BMB
Reports

BMB Rep. 2014; 47(2): 98-103
www.bmbreports.org

Anti-oxidizing effect of the dichloromethane and hexane fractions from *Orostachys japonicus* in LPS-stimulated RAW 264.7 cells via upregulation of Nrf2 expression and activation of MAPK signaling pathway

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Orostachys japonicus **shows various biological activities. However, the molecular mechanisms remain unknown in LPS-stimulated macrophages. Here, we investigated the anti-oxidizing effect of the dichloromethane (DCM) and hexane fractions from *O. japonicus* (OJD and OJH) against oxidative stress in RAW 264.7 cells stimulated by LPS. OJD and OJH significantly increased the expression of heme oxygenase-1 (HO-1) in a dose- and time-dependent manner. Additionally, it was found that the expression of HO-1 was stimulated by Nrf2 activated via degradation of Keap1. ERK and p38 inhibitors repressed HO-1 induced by OJD and OJH in LPS-stimulated cells, respectively. In conclusion, these results suggest that OJD and OJH may block oxidative damage stimulated by LPS, via increasing the expression of HO-1 and Nrf2, and MAPK signaling pathway. [BMB Reports 2014; 47(2): 98-103]**

INTRODUCTION

LPS, a component of the outer membrane of Gram-negative bacteria, is considered to be the most potent activator of macrophages. LPS-induced activation of macrophages shows some results in the production of inflammatory molecules and various reactive oxygen species (ROS) (1). An increase in intracellular ROS level has been shown to damage tissues and cells via lipid peroxidation, protein cross-linkage, and DNA breakage processes, which is effectively prevented by many kind of anti-oxidants and free radical scavengers (2). Heme oxygenase-1 (HO-1), as a stress-inducible enzyme, has a potent anti-inflammatory and anti-oxidant function (3). HO-1 is well known for using heme as a substrate, to produce bilirubin/biliverdin and carbon monoxide (CO). The subsequent metabolites of heme catabolism appear to play vital roles in regulating biological responses, including cell survival, oxidative stress, and inflammation (4). HO-1 is mediated by activation of relevant cytoplasmic transcription factors, such as nuclear factor E2-related factor 2 (Nrf2), activator protein (AP)-1, and cAMP-responsive element-binding protein (CREB). Nrf2 is a redox-sensitive transcription factor, which is a member of the cap-'n'-collar subfamily of basic leucine zipper (bZIP). Activated Nrf2 binds to antioxidant response elements (ARE) located in the promoter region of genes encoding many antioxidant enzymes including NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST), and HO-1 (5). The HO-1 expression and Nrf2 transcription factor are induced through the major MAPK signaling pathway (6).

Orostachys japonicus A. Berger (Crassulaceae), also referred to as Wa-song in Korea, is a perennial herb and a medicinal plant (7). It has been traditionally used as a general anti-inflammatory remedy to treat hepatitis, boils, and hemorrhoids; as a hemostatic agent to treat hematemesis, epistaxis, and hematochezia; and as an anti-cancer remedy (8). It has potent anti-inflammatory, antidote, anti-febrile, antioxidant and anti-cancer properties (9). However, mechanisms underlying the anti-oxidizing effect of OJD and OJH remain to be elucidated. In the present study, we investigated the anti-oxidizing effect of OJD and OJH in LPS-stimulated RAW 264.7 cells and examined whether OJD and OJH could inhibit oxidant responses via up-regulation of Nrf2 and activation of the MAPK signaling pathway.

RESULTS

Effect of OJD and OJH on cell viability

To assess whether the tested OJD and OJH affected cell via-

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http://dx.doi.org/10.5483/BMBRep.2014.47.2.088

Received 19 April 2013, Revised 14 May 2013, Accepted 13 June 2013

Keywords: Anti-oxidizing effect, HO-1, MAPK, Nrf2/Keap1, *Orostachys japonicus*

ISSN: 1976-670X (electronic edition)

bility, RAW 264.7 cells were incubated with LPS in the presence of OJD or OJH (0, 25, 50, 75, 100 and 150 μg/ml). As shown in Fig. 1A and B, cell viability was not affect by a 24 h treatment with OJD and OJH. However, OJD and OJH were cytotoxic at increasing concentration. The data showed as previously described by our team (10, 12).

