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6-Shogaol Attenuates H_2O_2-induced Oxidative Stress via Up-regulation of Nrf2-mediated γ-Glutamylcysteine Synthetase and Heme Oxygenase Expression in HepG2 Cells

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Abstract The signaling pathway by which 6-shogaol protects HepG2 cells against H_2O_2-induced oxidative stress was investigated. Cellular anti-oxidant activities, the GSH level, and anti-oxidant response element (ARE) promoter activity were analyzed. Activated protein kinases and nuclear transcription factor-erythroid 2-related factor 2 (Nrf2) accumulation in the nucleus, and phase II detoxification and anti-oxidant enzymes were analyzed using western blotting. 6-Shogaol enhanced cellular anti-oxidant activities, the GSH level, and ARE promoter activities. Nrf2 accumulation in the nucleus, c-jun *N*-terminal kinase (JNK) activation, and γ-glutamylcysteine synthetase (GCS) and heme oxygenase-1 (HO-1) expressions were increased by 6-shogaol. Blockage of the JNK signaling pathway removed the elicitation effect of 6-shogaol on JNK activation, Nrf2 accumulation in nucleus, and GCS and HO-1 expression, but partially suppressed cellular anti-oxidant activities and ARE promoter activities. 6-shogaol exerts an indirect cellular anti-oxidant activity based on up-regulation of GCS and HO-1 via a JNK-mediated Nrf2 signaling pathway.

Keywords: 6-shogaol, oxidative stress, c-jun *N*-terminal kinase-mediated, γ-glutamylcysteine synthetase, heme oxygenase-1

Introduction

Oxidative stress resulting from a cellular imbalance between production and elimination of reactive oxygen species (ROS), including the superoxide anion and hydrogen peroxide (H_2O_2), is a major cause of cellular damage and death in different liver disease. H_2O_2 can be produced by superoxide dismutase (SOD) from superoxide anions formed via an enzymatic process. In addition, H_2O_2 is known as an important cause of oxidative damage due to a longer half-life than other ROS, and can be transformed into a highly reactive hydroxyl radical by Fenton reactions in the presence of reduced ferrous or cuprous ions (1). To abrogate oxidative stress, several non-enzymatic and enzymatic factors in biological tissues, called anti-oxidants, prevent ROS formation or scavenge it. Anti-oxidants can be divided into direct and indirect varieties depending on the mechanism by which protection against oxidative stress is delivered (2). Direct anti-oxidants modify reactive oxygen and nitrogen radicals by donating hydrogens or electrons, while indirect anti-oxidants may not be oxido-reductively active. Indirect anti-oxidants exert effects via up-regulation of phase II detoxification and anti-oxidant enzymes (2). Some anti-oxidants such as phenolic Michael reaction acceptors can exert anti-oxidant effects via both direct and indirect methods (3) and are called bi-functional anti-oxidants.

For the induction of phase II detoxification and anti-oxidant enzymes by indirect anti-oxidants, Nrf2 has to be activated after being detached from the Nrf2-Kelch-like ECH associated protein 1 (Keap1) complex (4). Depending on Keap1 involvement in Nrf2 activation, there are two kinds of mechanisms (5,6). In Keap1-dependent Nrf2 activation, various inducers react with Keap1 to induce a change in its conformation resulting in Nrf2 activation (7). In Keap1-independent Nrf2 activation, the Nrf2 protein can be activated due to phosphorylation via mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), c-jun *N*-terminal kinase (JNK), p-38, phospha-tidylinositol 3-kinase (PI3K/Akt), and protein kinase C (PKC) signal transduction pathways (8,9).

Ginger (*Zingiber officinale* Roscoe) has been used as an herbal medication for treatment of a wide variety of ailments for a long time. Several bioactive compounds, including gingerols, paradols, zingerones, and shogaols, have been identified in fresh ginger and 6-shogaol has been reported as a minor bioactive compound (10). In addition, 6-shogaol is readily formed from 6-gingerol, the most abundant bioactive compound of ginger, via hydration during heating because 6-gingerol is thermally labile due to a β-hydroxy keto moiety (11,12). Accordingly, 6-shogaol is a major constituent

after 6-gingerol in commercial ginger powders (13). Because 6-shogaol bears methoxyphenol and α,β-unsaturated carbonyl moieties as reactive functional groups, 6-shogaol has been reported to possess not only anti-oxidant (12,14,15) and anti-inflammatory activities (16,17), but also anti-carcinogenic activities (18-20).

Recently, several *in vitro* anti-oxidant activities of 6-shogaol, including DPPH, superoxide anion, and hydroxyl free radical scavenging effects, reducing power, and Trolox equivalent antioxidant capacity (TEAC), have been reported (6,11,21). 6-Shogaol has also shown potent anti-oxidant activities in a cell model using rat pheochromocytoma PC12 cells (15). In addition, the enhancement effects of 6-shogaol for anti-oxidant response element (ARE) promoter activity and phase II detoxification and anti-oxidant enzymes, including NAD(P)H quinone oxidoreductase (NQO1) and heme oxygenase 1 (HO-1) in mouse embryonic fibroblast cells, have been reported (22). Furthermore, up-regulation effects of 6-shogaol-rich extracts from ginger on the anti-oxidant defense system were observed in HepG2 cells and mice (23). Unfortunately, no study has elucidated the cellular anti-oxidant activities of 6-shogaol in hepatic cells and associated underlying mechanisms.

The objective of this study was to investigate the contribution of Nrf2-mediated phase II detoxification and anti-oxidant enzyme induction for 6-shogaol protection against H_2O_2-iuduced oxidative stress in hepatic HepG2 cells.

