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Dehydroglyasperin C Isolated from Licorice Caused Nrf2-Mediated Induction of Detoxifying Enzymes

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Our preliminary experiment demonstrated that a *n*-hexane/EtOH (9:1, volume) extract of *Glycyrrhiza uralensis* (licorice) caused a significant induction of NAD(P)H:oxidoquinone reductase (NQO1), one of the well-known phase 2 detoxifying enzymes. We isolated dehydroglyasperin C (DGC) as a potent phase 2 enzyme inducer from licorice. DGC induced NQO1 both in wild-type murine hepatoma Hepa1c1c7 and ARNT-lacking BPRc1 cells, indicating that the compound is a monofunctional inducer. The compound induced not only NQO1 but also some other phase 2 detoxifying/antioxidant enzymes, such as glutathione *S*-transferase, γ-glutamylcysteine synthase, glutathione reductase, and heme oxygenase 1. Similar to most monofunctional inducers, DGC caused the accumulation of Nrf2 in the nucleus in dose- and time-dependent manners and thereby activated expression of phase 2 detoxifying enzymes. It also resulted in a dose-dependent increase in the luciferase activity in the reporter assay, in which HepG2-C8 cells transfected with antioxidant response element (ARE)−luciferase construct were used, suggesting that the induction of phase 2 detoxifying and antioxidant enzymes could be achieved through the interaction of Nrf2 with the ARE sequence in the promoter region of their genes.

KEYWORDS: Dehydroglyasperin C; phase 2 detoxifying enzymes; Nrf2; licorice

INTRODUCTION

Licorice, the root of *Glycyrrhiza* spp. (Fabaceae), has been used since ancient Egyptian, Greek, and Roman times in the West and since the former Han era (from 206 BC to 8 AD) in ancient China in the East (*1*). In traditional Chinese medicine, licorice is one of the most frequently used ingredients. Licorice is promoted as a herb that can treat peptic ulcers, eczema, skin infections, cold sores, menopausal symptoms, liver disease, respiratory ailments, inflammatory problems, chronic fatigue syndrome, acquired immune deficiency syndrome (AIDS), and even cancer. In particular, glycyrrhizin (a triterpenoid saponin) and chalcones in licorice have been reported to act as antioxidants and electrophile scavengers, stimulate the immune system, inhibit nitrosation and the formation of DNA adducts with carcinogens, inhibit hormonal actions and metabolic pathways associated with the development of cancer, and induce phase 1 or 2 detoxification enzymes (*1*, *2*). In addition to its anti-carcinogenic and anti-mutagenic effects, there is scientific evidence that licorice had antioxidant, anti-obesity, and anti-microbial activities (*3*−*6*).

Our preliminary study showed that the methanolic extract of licorice induced the activity of NAD(P)H: quinone oxidoreductase 1 (NQO1), an anti-carcinogenic marker enzyme, in a dose-dependent manner in mouse hepatoma cells. Therefore, bioassay-guided fractionation was performed to isolate the compound(s) responsible for NQO1 induction. Because most phase 2 enzyme inducers act through the interaction with the heterodimer of NF-E2-related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) (*7*), we also investigated whether the NQO1 inducer purified from licorice promotes the translocation of Nrf2 into the nucleus and exerts its effect through interacting with antioxidant response element (ARE) (*8*), a cis-acting enhancer sequence that mediates transcriptional activation of genes in the cells exposed to phase 2 detoxifying enzyme inducers.

MATERIALS AND METHODS

Materials. All cell culture reagents and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaitherburg, MD). Hepa1c1c7 and BPRc1 cells were from American Type Culture Collection (Rockville, MD). Dried licorice roots of *Glycyrrhiza uralensis* were purchased from a local medicinal herb store (Daeguang Medical, Chuncheon, Republic of Korea). A voucher (number 326) is deposited at the Hallym University Regional Innovation Center (RIC) in Chuncheon, Republic of Korea. The structure identification of the compound was carried out by ^{1}H and ^{13}C nuclear magnetic resonance (NMR). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer (Billerica, MA) with tetramethylsilane (TMS) as an internal standard. IR spectra were recorded on a FTIR 4100 (Jasco, Inc., Easton, MD). Mass spectra were obtained on a PolarisQ ion trap GC/MS^{n} (Thermo Fisher Scientific, Inc., Waltham, MA).

