JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# Neuroprotective Effects of Dehydroglyasperin C through Activation of Heme Oxygenase-1 in Mouse Hippocampal Cells

Hyo Jung Kim,[†] Soon Sung Lim,[§] In Sil Park,[†] Ji Sun Lim,[†] Ji Yeon Seo,[†] and Jong-Sang Kim*[,†]

[†]School of Applied Biosciences (BK21 program) and Food Science and Biotechnology, Kyungpook National University, Daegu 702-701, Republic of Korea

[§]Department of Food Science and Nutrition, Hallym University, Chuncheon 200-702, Republic of Korea

**ABSTRACT:** Licorice, the root of the *Glycyrrhiza* species (*Glycyrrhiza uralensis* Fisher), is known to have antioxidant, anti-inflammatory, antiviral, and antitumor properties. The objective of this study is to explore the neuroprotective effect of dehydroglyasperin C (DGC) against glutamate-induced oxidative stress in mouse hippocampal HT22 cells. DGC significantly reduced cytotoxicity and reactive oxygen species (ROS) generation induced by glutamate in HT22 cells, whereas DGC did not restore glutathione depletion caused by glutamate. In addition, it was further investigated whether DGC affected the expression of heme oxygenase (HO)-1, one of the major cellular antioxidant defense systems, and it was found that DGC dose-dependently increased HO-1 expression. DGC-mediated cytoprotection of HT22 neuronal cells from glutamate insult was abrogated by either HO-1 inhibitor (Tin protoporphyrin, SnPP) or AKT inhibitor (LY294002). In conclusion, the present results demonstrate for the first time that DGC protects neuronal cells against glutamate-induced oxidative injury through the induction of HO-1 expression, which is, in turn, activated maybe through Nrf2-Keap1 and PI3K/AKT signaling pathways.

**KEYWORDS:** *dehydroglyasperin C, neuroprotection, heme oxygenase 1*

## INTRODUCTION

Licorice, one of the most frequently used ingredients in traditional oriental medicine, has been prescribed for treating peptic ulcers, eczema, skin infections, cold sores, menopausal symptoms, liver disease, respiratory ailments, inflammatory problems, chronic fatigue syndrome, AIDS, and even cancer.[1] In particular, glycyrrhizin (a triterpenoid saponin) and chalcones in licorice have been reported to act as antioxidants and electrophile scavengers, to stimulate the immune system, to inhibit nitrosation and the formation of DNA adducts with carcinogens, to inhibit hormonal actions and metabolic pathways associated with the development of cancer, and to induce phase I or II detoxification enzymes.[1,2] In addition to anticarcinogenic and antimutagenic effects, there is scientific evidence that licorice has antioxidant, antiobesity, and antimicrobial activities.[3−6] Our study revealed that dehydroglyasperin C (DGC; Figure 1) isolated from licorice has the ability to induce antioxidant enzymes such as NAD(P)H:quinine oxidoreductase-1 and heme oxygenase-1 (HO-1) through the Nrf2-mediated pathway.[7] In particular, DGC caused a significant induction of HO-1, which is a ubiquitous and redox-sensitive inducible stress protein and can exert potent indirect antioxidative function by degrading heme to CO, iron, and biliverdin.[8] Moreover, the byproducts have their own significance in essential cellular metabolism and contribute to the suppression of oxidative stress.[9,10]

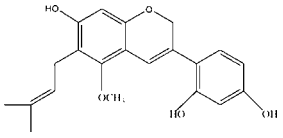


**Figure 1.** Chemical structure of dehydroglyasperin C.

DGC has good potential to protect against neuronal cell death induced by oxidative stress because it can induce phase 2 and/or antioxidant enzymes in a nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-mediated fashion. However, it has not been well understood whether pretreatment of neuronal cells with DGC, an effective antioxidant enzyme inducer, could inhibit neuronal cell damage caused by oxidative stress. It has been well established that the excitatory neurotransmitter glutamate plays a major role in determining certain neurological disorders which are characterized by increasing damage of cell components, including mitochondria, leading to generation and subsequent cell death.[11,12] Therefore, this study was conducted to examine if DGC protects neuronal cells from glutamate-induced oxidative stress.

## MATERIALS AND METHODS

**Materials.** All cell culture reagents and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD, USA). HT22 cells were kindly provided by Dr. Dong-Seok Lee, College of Natural Sciences, Kyungpook National University (Daegu, South Korea). DGC was prepared from dried licorice roots of *Glycyrrhizae uralensis* as described elsewhere.[13]

**Cell Culture.** Mouse hippocampal HT22 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100 units/mL penicillin/streptomycin at 37 °C in 5% $CO_2$.

