Research Article

6-Shogaol suppressed lipopolysaccharide-induced up-expression of iNOS and COX-2 in murine macrophages

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Ginger, the rhizome of *Zingiber officinale*, is a traditional medicine with carminative effect, antinausea, anti-inflammatory, and anticarcinogenic properties. In this study, we investigated the inhibitory effects of 6-shogaol and a related compound, 6-gingerol, on the induction of nitric oxide synthase (NOS) and cyclooxygenase-2 (COX-2) in murine RAW 264.7 cells activated with LPS. Western blotting and reverse transcription-PCR analyses demonstrated that 6-shogaol significantly blocked protein and mRNA expression of inducible NOS (iNOS) and COX-2 in LPS-induced macrophages. The *in vivo* anti-inflammatory activity was evaluated by a topical 12-*O*-tetradecanoylphorbol 13-acetate (TPA) application to mouse skin. When applied topically onto the shaven backs of mice prior to TPA, 6-shogaol markedly inhibited the expression of iNOS and COX-2 proteins. Treatment with 6-shogaol resulted in the reduction of LPS-induced nuclear translocation of nuclear factor-κB (NFκB) subunit and the dependent transcriptional activity of NFκB by blocking phosphorylation of inhibitor κB (IκB)α and p65 and subsequent degradation of IκBα. Transient transfection experiments using NFκB reporter constructs indicated that 6-shogaol inhibits the transcriptional activity of NFκB in LPS-stimulated mouse macrophages. We found that 6-shogaol also inhibited LPS-induced activation of PI3K/Akt and extracellular signal-regulated kinase 1/2, but not p38 mitogen-activated protein kinase (MAPK). Taken together, these results show that 6-shogaol downregulates inflammatory iNOS and COX-2 gene expression in macrophages by inhibiting the activation of NFκB by interfering with the activation PI3K/Akt/IκB kinases IKK and MAPK.

Keywords: Cyclooxygenase-2 (COX-2) / Mitogen-activated protein (MAPK) / NFκB / RAW 264.7 monocyte/macrophages / 6-Shogaol

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1 Introduction

Ginger (*Zingiber officinale*) has been widely used as a spice, and as a medicinal herb in traditional herbal medicine. Ginger is a natural dietary ingredient and spice with antioxidative, anti-inflammatory, and anticarcinogenic properties [1–3]. It contains several pungent constituents such as gingerols, shogaols, paradols, and gingerdiols. In

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Abbreviations: COX-2, cycoloxygenase-2; **IκB,** inhibitor κB; **IKK,** κB kinases; **iNOS,** inducible nitric oxide synthase; **MAPK,** mitogen-activated protein kinase; **NO,** nitric oxide; **NFκB,** nuclear factor-κB; **PGE_2,** prostaglandin E_2

the fresh ginger rhizome, the gingerols were identified as the major active components, and 6-gingerol is the most abundant constituent [4]. A recent report has shown that ginger could be useful in preventing the development of colorectal cancer [2], but Dias *et al*. [5] reported that ginger meal failed to reduce chemical-induced colon carcinoma in rats. However, at the cellular level, it is unclear which constituents of ginger work as cancer chemopreventive agents.

LPS, a component of the cell walls of Gram-negative bacteria, induces the activation of monocytes and macrophages and involves the production of proinflammatory cytokines [6]. Nitric oxide (NO) is produced endogenously by a family of NO synthases (NOSs) with a wide range of physiological and pathophysiological actions [7, 8]. NOS enzymes are classified into two groups. One group, (cNOS), is constitutively present in several cell types (*e. g*., neurons and endothelial cells) and is regulated predominantly at the post-transcriptional level by calmodulin in a Ca^{2+}-dependent manner [9]. The other group, the inducible form (inducible NOS, iNOS), which is expressed in various cell types, including vascular smooth muscle cells, macrophages, hepatocytes, and astrocytes, is induced in response to proinflammatory cytokines and bacterial LPS [10, 11]. Increased NOS expression and/or activity has been reported in human gynecological [12], breast [13], and central nervous system [14] tumors. Also, the accumulation of nitrotyrosine in inflamed mucosa of patients with ulcerative colitis and gastritis indicates NO is being produced and may be involved in the pathogenesis of these diseases [15]. The mechanism of the signal transduction cascade involved in the induction of iNOS in response to LPS and cytokines is an active area of investigation [16].

Cyclooxygenase-2 (COX-2) is an inducible enzyme catalyzing the conversion of arachidonic acid to prostaglandins. Recent studies have suggested that increased levels of prostaglandins and cyclooxygenase activity may play important roles in multiple epithelial cancers such as colon carcinoma [17, 18]. COX-2-derived bioactive lipids, including prostaglandin E_2 (PGE_2), are potent inflammatory mediators that promote tumor growth and metastasis by stimulating cell proliferation, invasion, and angiogenesis [19]. Therefore, high levels of prostaglandins may promote the development of malignancy [20].

