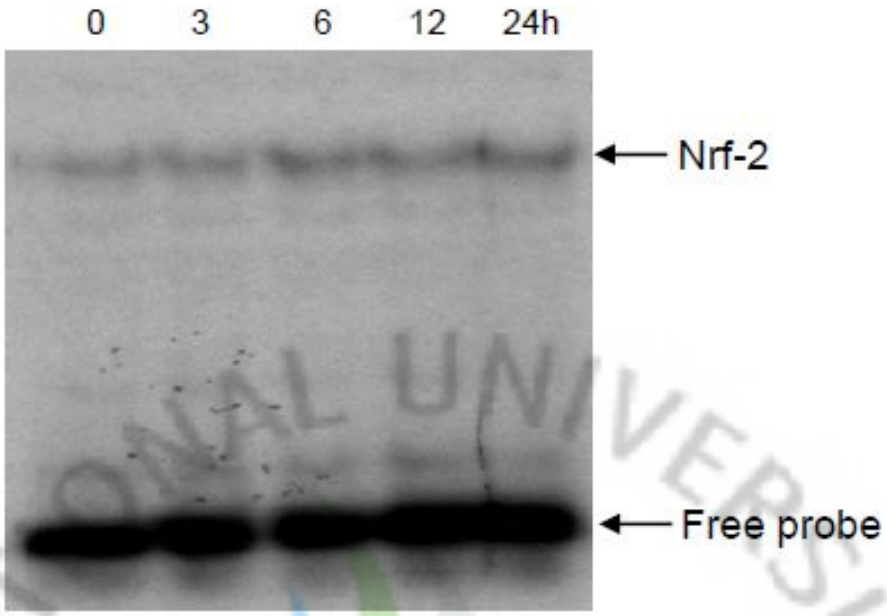

Figure 2D. Nuclear extracts prepared from cells were treated with 10 μg/ml of eckol for the indicated times. Electrophoretic mobility shift assay was performed for the detection of ARE-binding activity of Nrf2.

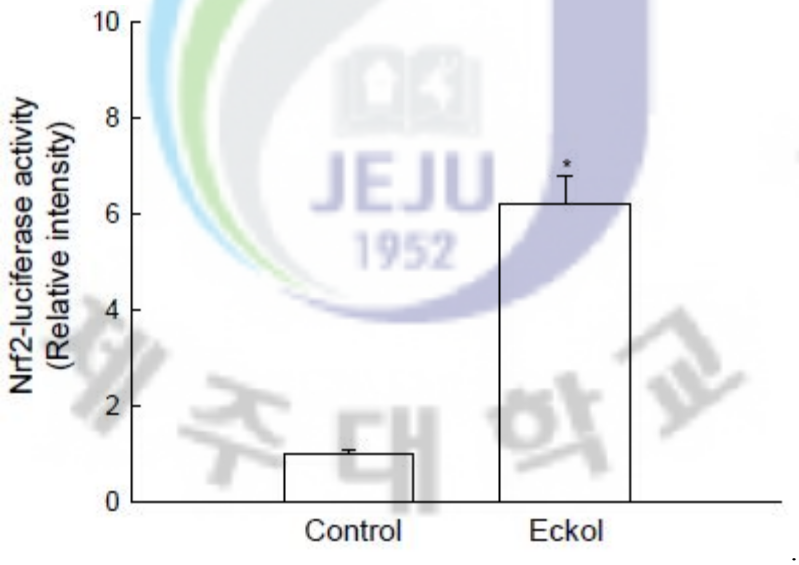

Figure 2E. V79-4 cells were transfected with an ARE-luciferase construct (1 μg per well) or control vector (1 μg per well). After overnight, cells were treated with eckol, cell lysates were mixed with a luciferase substrate, and the luciferase activity was measured by the luminometer. Data are expressed as the mean ± SEM in triplicate experiments. *Significantly different from control ($p<0.05$).