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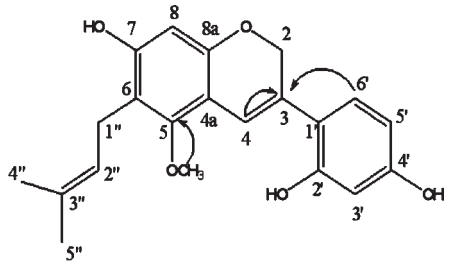

Figure 1. Structure of DGC.

Cell Culture. Hepa1c1c7 and its mutant (BPRc1) cells were plated at a density of 3×10^5 and 5×10^5 cells per 100 mm plate (Nunc, Rochester, NY) in 10 mL of α-MEM supplemented with 10% FBS, respectively. The HepG2-C8 cell line established in Dr. Kong's lab at Rutgers, The State University of New Jersey, New Brunswick, NJ, by transfecting human hepatoma HepG2 cells with pARE-TI-luciferase construct was used for the reporter assay (*9*). HepG2-C8 cells were maintained in modified Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, GlutaMax (Gibco number 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 °C. Cells were cultured for 48 h, exposed to various concentrations of sample for another 24 h, and followed by biochemical assays.

Extraction and Purification of NQO1 Inducer from Licorice. The *n*-hexane/ethanol extract of *G. uralensis* (90 g, 0.9% yield) was produced by dip extraction with *n*-hexane/ethanol at a ratio of 9:1 (v/v) of dried and ground roots of *G. uralensis* (1 kg). A portion of the extract (5.2 g) was subjected to flash column chromatography with silica gel (Macherey-Nagel Kieselgel, Darmstadt, Germany), eluted by gradient systems of *n*-hexane/ethyl acetate (10:0 → 5:5, v/v) to obtain 20 fractions. The fractions (0.7 g) showing NQO1-induction activity were combined and further purified by recrystallization to recover compound **1** (35 mg). The structure of the compound was elucidated as dehydroglyasperin C (DGC) by a comparison of the spectral data to an authentic sample as described in the literature (*10*).

Identification of DGC. ^{1}H and ^{13}C NMR spectra were recorded in dimethylsulfoxide (DMSO) on a Bruker Avance 600 MHz spectrometer (Billerica, MA). All ^{13}C multiplicities were deduced from 90° and 135° distortionless enhancement by polarization transfer (DEPT) experiments. IR spectra were recorded on a FTIR 4100 (Jasco, Inc., Easton, MD). Mass spectra were obtained on a PolarisQ ion trap GC/MS^n (Thermo Fisher Scientific, Inc., Waltham, MA). Vacuum liquid chromatography (VLC) separations were carried out on silica gel (Macherey-Nagel Kieselgel, Darmstadt, Germany). The analytical properties of DGC were as follows. UV ($C_{21}H_{22}O_5$) λ_{max} (MeOH): 289, 329. IR υ_{max} (cm^{-1}): 3351, 2967, 1699, 1616, 1514, 1167, 839. EI−MS: 354 $[M]^+$. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.62 (3H, s, H-5″), 1.7 (3H, s, H-4″), 3.14 (2H, d, J = 6.54, H-1″), 3.67 (3H, s, 5-OCH_3), 4.83 (2H, s, 2-CH_2), 5.13 (1H, t-like, H-2″), 6.14 (1H, s, H-8), 6.25 (1H, dd, J = 8.3, 2.4, H-5′), 6.32 (1H, s, J = 2.4, H-3′), 6.64 (1H, s, H-4), 7.04 (1H, d, J = 8.3, H-6′), 9.39 (1H, s, 4-OH), 9.54 (1H, s, 7-OH), 9.60 (1H, s, 2′-OH). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 18.12 (C-4″), 22.61 (C-1″), 25.94 (C-5″), 62.10 (C-5-OCH_3), 67.73 (C-2CH_2), 98.89 (C-8), 103.18 (C-3′), 107.30 (C-5′), 109.21 (C-4a), 114.36 (C-6), 115.06 (C-4), 117.02 (C-1′), 124.37 (C-2″), 127.97 (C-3), 129.12 (C-6′), 129.95 (C-3″), 152.74 (C-8a), 155.38 (C-5), 156.24 (C-7), 156.57 (C-2′), 158.46 (C-4′). The chemical structure of the compound was shown in **Figure 1**.