Effect of OJD and OJH on DPPH radical scavenging activity

The DPPH radical assay can accommodate many samples in a short time period and is sensitive enough to detect active ingredients at low concentration (13). As shown in Fig. 1C, the OJD and OJH displayed a dose-dependent DPPH radical scavenging activity. The extracts of *O. japonicus* scavenged DPPH radical with an IC_{50} value of 32.05 μg/ml and 205.76 μg/ml for OJD and OJH, respectively. In particular, radical scavenging of the OJD (100 μg/ml) showed similar to ascorbic acid (25 μg/ml).

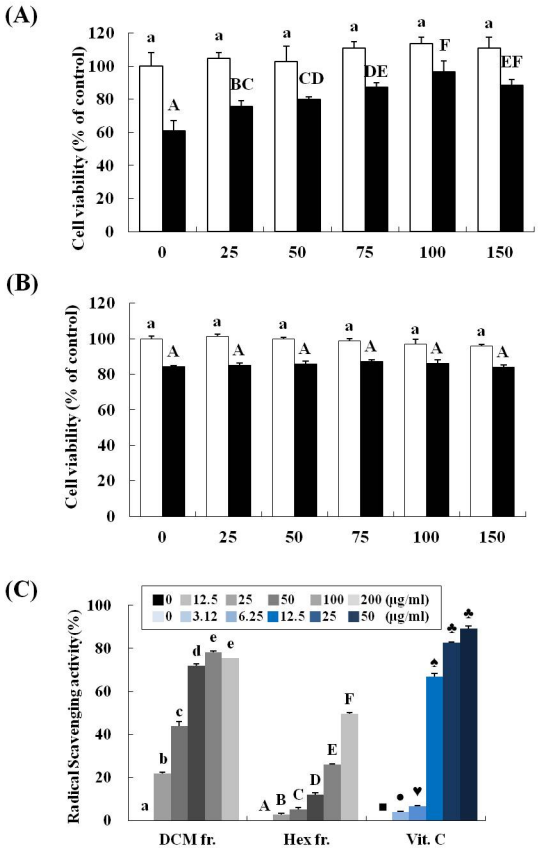

Fig. 1. Effect of OJD and OJH on cell viability and DPPH radical scavenging activities. Cells were incubated in the presence of (A) OJD, and (B) OJH, or in combination with LPS (1 μg/ml) for 24 h. Cell viability was determined by the MTS assay (open bar, LPS untreated; closed bar, LPS treated). These data were previously reported by Lee *et al* (10, 12). (C) DPPH radical scavenging activities of OJD and OJH form *O. japonicus*. A value sharing the same superscript is not significantly different at $P < 0.05$.

Effect of solvent fractions on the expression of HO-1 in LPS-stimulated cells

To investigate if *O. japonicus* induced the anti-oxidative enzyme HO-1, the expression of HO-1 was determined by Western blot analysis. Cells were stimulated with LPS in the

