

Synergistic Activation of the Nrf2-Signaling Pathway by Glyceollins under Oxidative Stress Induced by Glutathione Depletion

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ABSTRACT: Oxidative stress state such as depletion of the intracellular glutathione (GSH) is associated with the development of cancer. Some dietary phytochemicals have been shown to possess a cancer preventive effect, although the understanding of the involved mechanisms is still limited. Recent study has shown that glyceollins, phytoalexins derived from soybean by biotic elicitor, might have a cancer preventive effect through induction of detoxifying/antioxidant enzymes. The objective of this study was to investigate the effects of glyceollins on the Nrf2 signaling pathway under excessive oxidative stress induced by GSH depletion. In mouse hepatoma cells (Hepa1c1c7) subjected to the buthionine sulfoximine (BSO), an inhibitor of γ -glutamylcysteine synthetase (γ GCS), the intracellular GSH content was significantly lowered. On the other hand, incubation with glyceollins in the presence of BSO increased the level of GSH, expression of γ GCS, and nuclear translocation of NF-E2-related factor-2 (Nrf2), compared to the cells treated with BSO only. Nrf2-antioxidant responsive element (ARE)-reporter activity assay in HepG2-C8 showed that BSO increased the ARE-reporter activity in a dose-dependent manner, compared to vehicle-treated cells, whereas cotreatment with glyceollins caused further increase in reporter luciferase activity relative to BSO alone. Taken together, glyceollins synergistically activated the Nrf2 signaling pathway and subsequently the expression of phase 2/antioxidant enzymes in the presence of BSO, suggesting that BSO-induced oxidative stress and that glyceollins regulate the expression of phase 2/antioxidant enzymes through different mechanisms from each other.

KEYWORDS: *Nrf2, NQO1, antioxidant response element, glutathione, glyceollins*

■ INTRODUCTION

Oxidative stress may be the most inevitable of biological problems because it derives from a nonspecific type of reaction.¹ A series of reactions are responsible for the formation of reactive oxygen species (ROS) and for consequent damage of macromolecules such as DNA, protein, and lipid. Oxidative stress is induced by not only endogenous but also exogenous factors, including xenobiotics and drugs, leading to the generation of ROS and electrophiles, which have a profound influence on the survival, growth development, and evolution of all living organisms.^{2,3}

Nuclear factor E2-related factor 2 (Nrf2) is commonly recognized to antagonize oxidative stress and defend cells from oxidative damage.^{4–8} The Nrf2 transcription factor resides in cytoplasm until increased cellular oxidative stress or phase 2 enzyme inducers promote its nuclear translocation. Once translocated into the nucleus, Nrf2 binds to the *cis*-acting enhancer antioxidant response element (ARE) sequence (core sequence TGAG/CNNNGC) present in promoters of genes that code for proteins necessary for glutathione synthesis and electrophile detoxification.⁹ Nrf2 is rapidly degraded by the ubiquitin–proteasome pathway through association with Kelch-like ECH associating protein 1 (Keap1), a substrate adaptor

protein of the Cul3-based ubiquitin E3 ligase complex.^{10–13} Upon exposure to oxidative or electrophilic stress, reactive cysteine residues in Keap1 were thought to be covalently modified, leading to the liberation of Nrf2 from Keap1-mediated degradation. The stabilized Nrf2 is then translocated to the nucleus and interacts with a member of the small Maf family proteins.¹⁴ This complex activates the transcription of a wide range of cytoprotective genes (HO-1, NQO1, GST, peroxiredoxins, etc.) via a *cis*-acting DNA element known as the antioxidant/electrophile responsive element (ARE/EpRE).^{15,16} However, the precise mechanism of promoting nuclear translocation of Nrf2 by ROS has not been revealed so far.¹⁷

Some antioxidants as well as ROS have been reported to enhance the expression of phase 2 detoxifying enzymes through activating the Nrf2-signaling pathway and thereby protect cells from oxidative stress and exert cancer preventive action. The interaction between antioxidant and oxidative stress is of great importance to understand the synergy or antagonistic effects

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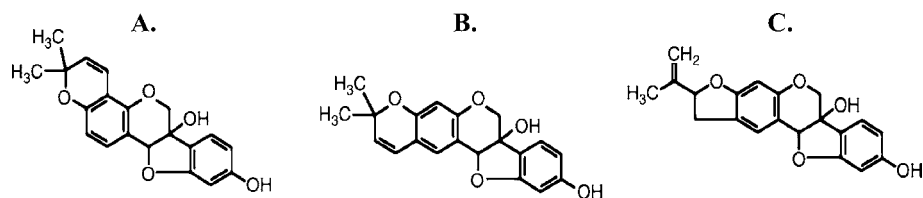


Figure 1. Molecular structures of the glyceollins: (A) glyceollin I; (B) glyceollin II; (C) glyceollin III.