NQO1 Activity Assay. NQO1 enzyme activity was measured by a spectrophotometric assay, in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm (*11*). *tert*-Butylhydroquinone (TBHQ, 20 μM or 3.3 μg/mL), a known NQO1 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 (*12*). All values are reported as mean ± standard deviation (SD) whenever possible.

Reporter Assay. HepG2-C8 cells (kindly provided by Dr. Tony Kong at Rutgers) 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 prior to sample treatment. After cells were cultured for another 16 h in the presence of varying concentrations of sample, cells were collected and the luciferase activity was assayed according to the protocol provided by the manufacturer (Promega Corp., Madison, WI) (*9*).

Preparation of the Nuclear Protein Extract. Nuclear and cytosolic protein extracts were prepared according to the method as described (*13*). Briefly, cells were cultured on 100 mm dishes to 90% confluence and treated with DGC for different periods. Cells were then washed with phosphate-buffered saline (PBS) twice and harvested by scraping in ice-cold PBS followed by centrifugation at 500*g* for 5 min. Cells were lysed with buffer A [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)−KOH (pH 7.9), 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] on ice for 20 min and then centrifuged at 14000*g* for 15 min at 4 °C. The supernatants were saved as the cytoplasmic fractions. The nuclear pellets were washed 3 times with buffer A and resuspended in buffer B (20 mM HEPES, 0.5 M KCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF at pH 7.9) for 30 min at 4 °C on a rotating wheel and then centrifuged at 14000*g* for 15 min at 4 °C. The nuclear fraction was subjected to immunoblot analysis using anti-Nrf2 and anti-Src-associated in mitosis 68 kDa (SAM68) antibodies.

Western Blot. Western blotting was performed on cytosolic fractions prepared from cultured cells to estimate the level of detoxifying enzymes according to a protocol described previously (*9*, *14*). The primary antibodies including anti-NQO1, anti-GST-pi, anti-HO1, anti-γGCS, anti-GR, anti-Nrf2, anti-β-tubulin, and horseradish-peroxidase-conjugated secondary antibody anti-goat or anti-rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunocytochemistry. Hepa1c1c7 and BPRc1 cells were plated in a 24-well plate containing sterile coverslips at a density of 2×10^4 cells/well. After 24 h of incubation, cells were exposed to the sample for another 6 h. Cells were washed with PBS, fixed with 4% paraformaldehyde, and blocked with 10% rabbit and goat serum. Cells were treated with anti-Nrf2 antibody and anti-Keap1 antibody with anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC), anti-goat IgG-conjugated Texas red, and 4′,6-diamidino-2-phenylindole (DAPI, 100 ng/mL). After washing with Tris-buffered saline (TBS), cell slides were treated with a drop of fluorescent mounting medium (Biomeda, Foster City, CA) and observed in an inverted fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan) at the 400× magnification.

Statistical Analysis. The statistical significance of data was tested by analysis of variance (ANOVA), followed by Duncan's multiple range test, using SPSS software (SPSS, Inc., Chicago, IL). $p < 0.05$ was considered to be statistically significant.

RESULTS

Effect of DGC on NQO1 Activity. To investigate the effect of DGC on NQO1 enzyme activity, we used mouse hepatoma Hepa1c1c7 and its mutant BPRc1 lacking arylhydrocarbon receptor nuclear translocator (ARNT). Both cell lines have usually been used together because they could differentiate monofunctional inducers, which affect only phase 2 enzymes in BPRc1 lacking ARNT, from bifunctional inducers, which are not able to induce phase 2 enzymes in the absence of functional ARNT. As shown in **Figure 2**, DGC enhanced NQO1 activity in a dose-dependent manner in the range of 0−2 μM, while it did not cause a further increase in the enzyme activity at the concentration of 4 μM or higher, probably because of cytotoxicity as evidenced by the cell growth inhibition assay (data not shown). Both Hepa1c1c7 and BPRc1 cells showed the morphology indicative of apoptosis, including the loss of cell volume, retraction of processes, and blebbing of the cell membrane at the dose of 8 μM DGC when exposed to the compound continuously or 72 h, as shown in **Figure 2A**.