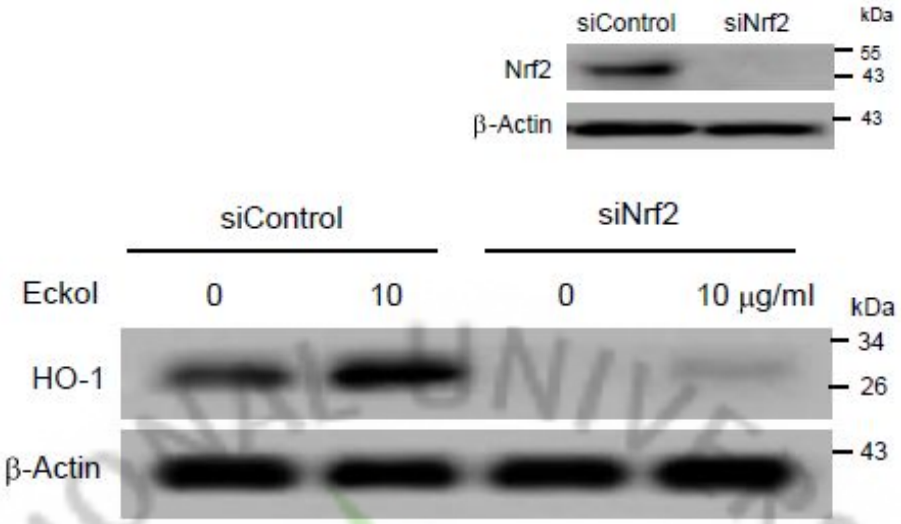

Figure 2F. Cells were transfected in 10-50 nM siControl and siNrf2 using lipofectamineTM 2000 based on the manufacturer's instruction. At 24 h after transfection, the cells were treated with 10 μg/ml of eckol for 24 h and the expression of HO-1 protein was examined by Western blot analysis.

Eckol activates Nrf2 via phosphorylation of Erk and Akt

To further elucidate the upstream signaling pathway involved in eckol-mediated Nrf2 activation and induction of HO-1, we examined the activation of Erk and Akt, both of which are major signaling enzymes involved in cellular protection against oxidative stress. Activation of Erk and Akt by eckol was assessed using Western blot with phospho-specific antibodies against Erk and Akt. As shown in Figs. 3A and B, exposure to eckol caused an increase in the phosphorylation of Erk, and Akt in a time- dependent manner. However, the phosphorylated forms of Jnk and p38 were not detected in eckol-treated cells (data not shown). We then analyzed whether Erk and Akt pathways were involved in the induction of Nrf2 activation and HO-1 expression. Cells were pre-incubated for 30 min with inhibitors of U0126 (an Erk kinase inhibitor) and LY294002 (a PI3K inhibitor) and then treated with eckol for additional 12 h. The effectiveness of these inhibitors was confirmed in eckol-treated cells with antibodies specific for phospho Erk and phospho Akt (data not shown). Increase in nuclear Nrf2 accumulation and HO-1 protein expression occurred following treatment with eckol as expected. Inhibition of the Erk and Akt pathways dramatically reduced the capacity of eckol to increase Nrf2 and HO-1 protein levels (Fig. 3C). To further confirm these observations, cells were transfected with siErk1 or siAkt. As shown in Fig. 3D, the expression of nuclear Nrf2 and HO-1 were markedly inhibited in siErk1 or siAkt-transfected cells regardless of eckol treatment. These results indicate that Erk and PI3K/Akt are required to induced HO-1 expression as well as nuclear accumulation of phosphorylated Nrf2.

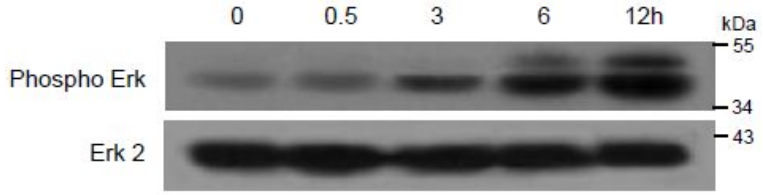

Figure 3A. Cell lysates were electrophoresed, and phospho-Erk, Erk were detected using their respective specific antibodies.

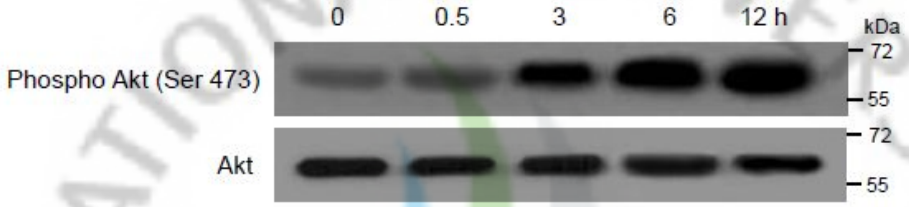

Figure 3B. Cell lysates were electrophoresed, and phospho-Akt, and Akt were detected using their respective specific antibodies.

Figure 3C. After treatment with U0126 or LY294002, cell lysates were electrophoresed and nuclear Nrf2 and HO-1 were detected using their respective specific antibodies.