Materials and Methods

Reagents 6-Shogaol, neocuproine, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), MTT, Triton X-100, Hank's balanced salt solution (HBSS), 2',7'-dichlorofluorescein-diacetate (DCFH-DA), N-acetylcysteine (NAC), DL-sulforaphane (SF), phosphate buffered saline (PBS) (pH 7.4), phenylmethanesulfonyl fluoride (PMSF), SP600125, HEPES, EDTA, dithiothreitol (DTT), Hoechst 33528, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Monochlorobimane (mBCl) was obtained from Molecular Probes (Eugene, OR, USA). The pGL4.37 (*luc2P*/ARE/Hygro) vector, the pGL4.74 (*hRluc*/TK) vector, FuGENE HD reagent, and a Dual-Glo luciferase assay kit were purchased from Promega (Madison, WI, USA). A Maxime PCR PreMix Kit and AccuPowerCycleScript RT PreMix were purchased from iNtRON Biotechnology and Bioneer (Seoul, Korea). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) including anti-p-JNK, γ-glutamylcysteine synthetase (GCS), PKC, PI3-kinase p85α, catalase, NQO1, HO-1, and p-PKC. Anti-Nrf2, p38MAPK, p44/42MAPK (ERK1/2), p-p44/42MAPK (p-ERK1/2), JNK, GST, and p-PI3-kinase p85α were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell cultures The human hepatoma cell line HepG2 (KCLB NO. 88065) was obtained from the Korea Cell Line Bank (Seoul, Korea). HepG2 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS in a humidified incubator (RCO3000T-5-VBC; Thermo Electron Co., Ashville, NC, USA) containing 5% CO_2 and 95% air at 37°C. Cell monolayers were grown in polystyrene culture dishes (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA), and cells were subcultured at a 1:3 ratio twice a week with a medium change.

Cell viability assay The tetrazolium dye colorimetric test (MTT) was used to determine the viability of HepG2 cells based on the ability of functional mitochondria to catalyze reduction of MTT to insoluble purple formazan, the concentration of which can be measured spectrophotometrically. HepG2 cells were first cultured in 96 well plates (5×10^5 cells/well) for 24 h, washed twice using PBS, then treated with 1-50 μM of 6-shogaol. After 24 h of incubation, MTT reagent was added to each well and the plate was incubated at 37°C for 2 h. The medium was removed and the plate was washed twice with PBS (pH 7.4). Intracellular insoluble formazan was dissolved in DMSO. The absorbance of each cell was then measured at 570 nm using an ELISA reader (A-5002; Tecan, Salzburg, Austria) and the percentage viability was calculated.

Cellular anti-oxidant activity Cellular oxidative stress due to ROS generated using H_2O_2 was measured spectrofluorometrically following the DCFH-DA method (24). DCFH-DA diffuses through the cell membrane and is hydrolyzed enzymatically by intracellular esterase to non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH), which is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. HepG2 cells were first cultured in 96 well plates (5×10^5/mL) with DMEM for 24 h. After incubation both with and without 10 μM SP600125 for 30 min, cells were incubated in 1-10 μM of 6-shogaol dissolved in DMSO for 30 min. The medium was then discarded and wells were gently washed twice with PBS.

HBSS, which is fluorescently stable, was then added to each well instead of DMEM and H_2O_2 was used as an oxidative stress inducer. After cells were treated with 1 mM H_2O_2 for 30 min, DCFH-DA was added to culture plates at a final concentration of 40 μM and cells incubated for 30 min at 37°C in the dark. After incubation, cells were washed with HBSS, and the DCF fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Tecan GENios fluorometric plate reader (Zurich, Switzerland).

Intracellular GSH level HepG2 cells were first cultured in 96 well plates (5×10^5/mL) with DMEM for 24 h. Subsequently, cells were incubated in 1-10 μM of 6-shogaol dissolved in DMSO for 30 min and exposed to 1 mM H_2O_2 for 30 min, after which the medium were discarded and wells were gently washed twice with PBS. HBSS, which is fluorescently stable, was then added to each well instead of DMEM. mBCl was then added to culture plates at a final concentration of 50 μM, followed by incubation for 30 min at 37°C in the dark. After incubation, cells were washed with HBSS and the mBCl fluorescence intensity was measured at an excitation wavelength of 380 nm and

an emission wavelength of 465 nm using a Tecan GENios fluorometric plate reader.

Transient transfection and an anti-oxidant response element (ARE)-luciferase assay The ARE assay was performed using FuGENE HD reagent. HepG2 cells were first cultured in 96 well plates (5×10^5/mL) with DMEM for 24 h. After cells were transiently transfected with different plasmids, cells were incubated with 1-10 μM of 6-shogaol dissolved in 10% DMSO for 30 min, then exposed to 1 mM H_2O_2 for 30 min. ARE-luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega) following manufacturer protocols after multi-well plates containing HepG2 cells were removed from the incubator. For measurement of ARE luciferase activities, a volume of Dual-Glo Luciferase reagent equal to the culture medium volume was added to each well and mixed. After 10 min, a volume of Dual-Glo Stop & Glo reagent equal to the original culture medium volume was added to each well and mixed for determination of the Renilla luciferase activity. Luminescence was measured using a Tecan GENios fluorometric plate reader. After the ratio of the ARE luciferase activity luminescence to the Renilla luciferase activity luminescence was calculated, the ARE luciferase activity was normalized to control.