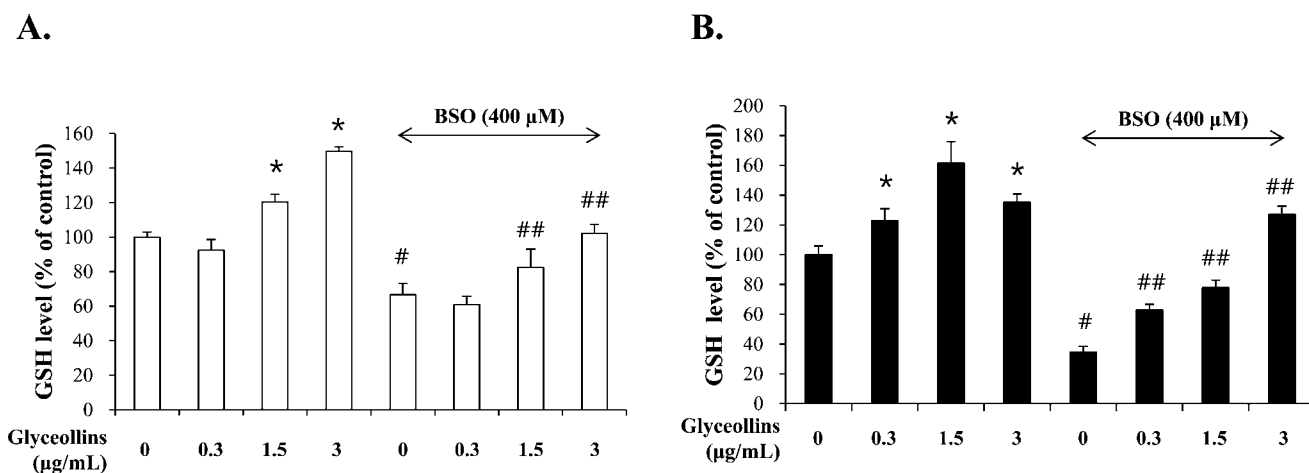


Figure 2. Effect of glyceollins on total GSH level in Hepa1c1c7 and BPRc1 cells in the absence and presence of BSO. Mouse hepatoma Hepa1c1c7 (A) and BPRc1 (B) cells were treated with glyceollins in the absence and presence of BSO 400 μ M, followed by measurement of intracellular level of total GSH. (*, #) $p < 0.05$, compared to the control; (##) $p < 0.05$, compared to BSO stimulation.

that affect an individual's health. For instance, it can be speculated that antioxidants will dampen the effect of oxidative stress loaded on cells and weaken the Nrf2 signaling amplitude enhanced by oxidative stress. Our previous study demonstrated that glyceollins (Figure 1), a family of prenylated isoflavonoids induced in soybean by fungal infection, had strong antioxidative activity and sensitively induced phase 2/antioxidant enzymes through the Nrf2-signaling pathway.⁶ Buthionine sulfoximine (BSO), an inhibitor of γ -glutamyl cysteine synthetase (γ GCS, also called γ -glutamyl cysteine ligase), was known to deplete the intracellular pool of glutathione and thereby cause oxidative stress.¹⁸ If glyceollins and BSO activate Nrf2 signaling via different mechanisms from each other, they are expected to show an additive or synergistic effect on Nrf2 signaling when treated together. Therefore, we examined the effect of antioxidant glyceollins on Nrf2 signaling activated by BSO-induced oxidative stress.

MATERIALS AND METHODS

Materials. Glyceollins were prepared as described previously.¹⁹ All cell culture reagents and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD, USA). Hepa1c1c7 and BPRc1 cells were from American Type Culture Collection (Rockville, MD, USA). All other chemicals were of reagent grade.

Cell Culture. Hepa1c1c7 and its mutant (BPRc1) cells were plated at densities of 3×10^5 and 5×10^5 cells per 100 mm plate (Nunc, Rochester, NY, USA) in 10 mL of α -MEM supplemented with 10% FBS, respectively. The HepG2-C8 cell line established in Dr. Kong's laboratory at Rutgers, The State University of New Jersey, by transfecting human hepatoma HepG2 cells with pARE-TI-luciferase construct was used for the reporter assay. HepG2-C8 cells were maintained in modified DMEM supplemented with 10% FBS, GlutaMax (Gibco 35050-061), 100 units/mL penicillin, and 0.5 mg/mL G418. Cells were normally starved overnight in 0.5% FBS-containing medium before treatment. The cells were normally

incubated for 3–4 days in a humidified incubator in 5% CO_2 at 37 $^\circ\text{C}$. Cells were cultured for 48 h and exposed to various concentrations of sample for another 24 h, followed by biochemical assays.