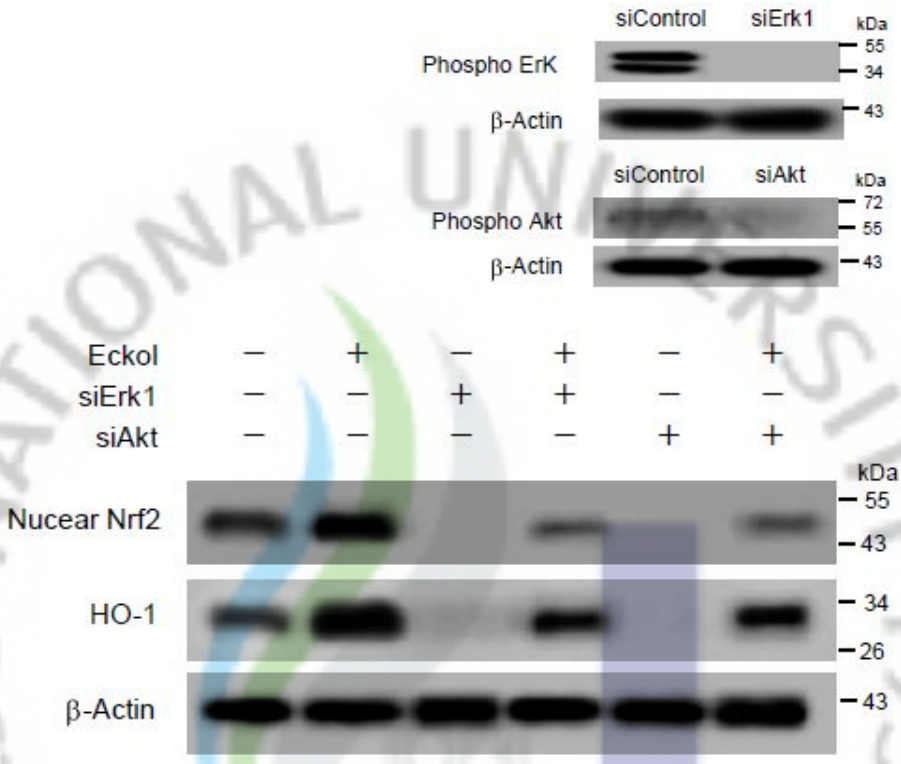

Figure 3D. Cells were transfected in 10-50 nM siControl and siErk1 or siAkt. At 24 h after transfection, the cells were treated with 10 μg/ml of eckol for 24 h and the expression of nuclear Nrf2 and HO-1 protein were examined by Western blot analysis.

Involvement of HO-1 in cell damage induced by oxidative stress

To determine whether the increased level of HO-1 activity enhanced by eckol confers cytoprotection against oxidative stress, V79-4 cells were pretreated with the HO-1 inhibitor ZnPP. ZnPP attenuated the protective effect of eckol on H_2O_2-induced cytotoxicity (Fig. 4A), and siHO-1-transfected cells exhibited similar results (Fig. 4B). Therefore, the cytoprotective effect of eckol is likely to be mediated through HO-1 induction. Likewise, siNrf2-transfected cells abolished the protective effect of eckol against H_2O_2-induced death (Fig. 4C), suggesting the involvement of Nrf2 transcription factor in eckol mediated HO-1 gene induction and cytoprotection.

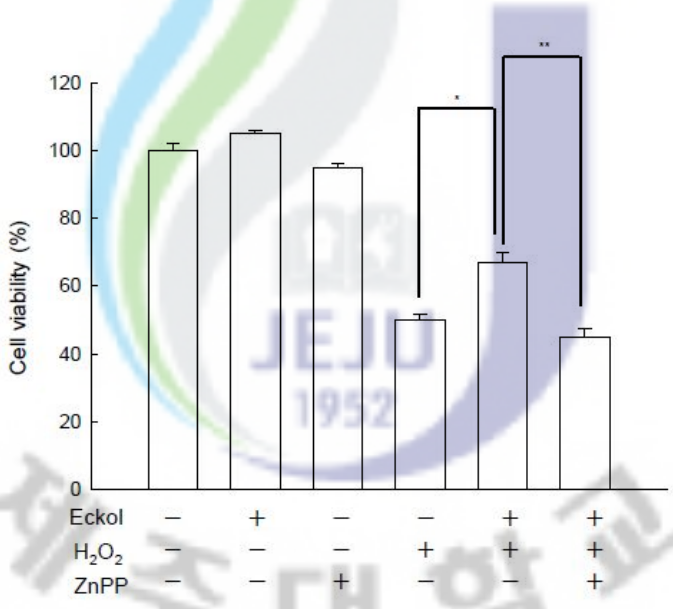

Figure 4A. Cells were pre-incubated with ZnPP at 10 μM for 6 h, followed by 1 h of incubation with eckol and exposure to 1 mM of H_2O_2 for 48 h. Cell viability was measured using the MTT assay. *Significantly different from H_2O_2 treated cells ($p<0.05$). **Significantly different from eckol plus H_2O_2 treated cells ($p<0.05$).