Western blot analysis HepG2 cells were grown in 6 well plates for 24 h, then incubated with 6-shogaol in the presence and absence of SP600125 at 37°C for 1 h. HepG2 cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, and 1 mM PMSF that contained a phosphatase inhibitor cocktail. For nuclear protein extraction, after harvested cells were rinsed twice with cold PBS, cells were scraped and centrifuged at 15,800×*g* for 2 min. The pellet was then re-suspended in 400 μL of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA (pH 7.9), 0.1 μM PMSF, and 1×protease inhibitor cocktail). After 15 min on ice, the lysate was centrifuged at 15,800×*g* for 2 min and the cytosolic extract supernatant was stored at 4°C. The nuclear pellet was re-suspended in buffer B (50 mM HEPES (pH 7.9), 50 mM KCl, 0.3 mM NaCl, 1 mM DTT, 1 mM EDTA (pH 7.9), 0.1 μM PMSF, and 10% glycerol) for 10 min at 4°C. The re-suspended pellet was centrifuged at 18,000×*g* for 10 min at 4°C and the nuclear extract supernatant was stored at 70°C. Lysed cells and extracted nuclear and cytosol proteins were subjected to electrophoresis using SDS-PAGE and transferred to nitrocellulose membranes, which were reacted with primary antibodies for 12 h, then incubated with appropriate horseradish peroxide-conjugated secondary antibodies for 1 h at room temperature. Proteins on membranes were detected using an EZ-Western Lumi Pico Detection Kit (DoGEN, Seoul, Korea) and visualized using an LAS4000 chemiluminescent image analyzer (Fuji, Tokyo, Japan).

Observation of Nrf2 and nuclear fluorescence imaging Observations were performed using confocal imaging described by Grindel *et al.* (25). Following treatment, HepG2 cells were rinsed twice with PBS and incubated for 15 min at room temperature with 3.7% paraformaldehyde in PBS. Cells were washed again with PBS, followed by incubation for 10 min at room temperature in 0.1% Triton X-100 in PBS. After washing with PBS, cells were blocked using 5% skimmed milk in PBS for 45 min at room temperature. The blocking solution was removed and replaced with a 1:500 Nrf2 primary antibody in 5% skimmed milk. Cells were then left overnight at 4°C, after which cells were washed 3x for 10 min in PBS. The washing solution was removed and replaced with 1:2,500 AlexaFlour 546 conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA) in PBS, and cells were incubated for 45 min at room temperature in the dark. Cells were then washed 3x for 10 min in PBS. Nuclear counterstaining was performed with Hoechst 33528. Images were acquired using an LSM5 live configuration Variotwo VRGB microscope (Zeiss, Jena, Germany) equipped with an oil immersion lens. Fluorescence imaging was achieved using a laser at 405 nm for blue, death-associated protein 1 (DAPI) nuclear staining, and at 535 nm for red, anti-Nrf2 excitation.

Statistical analysis All data are presented as a mean±standard deviation (SD). Statistical analysis was carried out using the SPSS statistical package (SPSS, Chicago, IL, USA) program and significance was verified using a one-way analysis of variance (ANOVA) followed by Duncan's test or Student's *t*-test at $p<0.05$.

Results and Discussion

6-Shogaol attenuated H_2O_2-induced oxidative stress via enhancement of the GSH level The cellular anti-oxidant activities of 6-shogaol against H_2O_2-induced oxidative stress were investigated using a cellular anti-oxidant activity assay. Cell viability over 95% was observed with both 1 mM H_2O_2 and 20 μM 6-shogaol (data not shown), HepG2 cells were incubated with 1-10 mM 6-shogaol for 30 min and exposed to 1 mM H_2O_2 for 30 min. Intracellular oxidative stress in HepG2 cells treated with H_2O_2 increased significantly ($p<0.001$) by 192.2%, compared with controls (Fig. 1A). However, 6-shogaol at 1-10 mM significantly ($p<0.05$) suppressed the cellular oxidative stress caused by H_2O_2 in a dose-dependent manner, compared with controls.

GSH is the most abundant non-protein thiol in cells and has several important functions, including defense against oxidative stress as a scavenger of H_2O_2 and a role as a redox state biomarker in cells. Accordingly, the GSH level in HepG2 cells treated with 6-shogaol was investigated. The GSH level was significantly ($p<0.001$) reduced by H_2O_2, compared with control. This decrease was significantly ($p<0.05$) dose-dependently reversed by 6-shogaol treatment, compared with controls (Fig. 1B). In addition, NAC was used as a positive anti-oxidant provided cysteine for GSH synthesis and significantly ($p<0.001$) enhanced the GSH level that was decreased by

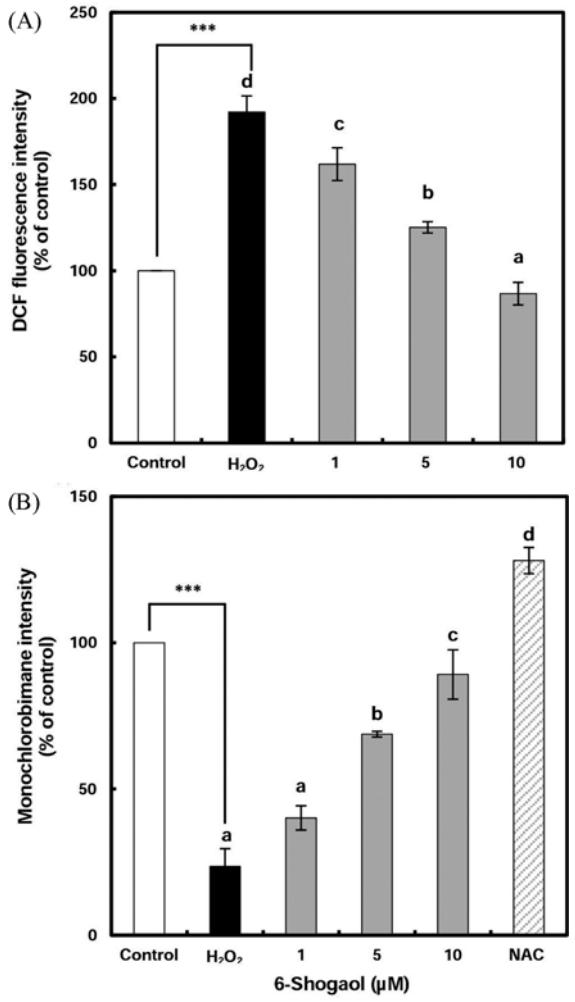

Fig. 1. Protective effects of 6-shogaol against H_2O_2-induced oxidative stress via enhancement of the GSH level. (A) Cellular anti-oxidant activity of 6-shogaol against H_2O_2-induced oxidative stress. (B) GSH level after 6-shogaol treatment. Data are expressed as percentages of values for untreated cells (mean±SD, n=3), ***p<0.001 vs. controls. Different corresponding letters indicate significant differences at p<0.05 based on Duncan's test. NAC, N-acetyl cysteine.

