Brassicaphenanthrene A from *Brassica rapa* protects HT22 neuronal cells through the regulation of Nrf2-mediated heme oxygenase-1 expression

HWAN LEE[1*], WONMIN KO[1*], ANISUZZAMAN CHOWDHURY[1], BIN LI[2], SAM CHEOL KIM[3], HYUNCHEOL OH[4], YOUN-CHUL KIM[4], EUN-RHAN WOO[1], NAM-IN BAEK[5] and DONG-SUNG LEE[1]

[1]College of Pharmacy, Chosun University, Dong-gu, Gwangju 61452, Republic of Korea; [2]Department of Pharmacy, Qingdao University of Science and Technology, Qingdao, Shandong 266042, P.R. China; [3]Department of Family Practice and Community Medicine, Chosun University College of Medicine, Dong-gu, Gwangju 61452; [4]College of Pharmacy, Wonkwang University, Iksan, Jeollabuk-do 54538; [5]Graduate School of Biotechnology and Department of Oriental Medicinal Biotechnology, Kyung-Hee University, Yongin, Gyeonggi-do 17104, Republic of Korea

Received April 30, 2019; Accepted July 9, 2019

DOI: 10.3892/mmr.2019.10824

Abstract. Brain cell damage that results from oxidative toxicity contributes to neuronal degeneration. The transcription factor nuclear factor-E2-related factor 2 (Nrf2) regulates the expression of heme oxygenase (HO)-1 and glutathione (GSH), and serves a key role in the pathogenesis of neurological diseases. *Brassica rapa* is a turnip that is unique to Ganghwa County, and is used mainly for making *kimchi*, a traditional Korean food. In the current study, brassicaphenanthrene A (BrPA) from *B. rapa* was demonstrated to exhibit protective effects against neurotoxicity induced by glutamate via Nrf2-mediated HO-1 expression. Similarly, BrPA increased the expression of cellular glutathione and glutamine-cysteine ligase genes. Furthermore, BrPA caused the nuclear translocation of Nrf2 and increased antioxidant response element (ARE) promoter activity. Nrf2 also mediated HO-1 induction by BrPA through the PI3K/Akt and JNK regulatory pathways. The results of the present study indicated the neuroprotective effect of BrPA, a natural food component from *B. rapa*.

Correspondence to: Professor Dong-Sung Lee, College of Pharmacy, Chosun University, 309 Pilmun-daero, Dong-gu, Gwangju 61452, Republic of Korea
E-mail: dslee2771@chosun.ac.kr

*Contributed equally

Key words: brassicaphenanthrene A, heme oxygenase-1, mouse hippocampal HT22 cell line, neuroprotection, nuclear factor-E2-related factor 2, oxidative stress

Introduction

Neurodegeneration refers to the gradual loss of neuron structure or function, including neuronal death. Diseases such as stroke, Parkinson's, Alzheimer's, and Huntington's disease result from neurodegeneration. Brain tissue is vulnerable to oxidative stress from either pathological or physiological causes, which results from the aging processes or neurodegenerative diseases (1). Oxidative stress in neurons is a major cause of neuronal apoptosis in neurodegenerative diseases (2).

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway is known to regulate various intracellular downstream signals such as apoptosis, cell growth, and differentiation, through oxidative stimulation (3). In addition, PI3K activates nuclear transcription of the nuclear factor-E2-related factor 2 (Nrf2), which promotes neural cell survival (4,5). Nrf2 has a leading role in the production of phase II enzymes regulated by antioxidant response elements (AREs) (6,7). Previous studies have reported the effect of Nrf2 activation and the subsequent HO-1 expression on nerve damage and oxidative stress in various models of neurodegenerative disorders (8,9). HO-1 is instrumental in the regulation of the biological responses that protect cells against oxidative stress-induced toxicity (10). In addition, HO-1 expression by inducers is one of the major cytoprotective mechanisms in HT22 cells with glutamate-induced oxidative toxicity (11). In addition, the activation of mitogen-activated protein kinase (MAPK) regulates the expression of several genes and proteins related to cytoprotective mechanisms, including the activation of HO-1 (12).

Brassica rapa is composed of numerous compounds, including flavonoids, phenylpropanoid derivatives, indole alkaloids, sterol glucosides, and fatty acids (13,14). However, there has been no study of the molecular targets of *B. rapa* and their anti-neurodegenerative biological activity. Therefore, as part of this screening research project to assess the neuroprotective potential of natural food components from *B. rapa*, we isolated a phenanthrene-derivative named BrPA and investigated its neuroprotective effects in HT22 cells.

Materials and methods

Materials. BrPA was isolated from *B. rapa* by a previously described method (15). All cell culture reagents were purchased from Gibco; Thermo Fisher Scientific, Inc.. All inhibitors were purchased from Calbiochem. Primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc.. All other chemicals were sourced from Sigma-Aldrich; Merck KGaA, unless otherwise stated.

Cell culture and MTT assay. Mouse hippocampal HT22 cells were donated by Professor Youn-Chul Kim of Wonkwang University. Mouse hippocampal HT22 cells were incubated in DMEM containing 1% antibiotic (Penicillin-Streptomycin) and 10% heat-inactivated FBS at 37˚C in a humidified 5% CO_2 and 95% air atmosphere. The media, antibiotics and FBS used for cell culture were purchased from Gibco; Thermo Fisher Scientific, Inc.. To test cell viability by using the MTT assay, mouse hippocampal HT22 cells were maintained and treated with BrPA (2.5-20 μM) in the absence or presence of 5 mM glutamate for 12 h. The cells were then treated with 500 μg/ml MTT and incubated at 37˚C for 1 h, subsequently the resulting formazan product in each well was dissolved in DMSO. The dissolved formazan was detected at a wavelength of 540 nm.

ROS generation assay. To check ROS generation, mouse hippocampal HT22 cells were incubated treat glutamate (5 mM) with or without BrPA (2.5-20 μM) or SnPP IX (HO inhibitor) (50 μM). Subsequently, the cells were incubated at 37˚C. for 12 h after glutamate treatment. After washing, Hank's balanced salt solution containing 10 μM 2',7'-dichlorofluorescein diacetate (DCFDA) was added to each well of the cell culture plate and stained in the dark for 60 min; subsequently, the medium was removed, the cells were washed twice, and extracted with 1% Triton X-100 in PBS at 37˚C for 10 min. The fluorescence of each sample at 490 nm and 525 nm was measured by using a SpectraMax Gemini XS (Molecular Devices).

