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6-Gingerol inhibits ROS and iNOS through the suppression of PKC-α and NF-κB pathways in lipopolysaccharide-stimulated mouse macrophages

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ABSTRACT

Inflammation is involved in numerous diseases, including chronic inflammatory diseases and the development of cancer. Many plants possess a variety of biological activities, including antifungal, antibacterial and anti-inflammatory activities. However, our understanding of the anti-inflammatory effects of 6-gingerol is very limited. We used lipopolysaccharide (LPS)-stimulated macrophages as a model of inflammation to investigate the anti-inflammatory effects of 6-gingerol, which contains phenolic structure. We found that 6-gingerol exhibited an anti-inflammatory effect. 6-Gingerol could decrease inducible nitric oxide synthase and TNF-α expression through suppression of I-κBα phosphorylation, NF-κB nuclear activation and PKC-α translocation, which in turn inhibits Ca^{2+} mobilization and disruption of mitochondrial membrane potential in LPS-stimulated macrophages. Here, we demonstrate that 6-gingerol acts as an anti-inflammatory agent by blocking NF-κB and PKC signaling, and may be developed as a useful agent for the chemoprevention of cancer or inflammatory diseases.

Chronic (or acute) inflammation is a multi-step process, which is mediated by activated inflammatory or immune cells. Macrophages play a central role in managing many different immunopathological phenomena, such as the overproduction of the pro-inflammatory cytokine, tumor necrosis factor (TNF)-α and inflammatory mediators [reactive oxygen species (ROS) and nitric oxide (NO)] generated by activated inducible nitric oxide synthase (iNOS) [1–3]. Protein kinase C (PKC) is one of the targets for lipopolysaccharide (LPS) and plays a key role in inflammation and tumor promotion [4]. Among the three groups of PKC isoforms, PKC-α (classical group) is translocated from the cytosol to the membrane when it is activated by physiological stresses. PKC-α then triggers the pro-inflammatory signal transduction cascade in inflammatory diseases [5,6]. Many studies have reported that PKC-α is responsible for the activation of the redox-sensitive transcription factor, nuclear factor-kappa B (NF-κB) [7]. NF-κB is a key element of the intracellular pro-inflammatory signaling cascades that control the expression of inflammation-related genes [8]. Expression of iNOS is transcriptionally regulated, particularly by NF-κB activation [9,10]. In mouse models, the iNOS gene promoter contains two NF-κB binding sites, both of which need to be bound to achieve full induction of iNOS during LPS stimulation.

Naturally occurring phenolic compounds are bioactive substances in plants that have been reported to possess beneficial effects on inflammatory diseases and are associated with antioxidant properties which combat chronic degenerative diseases in humans [11]. Polyphenols scavenge ROS and thereby directly exhibit anti-inflammatory activity by modulating important cellular signaling processes such as cellular growth, differentiation, and a host of other cellular features [12]. Ginger (*Zingiber officinale* Roscoe, Zingiber-aceae) is one of the most widely used spices and is a common condiment for a variety of foods and beverages. It has also been used in traditional oriental medicine in the management of ailments such as the common cold, digestive disorders, rheumatism, neurologia, colic and motion-sickness. The phenolic substance 6-gingerol, a major pungent principle of ginger, has been found to possess many interesting pharmacological and physiological activities, such as anti-inflammatory, analgesic, antipyretic, antihepatotoxic, and cardiotonic effects [13–16].

We hypothesized that 6-gingerol may inhibit the expression of pro-inflammatory genes and the production of inflammatory mediators such as NO by inhibiting the activation of NF-κB. Therefore, we examined whether 6-gingerol alters the activity of inflammation-related proteins in LPS-stimulated RAW264.7 cells. We first evaluated the production of intracellular ROS, the expression of inflammation related-signals (PKC-α, and NF-κB) and the expression of iNOS in order to understand the ability of 6-gingerol to inhibit inflammation. We found that 6-gingerol suppressed the

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expression of iNOS, TNF-α, and IL-10 as well as the production of NO in LPS-stimulated macrophages. This compound also blocked PKC-α expression and NF-κB activation and reduced the accumulation of intracellular ROS level in LPS-stimulated macrophages. Thus, these results indicate that 6-gingerol possesses anti-inflammatory potential by suppressing inflammatory cytokines and modulators through the suppression of redox-based NF-κB activation.

Material and methods

Cell culture. The RAW 264.7 cell line was obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL Life Technologies, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL Life Technologies Inc.) and maintained at 37 °C in a humidified incubator containing 5% CO_2. Lipopolysaccharide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). 6-Gingerol was purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were of analytical grade purity.

Cell viability assay. The mitochondrial-dependent reduction of MTT to formazan was used to measure cell respiration as an indicator of cell viability [17].