GSH Quantification. Total GSH and oxidized GSH (GSSG) were measured using a method described previously.²⁰ In brief, Hepa1c1c7 and BPRc1 cells were seeded onto 6-well plates (1×10^6 cells/well) and incubated with BSO (0, 100, 200, and 400 $\mu\text{g/mL}$) and glyceollins (0, 0.3, 1.5, 3, and 6 $\mu\text{g/mL}$). After 12 h of incubation, cells were washed with ice-cold PBS and collected using 1% sulfosalicylic acid. Cells were centrifuged at 13000g for 5 min, and supernatant was used for GSH measurement. The supernatant (10 μL) was transferred to a microplate and diluted with 90 μL of distilled H_2O . Immediately after the addition of 100 μL of a reaction mixture (0.32 mg/mL NADPH, 0.1 mg/mL DTNB, 0.1 M potassium phosphate (pH 7.5), and 1 U/mL GSH reductase), absorbance was measured at 412 nm using a microplate reader (Sunrise, Tecan, Grödig, Austria). GSH concentrations were calculated from a standard curve and normalized versus total protein levels.

Biochemical Assays. Quinone reductase (QR) activity was measured by a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm.¹⁰ *tert*-Butylhydroquinone (TBHQ, 20 μM or 3.3 $\mu\text{g/mL}$), a known QR inducer, was used as a positive control in all biochemical assays. The specific activity of enzymes was normalized to the protein concentration, which was determined in triplicate using the Lowry assay.¹¹ All values are reported as the mean \pm standard deviation (SD) whenever possible.

Assay of Reporter Gene Activity. HepG2-C8 cells were plated in 6-well plates at a density of 5×10^5 cells/well. After 16 h of incubation, cells were cultured in fresh modified DMEM with high glucose containing 0.5% FBS for 12 h before sample treatment. After cells were cultured for another 16 h in the presence of various concentrations of sample, cells were collected and the luciferase activity was determined according to the protocol provided by the manufacturer (Promega Corp., Madison, WI, USA).⁷ Briefly, after sample treatment, cells were washed twice with ice-cold PBS and harvested in reporter lysis buffer. The homogenates were centrifuged at 12000g for 2 min at 4 $^\circ\text{C}$. A 20

μL supernatant was assayed for luciferase activity using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). Luciferase activity was normalized against protein concentration.

Preparation of Nuclear Protein Extract. Nuclear and cytosolic protein extracts were prepared according to the method as described.¹² Briefly, cells were cultured on 100 mm dishes to 90% confluence and treated with samples for various times. After being washed, cells were harvested by scraping in ice-cold PBS and collected by centrifugation at 500g for 5 min. Cells were lysed with buffer A (10 mM HEPES–KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride) on ice for 20 min and then centrifuged at 14000g for 15 min at 4 °C. The supernatants were saved as the cytoplasmic fractions. The nuclear pellets were washed three times with buffer A and resuspended in buffer B (20 mM HEPES, 0.5 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, pH 7.9) for 30 min at 4 °C on a rotating wheel and then centrifuged at 14000g for 15 min at 4 °C. One part of the nuclear fraction was subjected to immunoblot analysis using anti-Nrf2, anti-Lamin B antibody.

Western Blot. This was performed on cytosolic fractions prepared from cultured cells to estimate the level of detoxifying enzymes according to a protocol described previously.^{13,14} The primary antibodies including anti-NQO1, anti-HO-1, anti- γGCS , anti-GR, anti-HIF-1 α , anti-PHD2, and anti- β -actin and horseradish peroxidase-conjugated secondary antibody anti-goat or anti-rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical Analysis. The statistical significance of QR enzyme activity and ARE-reporter assay data was tested by analysis of variance, followed by Duncan's multiple-range test, using SPSS software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Reversal of BSO-Induced Reduction of Glutathione Level by Glyceollins. The intracellular glutathione level was significantly reduced by BSO (400 μM), whereas it was increased by glyceollins in a dose-dependent manner in Hepa1c1c7 cells (Figure 2A). A similar trend was observed in BPRc1 cells carrying defective aryl hydrocarbon receptor nuclear transporter (ARNT or HIF-1 β) except that the intracellular level of glutathione reached the highest at 1.5 $\mu\text{g}/\text{mL}$ glyceollins and then tended to decrease at the concentration of the highest dose of 3 $\mu\text{g}/\text{mL}$, perhaps due to toxicity (Figure 2B).