Previous reports have shown a potential role for tyrosine kinase in LPS promoter that contain 24 transcriptional factor-binding sites, including those for nuclear factor-κB (NFκB) family, which appear to be essential for the enhanced iNOS and COX-2 gene expression seen in macrophages exposed to LPS [21, 22]. The p65 NFκB also seems to be responsible for inducing iNOS in astrocytes [23]. Activation of NFκB by LPS is induced by a cascade of events leading to the activation of inhibitor κB (IκB) kinases (IKKs), which in turn phosphorylates IκB and leads to the degradation of NFκB and its translocation to the nucleus [24]. In this pathway, NFκB transcriptional activity is independent of IκBα degradation and is regulated by phosphorylation of NFκB. Phosphorylation at Ser 536 on the p65 subunits is mediated by IKK during LPS stimulation [25]. Ser 536 phosphorylation is responsible for the recruitment of coactivators as p300, promoting the transcriptional activation of NFκB and subsequent production of inflammatory cytokines [26]. These kinases can be activated through phosphorylation by upstream kinases, including NFκB-inducing kinase, mitogen-activate protein kinase (MAPK), and protein kinase C [27, 28]. In addition, many studies imply cytokine in the induction of transcription activity of NFκB through extracellular signal-regulated 1/2 (ERK)1/2 (p42/44), p38 MAPK, and PI3K/AKT pathways [29 – 32]. Importantly, iNOS has been shown to be involved in regulating cycoloxygenase-2 (COX-2), which plays a pivotal role in colon tumorigenesis [33]. These observations clearly suggest that iNOS may exacerbate tumorigenesis. Collectively, suppression of enzyme induction and the activities of iNOS/COX-2 is an important approach to preventing carcinogenesis in several organs including the stomach and colon [34]. The recent emphasis on the role of NO in pathological conditions has led to discovery of new therapeutic agents. Although the broad spectrum of research on ginger and its extracts has concentrated on its action as a chemopreventive agent, the action mechanisms are not well established yet. Here we examine the effect of 6-shogaol and a related compound, 6-gingerol, on the generation of NO and PGE_2, the expression of iNOS and COX-2, and activation of NFκB, p38, p44/42 MAPK, and PI3K/Akt in LPS-stimulated RAW 264.7 macrophage cells. 6-Shogaol was found to protect against LPS-induced inflammation by blocking the activation of NFκB, p44/42 MAPK, and PI3K/Akt, thereby inhibiting the iNOS and COX-2 expression.

2 Materials and methods

2.1 Reagents

LPS (*Escherichia coli* 0127: E8), sufanilamide, naphthylethylenediamine dihydrochloride, and DTT were purchased from Sigma Chemical (St. Louis, MO). 6-Shogaol (>99%) was purchased from Fluka (St. Gallen, Switzerland).

2.2 Isolation of 6-gingerol and 6-shogaol from ginger extract

Fifty grams of commercial ginger extract (Sabinsa, Piscataway, NJ) was subjected to a Diaion HP-20 column (5 cm × 120 cm). The column was successfully eluted with water (3 L), 50% ethanol (3 L), 70% ethanol (3.5 L), and 95% ethanol (4 L), and the eluents were collected and concentrated. Eleven gram of 50% ethanol fraction containing gingerols and 19 g of 95% ethanol fraction containing shogaols were obtained. Five gram of 50% ethanol fraction was

further subjected to a normal phase silica gel column (5 cm × 120 cm) and eluted with hexane/ethyl acetate (9:1 v/v) (4 L). About 1.2 g of pure 6-gingerol was obtained. One gram of 95% ethanol fraction was subjected to a RP C-18 column (2 cm × 60 cm) and eluted with methanol/water (3:2 v/v) (4 L). About 190 mg of pure 6-shogaol was obtained as one of the compounds isolated. The identification of 6-gingerol and 6-shogaol were confirmed with HPLC analysis by comparing with standard compounds.

HPLC analyses were performed on a Hitachi LC 6200A intelligent pump (Hitachi, San Jose, CA) combined with a Waters 490 E programmable multiple wavelength pump and Waters 717 auto-sample injector (Waters Associates, Milford, MA, USA). 6-Gengerol and 6-shogaol were separated and identified using a Suplecosil LC-18 RP column (Sigma – Aldrich, St. Louis, MO, USA) (4.6 mm × 150 mm; 5 μm). The injection volume of sample was 50 μL with a flow rate of 1.0 mL/min with the following solvent system: A = water/acetonitrile/acetic acid (95:5:0.2 by volume), B = ACN/water/acetic acid (95:5:0.2 by volume); 0% B to 100% B in 36 min.

2.3 Cell culture

RAW 264.7 cells, derived from murine macrophages, were obtained from the American Type Culture Collection (Rockville, MD, USA). RAW 264.7 cells were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin. When the cells reached a density of $2-3 \times 10^6$ cells/mL, they were activated by incubation in medium containing *E. coli* LPS (100 ng/mL). Various concentrations of test compounds dissolved in DMSO were added together with LPS. Cells were either treated with 0.05% DMSO as vehicle control.

2.4 Determination of PGE_2

The culture medium of control and treated cells was collected, centrifuged, and stored at −70°C until tested. The level of PGE_2 released into culture medium was quantified using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Amersham, Arlington Heights, IL) [35].

2.5 Nitrite assay

The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction [36]. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture at 550 nm was measured with an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories, Chantilly, VA, USA).

2.6 Cytotoxicity assay

The RAW 264.7 cells were cultivated at a density of 2×10^5 cells in a six-well plate. The polyphenols studied were added to the medium 18 h after inoculation. The cells were harvested after 18 h. Viability was determined by trypan blue exclusion and microscopy examination.

2.7 Western blotting

The female ICR mice were topically treated on their shaved backs with 5-OH-HxMF in 200 μL of acetone and 30 min prior to 10 nmol 12-*O*-tetradecanoylphorbol 13-acetate (TPA) treatment. The mice were scarified by cervical dislocation at indicated time. For protein isolation from mouse skin, the dorsal skins of mice derived from different experiments were excised. After the fat from the dorsal skin were removed on ice, the skin samples were immediately placed in liquid nitrogen. The epidermal protein was homogenized on ice for 15 s with a Polytron tissue homogenizer and lysed in 0.5 mL ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 1% NP-40, and 10 μg/mL leupeptin) on ice for 30 min, followed by centrifugation at $10\,000 \times g$ for 30 min at 4°C. Afterward, the stimulated murine macrophage cell line RAW 264.7 cells were washed with PBS and lysed in an ice-cold RIPA buffer (Tris-HCl pH 7.2, 25 mM; SDS 0.1%; Triton X-100 1%; sodium deoxycholate 1%; NaCl 0.15 M; EDTA 1 mM) containing 1 mM of PMSF, 10 μg/mL of aprotinin, 1 mM of sodium orthovanadate, and 5 μg/mL of leupeptin. Protein concentrations were determined using the BCA method (Pierce, Rockford, IL, USA). Equal amount of total cellular protein (50 μg) were resolved by SDS-polyacrylamide minigels and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) as described previously [37]. The membrane was then incubated with the primary antibody of COX-2 or iNOS (Transduction Laboratories, Lexington, KY) and cytosolic fraction (for IκBα, p65). The membrane was blocked overnight at room temperature with blocking solution (20 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.2% Tween 20, 1% BSA, and 0.1% sodium azide), antiphospho (Ser 32)-specific IκBα (New England Biolabs, Ipwich, MA, USA), or anti-β-actin monoclonal antibodies (Oncogene Science, Uniondale, NJ) at room temperature for 1 h. The antiphospho-Akt (Ser473), antiphospho-p65 (Ser536), antiphospho-p38 (Thr180/Tyr182), antiphospho-ERK1/2 (Thr202/Tyr204), ERK, p38, and Akt antibodies obtained from Cell Signaling Technology (Beverly, MA) were used to determine the level of phosphorylated proteins. The membranes were subsequently probed with antimouse or antirabbit IgG antibody conjugated to