H_2O_2 treatment, compared with control.

Cellular anti-oxidant activities of some anti-oxidants can be exerted via both direct and indirect mechanisms. Direct anti-oxidant activity is a result of transfer of a hydroxyl group transferring hydrogens and/or donating electrons, while indirect anti-oxidant activity can be due to induction of phase II detoxification and anti-oxidant enzymes, including NQO1, GSH *S*-transferase (GST), GCS, HO-1, superoxide dismutase 1 (SOD1), and catalase (CAT). When 6-shogaol penetrates into HepG2 cells, a direct *in vitro* anti-oxidant activity is exerted (6,12,15). On the other hand, the enhancement effect of 6-shogaol on the GSH level in HepG2 cells was probably closely associated with an indirect anti-oxidant activity via induction of phase II detoxification and anti-oxidant enzymes. Thus, the combined effect of both direct and indirect anti-oxidant activities of 6-shogaol was probably responsible for cellular anti-oxidant activities.

6-Shogaol enhanced ARE promoter activities and induction of GCS and HO-1 For determination of whether 6-shogaol stimulated transcription of ARE-related genes in HepG2 cells as an indirect anti-oxidant, a luciferase reporter plasmid carrying ARE promoter was introduced into HepG2 cells and dose-response effects of 6-shogaol on ARE promoter activity were examined. H_2O_2 treatment had no significant (p<0.05) effect on ARE promoter activities, compared with controls, while 6-shogaol treatment caused a significant (p<0.05) dose-dependent induction of ARE promoter activities, compared with controls (Fig. 2A).

Sulforaphane was used as a positive control of ARE promoter activity induction. Therefore, the effects of 6-shogaol on the transcriptional and translational activities of phase II detoxification and anti-oxidant enzymes were examined after treatment with 6-shogaol. Expression levels of phase II anti-oxidant and detoxification enzymes after treatment with 1-10 μM 6-shogaol demonstrated that a dose-dependent increase in translational activity was observed only for GCS and HO-1 (Fig. 2B, 2C and 2D). Thus, the increase in ARE activity observed after 6-shogaol treatment was probably closely related with increases in GCS and HO-1 transcriptional activities, and the enhanced GSH level was probably attributable to induced GCS expression due to 6-shogaol treatment.

The induction effect of ARE-driven phase II detoxification and anti-oxidant enzymes due to 6-shogaol has been confirmed. Study of human embryonic kidney and mouse embryonic fibroblast cells showed enhanced ARE-dependent transcriptional activities and HO-1 expression after 6-shogaol treatment (22). In addition, the induction effect on HO-1, observed in 6-shoaol-rich extracts from ginger, was mediated by Nrf2 (23). Thus, augmented induction of ARE-related phase II detoxification and anti-oxidant enzymes appeared to be due to enhanced induction of GCS, which plays a crucial role as a rate-limiting enzyme responsible for GSH *de novo* synthesis (26). Consequently, 6-shogaol can augment the GSH level that is diminished by H_2O_2 treatment via up-regulation of GCS expression in HepG2 cells. Furthermore, the increased GSH level due to induction of GCS expression in HepG2 cells was also reported for the phytochemicals ajoene (27), quercetin (28), isorhamnetin (29), and kahweol/cafestrol (30).

6-Shogaol activates JNK, leading to Nrf2 accumulation in the nucleus For examination of Nrf2 induction by 6-shogaol, protein levels of Nrf2 in the nucleus and the cytosol were analyzed. 6-shogaol caused a time-dependent increase of Nrf2 accumulation in the nucleus, compared with control (Fig. 3A and 3B). In addition, this enhanced Nrf2 level in the nucleus was confirmed by fluorescence imaging and confocal microscopic analysis appeared in Fig. 3C. For translocation of Nrf2 existing as Nrf2-Keap1 complex in the cytosol into the nucleus, Nrf2 has to be activated by phosphorylation through Keap1-dependent or independent mechanism. Accordingly, the two types of Nrf2 activation mechanism depending or not on Keap1 can be attributable to the increased nucleus Nrf2 due to 6-