Furthermore, the BSO-induced decrease of the GSH level was restored by glyceollins in a dose-dependent manner in both Hepa1c1c7 and its mutant BPRc1 cells (Figure 2). These data are consistent with our previous study that found strong potential of glyceollins to induce phase 2/antioxidant enzymes such as γGCS , HO-1, NQO1, and GR.⁶ Glutathione synthesis is expected to be enhanced through activation of the Nrf2 signaling pathway by glyceollins even in the presence of BSO. That is, glyceollins could increase the total amount of glutathione in the cells by transcriptional activation even under inhibition of γGCS by BSO, independent of the signaling pathway generated by HIF-1 α /HIF-1 β heterodimer.

The activation of the Nrf2 signaling pathway by glyceollins has been suggested to be mediated by phosphoinositide-3-kinase (PI3K) and its downstream Akt as well as a structural change of Keap1 by covalent or noncovalent modification at cysteine residues.^{6,11,21} As expected from their structures containing a prenyl group, methyl groups present in glyceollins are likely to form a covalent bond with cysteine residues in Keap1 so as to change the three-dimensional structure, thereby facilitating release and nuclear translocation of Nrf2.²²

Antioxidant Enzyme Induction by Glyceollins in the Presence of BSO. The activity of NAD(P)H:oxidoreductase 1 (NQO1 or QR), one of the antioxidant enzymes, was significantly enhanced by BSO (Figure 3). The BSO-induced

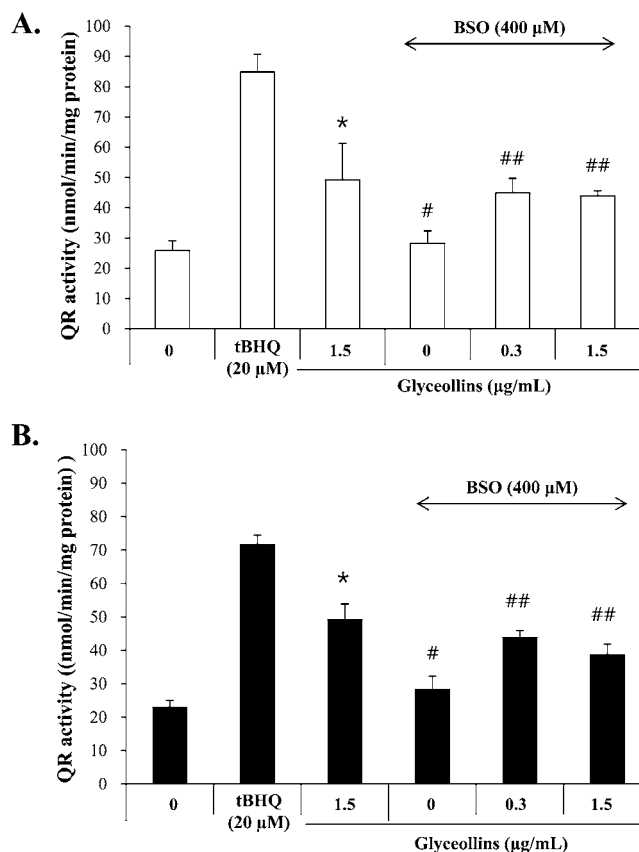


Figure 3. QR-inducing activity of glyceollins in Hepa1c1c7 and BPRc1 cells in the absence and presence of BSO. Mouse hepatoma Hepa1c1c7 (A) and BPRc1 (B) cells were treated with glyceollins in the absence and presence of BSO 400 μM for 24 h, followed by measurement of QR activity. Bars represent the mean \pm SD. (*, #) $p < 0.05$, compared to the control; (##) $p < 0.05$, compared to BSO stimulation.

QR activity was further increased by glyceollin treatment, although it did not show a dose-dependent pattern, maybe due to cytotoxicity of the combined chemicals.

Similar trends were observed for other antioxidant enzymes including heme oxygenase 1 (HO-1) and γGCS in Hepa1c1c7 cells (Figure 4). In BPRc1 cells, the expression of HO-1 was synergistically increased in the presence of both glyceollins and BSO. Overall, the expression of phase 2/antioxidant enzymes by BSO was consistently more pronounced than that of glyceollins, suggesting the higher sensitivity of cells to oxidative stress and strong tendency to restore normal redox state. Contrary to our initial hypothesis that antioxidative glyceollins might mitigate the phase 2/antioxidant enzyme induction by BSO, the compound did not counteract the BSO effect but rather tended to potentiate the cellular response to BSO. These findings indicate that glyceollins and BSO might have different targets, although both activate the Nrf2 signaling pathway upstream to phase 2 enzymes. For instance, glyceollins could interact with cysteine residue(s) in Keap1 protein to modify three-dimensional structure, releasing Nrf2 and facilitating its