Western blotting analysis and the extraction of cytoplasmic and nuclear cell fractions. The pelleted HT22 cells were washed with PBS, and then lysed in RIPA buffer. Equal amounts of proteins quantified by BCA assay were mixed in the sample loading buffer and separated by SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane. Non-specific binding to the membrane was blocked by incubation in a solution of skimmed milk. The membrane was incubated with primary antibodies at 4˚C overnight, and then reacted with a horseradish peroxidase-conjugated secondary antibody from Santa Cruz Biotechnology. To extract the cytoplasmic and nuclear cell components, HT22 cells were washed with PBS, pelleted, and lysed with NE-PER reagents (Pierce; Thermo Fisher Scientific, Inc.). All experimental procedures were performed in accordance with the manufacturer's data sheet. Cell lysate was used for western blotting as described above.

Transfections and luciferase assays. To construct ARE-Luciferase vector, the pGL2 promoter plasmid (Promega Corporation) was introduced into 5'-TGACTC AGCA-3' at the Nrf2 binding site to bind the ARE-luciferase vector. The cell lysate for the luciferase assay was mixed with luciferase substrate solution (Promega Corporation), and luciferase activity was measured using a luminometer. Luciferase activity was determined in triplicate for each experiment and normalized for each sample using β-galactosidase activity. In addition, siRNA transfections of Nrf2 were tested using a commercially available reagent Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, cells were transiently transfected with Nrf2 siRNA for 6 h and recovered in fresh media containing 10% FBS at 37˚C for 24 h. Cells transfected with Nrf2 siRNA were cultured with BrPA (20 μM) for 1.5 h (Nuclear Nrf2) or 12 h (HO-1, GST). In addition, HT22 cells were pre-treated with or without SnPP (50 μM) and Nrf2 siRNA, and then cultured with BrPA (20 μM), and later treated with glutamate (5 mM) for 12 h. The sequences of Nrf2 siRNA were sense: 5'-GAG GAUGGGAAACCUUACUTT-3', antisense: 5'-AGUAAG GUUUCCCAUCCUCTT-3'. The sequences of scramble siRNA were sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'.

Glutathione (GSH) determination. To evaluate GSH production, mouse hippocampal HT22 cells were assessed by using a glutathione assay kit from Cayman, and all experimental procedures were performed in accordance with the manufacturer's instructions.

Statistical analyses. All data were acquired from three independent experiments and expressed as the mean ± SD. Statistical analyses were computed by using GraphPad Prism v. 3.03 (GraphPad Software Inc.). The mean differences were derived using one-way ANOVA and Newman-Keuls post hoc test, with statistical significance accepted for values of $P<0.05$.

Results

Effects of BrPA on glutamate-induced cell toxicity and ROS generation. To investigate the cytotoxic potential of BrPA (Fig. 1A), we first examined the viability of mouse hippocampal HT22 cells treated with BrPA by using the MTT assay. No decrease in cell viability was observed at concentrations below 20 μM (Fig. 1B). To investigate if BrPA exhibited cytoprotective effects and ROS scavenging effects on glutamate-stimulated cytotoxicity and ROS production, respectively, HT22 cells pre-incubated with BrPA were treated with glutamate for 12 h. BrPA increased cytoprotective activity (Fig. 1C) and ROS scavenging in glutamate-induced HT22 cells (Fig. 1D).

Effects of BrPA on HO-1 expression in HT22 cells. We examined whether BrPA affected in HO-1 protein expression. As shown in Fig. 2A, it caused a significant upregulation in HO-1 protein expression, with a maximum increase caused by 20 μM. At this concentration, peaks in HO-1 expression were evident at 6 h and approximately 18 h (Fig. 2B). To determine if the expression of HO-1 induced by BrPA was transcribed, HT22 cells were treated with actinomycin D (a transcription inhibitor) or cycloheximide (a translational