horseradish peroxidase (Transduction Laboratories) and visualized using enhanced chemiluminescence (ECL, Amersham). The densities of the bands were quantitated with a computer densitometer (AlphaImager™ 2200 System). All the membranes were stripped and reprobed for β-actin (Sigma Chemical) as loading control.

2.8 RT-PCR

Total RNA was isolated from mouse macrophage RAW264.7 cell using Trizol Reagent according to the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA, USA). Changes in the steady-state concentration of mRNA in iNOS, COX-2, and G3PDH were assessed by reverse-transcription PCR (RT-PCR). Total RNA (2 μg) was converted to cDNA in a series of standard 10 μL reverse transcription reactions. DNA amplification was carried out in "Ready To Go" PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The initial conditions were 42°C for 60 min. Amplification 30 cycles of iNOS were 95°C for 40 s, 65°C for 60 s, and 72°C for 2 min, followed by a 10 min extension at 72°C. The thermal cycle conditions of COX-2 were initiated at 42°C for 60 min, then 30 cycles of amplification (94°C for 45 s, 55°C for 60 s, and 72°C for 2 min) were performed followed by 10 min extension at 72°C. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. Amplification of G3PDH served as a control for sample loading and integrity. PCR was performed on the cDNA using the following sense and antisence primer: iNOS, forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ (2944 – 2968), reverse primer 5′-GGCTGTCAGAGAGCCTCGTGG-CTTTGG-3′ (3416 – 3440); COX-2, forward primer 5′-GGAGAGACTATCAAGATAGTGATC-3′ (1094 – 1117), reverse primer 5′-ATGGTCAGTAGACTTTTACAGCTC-3′ (1931 – 1954); G3PDH, forward primer 5′-TGAAGG-TCGGTGTGAACGGATTTGGC-3′, reverse primer 5′-CATGTAGGCCATGAGGTCCACCAC-3′. Confirmation of the correct amplicons was obtained by direct DNA sequencing of the PCR products.

2.9 Transient transfection and luciferase assay

The luciferase assay was performed as described by George *et al*. [38] with some modifications. RAW 264.7 cells were seeded in a 60 mm dish. When the cells reached confluence, the medium was replaced with serum-free Opti-MEM (Gibco). The cells were then transfected with the pNFκB-Luc plasmid reporter gene (Stratagene, Jalla, CA, USA) using LipofectAMINE™ reagent (Gibo, NRL, Life Technologies). After 24 h of incubation, the medium was replaced with complete medium. After another 24 h, the cells were trypsinized and equal numbers of cells were plated in 12-well tissue culture plates for 18 h. The cells were then incubated with 100 ng/mL LPS and 6-shogaol and gingerol for 3 h. Each well was then washed twice with cold PBS and harvested in 150 μL of lysis buffer (0.5 M HEPES pH 7.8, 1% Triton N-101, 1 mM $CaCl_2$, and 1 mM $MgCl_2$). Luciferase activity was assayed by means of the LucLite™ luciferase reporter gene kit (Packard BioScience Company, Meriden, CT), with 100 μL of cell lysate used in each assay. Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence Counter (Packard 9912 V) in single photon counting mode for 0.1 min/well, following a 5 min adaptation in the dark. Luciferase activities were determined and normalized to protein concentrations.

2.10 Statistical analysis

Data are presented as means ± SE for the indicated number of independently performed experiments. One-way Student's *t*-test was used to assess the statistical significance between the LPS- and 6-shogaol plus LPS-treated cells. A p-value <0.05 was considered statistically significant.

3 Results

3.1 Inhibition of LPS-induced nitrite and PGE2 production by 6-gingerol and 6-shogaol in RAW 264.7 macrophages

To investigate the anti-inflammatory effects of 6-gingerol and 6-shogaol (Fig. 1A), we tested their effect on nitrite and prostaglandin production in LPS-activated macrophages. 6-Gingerol and 6-shogaol at 20 μM did not interfere with the reaction between nitrite and Griess reagents (data not shown). As shown in Fig. 2, 6-gingerol and 6-shogaol inhibited nitrite production by >50% at 3 μM. 6-Shogaol, at a concentration range of 1.5 – 20 μM, markedly and concentration-dependently suppressed nitrite production by 58 – 98%. A similar result was found in 6-gingerol with mild inhibitory effects, suppressed nitrite production by 44 – 74%. After treatment with LPS for 24 h, the medium concentration of PGE_2 had elevated significantly to 6.5 ng/mL. This increase was markedly inhibited by different concentrations of 6-shogaol (Fig. 3). In contrast, 6-gingerol slightly inhibited PGE_2 production. The data indicated that 6-shogaol was more active than 6-gingerol. Inhibition of nitrite production was not toxic, as determined by the trypan blue exclusion assay (Fig. 1B).