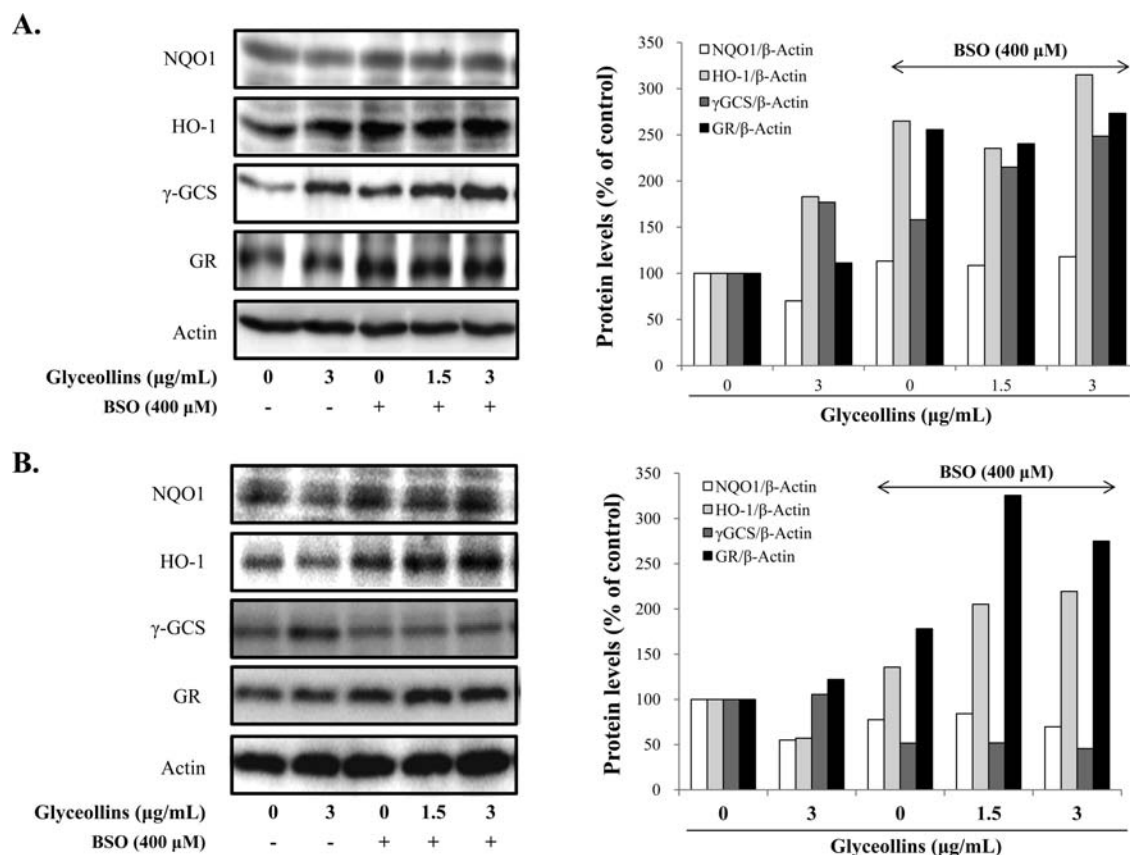


Figure 4. Effect of glyceollins on the expression of phase 2 detoxifying enzymes in the absence and presence of BSO. Hepa1c1c7 (A) and BPRc1 (B) cells were treated with different concentrations of glyceollins in the absence and presence of BSO (400 μM) for 24 h, followed by Western blot analyses. NQO1, NAD(P)H:(quinone acceptor) oxidoreductase 1 (also called QR); HO-1, heme oxygenase; γ-GCS, γ-glutamylcysteine synthase; GR, glutathione reductase. (#) $p < 0.05$, compared to the control.