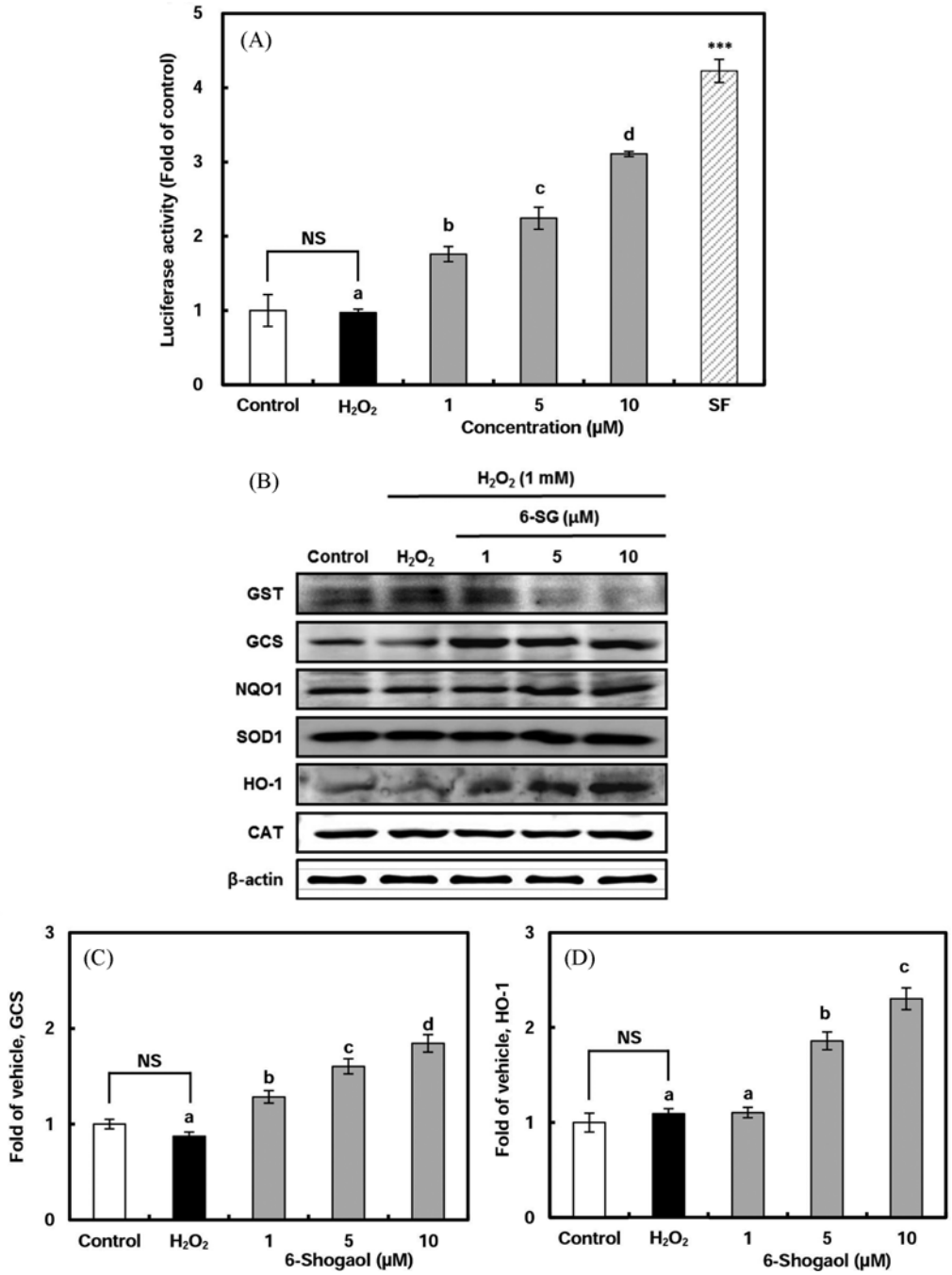

Fig. 2. Induction effect of 6-shogaol on the ARE luciferase activity and GCS and HO-1 expressions. HepG2 cells were treated with 10 μM 6-shogaol for 30 min, then exposed to 1 mM H_2O_2 for 1 h. (A) ARE luciferase activity. The ARE luciferase activity was determined and normalized to control. Sulforaphane (1 μM) was used as a positive control. (B) Expression level of phase II anti-oxidant and detoxification enzymes. (C) Protein level of GCS. (D) Protein level of HO-1. Data are expressed as mean±SD of 3 individual experiments, ***p<0.001 vs. control. Different corresponding letters indicate significant differences at p<0.05 based on Duncan's test. NS, not significant; SF, sulforaphane; 6-SG, 6-shogaol; SOD1, superoxide dismutase 1; HO-1, heme oxygenase-1; CAT, catalase; NQO1, NAD(P)H quinone oxidoreductase 1; GCS, γ-glutamylcysteine synthetase; GST, glutathione S-transferase.

shogaol treatment.

The previous study using human embryonic kidney cells reported that 6-shogaol can induce phase II detoxification and anti-oxidant enzymes through Nrf2 activation (22). 6-Shogaol phosphorylated Nrf2, thus causing to release it from the Keap1-Nrf2 complex and to translocate it from the cytosol into the nucleus, which indicates that 6-shogaol can activate Nrf2 via Keap1-independent way. Different Keap1-independent mechanism for Nrf2 activation, including phosphorylation by protein kinases, have been proposed in HepG2 cells (5). In consequence, the MAPK varieties including ERK, JNK, and