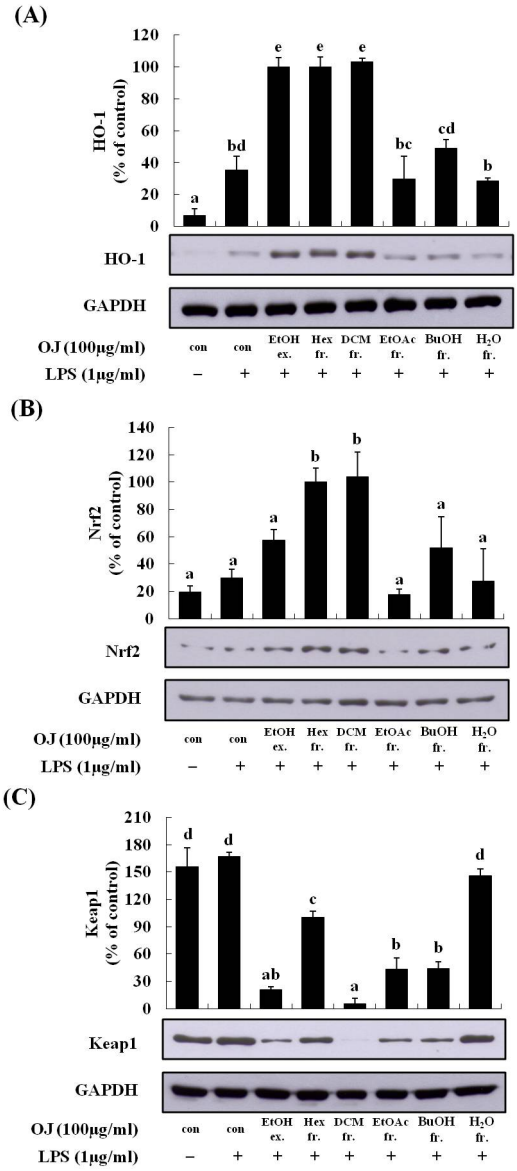

Fig. 2. Effect of various solvent factions from *O. japonicus* on expression of HO-1, Nrf2, and Keap1 protein in LPS-stimulated cells. Cells were pre-incubated with various solvent factions for 1 h, and then incubated with LPS (1 μg/ml) for 12 h. Total cell lysates of the proteins were subjected to Western blot analysis. The ratio of immunointensity between the (A) HO-1, (B) Nrf2, (C) Keap1, and GAPDH was calculated. A value sharing the same superscript is not significantly different at $P < 0.05$.

presence of organic solvent fractions for 12 h. As shown in Fig. 2A, the expression of HO-1 was significantly increased in the DCM and hexane fraction. Furthermore, we observed the expression of HO-1 by OJD and OJH through a wide range of conditions. As shown in Fig. 3, the expression of HO-1 was markedly increased in a dose- and time-dependent manner.

Effect of solvent fractions on the expression of transcription factor in LPS-stimulated cells

To explore the role of transcription factor Nrf2 activation in mediating the stimulation of HO-1 expression, expression of Nrf2 was evaluated by Western blotting analysis. Cells were stimulated with LPS in the presence of organic solvent fractions for 12 h. As shown in Fig. 2B, the expression of Nrf2 was significantly increased in the DCM and hexane fraction compared to the remnant faction. Keap1 functions as an inhibitory partner for the inactivation of Nrf2 in cellular stress by various stimulations (14). To further investigate the interaction between Nrf2 and Keap1, Keap1 was measured by Western blotting analysis, with treatment of organic solvent fractions. As shown in Fig. 2C, the expression of Keap1 was significantly decreased in the DCM and hexane fraction, most significantly in the DCM fraction. Also, as shown in Fig. 3, activation of Nrf2 by OJD and OJH was dramatically increased in a dose- and time-dependent manner. However, expression of Keap1 was decreased in a dose- and time-dependent manner.

Effect of OJD and OJH on specific MAPK inhibitors in LPS-stimulated cells

To determine which of the activated MAPK are instrumental in Nrf2-mediated increase in HO-1 gene expression by OJD and OJH, the cells were pretreated with inhibitors for various kinase (SB203580, p38 inhibitor; SP600125, JNK inhibitor; U0126, ERK inhibitor) for 30 min followed by treatment of each fraction and LPS for 12 h, and protein levels of HO-1 were assayed for Western blotting analysis. As shown in Fig. 4A, treatment of the cells with OJD and U0126 inhibitor significantly inhibited HO-1 expression. However, treatment of the cells with SB203580 or SP600125 inhibitor make no significant difference with OJD (100 μg/ml) and LPS. To confirm the effect of OJH, we examined the expression of HO-1 in the same treatment by Western blotting assay. As shown in Fig.