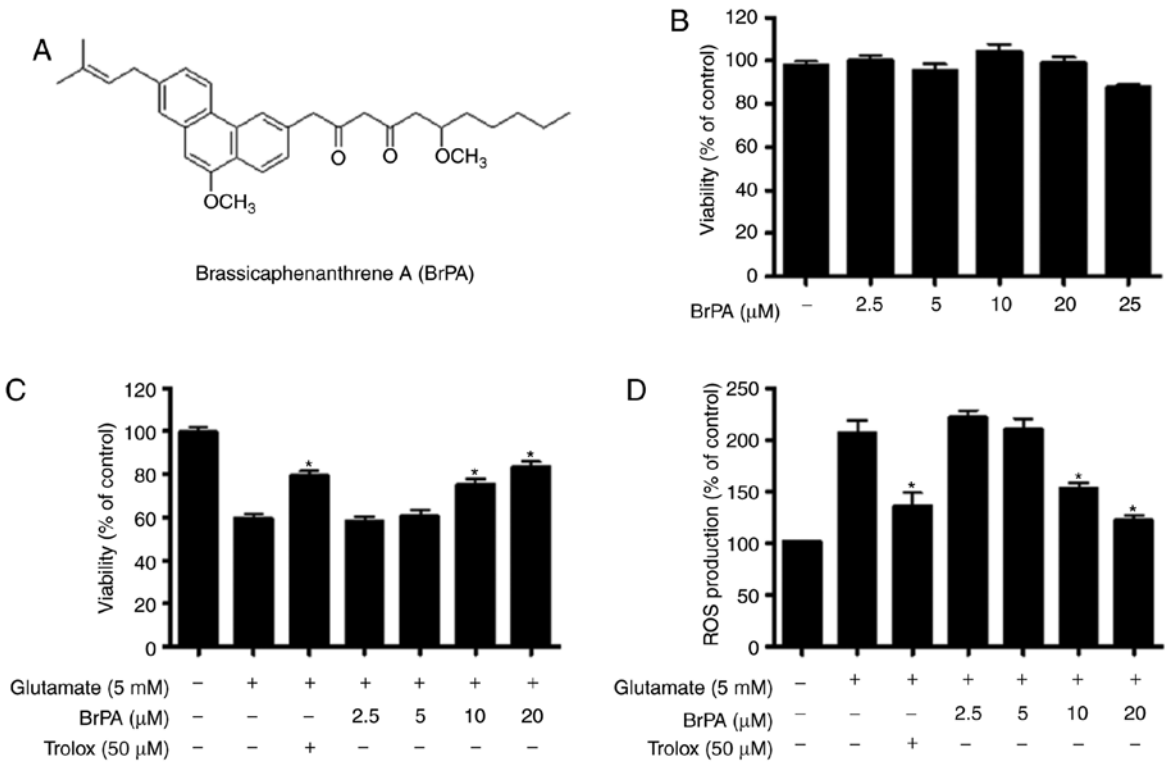

Figure 1. BrPA decreased neuronal toxicity induced by glutamate stimulation in HT22 cells. (A) The structure of BrPA. The viability of cells subjected to different concentrations of BrPA (12 h, 2.5-25 μM) was determined by (B) MTT assay. (C) Cells were then cultured with BrPA (12 h, 2.5-20 μM) and incubated with glutamate (5 mM) for 12 h. In the subsequent experiments, (D) DCFDA assay also confirmed the protection of BrPA under glutamate insults in HT22 cells. Results are indicated as mean ± standard deviation values (n=3). The positive control used was Trolox (50 μM). *P<0.05 vs. glutamate. BrPA, brassicaphenanthrene A.

inhibitor). Both treatments completely blocked HO-1 expression (Fig. 2C and D).

Effects of BrPA on cellular GSH levels and GSH synthesis enzymes. GSH was depleted in HT22 cells treated with glutamate, and cells treated with glutamate and BrPA dose-dependently prevented the glutamate-induced GSH depletion (Fig. 3A). In addition, glutathione S-transferase (GST) levels were also upregulated by treatment with BrPA (Fig. 3B). The effects of BrPA on glutamate-cysteine ligase (GCL) expression were also examined. GCL is a rate-limiting enzyme involved in GSH synthesis, consisting of a catalytic heavy subunit, glutamate-cysteine ligase catalytic subunit (GCLC), modulatory light subunit, and a glutamate-cysteine ligase modifier subunit (GCLM). As shown in Fig. 3B, BrPA treatment increased GCLC and GCLM in a dose-dependent manner.

Effects of BrPA on the regulation of Nrf2-related signaling. We investigated the translocation of Nrf2 to the nucleus of HT22 cells treated with 20 μM BrPA for 0.5, 1, and 1.5 h. Nrf2 levels gradually increased in the nuclear fraction, but were decreased in the cytoplasmic fraction of cells treated with BrPA, suggesting that BrPA promoted the nuclear translocation of Nrf2 (Fig. 4A). In addition, BrPA increased the expression of total Nrf2 (Fig. 4A). BrPA was modulated in transiently transfected HT22 cells with the ARE-luciferase plasmid and the modulating effect of luciferase activity was measured by using the ARE activation assay. BrPA upregulated ARE-driven luciferase activity (Fig. 4B), and this activation was closely correlated with the increase in HO-1 and expression of GST (Figs. 2 and 3). Similarly, we tested BrPA-induced HO-1 and GST expression by using siRNA to silence Nrf2. Transient transfection of HT22 cells with siRNA Nrf2 was performed and the cells were treated with 20 μM BrPA for 12 h (HO-1 and GST) or 1.5 h (nuclear Nrf2). Nrf2 siRNA significantly blocked BrPA-induced Nrf2 nuclear translocation. Transient transfection with Nrf2 siRNA reduced BrPA-induced GST and HO-1 expression (Fig. 4C).

Effects of BrPA on cell protection through the Nrf2-regulated HO-1 expression. In this experiment, we examined whether the BrPA-induced protein expression of HO-1 was involved in cytoprotection or ROS inhibition in HT22 cells. The cells were treated with 20 μM BrPA for 12 h in the presence or absence of SnPP IX, an inhibitor of HO-1 activity. SnPP IX markedly inhibited BrPA-mediated protection (Fig. 5A). The expression of HO-1 by BrPA was also important for the inhibition of glutamate-induced ROS production (Fig. 5B). Similarly, we evaluated the role of BrPA-induced Nrf2 translocation in cell protection and ROS inhibition. The transient transfection of HT22 cells with Nrf2 siRNA was performed and the cells were treated with 20 μM BrPA and stimulated with glutamate. As shown in Fig. 5, when the cells were transfected with Nrf2 siRNA, the cytoprotective and ROS inhibitory effects of BrPA were decreased.

Involvement of the MAPK pathway in BrPA-induced HO-1 expression. When oxidative stress increases within a cell,

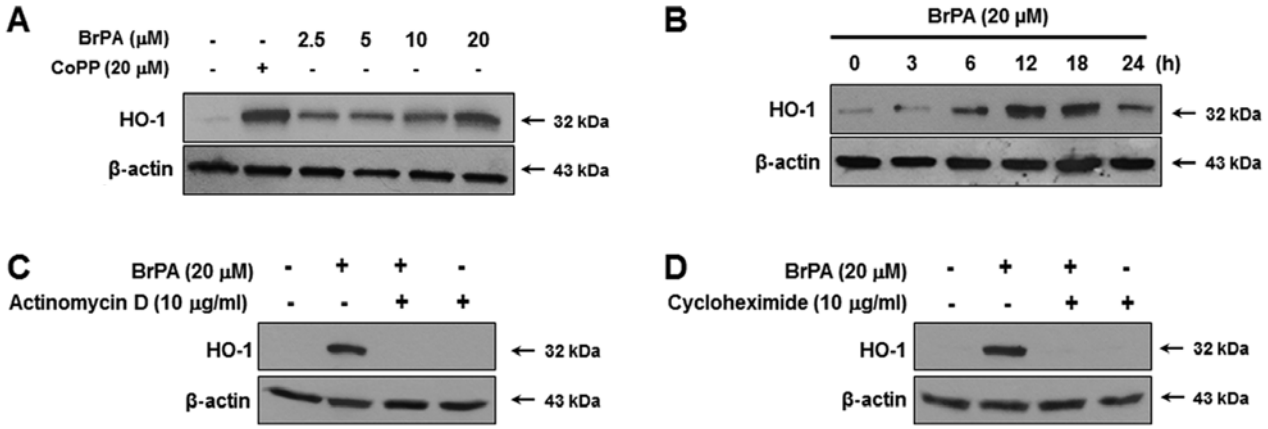

Figure 2. BrPA modulated the protein expression of HO-1 in HT22 cells. HO-1 expression during transcription and translation were analyzed using western blot analysis. The cells were incubated for 12 h with indicated concentrations of BrPA (A), or specified time periods with 20.0 μM of BrPA (B) in HT22 cells. The cells were incubated with (C) actinomycin D (5 μg/ml) or (D) cycloheximide (10 μg/ml) for 1 h, and cultured with BrPA (20 μM) for 12 h. The positive control used was CoPP (20 μM). BrPA, brassicaphenanthrene A; H heme oxygenase.