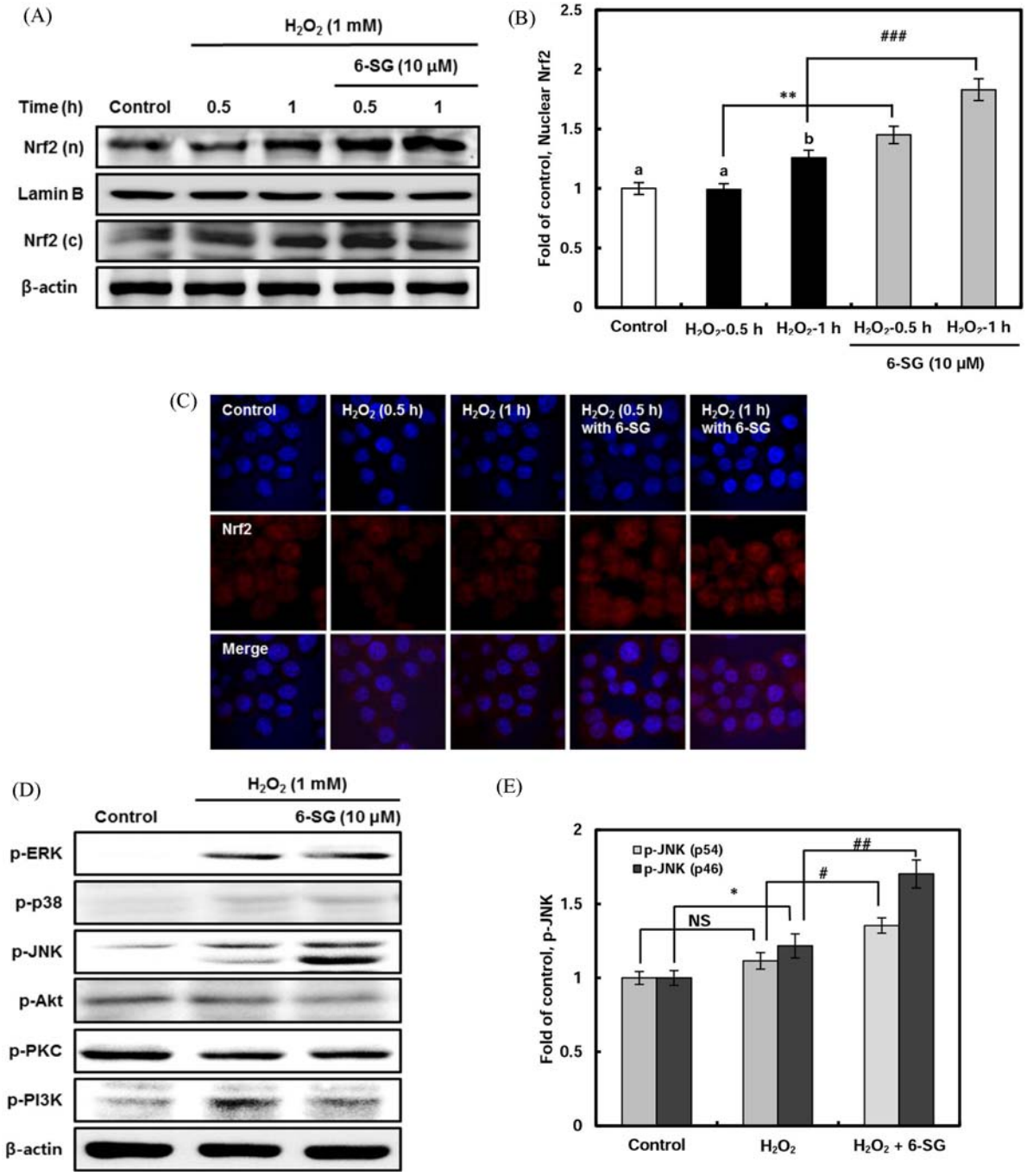

Fig. 3. Effects of 6-shogaol on the Nrf2 level in the nucleus via activation of JNK protein kinase. HepG2 cells were treated with 10 μM 6-shogaol for 30 min, then exposed to 1 mM H_2O_2 for 0.5 h or 1 h. (A) Nrf2 expression based on Western blotting. (B) Protein level of Nrf2 in the nucleus. (C) Immunofluorescence staining of HepG2 cells. (D) Expression of phosphorylated protein kinases based on Western blotting. (F) Protein level of activated JNK. Data are expressed as mean±SD of 3 individual experiments, *p<0.05 vs. control, **p<0.01 vs. H_2O_2-0.5 h, $^{\#}p$<0.05, $^{\#\#}p$<0.01 vs. H_2O_2, $^{\#\#\#}p$<0.001 vs. H_2O_2-1 h. NS, not significant. Different corresponding letters indicate significant differences at p<0.05 based on Duncan's test. 6-SG, 6-shogaol.

p38, Akt, and PKC as upstream signals may be involved in Nrf2 activation via phosphorylation (8).

For investigation of which protein kinases among ERK, JNK, p38, PKC, and Akt were involved in activation of Nrf2 via post-translational phosphorylation, protein kinase phosphorylation levels due to 6-shogaol were analyzed (Fig. 3D and 3E). An effect of 6-shogaol promotion of protein kinase phosphorylation was observed only for the JNK varieties p46 and p54. Thus, 6-shogaol can activate JNK via induction of phosphorylation that, in turn, phosphorylates Nrf2 as a downstream signal, which results in Nrf2 activation and translocation to the nucleus.