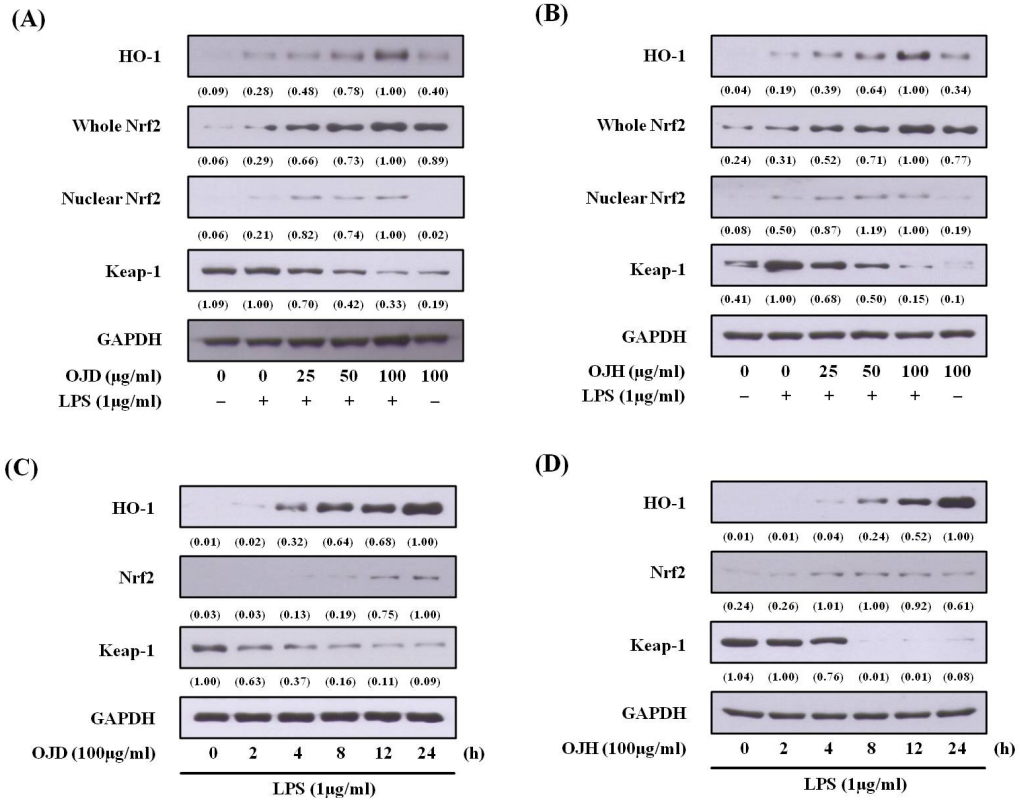

Fig. 3. Effect of OJD and OJH on expression of HO-1, Nrf2, and Keap1 protein in LPS- stimulated cells. Cells were pre-incubated with OJD and OJH for 1 h, and then incubated with LPS (1 μg/ml) for (A) and (B) 12 h, and (C) and (D) different times. Total cell lysates of the proteins were subjected to Western blot analysis. The ratio of immunointensity between the HO-1, Nrf2, Keap1 and GAPDH was calculated. A value sharing the same superscript is not significantly different at $P < 0.05$.

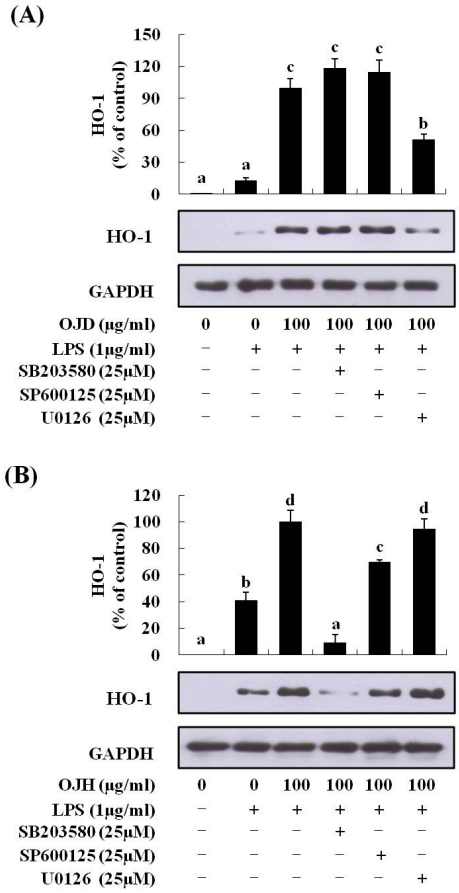

Fig. 4. Effect of OJD and OJH on MAPK inhibitors in LPS-stimulated cells. Cells treated with LPS (1 μg/ml) for 12 h in the presence of MAPK inhibitors and (A) OJD, and (B) OJH (100 μg/ml). Total lysates of the proteins were subjected to Western blot analysis. The ratio of immunointensity between the HO-1 and GAPDH was calculated.

4B, treatment of the cells with OJH and SB203580 inhibitor profoundly inhibited HO-1 expression.