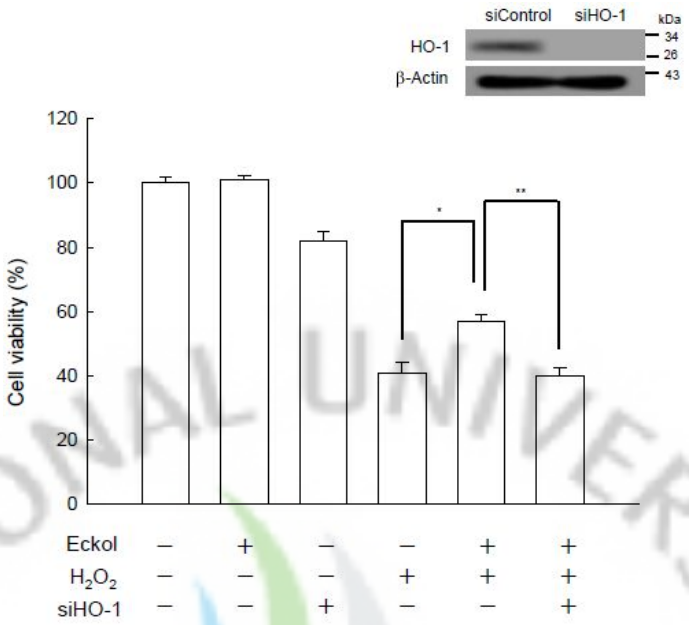

Figure 4B. Cells were transfected in 10-50 nM siControl and siHO-1. At 24 h after transfection, the cells were treated with 10 μg/ml of eckol for 48 h and cell viability was measured using the MTT assay. *Significantly different from H_2O_2 treated cells ($p<0.05$). **Significantly different from eckol plus H_2O_2 treated cells ($p<0.05$).

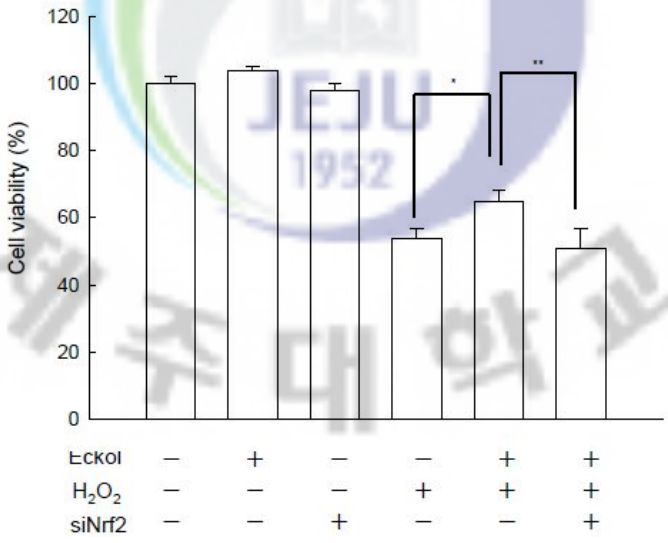

Figure 4C. Cells were transfected in 10-50 nM siControl and siNrf2. At 24 h after transfection, the cells were treated with 10 μg/ml of eckol for 48 h and cell viability was measured using the MTT assay. *Significantly different from H_2O_2 treated cells ($p<0.05$). **Significantly different from eckol plus H_2O_2 treated cells ($p<0.05$).

Involvement of Erk and Akt pathways in eckol-induced cytoprotection against oxidative stress

We then examined whether eckol could attenuate oxidative cell death through activation of the Erk and Akt signaling. U0126 and LY294002 abolished the eckol-induced increases in the expression of phospho-Erk and -Akt (Fig. 5A). U0126 and LY294002 attenuated the protective effect of eckol against H_2O_2-induced cytotoxicity (Fig. 5B), suggesting the involvement of Erk and Akt signaling in eckol-mediated HO-1 induction as well as in cytoprotection against oxidative stress.

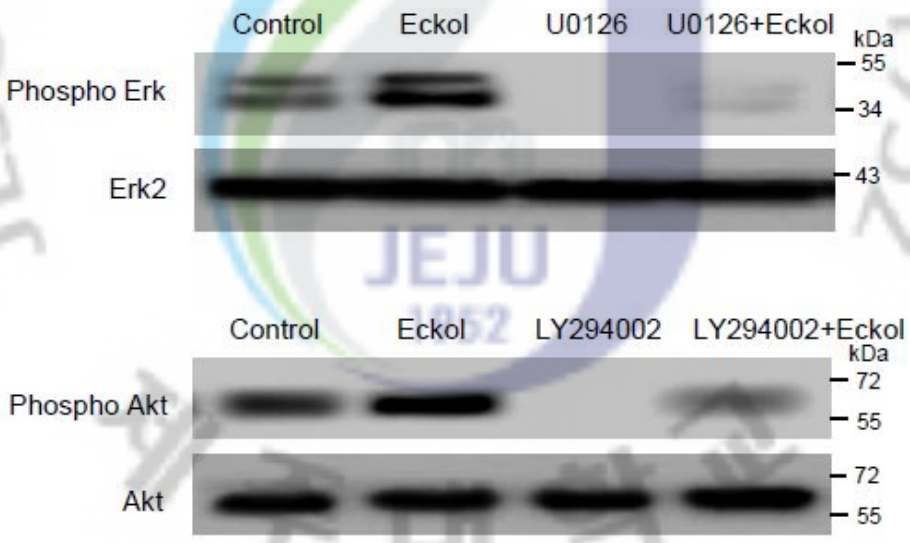

Figure 5A. Cells were incubated with eckol in presence of U0126 or LY294002 for 6 h. Cell lysates were electrophoresed and the membrane was detected by a specific antibody.