Induction of Phase 2 Enzymes by DGC. To examine whether DGC regulates the expression of other phase 2 detoxifying enzymes than NQO1, Hepa1c1c7 and BPRc1 cells preincubated for 48 h in DGC-free media were exposed to various concentrations

of DGC for 24 h, followed by Western blot analysis for the enzymes. The expression of phase 2 detoxifying enzymes and antioxidant enzymes, including NQO1, γ-glutamylcysteine synthase (GCS), glutathione *S*-transferase (GST), heme oxygenase 1 (HO1), and glutathione reductase (GR), was induced by DGC in a dose-dependent fashion in both Hepa1c1c7 and BPRc1 cells (**Figure 3**).

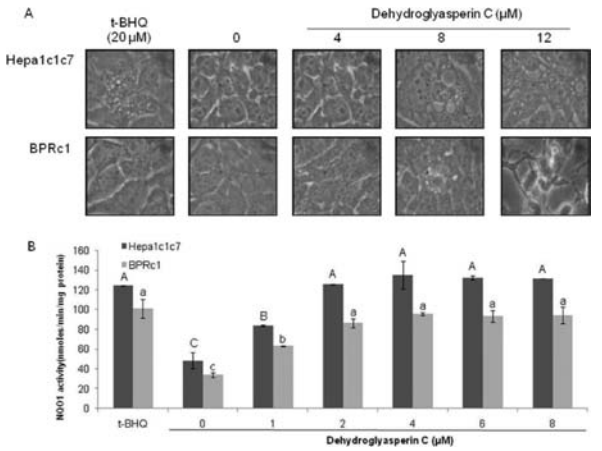

Figure 2. Induction of NQO1 by DGC in Hepa1c1c7 and BPRc1 cells. Hepa1c1c7 (black bar) and BPRc1 (gray bar) cells were treated with various doses of DGC for 24 h, followed by assaying for QR activity, as described in the Materials and Methods. Bars represent mean ± SD. Means without a common letter differ, $p < 0.05$. (A) Cell morphology after exposing cells to DGC for 72 h and (B) NQO1 enzyme activity.

ARE-Mediated Transcriptional Activation by DGC. To investigate whether DGC induces phase 2 enzymes through interacting ARE, cis-element in the promoter region of phase 2 enzymes, HepG2-C8 cells that contain reporter ARE−luciferase gene were treated with various doses of DGC for different periods. Luciferase activity in the cells was maximal when exposed to 8 μM of the compound for 12 h and decreased gradually with prolonged exposure, such as 16, 20, and 24 h (**Figure 4A**). The reporter activity showed a proportionate increase in response to the 12 h exposure to DGC in the range of 1−8 M (**Figure 4B**).

Nuclear Translocation of Nrf2 by DGC. It has been known that Nrf2 modulated the expression of phase 2 enzyme genes. That is, upon activation by various factors, such as oxidative stress, the Nrf2−Keap1 complex is dissociated through the modification of Nrf2 or Keap1 and Nrf2 translocates into the nucleus, binds to ARE, a specific DNA sequence in the promoter region of genes of some antioxidant and detoxifying enzymes, and increases the transcription of the related genes (*15*). Nuclear translocation of Nrf2 was increased by treatment with DGC for the first 6 h in a time-dependent manner, following attenuation by prolonged exposure, such as 9 and 12 h (**Figure 5**). Migration of Nrf2 into the nucleus was also enhanced in proportion to the dose of DGC in both Hepa1c1c7 and BPRc1 cells (**Figure 6**). Increased nuclear translocation of Nrf2 by DGC was also confirmed by immunocytochemistry, as shown in **Figure 7**.