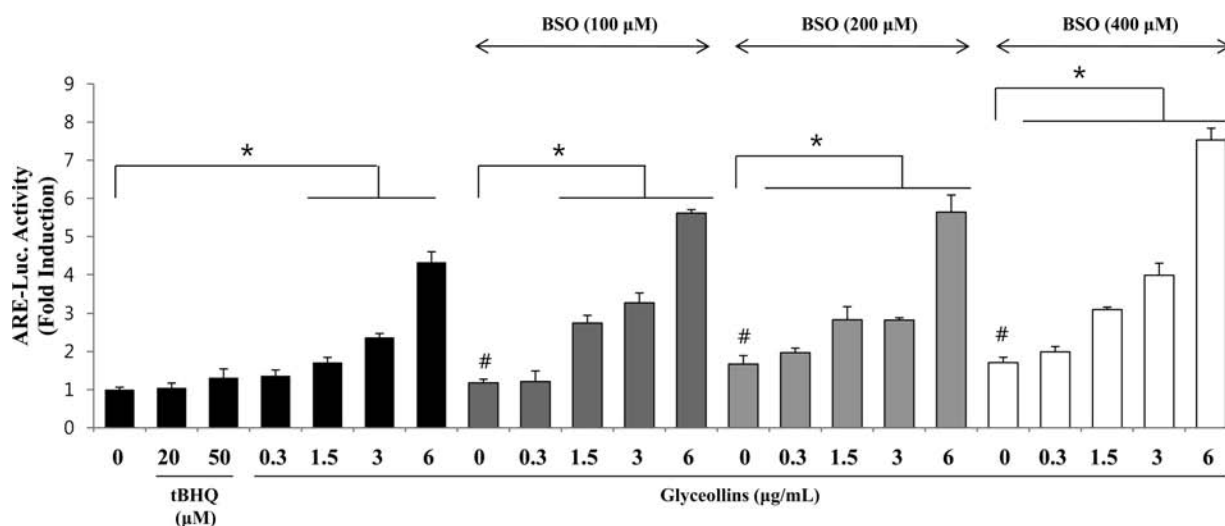


Figure 5. Effect of glyceollins and BSO on the ARE-luciferase activity. HepG2-C8 cells containing transfectants carrying the ARE-luciferase gene construct were incubated with glyceollins in the absence and presence of various doses of BSO for 16 h. Bars represent the mean \pm SD.

migration into the nucleus,²² whereas BSO might change the structure of Keap1 through redox potential in the cells.¹⁷

Activation of ARE by Glyceollins in the Presence of BSO. A reporter assay conducted using HepG-C8 cells carrying plasmid with the ARE-luciferase gene demonstrated that glyceollins and BSO synergistically could activate transcription of the genes with an ARE sequence such as NQO1, HO-1,

glutathione reductase, and some other antioxidant enzymes. Glyceollins induced the luciferase activity under ARE sequence in the promoter region in a dose-dependent manner, whereas BSO treatment also increased ARE-luciferase activity at a concentration of 200 μM or above (Figure 5). Furthermore, the combined treatment with BSO and glyceollins led to even higher induction of the reporter activity than treatment with a