3.2 6-Gingerol and 6-shogaol inhibition of LPS-induced iNOS and COX gene expression

We next investigated whether 6-shogaol and 6-gingerol might affect levels of iNOS and COX-2 proteins. As shown in Fig. 4A, treatment with 6-gingerol suppressed the expression of iNOS protein significantly when compared with

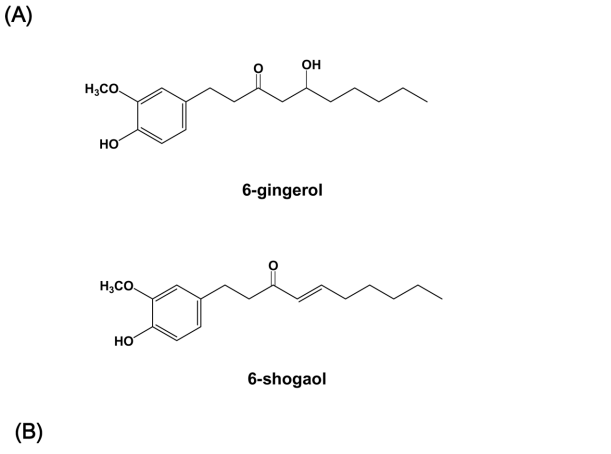

Figure 1. (A) The chemical structures of 6-gingerol and 6-shogaol. (B) Cytotoxic effects of 6-gingerol and 6-shogaol in RAW 264.7 cells. Cytotoxicity was estimated by trypan blue exclusion using a hemocytometer chamber. The values are expressed as means ± standard error of triplicate tests.

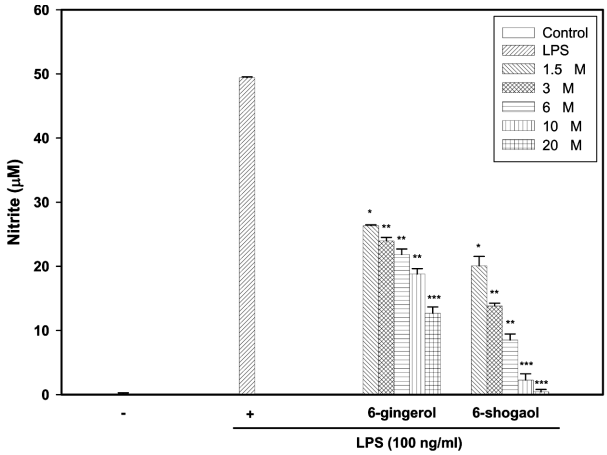

Figure 2. Effects of 6-gingerol and 6-shogaol on LPS-induced nitrite production in RAW 264.7 macrophage. The cells were treated with 100 ng/mL of LPS only or with different concentrations of 6-gingerol and 6-shogaol for 24 h. At the end of incubation time, 100 μL of the culture medium was collected for nitrite assay. The values are expressed as means ± standard error of triplicate tests. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate statistically significant differences from the LPS-treated group.

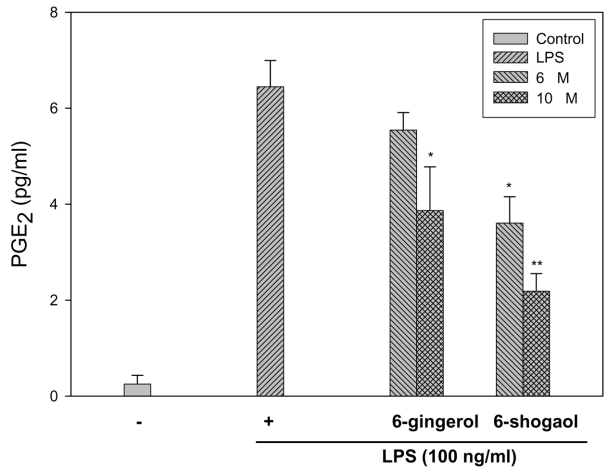

Figure 3. Effects of 6-gingerol and 6-shogaol on LPS-induced PGE_2 production in RAW 264.7 macrophage. The cells were treated with 100 ng/mL of LPS only or with different concentrations of 6-gingerol and 6-shogaol (6 and 10 μM) for 24 h. At the end of incubation time, 100 μL of the culture medium was collected for PGE_2 assay. The values are expressed as means ± standard error of triplicate tests. *$p < 0.05$, **$p < 0.01$ indicate statistically significant differences from the LPS-treated group.

LPS treatment alone. The reduced expression of iNOS protein was consistent with reductions in total nitrite in culture media. Interestingly, treatment with 6-gingerol did not suppress of COX-2 protein when compared with LPS treatment alone. As shown in Fig. 4B, 6-shogaol strongly and concentration-dependently suppressed the protein levels of both iNOS and COX-2. These data suggest that transcriptional events are involved in 6-shogaol's inhibition of LPS-induced expression of iNOS and COX-2. Changes in amounts of iNOS and COX-2 enzymes could reflect altered protein synthesis or degradation. RT-PCR was done to investigate whether 6-gingerol and 6-shogaol suppressed LPS-mediated induction of iNOS and COX-2 *via* a pre-translational mechanism. The amplification of cDNA with primers specific for mouse *iNOS* and *COX-2* (*G3PDH* as control gene) is shown in Fig. 5. 6-Shogaol was the most potent inhibitor of expression of *iNOS* and *COX-2* in LPS-activated macrophages, as measured by densitometer scans (Fig. 5, bottom), whereas 6-gingerol (10 μM) only inhibited the expression of *iNOS* but not *COX-2*. These data suggest that 6-shogaol may inhibit the expression of iNOS and COX-2 at the transcription levels. Therefore, we wanted to study the inhibitory effect of 6-shogaol on iNOS and COX-2 activities in LPS-activated macrophages in details.