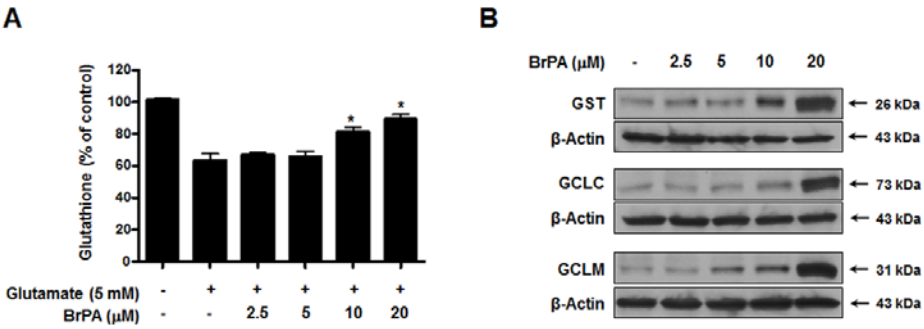

Figure 3. BrPA reduced glutamate-induced glutathione depletion, and (B) upregulated the protein expression of GST, GCLC, and GCLM. HT22 cells were pre-treated with BrPA (2.5-20 μM) for 12 h, and then (A) treated with glutamate (5 mM) for a further 12 h. The results are presented as the mean ± standard deviation (n=3). *P<0.05 vs. glutamate. BrPA, brassicaphenanthrene A.

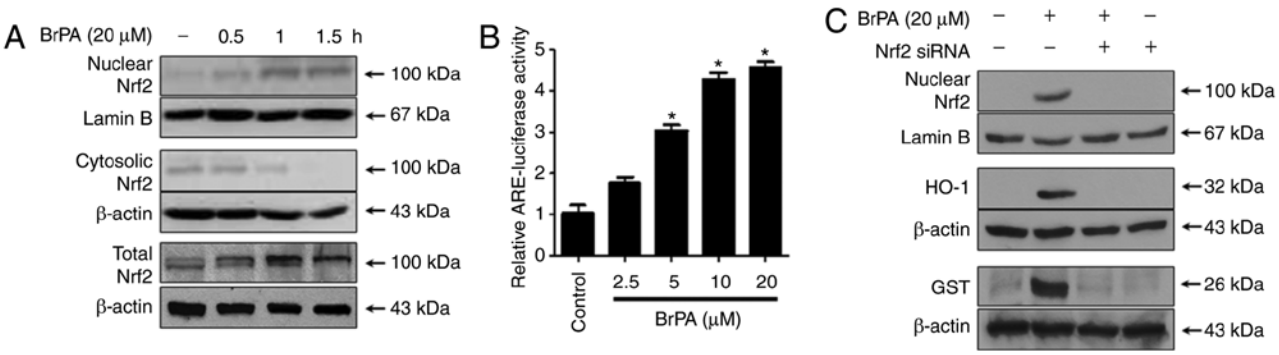

Figure 4. BrPA enhanced the nuclear translocation of (A) Nrf2, (B) ARE activation, and also HO-1 and GST expression via the (C) Nrf2 pathway in HT22 cells. Cells were cultured with BrPA (20 μM) for the indicated times (0.5, 1 and 1.5 h). ARE-luciferase or control vector-transfected quiescent cells and then incubated with BrPA (2.5-20 μM) for 1 h. Cells transfected with Nrf2 siRNA were cultured with BrPA (20 μM) for 1.5 h (Nuclear Nrf2) or 12 h (HO-1; GST). The results are presented as the mean ± standard deviation (n=3). *P<0.05 vs. control. BrPA, brassicaphenanthrene A; Nrf2, nuclear factor-E2-related factor 2; HO, heme oxygenase; si, small interfering.

MAPK signaling is activated. The activation of the MAPK pathway affects the expression of HO-1 (16). The largest increase in BrPA-induced expression of HO-1 occurred at a concentration of 20 μM BrPA (Fig. 2). BrPA (20 μM) activated the JNK pathway and upregulated JNK phosphorylation. As shown in Fig. 6A, the phosphorylation of JNK was observed 30 min after BrPA treatment. In contrast, p38 and ERK kinases were not phosphorylated in any of the time periods tested. In addition, specific inhibitors of MEK 1/2 (the kinases upstream of ERK 1/2; PD98059), JNK (SP600125), and p38 (SB203580) were used to confirm the association of BrPA-induced MAPK regulation and HO-1 expression with cytoprotective effects. The JNK inhibitor (SP600125) markedly attenuated BrPA-increased HO-1 expression, whereas the p38 and ERK