**Cytotoxicity of Glutamate.** HT22 cells were plated into 96-well plates with DMEM at an initial concentration of $1 \times 10^4$ cells/well and incubated in an atmosphere consisting of 5% $CO_2$ in air at 37 °C for 24 h. Then the medium was removed and replaced by DMEM containing

Received: February 10, 2012
Revised: May 7, 2012
Accepted: May 11, 2012
Published: May 11, 2012

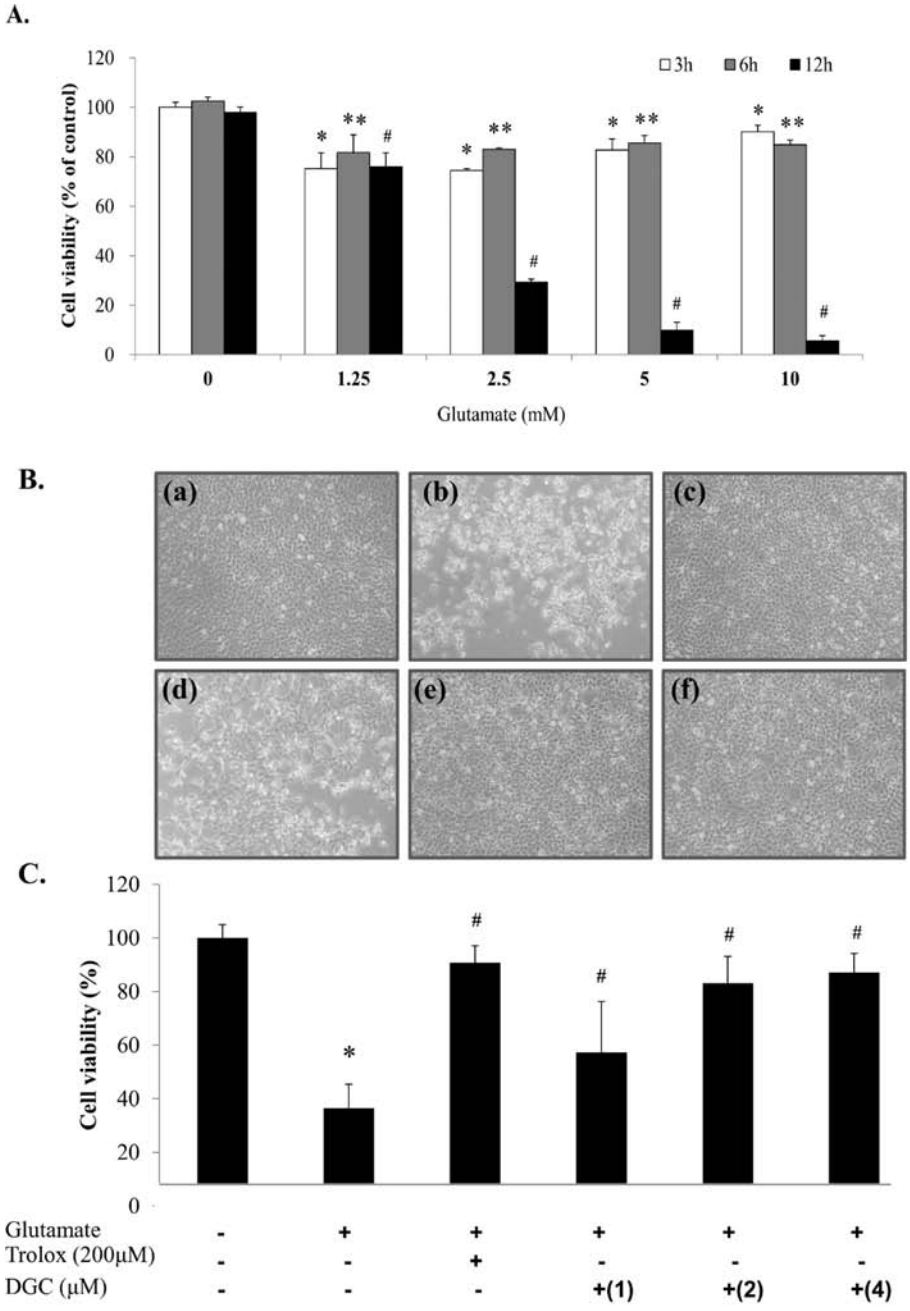


**Figure 2.** Effect of glutamate, DGC, and Trolox on proliferation of mouse neuroblastoma. (A) HT22 cells were incubated in the presence of various concentrations of glutamate for 3, 6, and 12 h, followed by measurement of cell viability by MTT assay. The results are expressed as the mean ± SD of three separate independent experiments. (∗, ∗∗, #) $p < 0.05$, compared to the control. (B, C) In separate experiments, cell viability was measured in the presence of glutamate and Trolox or DGC [(B) a, control; b, glutamate (5 mM); c, glutamate + Trolox (200 $\mu$M); d, glutamate + DGC (1 $\mu$M); e, glutamate + DGC (2 $\mu$M); f, glutamate + DGC (4 $\mu$M)]. (∗) $p < 0.05$, compared to the control; (#) $p < 0.05$, compared to the glutamate stimulation ($n = 3$).

various concentrations of DGC in the presence of 5 mM glutamate. In separate experiment, the cells were treated with glutamate and DGC in the absence and presence of 50 $\mu$M SnPP, an inhibitor of HO-1. The cell culture was terminated on day 1 by removing the medium from the wells and adding 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/mL in phenol red free culture medium). After 2 h, the solution was removed, and 200 $\mu$L of DMSO was added. After 10 min, the absorbance was determined at 540 nm by a microplate reader (Tecan, Australia). The cell proliferation of each group was calculated as the absorbance of the treatment group relative to the control.