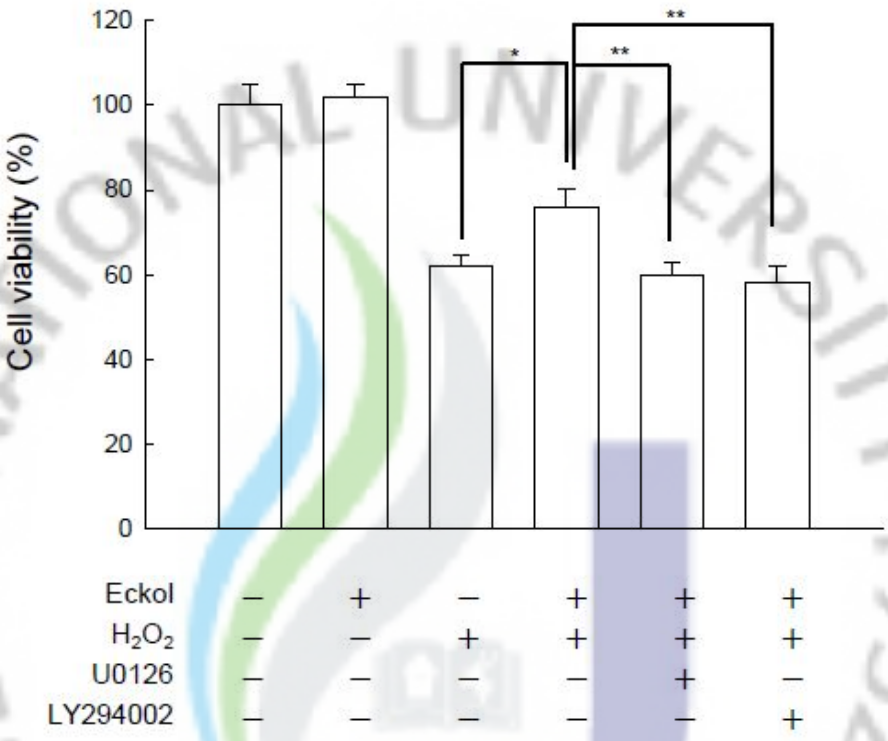

Figure 5B. Cell viability was measured using the MTT assay. *Significantly different from H_2O_2 treated cells ($p<0.05$). **Significantly different from eckol plus H_2O_2 treated cells ($p<0.05$).

DISCUSSION

In this study, we found that eckol up-regulated HO-1 expression by targeting the ARE sequences present in the HO-1 gene promoter. The regulation of ARE by eckol was mediated through an activation of Nrf2 transcription factor in a Erk and PI3K/Akt-dependent manner. Most studies on the regulation of phase II gene expression have focused on the role of the MAPK and Akt pathways. Our results indicate that Erk and Akt pathways are activated by eckol, and they participate in the induction of HO-1. However, Jnk and p38 were not involved in the induction of HO-1. The role of MAPK in ARE activation remains controversial. HO-1 gene was activated by the NAD(P)H oxidase inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride via a p38-dependent signaling pathway in monocytes (Wijayanti et al., 2005). However, genetic deficiency of p38 induced HO-1 gene expression via a Nrf2-dependent mechanism in monocytic cells (Naidu et al., 2009). In addition, the Erk pathway is dispensable for HO-1 induction in PC-12 cells in response to carnosol or in rat hepatoma cells treated with arsenite (Martin et al., 2004; Kietzmann et al., 2003), but it is essential in HO-1 up-regulation in our present study and in the arsenite-induced HO-1 in LMH cells (Elbirt et al., 1998). One possible explanation for such contradictory observations may be related to different types of inducers and cells used as well as the relative response of signaling pathways to a given stimulus.

The transcription factor Nrf2 is a member of the Cap-N-Collar family of basic leucine transcription factors, and plays an essential role in the ARE-mediated expression of HO-1 in response to oxidative stress (Itoh et al., 1997; Hayes et al., 2000; Chan and Kan, 1999; Chan et al., 2001; Kim et al., 2001; Kwak et al., 2002). Under normal physiological conditions, Nrf2 is sequestered by the cytoskeleton-associated cytoplasmic 'Kelch-like ECH-associated protein 1 (Keap 1)' which hampers the nuclear translocation of Nrf2. However, upon stimulation by electrophilic agents or reactive oxygen species, Nrf2 dissociates from its cytoplasmic docking protein Keap1, translocates into the nucleus and binds to the ARE site