DISCUSSION

Licorice has been reported to have numerous pharmacological activities, such as anti-ulcer, anti-allergic, anti-hepatitis, anticancer, and hepatoprotective effects (*1*). Because licorice has been

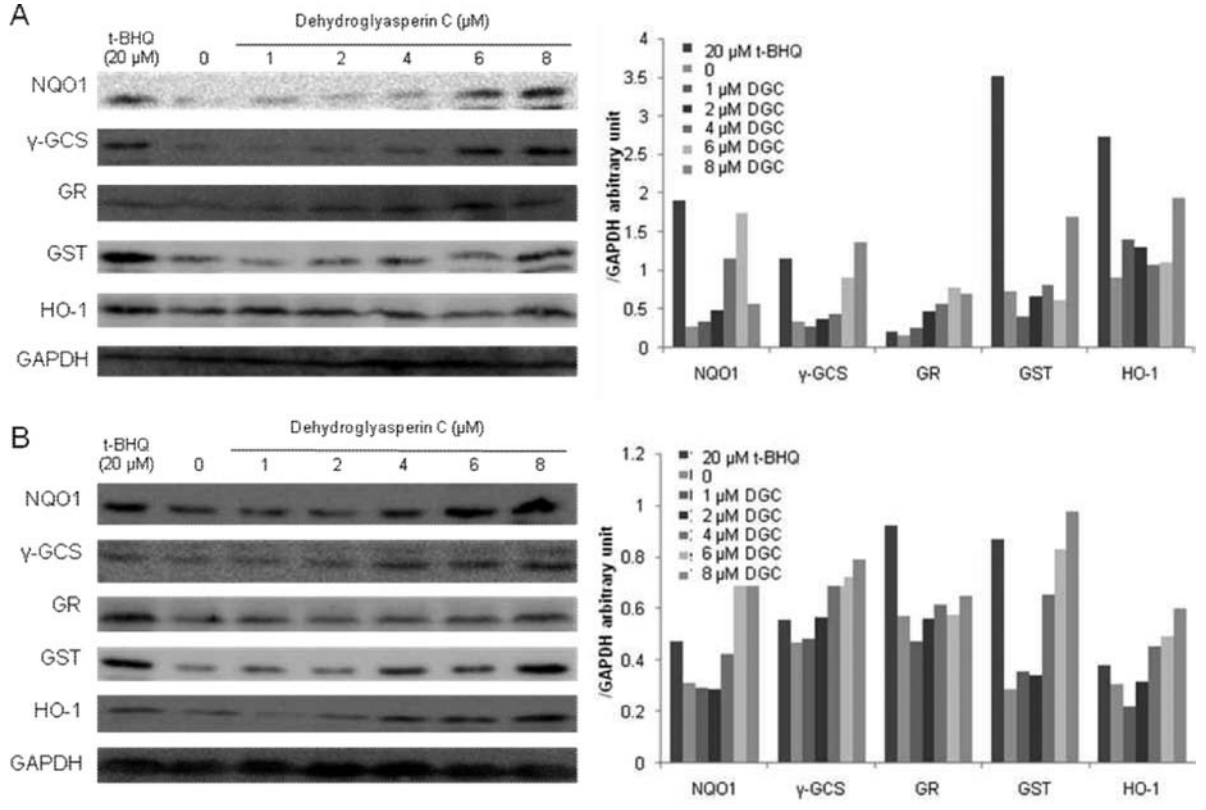

Figure 3. Effect of DGC on the expression of phase 2 detoxifying enzymes, including NQO1, γ-GCS, GR, GST-pi, and HO-1 in (A) Hepa1c1c7 and (B) BPRc1 cells. The cells preincubated for 48 h in DGC-free media were treated with various doses of DGC for 24 h, followed by immunoblot analyses, as described in the Materials and Methods. NQO1, NAD(P)H:(quinone acceptor) oxidoreductase 1; GST, glutathione *S*-transferase; HO-1, heme oxygenase; γ-GCS, γ-glutamylcysteine synthase; GR, glutathione reductase.