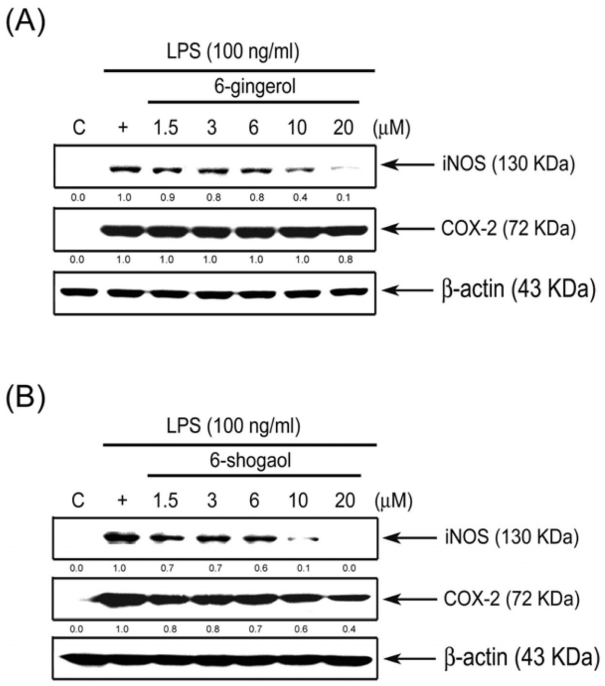

Figure 4. Effects of 6-gingerol and 6-shogaol on LPS-induced iNOS and COX-2 protein levels in RAW 264.7 cells. (A) The cells were treated with different concentrations of 6-gingerol and (B) 6-shogaol for 24 h. Equal amounts of total proteins (50 μg) were subjected to 10% SDS-PAGE. The expression of iNOS, COX-2 and β-actin protein was detected by Western blot using specific antibodies. Quantification of iNOS and COX-2 protein expression was normalized to β-actin using a densitometer. These experiments were repeated three times with similar results.

3.3 Reduction of nuclear NFμB level and NFμB activation by 6-shogaol treatment in LPS-stimulated macrophages

Because activation of NFκB is critical for induction of both iNOS and COX-2 by LPS or other inflammatory cytokines, we used nuclear accumulation to test whether 6-shogaol would perturb the distribution of NFκB subunits. Nucleus and cytosolic extracts were prepared and subjected to immunoblot analysis. As shown in Fig. 6A, coincubation with LPS plus 6-shogaol decreased NFκB proteins in the nucleus. PARP, a nuclear protein, and β-actin, a cytosolic protein, were used as controls to confirm that there was no contamination during extraction of each fraction. In an additional study, transient transfection with a NFκB-dependent luciferase reporter plasmid was done to confirm whether 6-shogaol and 6-gingerol inhibited NFκB-binding activity in LPS-induced macrophages. As shown in Fig. 6B, 6-shogaol strongly inhibited LPS-induced NFκB transcriptional activity. However, 6-gingerol significantly inhibited LPS-induced NFκB transcriptional activity as well. The phosphorylation of p65/RelA at serine 536 in LPS-mediated induction was also inhibited by the 6-shogaol in a dose-dependent manner (Fig. 6C).

3.4 Inhibitory effects of 6-shogaol on LPS-induced phosphorylation and degradation of IμBa

Because the LPS-mediated translocation of NFκB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα, we examined the phosphorylated and un-phosphorylated protein levels of IκBα by immunoblot analysis. It was found that the treatment with LPS caused the serine-phosphorylation of IκBα protein, as evidenced by the presence of anti-Ser32-phospho-specific IκBα antibody after 15 – 120 min and the degradation of IκBα after 30 min. Levels of IκBα gradually recovered after 45 – 120 min (Fig. 7A). As shown in Fig. 7B, treatment with 6-shogaol effectively sustained the IκBα protein content. The pattern of inhibition on IκBα phosphorylation by 6-shogaol was paralleled to the pattern of inhibition on its degradation. These results suggest that blocking the phosphorylation and the degradation of IκBα protein, 6-shogaol can inhibit the production of NO and PGE_2, thus preventing the translocation and activation of NFκB in the nucleus (Fig. 6).

3.5 Effects of 6-shogaol on activation of ERK (p44/42), p38 MAP kinase, PI3K, and Akt

Because p38 and p44/42 MAPK have been shown to be involved in the LPS-mediated induction of iNOS and COX-2 in mouse macrophages [39, 40] and cytokine activation of

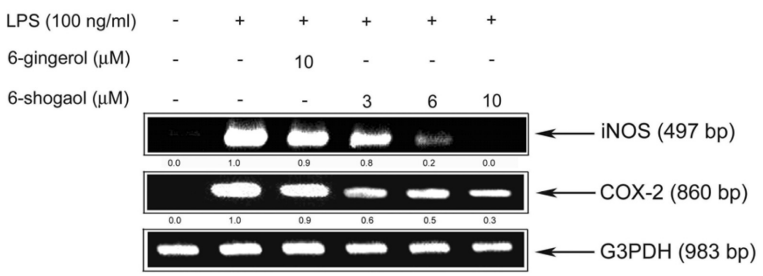

Figure 5. RT-PCR analysis of the expression of iNOS and COX-2 mRNA. Cells were treated with LPS (100 ng/mL) and 6-gingerol (10 μM) and 6-shogaol (3 – 10 μM) for 5 h, and total RNA was subjected to RT-PCR with the primers iNOS or COX-2 with G3PDH as internal control. The PCR product was resolved in 1.5% agarose gel. Quantification of iNOS and COX-2 RNA expression were performed by densitometric analysis of the agarose gel. This experiment was repeated three times with similar results.