**Determination of Intracellular ROS Level.** Oxidative stress was quantified in cells by 2,7-dichlorofluorescein (DCF) assay according to the method of Wang and Joseph,[14] with slight modifications. Briefly, HT22 cells were grown in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS at 37 °C in an atmosphere of 5% $CO_2$/95% air under saturating humidity and passaged every other day (1:4 split ratio) by trypsinization with 0.25% trypsin/0.02% EDTA sodium salt solution (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cells ($1 \times 10^5$ cells/well) were seeded into a black-bottom 96-well plate and cultivated for 24 h. After cell attachment, the cells were incubated in the presence of 0−10 mM glutamate for 3, 6, and 12 h. The stimulated cells were washed with PBS and incubated for 30 min with


dx.doi.org/10.1021/jf300548b | J. Agric. Food Chem. 2012, 60, 5583−5589

dichlorofluorescein diacetate (DCFDA) dissolved in DMSO (final concentration = 50 $\mu$M). Fluorescence was measured at 0 and 40 min using an excitation of 485 nm and an emission of 535 nm, in a fluorescence microplate reader (Infinite 200, Tecan, Grodig, Austria). Most of the steps including incubation of the reaction mixture containing dye and oxidant, washing, and fluorometric determination were performed in the dark. The intensity of fluorescence was calculated as $[(F_{40\,\mathrm{min}} - F_{0\,\mathrm{min}})/F_{0\,\mathrm{min}}] \times 100$ as described elsewhere.[15] Results are expressed as relative intensity of fluorescence (in percentage of control). In separate experiments, the cells were incubated with glutamate and DGC in the absence and presence of SB203580 (10−30 $\mu$M), SP600125 (10−30 $\mu$M), PD98059 (10−30 $\mu$M), LY294002 (10−30 $\mu$M), and SnPP (30−50 $\mu$M), which are inhibitors for MAPK (SB203580, SP600125, PD98059), PI3K (LY294002), and HO-1 (SnPP), respectively.

**Western Blot.** HT22 cells were treated with either DGC (0−4 $\mu$M) alone or together with inhibitor for MAPK or PI3K. The collected cells were homogenized in precooled RIPA buffer (pH 7.4) containing 50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, Triton-X 1%, sodium deoxycholate 0.5%, and 0.1% SDS. The homogenates were cleared by centrifugation at 7600*g* for 5 min at 4 °C, and the supernatants were denatured in sample buffer for 5 min at 95 °C. Proteins were separated by electrophoresis on a 10% SDS−polyacrylamide gel for 1.5 h at 70−150 V and transferred onto nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany) for 2 h at 250 mA. Membranes were incubated with antibodies to HO-1, Akt or p-Akt, and $\beta$-actin at dilutions of 1:1000 overnight at 4 °C. Nuclear fraction was also prepared from the cultured cells and used for measuring the nuclear level of Nrf2 by Western blot. The bands were detected using a chemiluminescence kit (Pierce, Cheshire, U.K.). Densitometry analysis was performed with Lab Image software (Scion Corp., Frederick, MD, USA).

## RESULTS

**Effect of DGC on Glutamate-Induced Cytotoxicity.** Treatment of HT22 cells with glutamate at 2.5 mM or higher concentrations for 12 h dramatically decreased the cell survival rate, but cell survival was dependent on the exposure time more than the dose of the compound (Figure 2A). The cell growth suppression caused by glutamate (5 mM) was restored by 200 $\mu$M Trolox or DGC in a dose-dependent manner as shown in Figure 2B,C.

**Suppression of Glutamate-Induced ROS Production by DGC.** Exposure of HT22 cells to glutamate (5 mM) for 12 h resulted in a 60−80% increase in ROS production, compared to untreated control. However, DGC (2 $\mu$M) lowered the intracellular ROS level induced by glutamate in a dose-dependent fashion (Figure 3).

**Effect of Inhibitors for MAPK, PI3K, and HO-1 on Glutamate-Induced ROS Production.** Whereas the ROS level enhanced by glutamate insult was decreased by DGC, the addition of inhibitors for MAPK, PI3K, and HO-1 dampened the suppressive effect of DGC on ROS production. In particular, the inhibitors for PI3K and HO-1 abolished ROS suppression by DGC in dose-dependent manners (Figure 4).

**Effect of Inhibitors of HO-1, PI3K, and MAPKs on Glutamate-Induced Cell Death.** The growth inhibition of HT22 cells caused by glutamate was restored to the control level by DGC. The effect of DGC was nullified by cotreatment with 50 $\mu$M SnPP, a HO-1 inhibitor (Figure 5). MAPK inhibitors including PD, SB, and SP did not have further effect on the cell proliferation pattern shown in the presence of both glutamate and DGC (Figure 6A). However, a PI3K inhibitor (LY294002) weakened the protective effect of DGC on glutamate-induced cell death (Figure 6B,C).