known to be included in many Chinese herbal medicines, we hypothesized that it should contain components not only exhibiting pharmacological activity but also alleviating side effects that the other herbal ingredients might have. We, therefore, attempted to isolate component(s) that induce phase 2 detoxification enzymes by bioassay-guided fractionation. Among three compounds screened by evaluating NQO1 inducing activity in BPRc1 cells, DGC showed the highest potential to induce NQO1 enzyme activity, suggesting that it is a good monofunctional inducer (*16*). The other two compounds, isoangustone A and dehydroglyasperin D, barely affected NQO1 enzyme activity, although its structure was similar to DGC (data not shown). The presence of the OH group at C7 appears to play an important role in the induction of phase 2 detoxifying enzymes because substitution of the OH group at C7 with the methoxyl group significantly attenuated the enzyme-inducing activity. The expression of most phase 2 detoxifying enzymes has been known to be upregulated by the interaction between ARE in those genes and Nrf2, of which nuclear translocation was stimulated by external and internal stimuli, such as phase 2 enzyme inducers (*17*). That is, upon binding the cell membrane, phase 2 enzyme inducers generate a certain signal that, in turn, is transmitted to the Nrf2−Keap1 complex, facilitating dissociation of Nrf2 from Keap1 and migration of Nrf2 into the nucleus (*15*). It is also possible for phase 2 enzyme inducers to liberate Nrf2 from the complex by directly influencing the Nrf2−Keap1 complex. For instance, modification of cysteine residues in Keap1 has been reported to reduce the affinity to Nrf2 and promote proteosomal degradation of Keap1, while Nrf2 is translocated into the nucleus. Some inducers appear to act by stimulating the PI3K-Akt-mTOR signaling pathway that, in turn, leads to phosphorylation of Nrf2 (*18*, *19*). The phosphorylation of Nrf2 can cause conformational change, facilitating the separation of Nrf2 from Keap1 and its migration into the nucleus to activate the transcription of its target genes. There is also the possibility that DGC may directly modify Keap1 and cause dissociation of Nrf2 from Keap1 and the translocation of Nrf2 into the nucleus, which will lead to the induction of phase 2 enzymes (*17*). However, it is also possible that it may disturb the intracellular redox status, which will transmit the signal to "sensor(s)", activate a certain kinase, such as guanosine 3′,5′-monophosphate (cGMP)-dependent protein kinase (PKG), relay its signal to the Nrf2−Keap1 complex, and lead to the dissociation of Nrf2 from the complex (*20*, *21*). It is likely that the nuclear accumulation of Nrf2 is regulated at multiple levels, allowing for rapid yet precise regulation of Nrf2-dependent transcription in response to perturbations of the intracellular redox environment (*22*).

Indeed, a structurally diverse array of plant-derived phytochemicals, including isothiocyanates, coumarins, indoles, sesquiterpenes, and lactones, has been shown to be efficacious in the

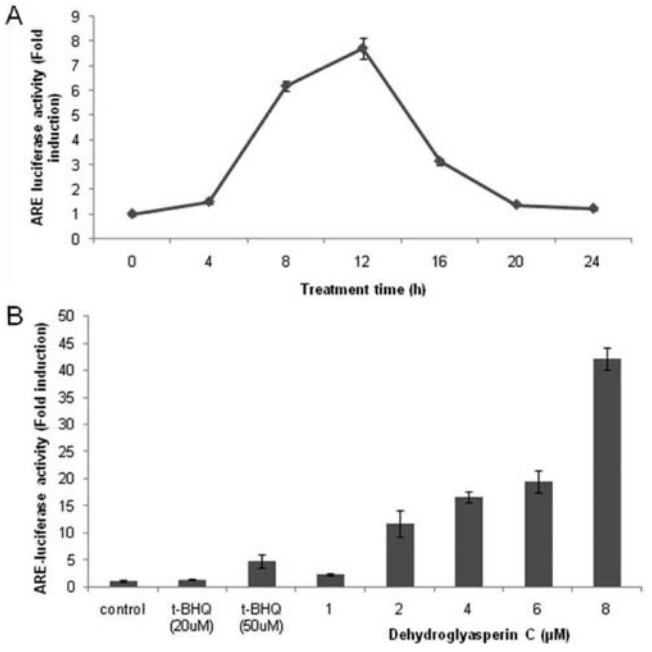

Figure 4. Time- and dose-dependent induction of ARE−luciferase activities by DGC in HepG2-C8 cells, which were generated by transfecting plasmid containing ARE−luciferase gene into HepG2 cells. (A) Cells were exposed to 8 μM DGC for 0−24 h, followed by the reporter assay. (B) Cells were exposed for 12 h to DGC in the range of 0−8 μM, followed by the reporter assay. Bars represent mean ± SD. Means without a common letter differ, $p < 0.05$.