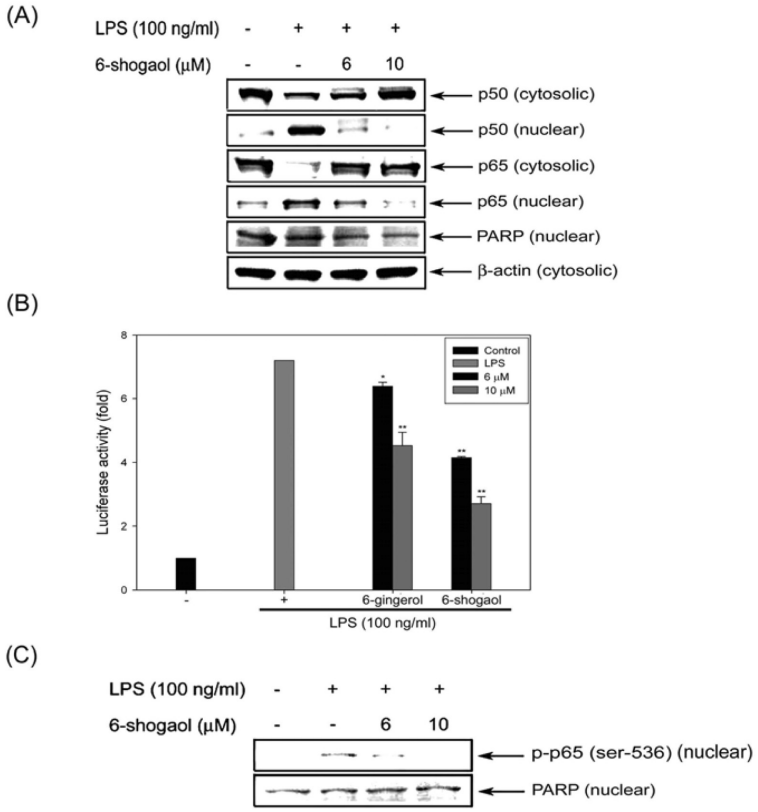

Figure 6. Effects of 6-shogaol on LPS-induced p65 and p50 translocation and NF-κB activation in RAW264.7 cells. (A) Cells were treated with LPS (100 ng/mL) with or without 6-shogaol (6 or 10 μM) for 45 min. Then cytosolic and nuclear fractions were prepared and analyzed by Western blotting. (B) The cells were transiently transfected with 2 μg of pNF-κB-Luc reporter gene, and then treated with LPS (100 ng/mL) with or without flavonoids for 12 h. Cells were harvested and the levels of luciferase activities were determined as described in Section 2. Results show the means ± SE of three experiments. * $p < 0.05$, ** $p < 0.01$ *versus* LPS treatment. (C) Nuclear extracts fractions were analyzed for the level of phospho-p65 (ser-536) by Western Blotting.

PI3K/Akt pathway leads to the phosphorylation and activation of the NFκB [41], we investigated the effects of 6-shogaol on the activation of p38, p44/42 MAPK, and PI3K/Akt in LPS-stimulated macrophages. Activation of MAPK requires phosphorylation of threonine and tyrosine residues [42]. Using immunoblot analysis with antiphospho-specific antibody, the activation of p38 and p44/42 was found to peak after 20–40 min of treatment with LPS and returned to basal level 60 min afterwards (data not shown). When the cells were cotreated both 6-shogaol and LPS for 30 min, shogaol was found to attenuate the LPS-stimulated activation of p44/42 MAPK (Fig. 8A), but not affect LPS's activation of p38 (Fig. 8B).We used immunoblot analysis with anti-p85 antibody to investigate whether PI3K pathway was involved in the 6-shogaol's inhibition of LPS-induced RAW 264.7 macrophages. We detected PI3K activity. The LPS-stimulated activation of PI3K was attenuated by 6-shogaol (in 10 μM) (Fig. 8C). To further evaluate the involvement of Akt, a downstream target of PI3K, in LPS-induced responses, we used immunoblot analysis with antiphospho-Akt antibody to examine Akt activity in macrophage cells. When the cells were cotreated with 6-shogaol and LPS for 30 min, the LPS-stimulated activation of Akt was attenuated by 6-shogaol in a dose-dependent manner (Fig. 8C). More importantly, no changes were observed in total Akt content in cells treated with both LPS and 6-shogaol as compared with vehicle-treated control. These results of our immunoblot analyses suggest that 6-shogaol's inhibition of

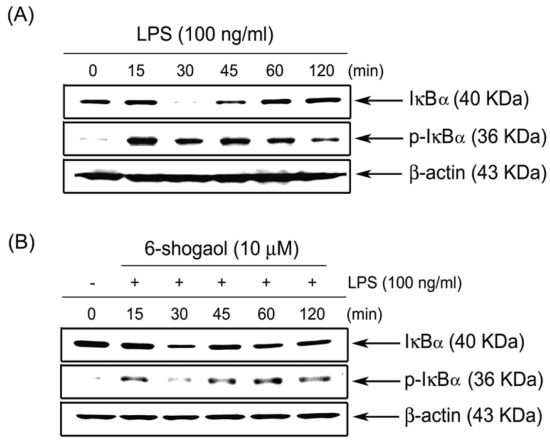

Figure 7. Effects of 6-shogaol on LPS-induced phosphorylation and degradation of IκBα. (A) RAW 264.7 cells were treated with LPS (100 ng/mL) for different times. Total cellular lysates were prepared for Western blot analysis. (B) Cells were treated with LPS (100 ng/mL) and 6-shogaol (10 μM) for different time, and the cellular lysates were prepared and analyzed for content of IκBα, P- IκBα, and β-actin by Western blot. These experiments were repeated three times with similar results. The values below the figure represent change in protein expression of the bands normalized to β-actin.

iNOS and COX-2 expression might block LPS-induced NFκB activation by inhibiting ERK1/2, but not p38, and PI3K/Akt/IKK pathway, which interrupts the degradation of IκBα.