**Effect of DGC on the Expression of HO-1, Phosphorylation of Akt, and Nuclear Translocation of Nrf2.** To examine the connection between the ability of DGC to induce antioxidant/phase 2 enzymes and its suppression of glutamate-induced ROS generation and neuronal cell death, the expression of HO-1 and its upstream regulator Nrf2 and phosphorylated Akt was analyzed using Western blot. The expression of HO-1 was enhanced upon exposure to DGC in a dose-dependent manner (Figure 7A). The nuclear level of Nrf2, a known upstream regulator of HO-1, was also increased in the cells

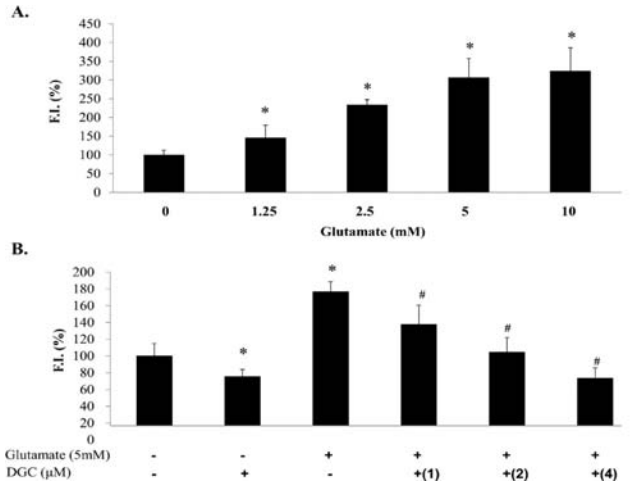


**Figure 3.** Intracellular ROS level as assessed by DCF fluorescence probe. The cells were treated with different doses of glutamate for 6 h, followed by incubation with DCFDA for 30 min and measurement of fluorescence (Ex. 485 nm, Em. 535 nm) (A). The results are expressed as the mean ± SD of three separate independent experiments. (∗, ∗∗, #) $p < 0.05$, compared to the control. The same experiment was conducted except cells were pretreated with DGC for 24 h before exposure to 5 mM glutamate for 12 h (B). (∗)$p < 0.05$, compared to the control; (#) $p < 0.05$, compared to the glutamate stimulation ($n = 3$).

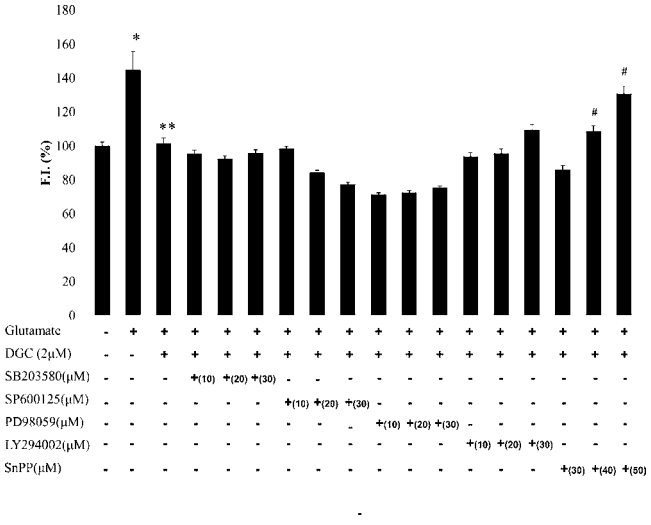


**Figure 4.** Effects of inhibitors for MAPK, PI3K, and HO-1 on glutamate-induced ROS generation. The cells were pretreated with DGC for 2 h and subsequently incubated in the presence of glutamate and one of the following inhibitors of MAPK (SB203580, SP600125, PD98059), PI3K (LY294002), and HO-1 (SnPP), prior to visualization of intracellular ROS by adding fluorescent probe DCFDA. The results are expressed as the mean ± SD of three separate independent experiments. (∗) $p < 0.05$, compared to the control; (∗∗) $p < 0.05$, compared to the glutamate stimulation; (#) $p < 0.05$, compared to glutamate plus DGC.