(Kobayashi and Yamamoto, 2005). This leads to the *de novo* synthesis of antioxidant enzymes that efficiently protect cells from oxidative stress. The actual mechanism of dissociation of Nrf2 from Keap1 is largely unresolved, but it is thought to involve the phosphorylation of Nrf2 (Itoh et al., 1999). It is reported that Erk and PI3K/Akt phosphorylates Nrf2 which may facilitate the release of Nrf2 from the Keap1-Nrf2 complex, allowing activated Nrf2 to translocate into the nucleus where it forms a heterodimer with the small Maf protein (Li et al., 2007; Chan et al., 2001; Kim et al., 2001; Kwak et al., 2002). Eckol increases the nuclear levels of the Nrf2, its binding to the ARE, and its transcriptional activity. The mechanisms leading to nuclear translocation of Nrf2 include its release from Keap1 in the cytosol. However, because the half-life of Nrf2 transcription factor is very short, these mechanisms should necessarily rely on the stabilization of the Nrf2 protein (Jain et al., 2008; Lee et al., 2007). Our results demonstrate that eckol increased the level of Nrf2, suggesting that eckol may retard Nrf2 degradation. The increase in Nrf2 protein level induced by eckol was dependent on the activation Erk and PI3K because U0126 and LY294002 decreased the eckol-induced accumulation of Nrf2 protein through inhibition of Erk and Akt phosphorylation. Eckol treated cells clearly induced the expression of phosphorylated Nrf2 in the purified phosphoproteins. The increased phosphorylated Nrf2 might play a role in its activation or nuclear translocation.

Eckol, a phlorotannin component present in some brown algae, is a phytoalexin containing defensive or protective functions against herbivores. Recently, we reported that eckol attenuates oxidative stress induced cell damage by activating Erk and nuclear factor kappa B (Kang et al., 2005) and that it protected V79-4 lung fibroblast cells against gamma-ray radiation-induced apoptosis by scavenging of ROS and inhibiting of the Jnk pathway (Zhang et al., 2008).

In summary, the present results suggest that eckol protects V79-4 cells against oxidative stress-induced cell death. This occurs via an elevated activation of Erk and PI3K/Akt, which appears to be responsible for nuclear translocation of Nrf2, its subsequent binding to ARE,

and the up-regulating of HO-1 gene expression (Fig. 6).

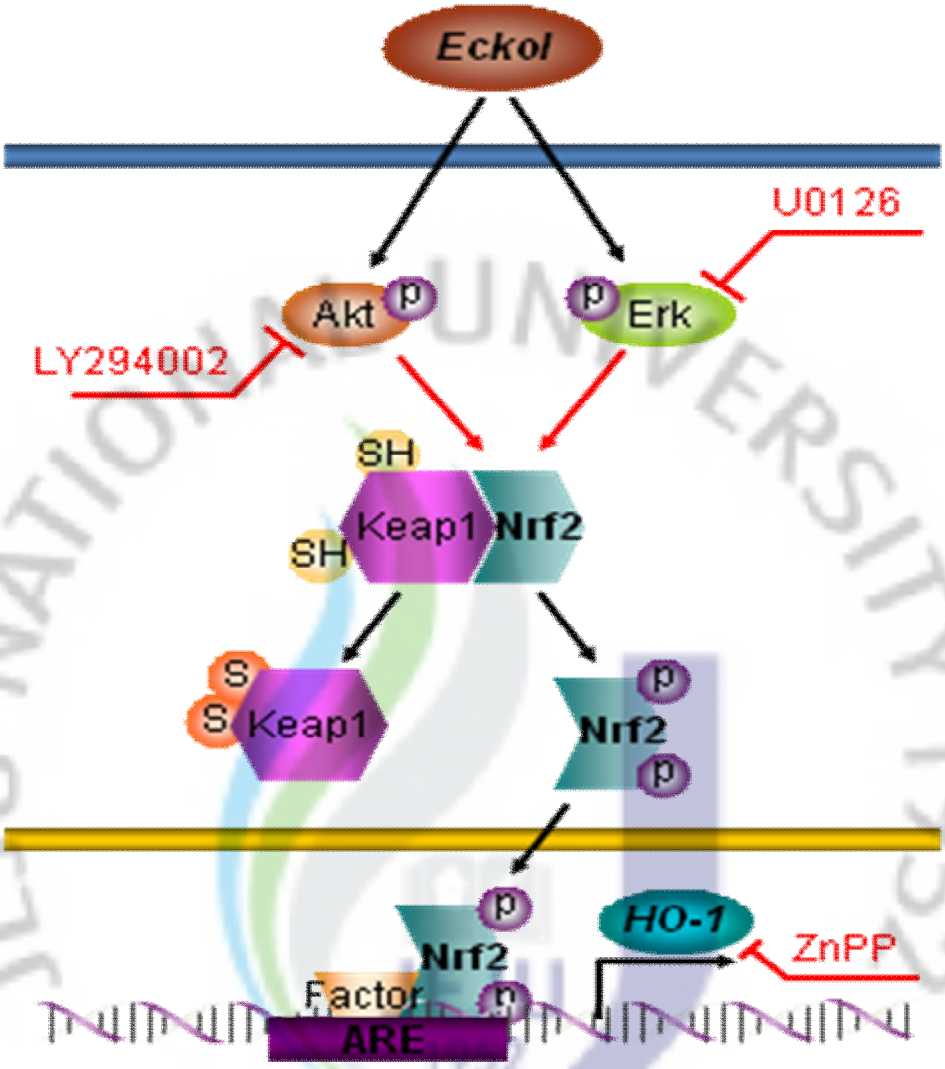

Figure 6. A proposed pathway for eckol-induced HO-1 via up-regulation of Erk, PI3K/Akt and Nrf2, explaining the cytoprotective effect against oxidative stress in V79-4 cells.