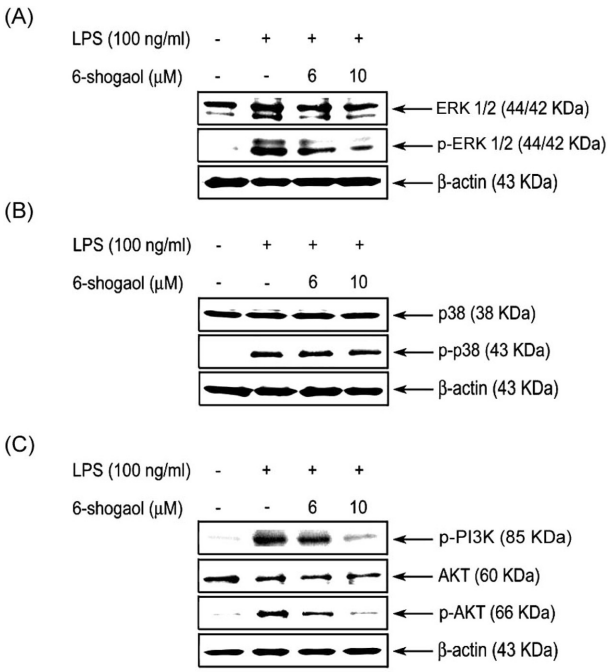

Figure 8. Inhibition of ERK1/2, p38 MAPK, and PI3K/Akt by 6-shogaol in LPS-activated RAW macrophages. RAW 264.7 cells were treated with LPS (100 ng/mL) with or without 6-shogaol (6 or 10 μM) for 30 min. Cells extracts were then prepared and analyzed for (A) p44/42 and P-p44/42, (B) p38 and P-p38, or (C) P-PI3K, Akt, and P-Akt by Western blot. These experiments were repeated three times with similar results.

3.6 Inhibitory effects of 6-gingerol and 6-shogaol on TPA-induced iNOS and COX-2 expression in mouse skin

In order to examine whether 6-gingerol and 6-shogaol can inhibit iNOS and COX-2 expression in TPA-treated mouse skin, we topically applied the 6-gingerol or 6-shogaol 30 min prior to TPA treatment. Upon treatment with TPA for 4 h, the expression of iNOS was markedly suppressed by 6-gingerol and 6-shogaol pretreatment (Fig. 9). In addition, TPA-induced upregulation of COX-2 was significantly suppressed by 6-gingerol (2.5 μmole) treatment. Interestingly, 6-shogaol strongly suppressed the expression of COX-2 protein induced by TPA-induced COX expression. The data indicated that 6-shogoal was markedly more active than 6-gingerol.

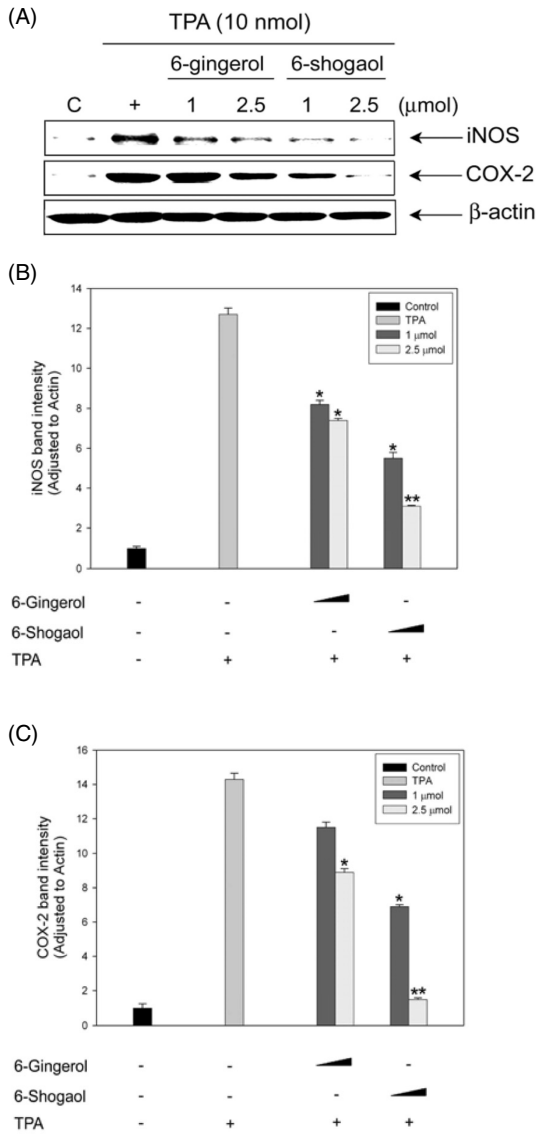

Figure 9. Inhibitory effects of 6-gingerol and 6-shogaol on TPA-induced iNOS and COX-2 expression in mouse skin. (A) Mice were treated topically with 0.2 mL acetone or 6-gingerol and 6-shogaol (1 and 2.5 μmol) in the same volume of acetone 30 min prior to 10 nmol TPA, and animals were killed 2 and 4 h, respectively after the TPA treatment. The epidermal proteins were analyzed for iNOS and COX-2 by Western blotting analysis. The western blot is representative of at least three independent experiments. Quantification of (B) iNOS and (C) COX-2 expression were normalized to β-actin using a densitometer. The values are expressed as means ± SE of triplicate tests, indicating statistically significant differences from the positive group (*$p < 0.05$ and **$p < 0.01$; Student's *t*-test).

4 Discussion

In the present study, we examined the effects and mechanisms of 6-shogaol and 6-gingerol on LPS-induced expression of iNOS and COX-2. Recent reports indicated that compounds having α,β-unsaturated ketone moiety contribute to the inhibition of ERK2 activity but not for other MAPKs such as JNK1 and p38MAPK. On the other hand, MEK1 and MKK7, kinase of the MAPKK family which contain a cysteine residue corresponding to Cys166 of ERK2 can also be inhibited by the compounds [43]. The authors suggested that the covalent binding to the common cysteine residue in the ATP-binding site is likely to play a crucial role in the inhibitory activity for these MAPK. Pre-