dx.doi.org/10.1021/jf300548b | J. Agric. Food Chem. 2012, 60, 5583−5589

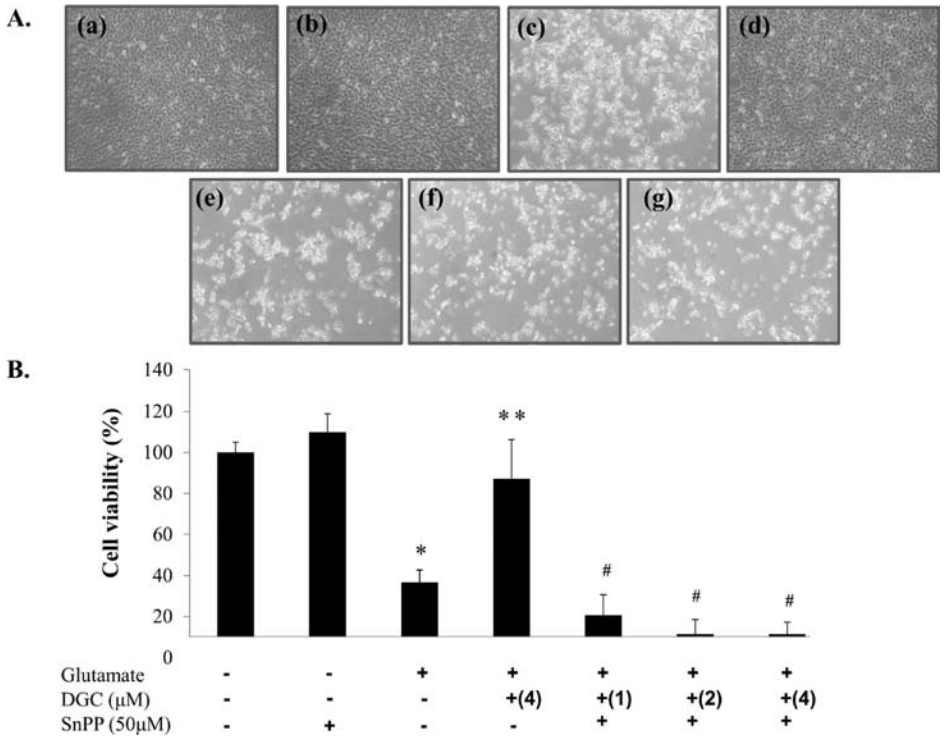


**Figure 5.** Effect of HO-1 inhibitor and DGC on glutamate-induced cytotoxicity. The cells were pretreated with DGC for 2 h and subsequently incubated with glutamate in the absence and presence of SnPP, a HO-1 inhibitor, followed by MTT assay to measure cell viability. (A) Cell morphology (a, control; b, SnPP (50 $\mu$M); c, glutamate (5 mM); d, glutamate + DGC (4 $\mu$M); e, glutamate + DGC (1 $\mu$M) + SnPP (50 $\mu$M); f, glutamate + DGC (2 $\mu$M) + SnPP (50 $\mu$M); g, glutamate + DGC (4 $\mu$M) + SnPP (50 $\mu$M); magnification, 40×). (B) Cell viability as expressed in the percentage of untreated control. The results are expressed as the mean ± SD of three separate independent experiments. (*) $p < 0.05$, compared to the control; (**) $p < 0.05$, compared to the glutamate stimulation; (#) $p < 0.05$, compared to glutamate plus DGC.

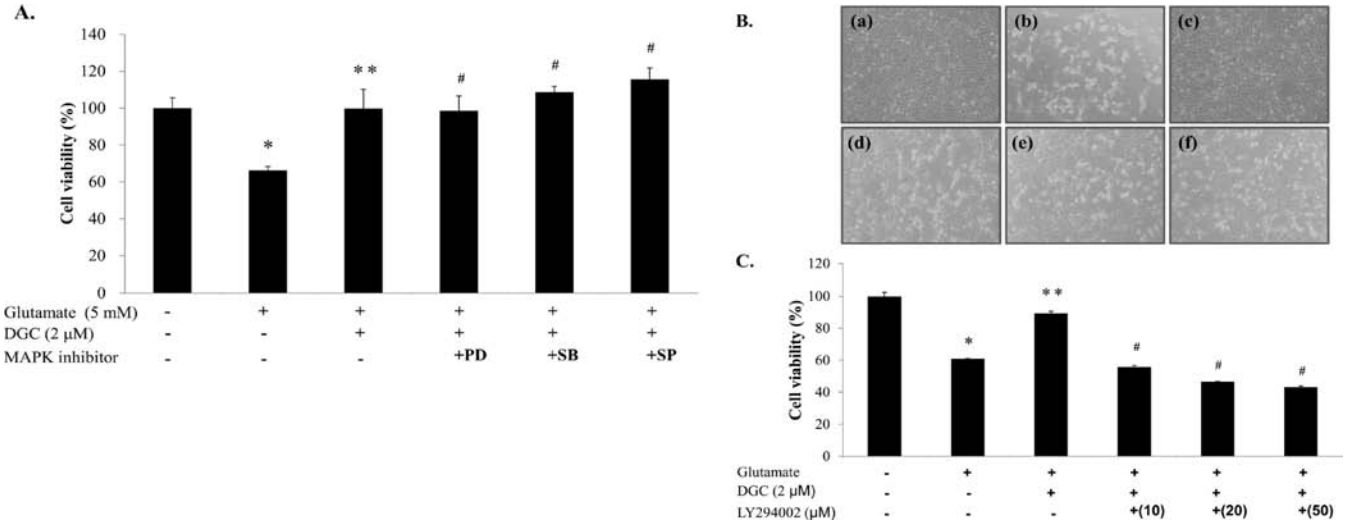


**Figure 6.** Effects of MAPK (A) and PI3K inhibitors (B, C) on neuroprotection by DGC from glutamate-induced cytotoxicity. The cells were pretreated with DGC for 2 h and subsequently incubated with glutamate in the absence and presence of MAPK and PI3K inhibitors [(B) a, control; b, glutamate (5 mM); c, glutamate + DGC (2 $\mu$M); d, glutamate + DGC (2 $\mu$M) + LY294002 (10 $\mu$M); e, glutamate + DGC (2 $\mu$M) + LY294002 (20 $\mu$M); f, glutamate + DGC (2 $\mu$M) + LY294002 (50 $\mu$M)], followed by MTT assay to measure cell viability. The results are expressed as the mean ± SD of three separate independent experiments. (*) $p < 0.05$, compared to the control; (**) $p < 0.05$, compared to the glutamate stimulation; (#) $p < 0.05$, compared to glutamate plus DGC.

treated with DGC (Figure 7B). Up-regulation of HO-1 by DGC was not affected by MAPK inhibitors such as SB203580, SP600125, and PD98059 but was suppressed by PI3K inhibitor LY294002, suggesting the involvement of the PI3K signaling pathway in the regulation of the intracellular level of HO-1 (Figure 7C).