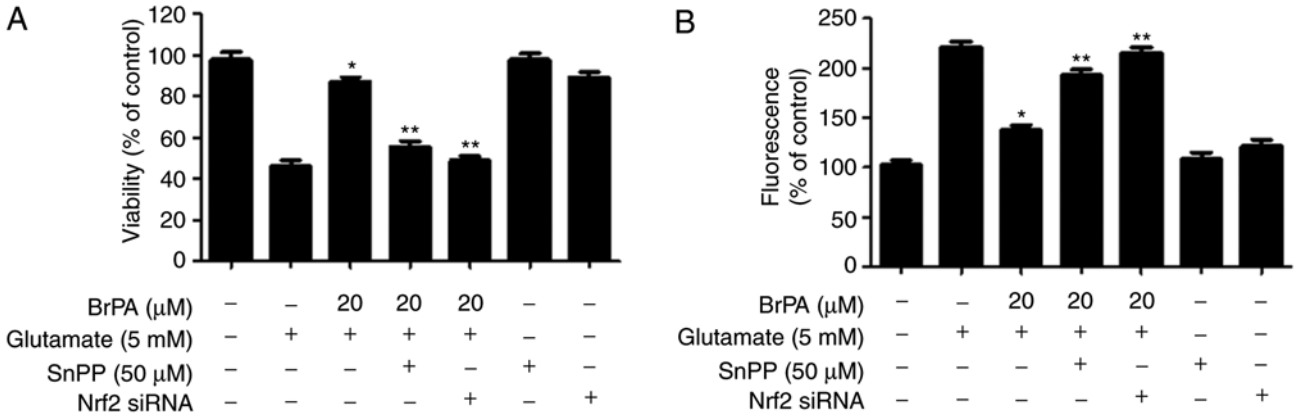

Figure 5. BrPA-induced HO-1 expression inhibited glutamate-induced cell death (A) and ROS production (B). HT22 cells were pre-treated with or without SnPP (50 μM) and Nrf2 siRNA, and then cultured with BrPA (20 μM), and later treated with glutamate (5 mM) for 12 h (A). Subsequently, the ROS-sensitive fluorophore DCF (10 μM) was added. Results are presented as the mean ± standard deviation (n=3). *P<0.05 vs. glutamate. **P<0.05 vs. glutamate with BrPA (20 μM). HO, heme oxygenase; BrPA, brassicaphenanthrene A; ROS, reactive oxygen species; si, small interfering.

inhibitors were ineffective (Fig. 6B). As expected, the JNK inhibitor (SP600125) reduced the cytoprotective effect of BrPA, but not the p38 and ERK inhibitors (Fig. 6C).

Involvement of the PI3K/Akt pathway in the BrPA-induced HO-1 expression. Phosphatidylinositol 3-kinase (PI3K), which is regulated by various phytochemicals, is known to be related to the expression of HO-1 (17). To verify an association between BrPA-induced Akt activation and HO-1 expression, Akt phosphorylation mediated by BrPA was examined in HT22 cells by using an anti-phospho-Akt antibody. After treatment with BrPA, Akt was phosphorylated for 15-60 min, after which the phosphorylation decreased gradually (Fig. 7A). The inhibitor of the PI3K pathway, LY294002, inhibited BrPA-enhanced cytoprotection (Fig. 7B). Furthermore, pre-treatment with LY294002 attenuated BrPA-induced phosphorylation of Akt and JNK (Fig. 7C). Pre-treatment with LY294002 caused a similar attenuation of the nuclear translocation of BrPA-induced Nrf2, and of HO-1 expression in the cells (Fig. 7D).

Discussion

B. rapa ssp. *campestris* is a staple constituent of *kimchi*, a traditional Korean food. It is a type of turnip commercially cultivated in Ganghwa Island, Korea (18). *B. rapa* is known to relieve chronic constipation and liver illnesses and ameliorate kidney function (19). However, no study has yet been conducted on the molecular mechanisms of the neuroprotective effects on the pharmacological activities of its constituents. This study has provided evidence that brassicaphenanthrene A (BrPA) isolated from the roots of *B. rapa* exerted neuroprotection through upregulation the Nrf2/MAPK pathway, following the activation of HO-1 expression. This study has presented the first evidence that natural extracts from the roots of *B. rapa* can exert antioxidant effects against glutamate-induced neurotoxicity by the activation of Nrf2/HO-1 signaling in HT22 cells.

BrPA resulted in a significant dose-dependent reduction in glutamate-induced reactive oxygen generation in immortalized mouse hippocampal HT22 cells. It is known that neuronal cells, such as primary neuronal cells and tissue slices, can be damaged by oxidative glutamate toxicity (20). Therefore, glutamate-induced oxidative damage is one of the key contributors to pathological neuronal cell damage (21). To combat this, natural compounds have an intrinsic antioxidant effect on glutamate-induced oxidative toxicity and can provide a strategy for the development of therapeutic agents, involving the induction of an intracellular cascade of protective pathways. In our previous studies, certain plant-derived chemicals were confirmed to exert a protective effect against glutamate-stimulated oxidative cell death in HT22 cells (22-24).

HO-1 is highly expressed in oxidative conditions in neurons (25). Cerebellar granule cells are obtained from neurons of transgenic mice designed to overexpress HO-1 and appear to be relatively resistant to glutamate- and H_2O_2-mediated oxidative stress *in vitro* (26,27). The dose-dependent induction of HO-1 expression is evidence of the induction of HO-1 by BrPA in HT22 cells. In addition, we observed that both cycloheximide and actinomycin D completely blocked HO-1 expression. These results suggested that BrPA-mediated HO-1 expression required *de novo* RNA and protein synthesis. Furthermore, the pre-incubation of HT22 cells with BrPA resulted in improved resistance to glutamate-induced oxidative stress; this effect was partially attributable to the induction of HO-1 as SnPP IX, which prevents HO enzyme activity and markedly decreases BrPA-induced cytoprotection. The induction of HO-1 expression was also needed to attenuate glutamate-induced ROS production. Our results demonstrated that in our experimental conditions, BrPA-induced cytoprotective effects were mediated through HO-1 expression. Furthermore, several researchers have reported that phytochemicals such as resveratrol (28), curcumin (29), and epigallocatechin-3-gallate (30) showed a noteworthy therapeutic advantage in oxidative stress-induced neuronal damage, via the induction of HO-1.

Nrf2 plays a crucial role in the oxidative stress-related induction of ARE-mediated expression of antioxidant enzymes and other inducible genes via various stimuli (7). Among phase 2 detoxifying and antioxidant enzymes, GSH and GST are well known as pivotal antioxidants that induce