vious studies suggested that α,β-unsaturated carbonyls are very susceptible to nucleophilic addition reactions with thiols such as glutathione; the most abundant nonprotein thiol *in vivo* [44]. Moreover, NFκB activation could be inhibited by the α,β-unsaturated carbonyl group [45]. IKK consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ/NEMO. It is suggested that IKKα exhibits a catalytic activity of p65 phosphorylation (Ser536), whereas IKKβ is largely responsible for phosphorylation of both IκBα (Ser32 and p65) [46]. Previous studies indicated that the anti-inflammatory agents aspirin and sodium salicylate specifically inhibit IKKβ activity *in vitro* and *in vivo* through conjugate to IKKβ to reduce ATP binding [47]. We suggested that the α,β-unsaturated carbonyl group in 6-shogaol, but not in 6-gingerol, may be responsible for the potent depletory of these thiols in these protein kinase [48]. In this study, we found that 6-shogaol was stronger inhibitor of iNOS and COX-2 expression in LPS-activated macrophages than that of 6-gingerol (Fig. 4). Therefore, we suggested that the α,β-unsaturated carbonyl group in 6-shogaol might influences the conformation of the molecule and modulates its inhibitory effect. This could provide new information for the design of anti-inflammatory agents and the study of these functional groups in the future.

Because there is a causal relationship between inflammation and cancer, iNOS and COX-2 are considered potential molecular targets for chemoprevention [49, 50]. Our present results suggest that 6-shogaol's inhibition of the p44/42 MAPK and PI3K/Akt signaling pathway may partially explain how it reduces the induction of iNOS and COX-2 protein. In contrast, 6-shogaol did not affect the p38 MAPK signaling cascade elicited by LPS in macrophages. The possible mechanism is that 6-shogaol downregulates inflammatory iNOS and COX-2 gene expression in macrophages by inhibiting the activation NFκB by interfering with the activation of PI3K/Akt and MAPK.

Activation of NFκB is necessary for LPS induction of the iNOS and COX-2 promoter [51]. NFκB is composed mainly of two proteins, p50 and p65. In resting cells, the NFκB heterodimer is held in the cytosol through interaction with IκB inhibitory proteins [51]. With exposure to proinflammatory stimuli, IκB becomes phosphorylated, ubiquitinated, and then degraded. Thus, the liberated NFκB dimers are translocated to the nucleus, where the transcription of target gene is induced. Our results show that 6-shogaol reduces iNOS and COX-2 expression by blocking transcription of its gene, a conclusion supported by the observation that it reduced the steady state of iNOS mRNA levels, and promoter activity (as assessed by leuciferase activity assay). Phosphorylation plays an important role in activating protein tyrosine kinase. Many signaling pathways, including PI3K/Akt and MAPK, have been proposed to respond to LPS stimulation [52]. PI3K activation leads to phosphorylation of phosphatidylinositides, which then activate the downstream main target, Akt, which appears to play various

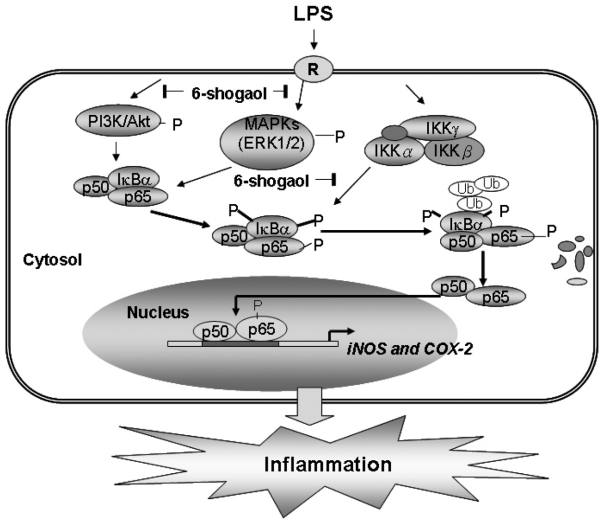

Figure 10. A schematic representation of suppression of LPS-induced NFκB activation and iNOS and COX-2 expression by 6-shogaol in mouse macrophages. LPS activates p38, ERK1/2 MAPK, IκB kinases and PI3K/Akt, which in turn, phosphorylation IκBα and p65 thereby contributing to the activation of NFκB and subsequent induction of iNOS and COX-2. Each of these events can be blocked by 6-shogaol. LPS, lipopolysaccharide; R, LPS receptor; P, phosphate group; Ub, ubiquitin; ⊥, site of inhibition.

important roles in regulating cellular growth, differentiation, adhesion, and the inflammatory reaction [53]. Activation of PI3K/Akt plays an important role in the expression of iNOS and COX-2 in vascular smooth muscle cells, peritoneal macrophages, and mesangial cells [54, 55]. Our findings showed the PI3K/Akt pathway to be involved in 6-shogaol's inhibition of expression of NFκB-related iNOS and COX-2. The p38 MAPK is an important mediator of stress-induced gene expression [42]. In particular, p38 MAPK is known to play a key role in the LPS-induced signal transduction pathway. In this study, we found that incubation of RAW 264.7 cells with LPS brought about the activation of p38 and p44/42 MAPK. We also found that cotreatment of 6-shogaol only blocked the activation of p44/42 MAPK, not p38. These results suggest that 6-shogaol suppresses LPS-induced NFκB translocation by inhibiting the activation of p44/42 MAPK and subsequently decreasing the protein levels of iNOS and COX-2 (Fig. 10).

Based on our findings, 6-shogaol shows great potential as a novel chemopreventive agent and may be used in the future to treat inflammation-associated tumorigenesis. We observed that 6-shogaol was able to inhibit LPS-stimulated expression of iNOS and COX-2 by regulating the signaling pathway, particularly affecting the activation of PI3K/Akt, p44/42 MAPK, the degradation and phosphorylation of IκBα, and the phosphorylation and translocation of NFκB. Both iNOS and COX-2 have been reported to contribute to tumor growth, and coexpression of two has been observed

in malignancies [56]. The possible relationship between the structural properties of ginger's constituent and their anti-inflammatory and anticarcinogenic activities deserves further investigation.

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The authors have declared no conflict of interest.

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