Furthermore, phosphorylation of Akt, which is downstream of PI3K, was dose-dependently increased by DGC, confirming the

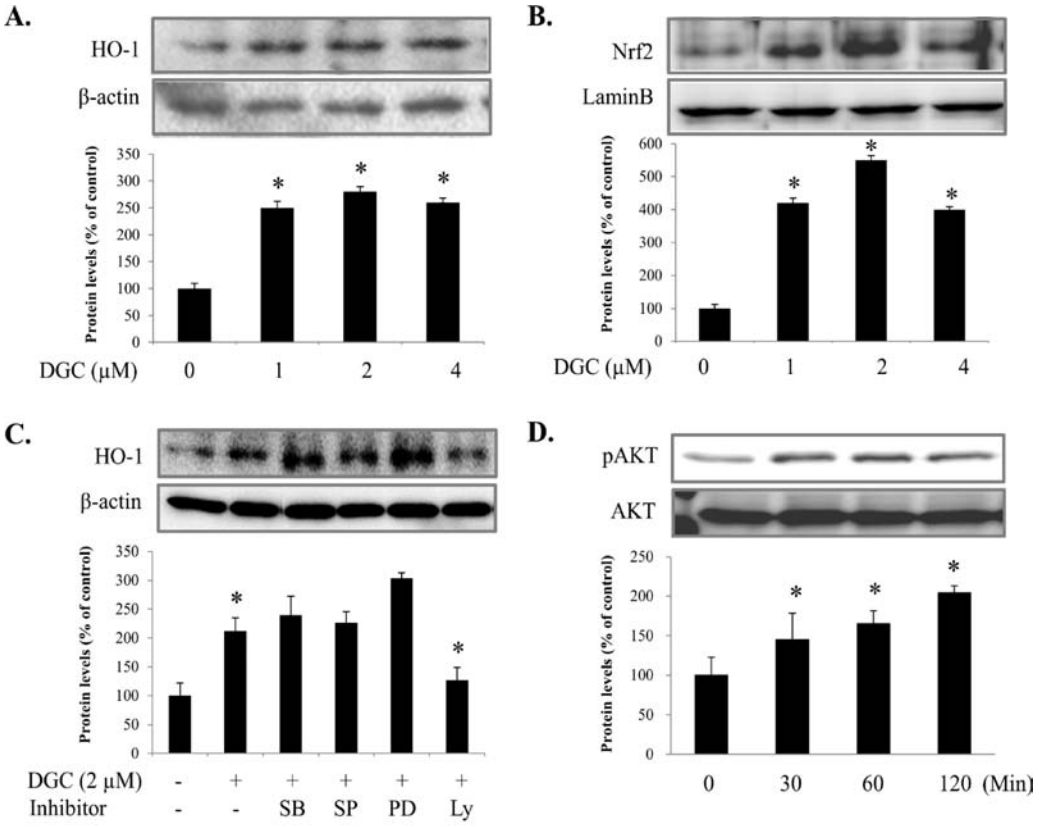


**Figure 7.** Effect of DGC on cellular HO-1 and nuclear Nrf2 levels, phosphorylation of Akt. The cells were incubated with various concentrations of DGC in the absence and presence of MAPK and PI3K inhibitors, followed by Western blot for HO-1 (A, C), nuclear Nrf2 (B), and phosphorylated Akt (D). The results are expressed as the mean ± SD of three separate independent experiments. (*) $p < 0.05$, compared to the control.

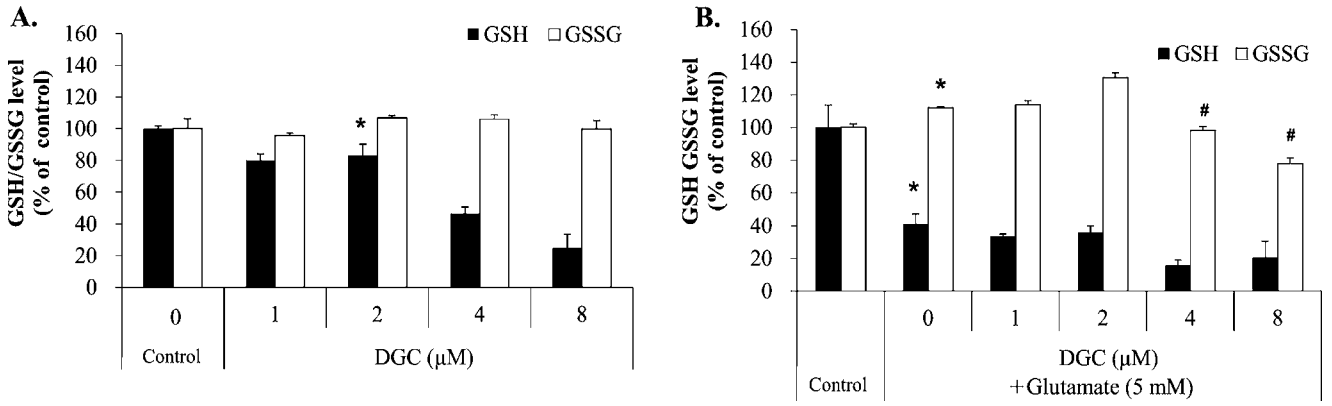


**Figure 8.** Change of levels of reduced and oxidized glutathione by DGC in the absence and presence of glutamate. The cells were incubated with various concentrations of DGC in the absence (A) and presence (B) of 5 mM glutamate, followed by measuring reduced and oxidized levels of glutathione. The results are expressed as the mean ± SD of three separate independent experiments. (*) $p < 0.05$, compared to the control; (#) $p < 0.05$, compared to the glutamate stimulation.

involvement of the PI3K signaling pathway in increased expression of HO-1 (Figure 7D).