DISCUSSION

To our knowledge, this is the first study to demonstrate the anti-oxidant effect of the DCM and hexane fractions from *O. japonicus* in LPS-stimulated RAW 264.7 cells. To evaluate these anti-oxidant effects, the cells were pretreated with fractions from an organic solvent; then, oxidative stress was induced by LPS stimulation. The LPS-induced activation of macrophages resulted in the production of bioactive lipids, reactive oxygen species, and in particular, inflammatory cytokines (1). Inducible nitric oxide synthase (iNOS) stimulated by LPS catalyzed the production of nitric oxide (NO) from L-arginine (15). Superoxide (O_2^-) and NO promoted the production of peroxynitrite ($ONOO^-$), a highly cytotoxic oxidant that is known to releases heme from intracellular heme proteins (16). Heme oxygenases (HOs) are enzymes responsible for catalyzing heme degradation, and producing 4 metabolites: iron, carbon monoxide (CO), biliverdin, and bilirubin (2). There are 3 types of HOs including HO-1, HO-2, and HO-3. HO-1 is localized in the non-neural tissues, and is induced in response to oxidative stress, nitrosative stress, and the release of cytokines. In contrast, HO-2 and HO-3 are mostly found in neural tissues, and are constitutively expressed (17). The HO-1 enzyme is believed to beneficially play a cytoprotective role in a variety of pathological models such as that of inflammation, or an oxidative disorder. The anti-oxidant properties of HO-1 are related to the inhibition of formation of adhesion molecules, such as NO, and reduction of oxidative stress CO produced through the degradation of heme decreases the production of inflammatory mediators, such as cytokines and NO (18). Previous studies have shown that OJD and OJH effectively decreased NO production in LPS-stimulated RAW 264.7 cells (10, 12). In the present study, OJD and OJH significantly induced HO-1 expression in LPS-stimulated RAW 264.7 cells in a dose- and time-dependent manner (Figs. 2A and 3). These results suggest that OJD and OJH exert strong cytoprotective effect, through increased expression of HO-1 and suppression of NO production, thereby inhibiting $ONOO^-$ formation.

Many reports suggest that HO-1 induction is dependent on the activation or nuclear translocation of Nrf2. The transcription factor Nrf2 plays an important role in the upregulation of the expression of anti-oxidant enzymes, such as HO-1, NQO1, and GST (19). Under quiescent conditions, Nrf2 is located in the cytoplasm as an inactive complex, with a Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1) homodimer which facilitates the ubiquitination of Nrf2, via the Cul3 E3 ligase and proteasomal degradation (20). On the other hand, oxidative stimulation can block Nrf2 degradation by the 26S proteasome. Nrf2 releases from the Keap1 complex, and translocates to the nucleus, for activation of ARE-containing genes (21). Keap1 interacts with LC3 in a stimulation-inducible manner, and this interaction is mediated by p62. Keap1 is involved in a selective autophagic degradation pathway that degrades ubiquitinated protein aggregates (22). Thus, OJD- and OJH-induced Nrf2-ARE activation may be linked to the relative levels of Nrf2-Keap1 expression. For this reason, we examined the effects of OJD and OJH on the endogenous level of Nrf2 and Keap1 expression. We found that OJD and OJH significantly enhanced the expression of Nrf2 protein in a dose- and time-dependent manner in LPS-stimulated cells (Figs. 2B and 3). In contrast, OJD and OJH reduced the expression of the Keap1 protein under the same treatment (Figs. 2C and 3). Our results indicate that OJD and OJH may stimulate Nrf2- mediated ARE activation by increasing Nrf2 protein levels, but reducing Keap1 at the same condition.

MAPKs are a highly conserved family of protein serine/threonine kinases and include the ERK1/2, JNK, and p38 subgroups.

MAPK signaling represents a central regulatory pathway for Nrf2 activation associated with inducible expression of antioxidant enzymes, including HO-1 (6, 23, 24). Our previous studies showed that OJD inhibited phosphorylation of JNK and p38, but, increased ERK phosphorylation in a dose-dependent manner (10). We also found that OJH slightly increased phosphorylation of ERK and p38 in a dose-dependent manner while JNK levels were unchanged (12). To further confirm that the MAPK signaling pathway was involved in the anti-oxidant effect of OJD and OJH on LPS-induced oxidative stress, specific MAPK inhibitors were used (SP600125, JNK inhibitor; SB203580, p38 inhibitor; U0126, ERK inhibitor). Only the ERK inhibitor significantly attenuated OJD-induced HO-1 upregulation, and only the p38 inhibitor profoundly inhibited OJH-induced HO-1 expression. These results indicate that the anti-oxidant mechanism of OJD and OJH-induced HO-1 upregulation involves the phosphorylation of ERK and p38, respectively (Fig. 4).