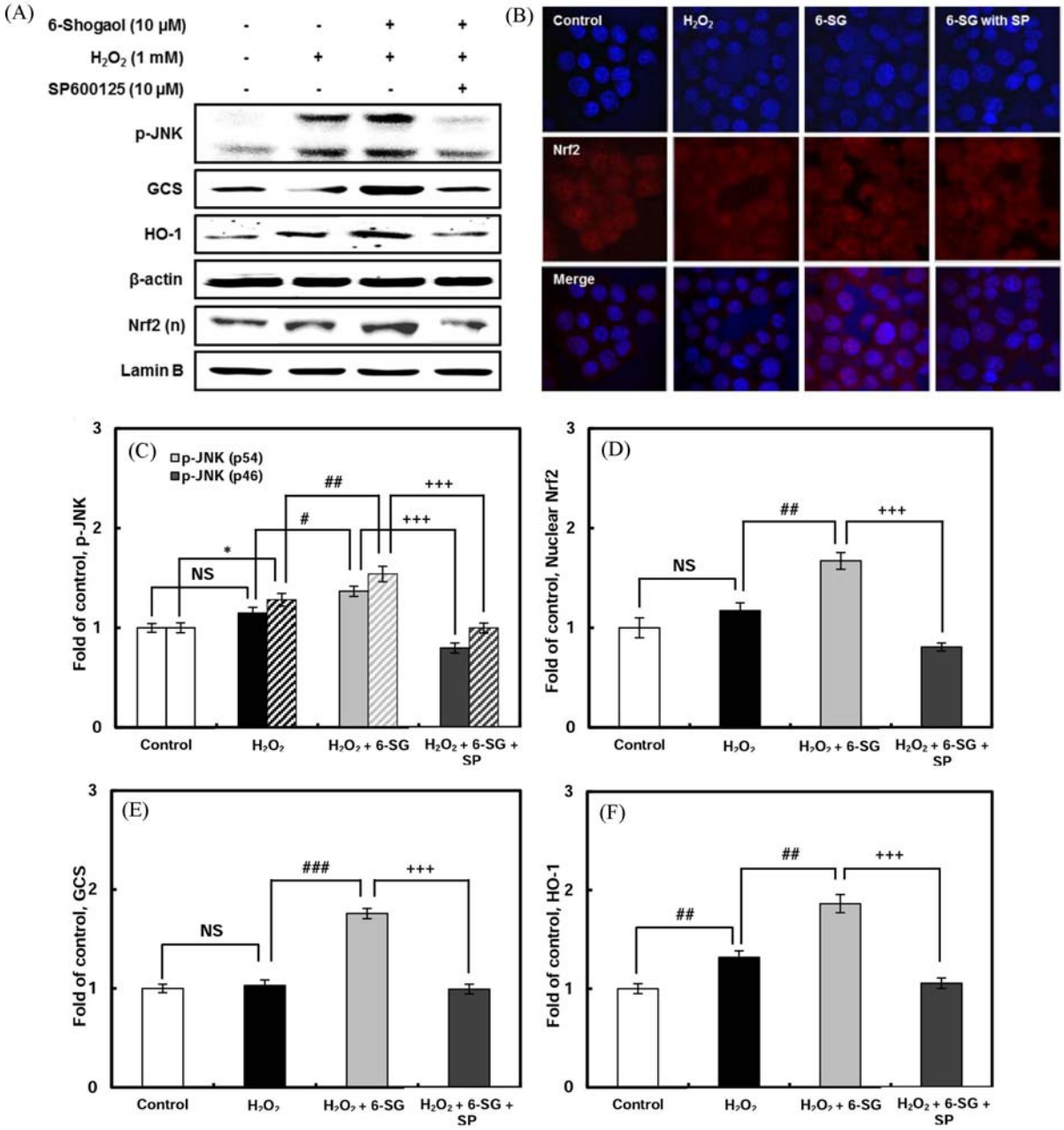

Fig. 4. Suppressive effect of the JNK inhibitor SP600125 on the JNK/Nrf2/GCS-HO-1 signaling pathway induced by 10 μM 6-shogaol. HepG2 cells were sequentially treated with the JNK inhibitor SP600125 and 10 μM 6-shogaol for 30 min, respectively, then exposed to 1 mM H_2O_2 for 1 h. (A) Expression of phosphorylated JNK, and Nrf2 in the nucleus, and GCS and HO-1 using Western blotting. (B) A confocal microscopic fluorescence image of immunofluorescence staining of HepG2 cells. Nuclear counterstaining was performed with Hoechst 33528. (C) Protein level of activated JNK. (D) Nrf2 accumulation in the nucleus. (E) Protein level of GCS. (F) Protein level of HO-1. Data are expressed as mean±SD of 3 individual experiments, *p<0.05 vs. control, , $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. H_2O_2, ^{+++}p<0.001 vs. H_2O_2 + 6-SG. NS, not significant; 6-SG, 6-shogaol; SP, SP600125.

Blockage of the JNK signaling pathway by a JNK inhibitor completely abolishes GCS and HO-1 expressions via the JNK-Nrf2 signaling pathway The JNK signaling pathway coupled with Nrf2-mediated GCS and HO-1 induction was investigated using the JNK inhibitor SP600125, treatment with which abolished the p46 and p54 JNK activation that was induced by 6-shogaol (Fig. 4A and 4C). In addition, based on Western blotting and confocal microscopic analysis of Nrf2 (Fig. 4B and 4D), the nuclear accumulation of Nrf2 that was increased by 6-shogaol was significantly (p<0.001) reduced by the JNK inhibitor, compared with controls. Thus, JNK activation due to phosphorylation by 6-shogaol can activate Nrf2 via post-translational phosphorylation and allow translocation of Nrf2 to the nucleus. Furthermore, SP600125 treatment significantly (p<0.001) reduced both GCS and HO-1 expressions that were significantly

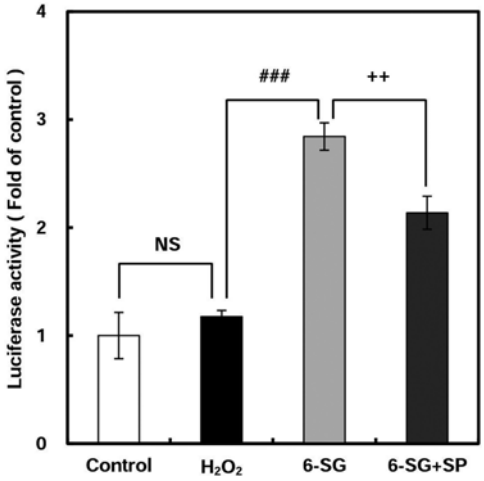

Fig. 5. Suppressive effect of the JNK inhibitor SP600125 on the ARE luciferase activity induced by 10 μM 6-shogaol. HepG2 cells were sequentially treated with the JNK inhibitor SP600125 and 10 μM 6-shogaol for 30 min, respectively, then exposed to 1 mM H_2O_2 for 1 h. The ARE luciferase activity was then determined and normalized to controls. Data are expressed as mean±SD of 3 individual experiments, $^{###}p<0.001$ vs. H_2O_2, $^{++}p<0.01$ vs. 6-SG. NS, not significant; 6-SG, 6-shogaol; SP, Sp600125.

(p<0.001) increased by 6-shogaol, compared with controls (Fig. 4E and 4F).

6-Shogaol phosphorylated JNK for induction of post-translational Nrf2 phosphorylation, which is required for Nrf2 accumulation in the nucleus, leading to induction of both GCS and HO-1 expressions. Thus, Nrf2 activation coupled with the JNK signaling pathway that caused GCS and HO-1 expressions appeared to be Keap1-independent. Apparently, 6-shogaol activates Nrf2 via JNK mediation, leading to induction of phase II detoxification and anti-oxidant enzymes, including GCS and HO-1 in hepatic HepG2 cells.