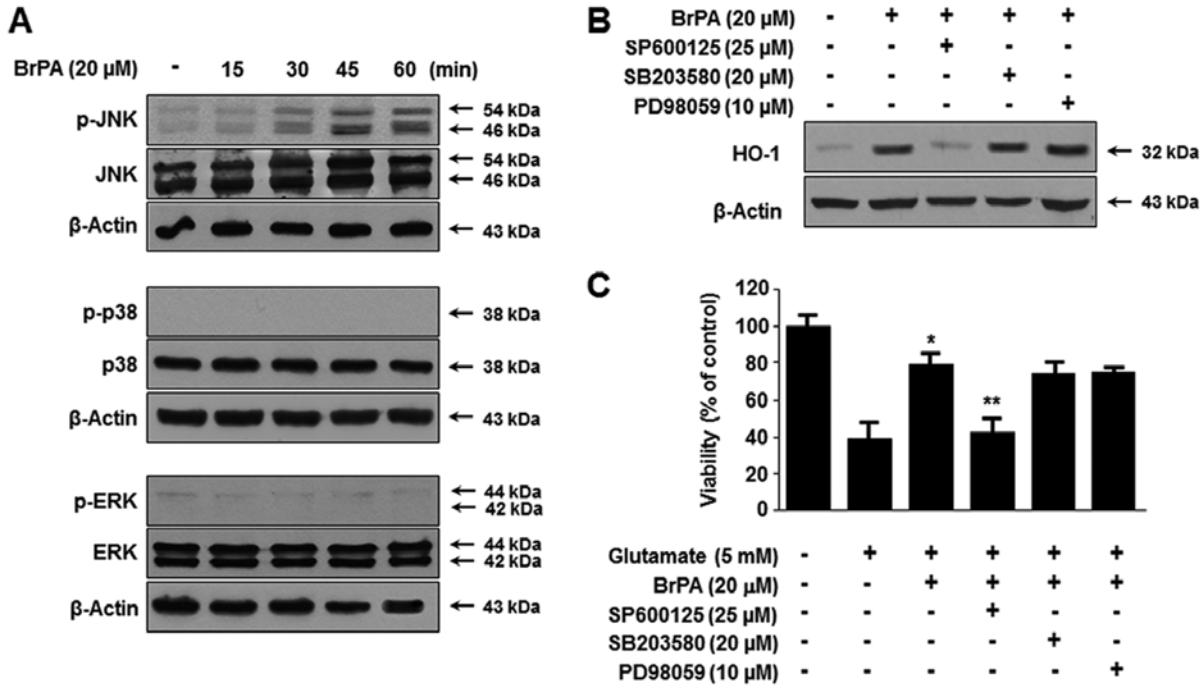

Figure 6. BrPA upregulated HO-1 expression via the phosphorylation of JNK MAPK. HT22 cells were cultured with BrPA (20 μM) for the indicated time period (0-60 min), and the expression of phosphorylated JNK, p38, or ERK MAPK was determined using (A) western blotting. The cells were pre-incubated with specific inhibitors, SP600125 (25 μM, JNK inhibitor), SB203580 (20 μM, p38 inhibitor), or PD98059 (10 μM, ERK inhibitor) for 1 h, and then (B) cultured with BrPA (20 μM) for 12 h. Each specific inhibitor was treated for 1 h in cells exposed or not exposed to BrPA (20 μM) for 12 h, and the cells were then (C) exposed to 5 mM glutamate for 12 h. The results are presented as the mean ± standard deviation (n=3). *P<0.05 vs. glutamate. **P<0.05 vs. glutamate with BrPA (20 μM). BrPA, brassicaphenanthrene A; HO, heme oxygenase.

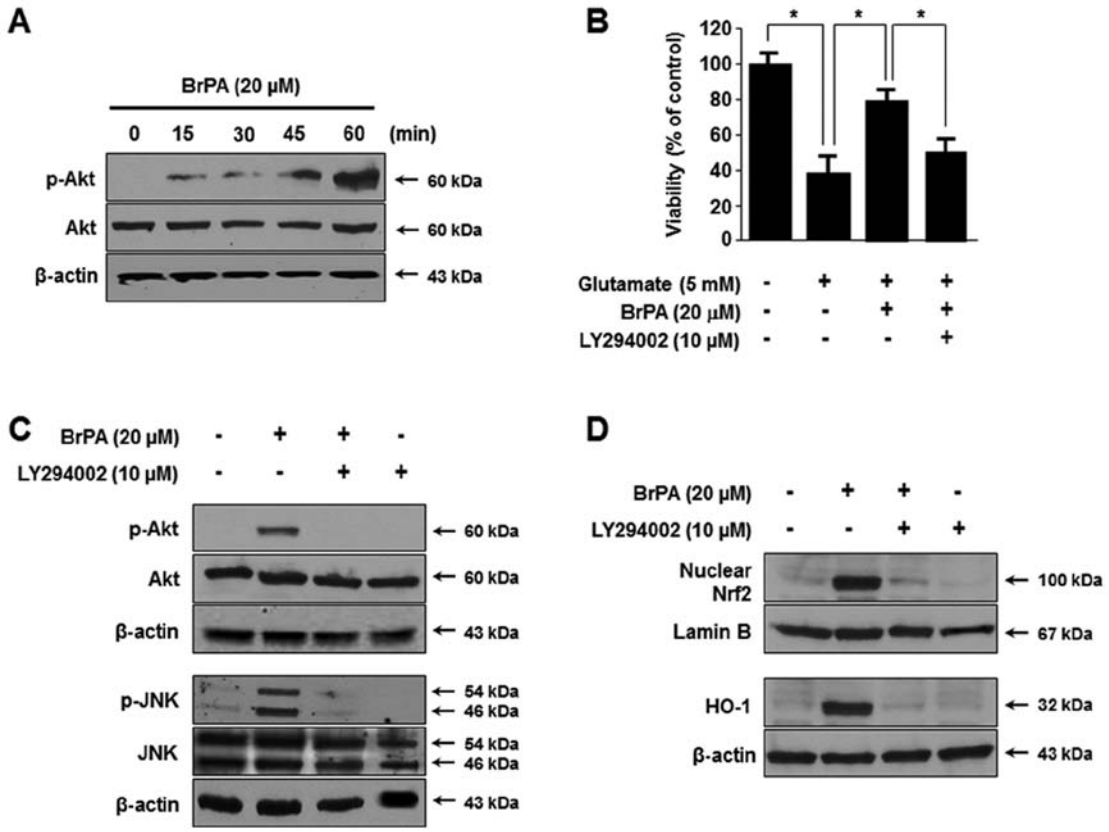

Figure 7. BrPA induced HO-1 expression via the PI3K/Akt signaling pathways. HT22 cells were cultured with BrPA (20 μM) for the indicated time period (0-60 min), and phosphorylated Akt was determined by (A) western blot analysis. The cells were not treated or pre-treated with the specific inhibitor LY294002 (1 h; 10 μM), and then incubated with BrPA (20 μM) for 12 h, and later exposed to (B) 5 mM glutamate for 12 h. BrPA (20 μM) not treated or treated with (C) inhibitor LY294002 (1 h, 10 μM) for 30 min, (D) 1.5 h (nuclear Nrf2), or 12 h (HO-1). The results are presented as the mean ± standard deviation (n=3). *P<0.05. BrPA, brassicaphenanthrene A; HO, heme oxygenase.