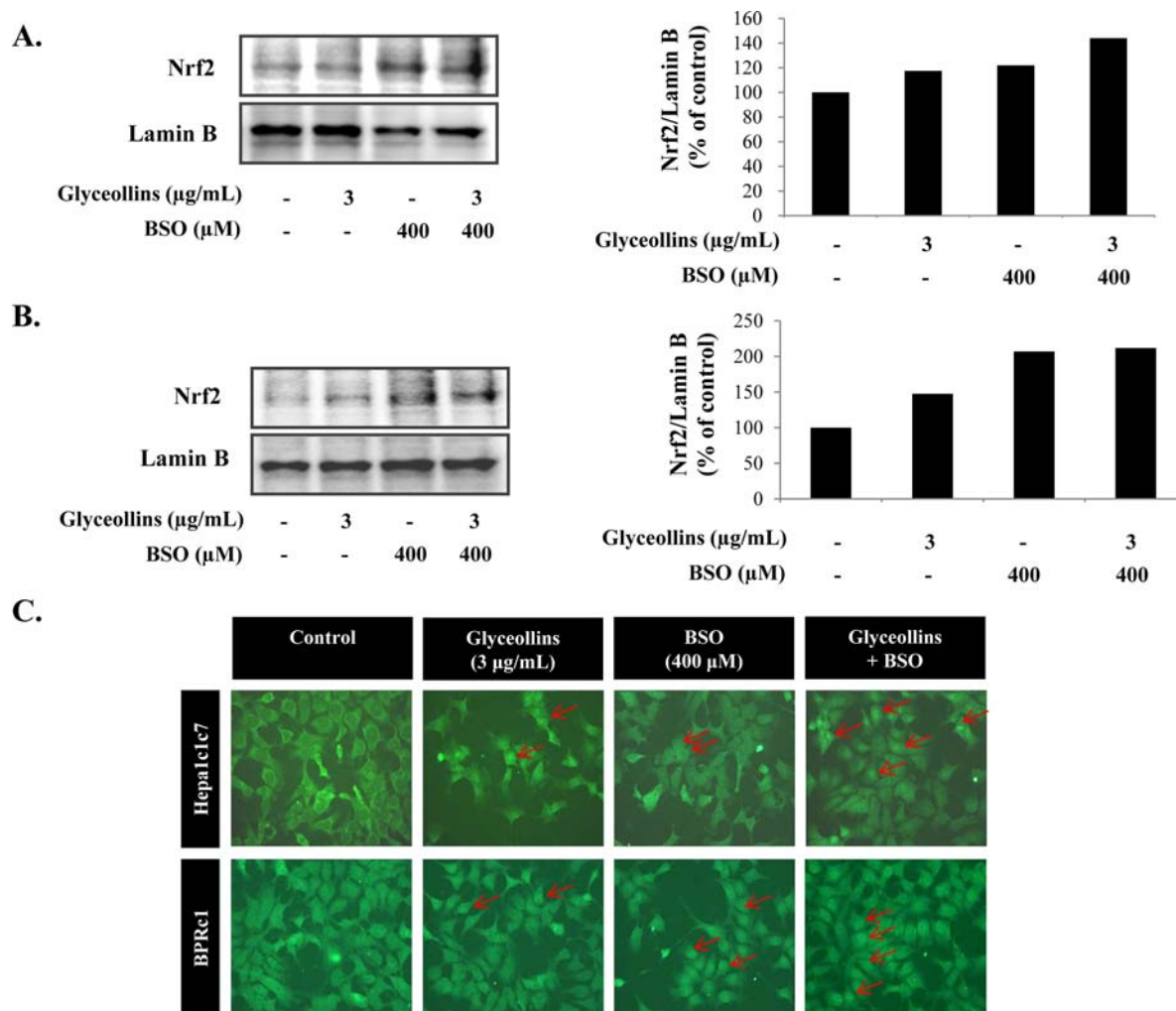


Figure 6. Nuclear translocation of Nrf2 by glyceollins and BSO. Hepa1c1c7 (A) and BPRc1 (B) cells were treated with either glyceollins or BSO or both, followed by preparation of nuclear fraction and measurement of Nrf2 level in the nucleus by Western blot. Antibody against lamin B was used as loading control of nuclear extract. Ethanol-fixed hepatoma cells were analyzed by immunocytochemistry, and the levels of Nrf2 translocated into the nucleus were compared between untreated and treated with glyceollins and/or BSO (C). Arrows indicate the nuclear Nrf2 as observed under fluorescence microscope after immunocytochemistry.

single compound, consistent with the Western blot data of the compounds on phase 2 enzyme expression (Figure 4). The data suggest that glyceollins and BSO promote reporter gene activity, which is, in turn, regulated by Nrf2, through different pathways.

Nuclear Translocation of Nrf2 by Glyceollins in the Presence of BSO. The nuclear level of Nrf2 was significantly increased by BSO in both Hepa1c1c7 (Figure 6A) and BPRc1 (Figure 6B) cells, whereas it was slightly increased in BPRc1 cells treated with glyceollins alone. BSO caused an even further increase in the nuclear Nrf2 level in the presence of glyceollins in Hepa1c1c7 cells but not BPRc1 cells. Green fluorescence photographs taken by the FITC filter cube (Figure 6C) appeared to be consistent with the data from Western blot. Thus, it is evident that activation of Nrf2 plays a pivotal role in glyceollins and BSO-induced expression of phase 2 enzymes in mouse hepatoma cells.

The expression of phase 2 and antioxidant enzymes is well established to be regulated by the Nrf2 signaling pathway. The binding of Nrf2 with Keap1 serves as an adaptor for the cullin3/ring box 1 (Cul3/Rbx1) E3 ubiquitin ligase complex, which elicits the ubiquitination of Nrf2 that then is degraded by

the 26S proteasome.^{3,23,24} The cytosolic Nrf2 activated by a stimulus is released from Keap1 and migrates into the nucleus. Once translocated into the nucleus, Nrf2 binds to the ARE sequence in the promoter region of phase 2 enzymes, resulting in transcriptional activation.^{25,26}

Increased PHD2 Expression by Glyceollins in the Presence of BSO. HIF-1 and Nrf2 represent oxygen- and redox state-dependent transcription factors.^{27,28} HIF-1 is important in adapting cells to conditions of limited oxygen supply by affecting oxygen transfer, angiogenesis, glycolytic metabolism, proliferation, and apoptosis, thus favoring survival under hypoxic conditions.²⁹ Under intermittent hypoxic conditions, NADPH oxidase 1 (NOX1) was demonstrated to be responsible for ROS production and to activate Nrf2 by protein stabilization. It has been reported that Nrf2 inhibition suppresses tumor angiogenesis by inhibiting hypoxia-induced accumulation of HIF-1 α .³⁰ In contrast, the exposure of cells to BSO, which was previously shown to activate the Nrf2 signaling pathway, caused a significant induction of HIF prolyl 4-hydroxylase D2 (PHD2) and thereby decreased the HIF-1 α level through ubiquitinylation (Figure 7).