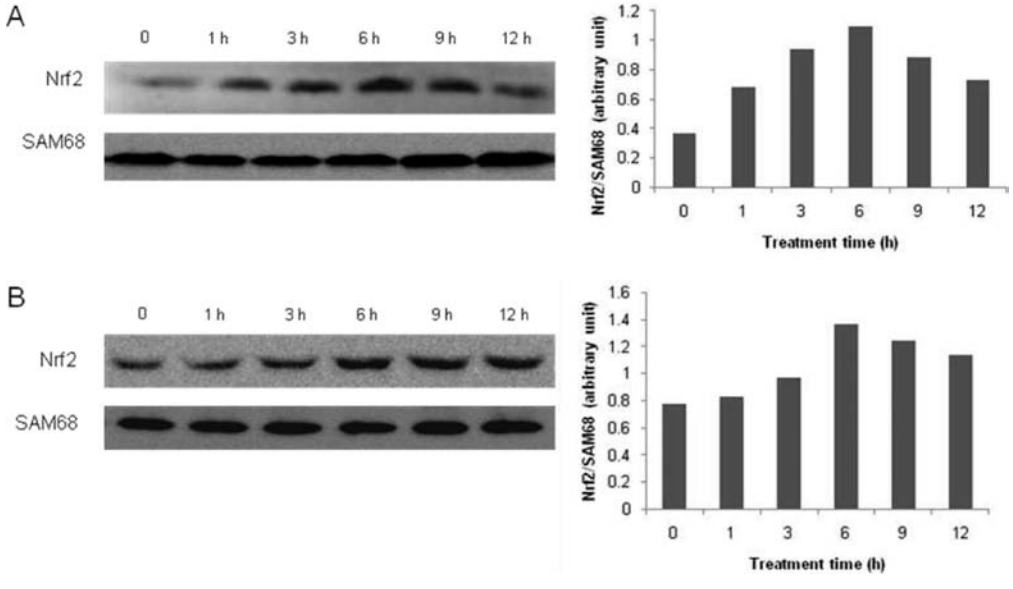

Figure 5. Time-dependent nuclear accumulation of Nrf2 by DGC in Hepa1c1c7 and BPRc1 cells. (A) Hepa1c1c7 and (B) BPRc1 cells preincubated for 48 h were treated with 8 μM DGC for indicated times. The antibody against Sam 68 was used as a loading control of the nuclear extract.