## DISCUSSION

Oxidative stress has been implicated in numerous chronic diseases such as Alzheimer's disease, cancer, heart disease, diabetes, and others. Natural antioxidants, therefore, have been considered as valuable food components in preventing chronic diseases. Our previous study showed that DGC, one of the prenylflavonoids isolated from licorice, had a strong potential to induce antioxidant/phase 2 detoxifying enzymes.[16] The compound was also found to exhibit stronger antioxidant activity in an in vitro assay,[13] compared to other prenylflavonoids found in licorice such as isoangustone A and dehydroglyasperin D (unpublished data). Therefore, we hypothesized that DGC could repress glutamate-induced ROS production and thereby protect neuronal cells from glutamate insult. This study showed that DGC effectively inhibited ROS production and cell death induced by glutamate. The reversal of glutamate-induced cell death by DGC could be


dx.doi.org/10.1021/jf300548b | J. Agric. Food Chem. 2012, 60, 5583−5589

explained by either direct radical scavenging activity of the compound or indirect effect that induces antioxidant enzymes such as HO-1. Whereas the possibility of direct antioxidative action of the compound is not excluded, it is more likely that DGC exerts a protective effect against glutamate toxicity on neuronal cells through Nrf2-mediated HO-1 expression because the inhibition of HO-1 or its upstream PI3K abrogated the cytoprotective effect of DGC against glutamate-induced toxicity.

HO-1, an inducible and cytoprotective enzyme, catalyzes the rate-limiting step in heme degradation, resulting in the generation of biliverdin, free iron, and carbon monoxide.[16,17] That is, heme and its oxidized form hemin are strong prooxidants and are normally metabolized by HO-1 and HO-2 enzymes into biliverdin, carbon monoxide, and iron, another prooxidant. Whether inhibiting or promoting heme catabolism is protective seems to be cell type-specific, as up-regulation of the HO enzymes yielded opposing effects in neurons and astrocytes.[18−20] Whereas overexpression of HO-1 protected SN56 neuron-like cells from $H_2O_2$-induced death,[21] PC12 cells cultured on HO-1-overexpressing astrocytes were more prone to oxidative injury.[22] A recent study demonstrated that sulforaphane, one of the well-known phase 2 enzyme inducers, protected immature hippocampal neurons against death caused by exposure to hemin or to oxygen and glucose deprivation, suggesting that sulphoraphane, a typical ARE/Nrf2 pathway activator, protects immature neurons from death caused by oxidative stress.[20] Our study also showed that DGC did not affect the expression of the other antioxidant/phase 2 enzymes such as NAD(P)H:quinone oxidoreductase, glutathione *S*-transferase, γ-glutamylcysteine ligase, and glutathione reductase, known to be induced by ARE-Nrf2 and PI3K/Akt signaling pathway were not significantly upregulated (unpublished data), accordingly causing a limited change or reduction in the levels of total and reduced glutathione (Figure 8). Taken together, the data strongly suggest the major role of HO-1 in the neuroprotective effect of DGC.

Although DGC showed strong neuroprotective potential using in vitro model systems, it is not clear whether it exerts the same effect in vivo or not because most flavonoids are usually extensively metabolized in the body and their physiological activities could be altered significantly. Even so, there are a number of reports that antioxidant phytochemicals contribute to benefits of human health. In conclusion, DGC merits further in vivo study to evaluate its potential as an agent for preventing Alzheimer's disease incidence or progression.

## AUTHOR INFORMATION

### Corresponding Author

*¶Phone: 82-53-950-5752. Fax: 82-53-950-6750. E-mail: vision@knu.ac.kr.

### Funding

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0009782).

### Notes

The authors declare no competing financial interest.

## REFERENCES

(1) Shibata, S. A drug over the millennia: pharmacognosy, chemistry, and pharmacology of licorice. *Yakugaku Zasshi* **2000**, *120*, 849−862.

(2) Thimmulappa, R. K.; Rangasamy, T.; Alam, J.; Biswal, S. Dibenzoylmethane activates Nrf2-dependent detoxification pathway and inhibits benzo(a)pyrene induced DNA adducts in lungs. *Med. Chem.* **2008**, *4*, 473−481.

(3) Craig, W. J. Health-promoting properties of common herbs. *Am. J. Clin. Nutr.* **1999**, *70*, 491S−499S.

(4) Mae, T.; Kishida, H.; Nishiyama, T.; Tsukagawa, M.; Konishi, E.; Kuroda, M.; Mimaki, Y.; Sashida, Y.; Takahashi, K.; Kawada, T.; Nakagawa, K.; Kitahara, M. A licorice ethanolic extract with peroxisome proliferator-activated receptor-γ ligand-binding activity affects diabetes in KK-Ay mice, abdominal obesity in diet-induced obese C57BL mice and hypertension in spontaneously hypertensive rats. *J. Nutr.* **2003**, *133*, 3369−3377.