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국문요약

이 연구에서는 감태(*Ecklonia cava*)로부터 분리 정제한 Phlorotannin 화합물인 eckol의 세포 보호효과와 그 기작에 대해 밝히고자 하였다. 세포 보호기작 연구에 있어서 중요한 항 산화효소로 알려진 Heme oxygenase-1(HO-1)의 산화적 손상으로부터의 보호 작용을 검색하였다. Eckol은 Chinese hamster lung fibroblast인 V79-4 세포주에서 HO-1 단백질량과 HO-1의 mRNA의 수준을 증가 시켰고, HO-1의 효소활성도 증가 시켰다. NF-E2-related factor 2(Nrf2)는 antioxidant response element(ARE)와 결합함으로써 HO-1의 주 조절자가 된다고 알려져 있다. Eckol을 처리하였을 때 Nrf2의 핵 내로의 이동을 유도하였고, ARE와의 결합력과 Nrf2의 전사활성을 증가시켰다. 그 다음으로 mitogen activated protein kinase(MAPK)인 Erk와 phosphatidylinositol 3-kinase(PI3K)의 직속하위인자인 PKB/Akt는 HO-1의 발현을 조절하는 ARE의 활성을 유도한다고 보고되어 있다. Eckol은 Erk와 Akt 둘 다의 활성을 증가 시켰으며 Erk의 활성 억제재인 U0126과 PI3K 활성 억제재를 처리 하였을 때 핵 내의 활성형 Nrf2의 억제와 HO-1의 발현을 억제하였으며, mRNA 수준에서의 Erk와 Akt의 억제하기 위해 siErk1과 siAkt를 이식하였을 때 역시 같은 결과를 얻을 수 있었다. 마지막으로 HO-1의 산화적 손상으로부터의 보호효과를 확인하기 위해 hydrogen peroxide로 세포 손상을 유도하였으며 HO-1 특이적 억제재인 ZnPP 처리와 siHO-1 이식으로 Eckol에 의해 유도된 세포보호효과가 상쇄되었다. 이 외에도 U0126과 LY294002 처리로 eckol에 의해 유도된 산화적 손상으로부터의 보호효과가 억제되었다. 이 연구는 eckol이 Erk와 PI3K/Akt를 통하여 Nrf2 활성에 매개된 HO-1의 증가에 의해 산화적 손상을 억제시킨다는 내용을 증명하였다.

검색어: Eckol; Heme oxygenase-1; NF-E2-related factor 2; Oxidative stress; Cytoprotection; Extracellular regulated kinase; Phosphatidylinositol 3-kinase

감사의 글

길다면 길고 짧다면 짧은 2년의 석사과정이 주마등처럼 제 머리 속을 스치듯 지나갑니다. 그 기간을 돌아보면 참으로 많은 일들이 있었던 것 같습니다. 그런데 막상 돌이켜 보면 뭐하나 제대로 한 것이 없어 후회만 되고, 시간이 지날수록 모르는 것이 너무 많다는 생각이 들어 과연 석사 학위를 받아도 될지 모르겠다는 생각이 듭니다. 그렇지만 저는 감히 지식은 평생 동안을 배워도 부족하다는 말과 아는 것이 많을수록 모르는 것이 더욱 많아진다는 말을 상기하며 그래도 많이 배워서 이런 생각도 하는구나 라고 제 스스로를 위안해 봅니다. 아직은 갈 길이 멀었지만 그래도 제가 이 정도 까지 왔다는 것에 저 자신에게 감탄하며 이 논문을 빌어 그 동안 제에게 많이 가르쳐 주시고 제가 연구와 공부에 몰두할 수 있도록 도와주신 분들께 감사의 말씀을 전하고 싶습니다.

먼저 제가 무슨 일을 할까 하고 고민하고 있을 때 이쪽 길을 걷도록 인도해주시고 엄한 교수님이시지만 학교선배님처럼 때로는 친한 형처럼 저에 인생의 등대가 되어주신 제가 너무도 닮고 싶은 김기영 교수님, 항상 제 일을 당신의 일처럼 보듬어 주시고 나태해지지 않도록 격려와 충고를 아끼지 않으신 그 가르침에 대해 진심으로 감사를 드립니다. 그리고 석사기간 동안 의학과 생화학 교실에서 생활하며 논문에 대한 연구를 수행하는 동안 자신의 학생처럼 아니 그보다 더 친밀한 어머니 같은 애정으로 많은 가르침과 연구 지도를 해주시며 배움의 길로 인도해 주신 현진원 교수님께도 진심 어린 감사의 말씀을 전합니다. 그리고 바쁘신 와중에도 아랑곳하지 않으시고 부족한 논문을 격려와 지도로 세심하게 심사해주신 강희경 교수님께도 진심으로 감사를 드립니다. 대학원 석사과정 동안 항상 아낌없는 격려와 거침없는 지도를 해주신 이기완 교수님, 최광식 교수님, 이경준 교수님, 정준범 교수님, 이영돈 교수님, 송춘복 교수님, 이제희 교수님, 허문