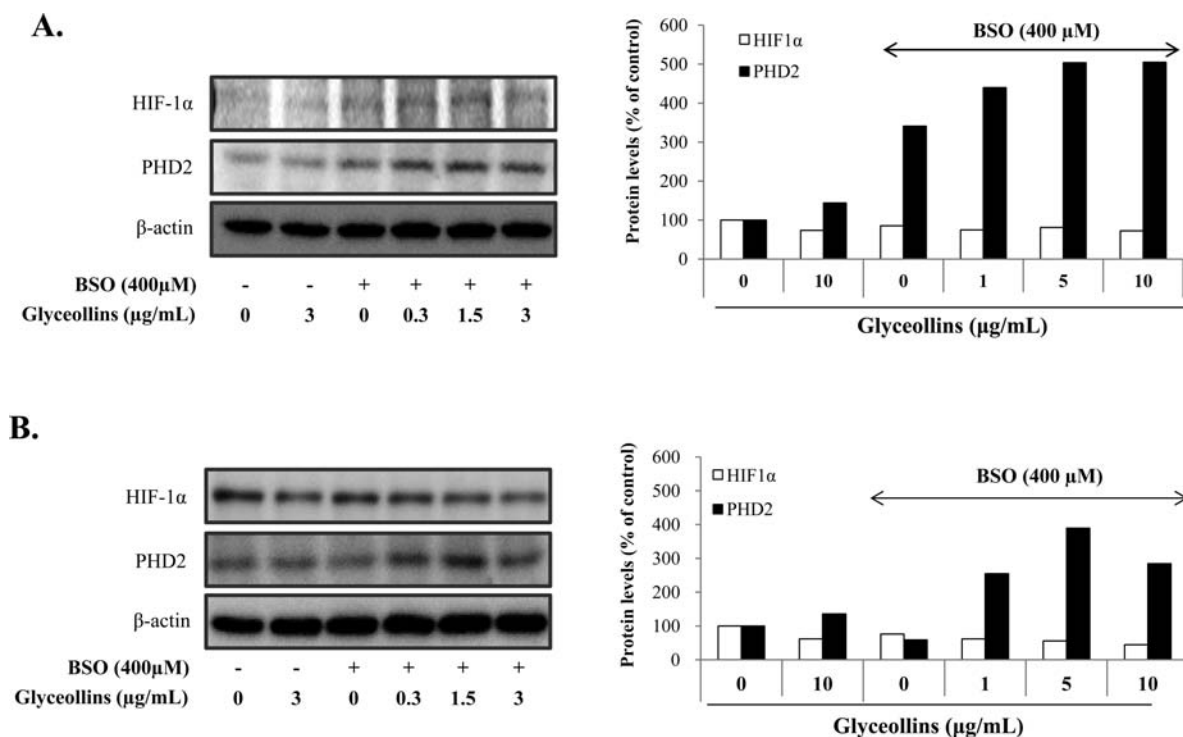


Figure 7. Effect of BSO and glyceollins on PHD2 and HIF-1 α levels in Hepa1c1c7 (A) and BPRc1 (B). The cells were treated with various concentrations of glyceollins in the absence and presence of 400 μ M BSO, prior to measuring the levels of HIF-1 α and PHD2.

In particular, the expression of PHD2 was increased synergistically in BPRc1 with defective HIF-1 β (arylhydrocarbon nuclear transporter) by BSO and glyceollins, resulting in subsequent reduction of HIF-1 α , which mainly regulates the transcription of angiogenesis factors such as vascular epithelial growth factor (VEGF) and glycolytic enzymes.

BSO sensitizes cancer cells to chemotherapy, and the use of BSO as an anticancer agent is currently in clinical trials.³¹ Current data suggest that phase 2 enzyme-inducing phytochemicals such as glyceollins might augment the inhibition of angiogenesis by BSO through PHD2 induction and subsequent down-regulation of HIF-1 α and thereby suppress tumor development under hypoxic conditions characterized by most tumors.

In conclusion, soybean-derived glyceollins, which hold antioxidant activity, could boost the Nrf2 signaling pathway instead of dampening the activation of the Nrf2 signaling pathway by BSO-induced oxidative stress. Activation of the Nrf2 signaling pathway by glyceollins, therefore, appeared to be mediated by a different mechanism from BSO-induced oxidative stress. Furthermore, these results suggest that some antioxidants such as glyceollins could exert augmented chemopreventive activity in combination with oxidative stress caused by various endogenous and exogenous stimuli such as exercise or inflammation.

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Notes

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