(5) Gumpricht, E.; Dahl, R.; Devereaux, M. W.; Sokol, R. J. Licorice compounds glycyrrhizin and 18β-glycyrrhetinic acid are potent modulators of bile acid-induced cytotoxicity in rat hepatocytes. *J. Biol. Chem.* **2005**, *280*, 10556−105563.

(6) Wittschier, N.; Faller, G.; Hensel, A. Aqueous extracts and polysaccharides from liquorice roots (*Glycyrrhiza glabra* L.) inhibit adhesion of Helicobacter pylori to human gastric mucosa. *J. Ethnopharmacol.* **2009**, *125*, 218−223.

(7) Seo, J. Y.; Lee, Y. S.; Kim, H. J.; Lim, S. S.; Lim, J. S.; Lee, I. A.; Lee, C. H.; Yoon Park, J. H.; Kim, J. S. Dehydroglyasperin C isolated from licorice caused Nrf2-mediated induction of detoxifying enzymes. *J. Agric. Food Chem.* **2010**, *58*, 1603−1608.

(8) Ren, J.; Fan, C.; Chen, N.; Huang, J.; Yang, Q. Resveratrol pretreatment attenuates cerebral ischemic injury by upregulating expression of transcription factor Nrf2 and HO-1 in rats. *Neurochem. Res.* **2011**, *36*, 2352−2362.

(9) Nakao, A.; Neto, J. S.; Kanno, S.; Stolz, D. B.; Kimizuka, K.; Liu, F.; Bach, F. H.; Billiar, T. R.; Choi, A. M.; Otterbein, L. E.; Murase, N. Protection against ischemia/reperfusion injury in cardiac and renal transplantation with carbon monoxide, biliverdin and both. *Am. J. Transplant.* **2005**, *5*, 282−291.

(10) Berberat, P. O.; Katori, M.; Kaczmarek, E.; Anselmo, D.; Lassman, C.; Ke, B.; Shen, X.; Busuttil, R. W.; Yamashita, K.; Csizmadia, E.; Tyagi, S.; Otterbein, L. E.; Brouard, S.; Tobiasch, E.; Bach, F. H.; Kupiec-Weglinski, J. W.; Soares, M. P. Heavy chain ferritin acts as an antiapoptotic gene that protects livers from ischemia reperfusion injury. *FASEB J.* **2003**, *17*, 1724−1726.

(11) Atlante, A.; Calissano, P.; Bobba, A.; Giannattasio, S.; Marra, E.; Passarella, S. Glutamate neurotoxicity, oxidative stress and mitochondria. *FEBS Lett.* **2001**, *497*, 1−5.

(12) Coyle, J. T.; Puttfarcken, P. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **1993**, *262*, 689−695.

(13) Lee, Y. S.; Kim, S. H.; Kim, J. K.; Shin, H. K.; Kang, Y. H.; Park, J. H.; Lim, S. S. Rapid identification and preparative isolation of antioxidant components in licorice. *J. Sep. Sci.* **2010**, *33*, 664−671.

(14) Wang, H.; Joseph, J. A. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biol. Med.* **1999**, *27*, 612−616.

(15) Kim, H. J.; Suh, H. J.; Kim, J. H.; Park, S.; Joo, Y. C.; Kim, J. S. Antioxidant activity of glyceollins derived from soybean elicited with *Aspergillus sojae*. *J. Agric. Food Chem.* **2011**, *58*, 11633−11638.

(16) Lee, S. E.; Jeong, S. I.; Yang, H.; Jeong, S. H.; Jang, Y. P.; Park, C. S.; Kim, J.; Park, Y. S. Extract of *Salvia miltiorrhiza* (Danshen) induces Nrf2-mediated heme oxygenase-1 expression as a cytoprotective action in RAW 264.7 macrophages. *J. Ethnopharmacol.* **2010**, *139*, 541−548.

(17) Ghattas, M. H.; Chuang, L. T.; Kappas, A.; Abraham, N. G. Protective effect of HO-1 against oxidative stress in human hepatoma cell line (HepG2) is independent of telomerase enzyme activity. *Int. J. Biochem. Cell Biol.* **2002**, *34*, 1619−1628.

(18) Platt, J. L.; Nath, K. A. Heme oxygenase: protective gene or Trojan horse. *Nat. Med.* **1998**, *4*, 1364−1365.

(19) Wagner, K. R.; Sharp, F. R.; Ardizzone, T. D.; Lu, A.; Clark, J. F. Heme and iron metabolism: role in cerebral hemorrhage. *J. Cereb. Blood Flow Metab.* **2003**, *23*, 629−652.

(20) Soane, L.; Li Dai, W.; Fiskum, G.; Bambrick, L. L. Sulforaphane protects immature hippocampal neurons against death caused by exposure to hemin or to oxygen and glucose deprivation. *J. Neurosci. Res.* **2010**, *88*, 1355−1363.

(21) Le, W. D.; Xie, W. J.; Appel, S. H. Protective role of heme oxygenase-1 in oxidative stress-induced neuronal injury. *J. Neurosci. Res.* **1999**, *56*, 652−658.
(22) Song, L.; Song, W.; Schipper, H. M. Astroglia overexpressing heme oxygenase-1 predispose co-cultured PC12 cells to oxidative injury. *J. Neurosci. Res.* **2007**, *85*, 2186−2195.