수 교수님, 여인규 교수님, 전유진 교수님 크나큰 가르침과 하늘같은 은혜에 너무 감사합니다. 그리고 석사 생활을 의대에 와서 시작하게 되었는데 타과 학생인데도 불구하고 많은 관심을 가져주신 조문제 교수님, 고영상 교수님, 이근화 교수님, 김수영 교수님, 이영기 교수님, 박덕배 교수님 진심으로 감사 드립니다.

제가 처음 실험실에 왔을 때 아무 것도 모르던 저에게 군소리 없이 이것저것 많이 가르쳐 주신 문동오 선배님, 문옥씨 시간이 지날수록 그 고마움이 더 커지는 것이 어떻게 말로 표현할 수가 없네요. 정말 너무너무 감사 드립니다.

제 주위에서 격려와 대학원 생활에 대해서 가르쳐 주신 세진이형, 봉주형, 성삼이형, 철홍이형, 상우형, 맹진이형, 송원이형, 현성이형, 수진이형, 길남이형, 승홍이형, 만철이형, 주상이형, 성표형, 용운이형, 현실이누나 덕분에 수월한 대학원 생활을 해서 그 고마움을 어찌 표현을 해야할 지 모르겠습니다. 그리고 실험실에서 고생만 한 학부 동기이자, 석사 선배인 상혁이에게 항상 미안하고 고마워요. 무척추 연구실에 봉규, 사료학 연구실의 경용, 진이, 함덕 연구소에 형철이, 유전학 연구실에 영득이, 해양자원이용 연구실에 선희, 성명이, 긴내, 그리고 우리 학부 동기들, 석사 생활 이전부터 친하게 지내며 실험적으로 또 정신적으로 많이 도와주셔서 모두모두 고맙습니다. 또 이번에 의대에 조교로 와서 저와 같이 생활한 예쁜 후배 아름다슬이에게도 열심히 하라고 하고 싶습니다. 그리고 석사 동기인 지연누님, 지웅, 현기, 희중, 희도, 지금은 고향으로 돌아간 싸오 다들 고생하셨고, 아무쪼록 하는 일이 모두가 잘되었으면 합니다.

처음 의과대로 와서 아무것도 모르는 저에게 하나하나 친절히 가르쳐주시며 용기를 북돋아 주신 경아누나, 미경누나, 장예 감사의 말씀을 어찌 한 문장으로 끝낼 수 있겠습니까? 너무너무 감사합니다. 그리고 석사 동기인 동옥이와 지금은 졸업을 하여 여기엔 없지만 저를 잘 따라주고 실험을 가르쳐 줬던 지홍이에게도

고맙다는 말을 전하고 싶습니다. 그리고 같은 실험실에 있으면서 이것 저것 많이 가르쳐 주시고 도와주신 희경누나, 영미누나, 남재국 선생님, 진영이, 지금은 졸업하여 안 계신 지은누나 모두모두 감사합니다. 그리고 상철이형, 경진이형, 정일이형, 재희누나, 민경씨, 혜진씨, 연희씨, 윤실씨, 형준이, 정은누나, 수길이형, 지강이형, 명선이형(조교형), 그리고 행정실 선생님들 낯선 저를 친절하게 대해주시고 많은 것을 가르쳐 주셔서 감사합니다.

마지막으로 저를 낳으시고 길러주신 아버지, 어머니, 할머니, 할아버지 제가 공부한다고 하였을 때 어려운 집안 환경에도 불구하고 거절하지 않으시고 오히려 용기를 북돋아 주시고 항상 기도해 주셔서 감사합니다. 꼭 더욱 열심히 하여 부모님께 효도겠습니다. 그리고 지금은 대전에서 중학교 선생님을 하고 있는 우리 형 기환이형과 일본 홋카이도에서 장학금과 아르바이트로 번 돈으로 대학에 다니고 있는 우리 동생 경실이, 다들 뿔뿔이 흩어진 어려운 환경에도 항상 저를 믿고 응원해 주어서 너무너무 감사합니다. 나중에 꼭 성공하여 멋진 모습으로 가족이 재회하는 날을 기다려 봅니다.

옆에서 지켜봐 주시며 도와주신 분들이 아직도 많이 남았지만 일일이 감사의 말씀을 전하지 못하여 죄송할 다름입니다. 감사의 말씀을 못 드린 모든 분들께 마지막을 빌어 진심으로 감사하다는 말씀을 올립니다.