In conclusion, this study showed that OJD and OJH significantly increased the expression of HO-1, and that this effect is mediated by the activation of Nrf2, and the degradation of Keap1 via upregulation of ERK and p38 signaling pathways, respectively. These results also suggest that the DCM and hexane fractions of *O. japonicus* may be potent natural protective agents against oxidative disorders caused by LPS-stimulated inflammation.

MATERIALS AND METHODS

Cell line and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and antibiotics were purchased from Hyclone (Thermo Fisher Scientific Inc, Waltham, MA, USA). LPS, SB 203580 (p38 inhibitor), SP 600125 (JNK inhibitor), and U0126 (ERK inhibitor) were purchased from Sigma (St. Louis, MO, USA). Antibodies against HO-1, Nrf2, Keap1, Goat anti-rabbit IgG-HRP secondary antibody, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals were of analytical grade.

Cell culture

Murine RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in DMEM supplemented with 10% fetal bovine serum, 1% streptomycin/penicillin, at 37°C in a 5% CO_2 incubator. The cells were sub-cultured every 2~3 days at 1 : 5 split ratios.

Preparation of dichloromethane and hexane fraction from *O. japonicus*

Dried *O. japonicus* was provided by Geobugiwasong Ltd. (Miryang, Korea). Dichloromethane and hexane fraction from *O. japonicus* were fractioned as previously described by our team (9, 10). The extracted dichloromethane and hexane fraction were concentrated by rotary evaporation at 40°C up to dryness, and dissolved in dimethyl sulphoxide (DMSO) for the experiment.

GC-MS analysis

DCM and hexane fractions from *O. japonicus* were analyzed as previously described by our team (10, 12).

Cell viability assay

Cell viability was measured with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instruction. Briefly, RAW 264.7 cells (5×10^4 cells/well in 96-well culture plate) were incubated at 37°C (5% CO_2) with different concentration of OJH or in combination with LPS for 24 h. After incubation time, twenty micro liter of MTS[3-(4,5-dimethylthiaxol-2-yl)-5-(3carboxy-methoxyphenyl)-2(4-sulfophenyl)- 2H-tetrazolium, inner salt] was added to the culture plate and cultured for 2 h at 37°C, in a humidified 5% CO_2 atmosphere. The absorbance in the wells was 490 nm, using a fluorescence multi-detection reader (Synergy™ HT, BioTek, Winooski, VT, USA).

DPPH radical scavenging activity

The scavenging activity of extracts on the DPPH radical was estimated according to the method of Khlifi *et al.* (11). The extracts (0-200 μg/ml) were added to methanolic solution (0.1 ml) of DPPH radical (final concentration of DPPH was 0.2 mM). The absorbance at 517 nm was measured, after the solution was maintained in the dark for 30 min. Ascorbic acid was used as a positive control. A lower absorbance of the reaction mixture indicated higher DPPH radical scavenging activity, which was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = (1\text{-}Abs_{sample}/Abs_{control}) \times 100$$

Western blot analysis

Samples (40 μg of proteins) were separated on 10% SDS-PAGE (polyacrylamide gels) for electrophoresis (Bio-Rad, Philadelphia, PA, USA). After electrophoresis, the gel was transferred to PVDF membrane (Bio-Rad, Philadelphia, PA, USA). The membrane was blocked for 2 h with 5% nonfat milk in PBST buffer. After blotting, the membrane was incubated for 12 h at 4°C, with 1 : 1,000 dilution of the primary antibody. The membrane was washed three times with PBST buffer at 10 min intervals. After washing, the membrane was incubated for 2 h at 4°C, with 1 : 1,000 dilution of secondary antibody (HRP conjugated goat anti rabbit IgG), and again washed in PBST three times. Blot was detected using ECL detection kits (Santa Cruz, CA, USA) and exposure to X-ray film. The intensity of the bands was measured by densitometry analysis with PDQuest software (version 7.0, Bio-Rad, Philadelphia, PA, USA).

Statistical analysis

All experiments were repeated at least three times. The results

were represented as mean ± SD. All experimental data were treated by SPSS program (SPSS 12.0, SPSS Institute, and Chicago, IL, USA). One-way ANOVA with Duncan's multiple range tests was used to examine the difference between groups. A $P < 0.05$ was considered statistically significant.

ACKNOWLEDGEMENTS

This work was supported by a 2013 Inje University research grant.

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