the protection of various cells against oxidative injury through the reduction of ROS and nitrogen radicals (31). GCL is composed of two subunits that contain ARE sequences in their promoters: A modifier subunit (GCLM) and a catalytic subunit (GCLC). GCL is the rate-limiting enzyme in the synthesis of GSH (32). In addition, the expression of GCLM and GCLC is modulated by antioxidants and anti-inflammatory agents (33). In this study, BrPA-induced dose-dependent protection against GSH depletion induced by glutamate was observed, as well as the upregulation of GST and GCL levels. It has been reported that GSH, GST, and GCL, known to transcribe many oxidative stress-inducible genes, are also modulated via Nrf2 induction in response to exposure to electrophiles (34). Nrf2, which is bound to an inhibitor protein Keap-1, resides in the cytosol. Nrf2 released from Keap-1 by cell stimulation enters the nucleus and binds to the AREs in the promoter of the target gene (6,7). In this study, we found that BrPA markedly induced Nrf2 levels and efficiently promoted its translocation into the nucleus. Thus, BrPA-induced Nrf2 nuclear translocation is associated with an increase in its ARE transcriptional activity. Transient transfection with Nrf2 siRNA completely diminished BrPA-induced HO-1 and GST expression, and reversed the BrPA-induced decrease in cell protection and ROS inhibition. Overall, these observations indicated that Nrf2-dependent increases in the protein expression of GCL, GST, and HO-1 induced by BrPA, conferred cytoprotection against glutamate-induced oxidative damage in HT22 cells.

Moreover, the JNK pathways appear to be related to BrPA-mediated HO-1 expression. When cells were treated with a specific protein kinase inhibitor, the JNK pathway proved to have a decisive role in HO-1 induction. Previous studies reported that MAPK expression mediated the regulation of basic cellular processes such as transcription factor phosphorylation, proliferation, stress response, apoptosis, and immune defense. In addition, MAPK expression modulated the expression of several genes and proteins, such as HO-1 (16). Because the inhibitor SP600125 completely blocked HO-1 induction, BrPA-induced HO-1 expression was directly correlated with the JNK pathway.

The PI3K/Akt pathway is reportedly connected with the regulation of Nrf2 nuclear translocation and the expression of ARE-related phase II enzymes (35,36). The neuroprotective mechanisms of PI3K/Akt signaling may be related to the activation of Nrf2 transcription, which is widely viewed as a mediator of neuroprotection, as it upregulates various antioxidant enzymes (4,5). Therefore, we also observed if the activation of the PI3K/Akt and JNK pathways was related to BrPA-induced Nrf2-mediated HO-1 expression. The specific PI3K inhibitor LY294002 attenuated BrPA-induced Akt and JNK activation, Nrf2 nuclear translocation, HO-1 expression, and cytoprotective effects in HT22 cells. These results indicated that the PI3K/Akt cascade and JNK pathways, activated by BrPA, participate in the early-stage expression of HO-1 in HT22 cells. This study suggests that BrPA isolated from the roots of *B. rapa* effectively prevented oxidative neuronal cell death and BrPA-induced HO-1 regulation via PI3K/Akt, JNK, and Nrf2 signals, eventually playing a significant role in the protection of HT22 cells. The further studies are required to evaluate the nuclear translocation of some molecules using such as the immunofluorescence staining to confirm the detailed mechanism by BrPA.

In conclusion, these results indicated that BrPA isolated from the roots of *B. rapa*, effectively prevented glutamate-induced mouse hippocampal cell damage. In addition, HO-1 expression through the PI3K/Akt, JNK, and Nrf2 pathways regulated by BrPA, appeared to have an important role in neuronal cell protection. Further, this study has shown that BrPA exerts neuroprotective effects through the regulation of Nrf2-HO-1 signaling.

Acknowledgements

Not applicable.

Funding

The current study was supported by a research fund from Chosun University in 2016 (K207334001).

Authors' contributions

WK, NIB, YCK and DSL conceived of the study. HL, WK, AC, BL and DSL performed formal analysis. HL, WK, AC, BL and DSL performed investigation. NIB, HO, and YCK provided the resources. HL, WK, AC, BL and DSL curated the data. HL, WK, and DSL wrote and prepared the draft of the manuscript. HL, WK, SCK, ERW and DSL wrote, reviewed and edited the manuscript. SCK, HO, ERW, NIB, YCK and DSL performed the visualization. NIB, YCK and DSL supervised the study.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Hald A and Lotharius J: Oxidative stress and inflammation in Parkinson's disease: Is there a causal link? Exp Neurol 193: 279-290, 2005.
2. Simonian NA and Coyle JT: Oxidativec stress in neurodegenerative diseases. Annu Rev Pharmacol Toxiol 36: 83-106, 1996.
3. Manning BD and Cantley LC: AKT/PKB signaling: Navigating downstream. Cell 129: 1261-1274, 2007.
4. Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T, Harada T and Yamamoto M: High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. Toxicol Sci 59: 169-177, 2001.
5. Narasimhan M, Mahimainathan L, Rathinam ML, Riar AK and Henderson GI: Overexpression of Nrf2 protects cerebral cortical neurons from ethanolinduced apoptotic death. Mol Pharmacol 80: 988-999, 2011.

6. Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, Alam J and Motterlini R: Curcumin activates the heme oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. Biochem J 371: 887-895, 2003.
7. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, *et al*: An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236: 313-322, 1997.
8. De Vries HE, Witte M, Hondius D, Rozemuller AJ, Drukarch B, Hoozemans J and van Horssen J: Nrf2-induced: A promising target to counteract ROS-mediated damage in neurodegenerative disease antioxidant protection? Free Radic Biol Med 45: 1375-1383, 2008.
9. Lee DS and Jeong GS: Arylbenzofuran isolated from *Dalbergia odorifera* suppresses lipopolysaccharide-induced mouse BV2 microglial cell activation, which protects mouse hippocampal HT22 cells death from neuroinflammation-mediated toxicity. Eur J Pharmacol 728: 1-8, 2014.
10. Maines MD: Heme oxygenase: Function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J 2: 2557-2568, 1988.
11. Rössler OG, Bauer I, Chung HY and Thiel G: Glutamate-induced cell death of immortalized murine hippocampal neurons: Neuroprotective activity of heme oxygenase-1, heat shock protein 70, and sodium selenite. Neurosci Lett 362: 253-257, 2004.
12. Choi BM, Kim HJ, Oh GS, Pae HO, Oh H, Jeong S, Kwon TO, Kim YM and Chung HT: 1,2,3,4,6-Penta-O-galloyl-beta-D-glucose protects rat neuronal cells (Neuro 2A) from hydrogen peroxide-mediated cell death via the induction of heme oxygenase-1. Neurosci Lett 328: 185-189, 2002.
13. Schonhof I, Krumbein A and Brückner B: Genotypic effects on glucosinolates and sensory properties of broccoli and cauliflower. Nahrung 48: 25-33, 2004.
14. Bang MH, Lee DY, Han MW, Oh YJ, Chung HG, Jeong TS, Choi MS, Lee KT and Baek NI: Development of biologically active compounds from edible plant sources-XX: Isolation of lipids from the roots of *Brassica campestris* ssp. *rapa*. J Korean Soc App Biol Chem 50: 233-237, 2007.
15. Wu Q, Cho JG, Yoo KH, Jeong TS, Park JH, Kim SY, Kang JH, Chung IS, Choi MS, Lee KT, *et al*: A new phenanthrene derivative and two diarylheptanoids from the roots of *Brassica rapa* ssp. *campestris* inhibit the growth of cancer cell lines and LDL-oxidation. Arch Pharm Res 36: 423-429, 2013.
16. Kietzmann T, Samoylenko A and Immenschuh S: Transcriptional regulation of heme oxygenase-1 gene expression by MAP kinases of the JNK and p38 pathways in primary cultures of rat hepatocytes. J Biol Chem 278: 17927-17936, 2003.
17. Martin D, Rojo AI, Salinas M, Diaz R, Gallardo G, Alam J, De Galarreta CM and Cuadrado A: Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3'-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. J Biol Chem 279: 8919-8929, 2004.
18. Jung UJ, Baek NI, Chung HG, Bang MH, Jeong TS, Lee KT, Kang YJ, Lee MK, Kim HJ Yeo J and Choi MS: Effects of the ethanol extract of the roots of *Brassica rapa* on glucose and lipid metabolism in C57BL/KsJ-db/db mice. Clin Nutr 27: 158-167, 2008.
19. van Poppel G, Verhoeven DT, Verhagen H and Goldbohm RA: Brassica vegetables and cancer prevention. Epidemiology and mechanisms. Adv Exp Med Biol 472: 159-168, 1999.
20. Vornov JJ and Coyle JT: Glutamate neurotoxicity and the inhibition of protein synthesis in the hippocampal slice. J Neurochem 56: 996-1006, 1991.
21. Coyle JT and Puttfarcken P: Oxidative stress, glutamate, and neurodegenerative disorders. Science 262: 689-695, 1993.
22. Jeong GS, Li B, Lee DS, Byun E, An RB, Pae HO, Chung HT, Youn KH and Kim YC: Lavandulyl flavanones from *Sophora flavescens* protect mouse hippocampal cells against glutamate-induced neurotoxicity via the induction of heme oxygenase-1. Biol Pharm Bull 31: 1964-1967, 2008.
23. Jeong GS, Li B, Lee DS, Kim KH, Lee IK, Lee KR and Kim YC: Cytoprotective and anti-inflammatory effects of spinasterol via the induction of heme oxygenase-1 in murine hippocampal and microglial cell lines. Int Immunopharmacol 10: 1587-1594, 2010.
24. Li B, Lee DS, Jeong GS and Kim YC: Involvement of heme oxygenase-1 induction in the cytoprotective and immunomodulatory activities of 6,4'-dihydroxy-7-methoxyflavanone in murine hippocampal and microglia cells. Eur J Pharmacol 674: 153-162, 2012.
25. Dwyer BE, Nishimura RN and Lu SY: Differential expression of heme oxygenase-1 in cultured cortical neurons and astrocytes determined by the aid of a new heme oxygenase antibody. Response to oxidative stress. Brain Res Mol Brain Res 30: 37-47, 1995.
26. Le WD, Xie WJ and Appel SH: Protective role of heme oxygenase-1 in oxidative stress-induced neuronal injury. J Neurosci Res 56: 652-658, 1999.
27. Chen K, Gunter K and Maines MD: Neurons overexpressing heme oxygenase-1 resist oxidative stress-mediated cell death. J Neurochem 75: 304-313, 2000.
28. Chen CY, Jang JH, Li MH and Surh YJ: Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. Biochem Biophys Res Commun 331: 993-1000, 2005.
29. Yang C, Zhang X, Fan H and Liu Y: Curcumin upregulates transcription factor Nrf2, HO-1 expression and protects rat brains against focal ischemia. Brain Res 1282: 133-141, 2009.
30. Romeo L, Intrieri M, D'Agata V, Mangano NG, Oriani G, Ontario ML and Scapagnini G: The major green tea polyphenol, (-)-epigallocatechin-3-gallate, induces heme oxygenase in rat neurons and acts as an effective neuroprotective agent against oxidative stress. J Am Coll Nutr 28 (Suppl): 492S-499S, 2009.
31. Dickinson DA and Forman HJ: Cellular glutathione and thiols metabolism. Biochem Pharmacol 64: 1019-1026, 2002.
32. Rushworth SA, Ogborne RM, Charalambos CA and O'Connell MA: Role of protein kinase C delta in curcumin-induced antioxidant response element-mediated gene expression in human monocytes. Biochem Biophys Res Commun 341: 1007-1016, 2006.
33. Thimmulappa RK, Fuchs RJ, Malhotra D, Scollick C, Traore K, Bream JH, Trush MA, Liby KT, Sporn MB, Kensler TW and Biswal S: Preclinical evaluation of targeting the Nrf2 pathway by triterpenoids (CDDO-Im and CDDO-Me) for protection from LPS-induced inflammatory response and reactive oxygen species in human peripheral blood mononuclear cells and neutrophils. Antioxid Redox Signal 9: 1963-1970, 2007.
34. Rushworth SA and MacEwan DJ: The role of Nrf2 and cytoprotection in regulating chemotherapy resistance of human leukemia Cells. Cancers (Basel) 3: 1605-1621, 2011.
35. Chen C, Pung D, Leong V, Hebbar V, Shen G, Nair S, Li W and Kong AN: Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2: Effect of chemical structure and stress signals. Free Radic Biol Med 37: 1578-1590, 2004.
36. Shen G, Hebbar V, Nair S, Xu C, Li W, Lin W, Keum YS, Han J, Gallo MA and Kong AN: Regulation of Nrf2 transactivation domain activity. The differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein. J Biol Chem 279: 23052-23060, 2004.