On the other hand, blockage of the JNK signaling pathway by SP600125 in part abrogated ARE promoter activity, which was significantly (p<0.001) increased by 6-shogaol, compared with controls (Fig. 5). Thus, the GCS and HO-1 genes may be located in an ARE-dependent region.

Blockage of the JNK signaling pathway by the JNK inhibitor in part attenuated the cellular antioxidant activity of 6-shogaol via down-regulation of the GSH level Nrf2-mediated up-regulation of GCS and HO-1 induction via the JNK signaling pathway may contribute to the cellular anti-oxidant activity of 6-shogaol in HepG2 cells. Therefore, clarification of whether the JNK signaling pathway is involved in indirect anti-oxidant activity of 6-shogaol was attempted.

HepG2 cells were treated with 10 μM SP600125 as a JNK inhibitor for 30 min before 6-shogaol treatment. Intracellular oxidative stress levels in HepG2 cells that were significantly (p<0.001) increased by 192.2% due to H_2O_2 were significantly (p<0.001) reduced by 86.7% due to 6-shogaol treatment, compared with controls (Fig. 6A). However, treatment with a JNK inhibitor significantly (p<0.001) increased the

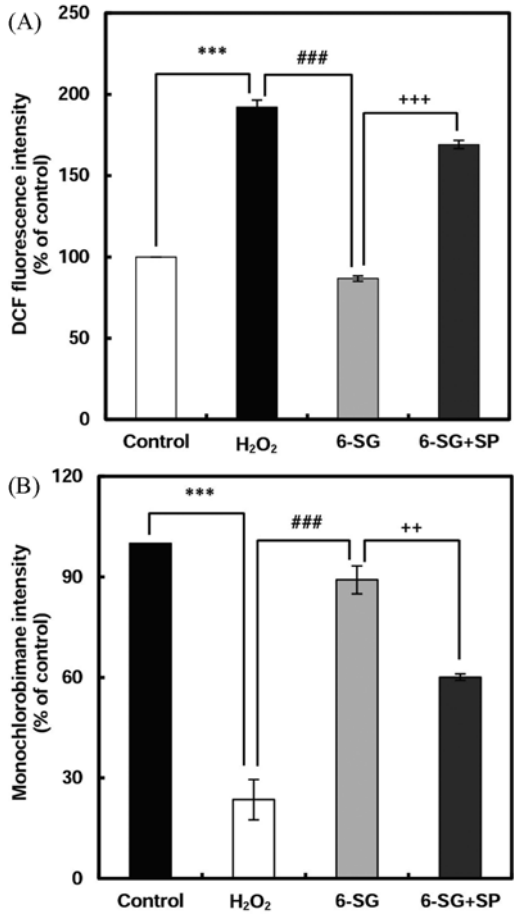

Fig. 6. Attenuating effect of the JNK inhibitor SP600125 on the cellular anti-oxidant activity and the GSH level induced by 6-shogaol. HepG2 were sequentially incubated with 10 ìM SP600125 and 10 μM 6-shogaol for 30 min, respectively, then exposed to 1 mM H_2O_2 for 30 min. (A) The cellular anti-oxidant activity determined using DCFH-DA (B) GSH level determined using mBCl. Data are expressed as percentages of values for untreated cells (mean±SD, n=3), $^{***}p<0.001$ vs. control, $^{###}p<0.001$ vs. H_2O_2, , $^{++}p<0.01$, $^{+++}p<0.001$ vs. 6-SG. 6-SG, 6-shogaol; SP, SP600125.

H_2O_2-induced oxidative stress level by 169.2%, compared with controls. Although treatment with the JNK inhibitor reduced the 6-shogaol cellular anti-oxidant activity against H_2O_2-induced oxidative stress, the JNK inhibitor did not completely inhibit the cellular anti-oxidant activity of 6-shogaol. In addition, the GSH level that was significantly (p<0.001) increased by 6-shogaol, compared with H_2O_2 treatment, was partially abolished via blockage of the JNK signaling pathway (Fig. 6B), indicating that metabolic processes and signaling pathways other than the JNK signaling pathway for up-regulation of the GSH level should not be ignored.

Cellular anti-oxidant activities were determined at 1 h after treatment with 6-shogaol using a cellular anti-oxidant capacity assay system. In a short period such as 1 h, 6-shogaol could scavenge H_2O_2 via donation of a hydrogen from hydroxyl groups in the ferulic acid moiety. Meanwhile, 6-shogaol markedly (p<0.001) induced JNK-mediated Nrf2 accumulation in the nucleus, ARE promoter activity,

and GCS and HO-1 expressions, indicating that 6-shogaol can exert an indirect anti-oxidant activity via induction of the phase II detoxification and anti-oxidant enzymes GCS and HO-1. Blockage of the JNK signaling pathway abrogated JNK activation, Nrf2 accumulation in the nucleus, and GCS and HO-1 expressions elicited by 6-shogaol, However, blockage did not completely suppress the induced ARE promoter activity or cellular anti-oxidant activities. Thus, other indirect anti-oxidant activities not coupled with the JNK signaling pathway and direct anti-oxidant activities probably accounted for some portion of the cellular anti-oxidant activities of 6-shogaol in HepG2 cells.

In conclusion, results of this study provide evidence that 6-shogaol exerts an indirect anti-oxidant activity via up-regulation of GCS and HO-1 expression via the JNK-Nrf2 signaling pathway. Accordingly, the cellular anti-oxidant activity displayed by 6-shogaol in HepG2 cells was probably partially due to enhanced GCS and HO-1 expressions coupled with the JNK-Nrf2 signaling pathway. However, more research is required for clear elucidation of the indirect anti-oxidant activity of 6-shogaol observed in HepG2 cells.

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