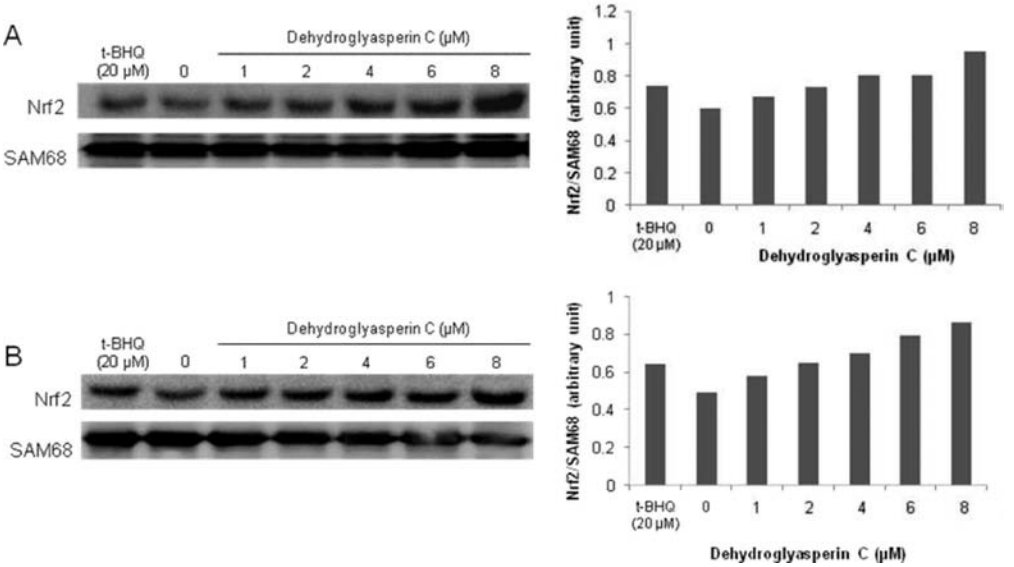

Figure 6. Dose-dependent nuclear accumulation of Nrf2 by DGC in Hepa1c1c7 and BPRc1 cells. (A) Hepa1c1c7 and (B) BPRc1 cells were treated with indicated concentrations of DGC for 6 h. (A) Hepa1c1c7 and (B) BPRc1 cells were treated with 8 μM DGC for different periods. The antibody against Sam 68 was used as a loading control of the nuclear extract.

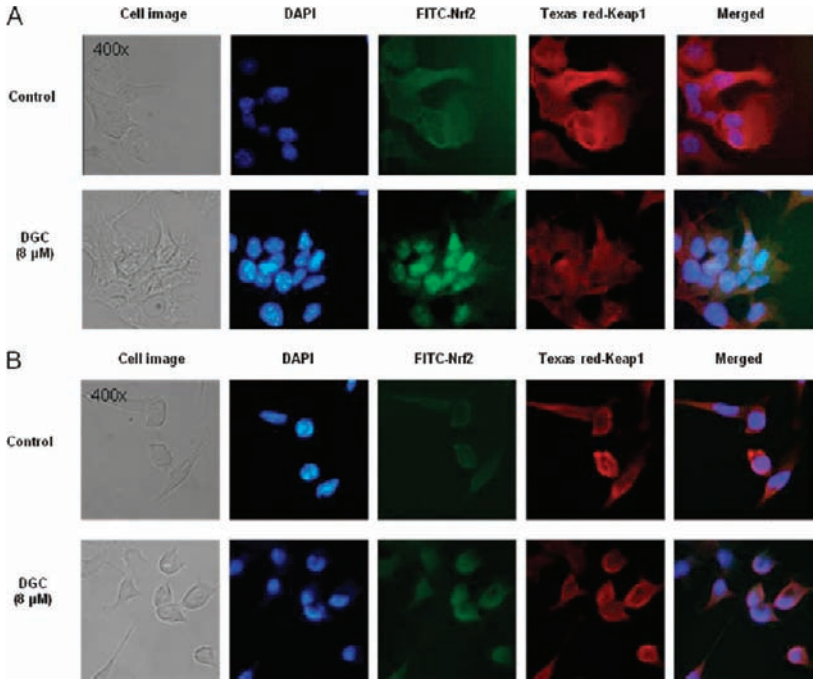

Figure 7. Confirmation of nuclear localization of Nrf2 in Hepa1c1c7 and BPRc1 cells by immunocytochemistry. (A) Hepa1c1c7 and (B) BPRc1 cells were treated with 8 μM DGC for 6 h, followed by immunocytochemistry.

prevention or reduction of cancer in both humans and laboratory animals (*23*−*29*). Despite their structural diversity, these phytochemicals, along with synthetic chemopreventive agents, such as 1,3-dithiolethiones, share chemically common features that are all electrophilic. Electrophiles have a striking propensity to react with sulfhydryls, leading to the suggestion that the cellular target(s) of these chemicals might be cysteine residues in one or more proteins that control key regulatory pathways leading to cancer. It has been suggested that Nrf2-activating compounds could be classified into three categories, including involvement of Keap1 and/or Keap1b and the other Keap1-independent groups (*17*). It is likely that DGC, which belongs to a family of prenylated flavonoids, activates Nrf2 by modifying cysteine residue(s) in Keap1 as many compounds in the same family do. However, the precise mechanism that DGC leads to the nuclear accumulation of Nrf2 remains to be elucidated.

In conclusion, DGC isolated from licorice showed phase 2 detoxifying enzyme-induction activity in Nrf2-mediated fashion and, thus, warrants further study as a potential cancer-preventive agent.

ABBREVIATION USED

DGC, dehydroglyasperin C; NQO1, NAD(P)H:oxidoquinone reductase; ARE, antioxidant response element; Nrf2, NF-E2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; GCS, γ-glutamylcysteine synthase; GST, glutathione *S*-transferase; HO1, heme oxygenase 1.

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