Determination of nitrite production. Nitric oxide (NO) levels were determined by using the Griess reaction to measure the level of nitrite, which is an indicator of NO synthesis. The supernatant of LPS-induced RAW 264.7 cell cultures was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). Absorbance at 550 nm was measured and concentrations were calculated against a sodium nitrite standard curve.

TNF-α measurement. The levels of TNF-α were measured by ELISA kits (R&D, Minneapolis, MN). The assay was carried out according to the manufacturer's instructions.

Western blot analysis. Protein extracts were prepared in lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin). Lysates (60 μg) were electroblotted onto a nitrocellulose membrane following separation on a 10 or 15% SDS–polyacrylamide gel. The immunoblot was incubated for 1 h with blocking solution (Tris-buffered saline/Tween 20, TBST) containing 5% skim milk (w/v) at room temperature, followed by incubation overnight at 4 °C with a 1:1000 dilution of primary antibodies against iNOS, PKC-α, IκBα, p-IκBα, or β-actin antibodies (Santa Cruz). The membrane was washed four times with TBST, incubated for 1 h with blocking solution at room temperature and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The membrane was washed four times in TBST and then developed by ECL.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. The total cellular RNA was prepared from 10^6 cells/sample according to the manufacturer's instructions provided for Trizol reagents (Invitrogen Corp.). The RT-PCR for each sample was conducted with 500 ng of total RNA using Super Script III Reverse Transcriptase (Invitrogen) and oligo dT18 primers. PCR products were fractionated on a 2% agarose gel. The primers used in this experiment were forward primer atg gct tgc ccc tgg aag ttt ctc and reverse primer cct ctg atg gtg cca tcg ggc atc tg for iNOS; forward primer cgt cgg atc cgc cat gcc tgg ctc acc act gct and reverse primer cgt ctc tag att agc ttt tca ttt tga tca for IL-10; and forward primer gac gtg ccg cct gga gaa acc and reverse primer ggg ggc cga gtt ggg gat ag for GAPDH. Following reverse transcription, PCR was performed with the initial denaturation at 94 °C for 2 min, followed by 10 cycles of primary and 15 cycles of secondary amplification. The extension step was performed at 72 °C for 10 min.

Flow cytometry analysis. Four hours after treatment with LPS and 6-gingerol, RAW 264.7 cells were washed twice with ice-cold PBS and re-suspended in 1× binding buffer (10 mM HEPES, pH 7.4, and 140 mM NaCl, 2.5 mM $CaCl_2$) at a density of 10^6 cells/ml. The cell suspension, containing approximate 10^5 cells, was stained with Fluo-4 (Sigma), DCFH-DA(Eugene, OR) and JC-1 (BD™ Mito-Screen Kit) to show Ca^{+2}, ROS and mitochondrial membrane potential, respectively. After incubation at 37 °C for 30 min, samples were analyzed by flow cytometry using a FACSCalibur system (BD Biosciences). Data were analyzed using the CellQuest software program (Becton Dickinson, USA).

Electrophoretic mobility-shift assay (EMSA). The preparation of nuclear extracts and the conditions for electrophoretic mobility-shift assay (EMSA) reactions have been previously described [18]. The 22-mer synthetic double-stranded oligonucleotides used as the NF-κB probes in the gel shift assay were 5'-AGTTGAGGGG ACTTTCCCAGGC-3', and 3'-TCAACTCCCCTGAAAGGGTCCG-5'.

Confocal microscopy. RAW264.7 cells were cultured directly on glass coverslips in 24-well plates for 12 h. After stimulation with 50 ng/ml LPS and/or 40 μM 6-gingerol, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS, and blocked with 1.5% normal donkey serum (Sigma). Coverslips were then rinsed three times over 15 min and incubated for 1 h at RT with PKC-α antibody (1:200) After incubation, coverslips were rinsed six times over 0.5 h in D-PBS and then incubated for 0.5 h at RT with orange-red fluorescence rhodamine conjugated goat anti-rabbit secondary antibody (Santa Cruz). Excess antibody was then removed by six rinses in PBS over 0.5 h. Images were captured with a BioRad MRC 1024 ES confocal microscope and analyzed using acquisition software (Laser- Sharp 2000, BioRad).

Statistical analysis. All data are presented as means ± SEM from three independent experiments. The significance of the differences at each sample was analyzed by ANOVA and Duncan's multiple-range test with significant difference set at $p < 0.05$.

Results

Exposure to LPS alone increased iNOS protein expression in mouse RAW 264.7 macrophages in a dose- and time-dependent manner when compared to the control (Fig. 1A and B). Interestingly, LPS (50 ng/ml) or a combination of LPS and 6-gingerol did not show significant cytotoxicity to RAW264.7 cells (Fig. 1F). Nitrite production was substantially higher in the presence of LPS than with the DMSO vehicle control (Fig. 1E). When treated with 6-gingerol, LPS-induced nitrite production was significantly inhibited in a dose-dependent manner ($p < 0.05$). Consistent with the results for nitrite production, 6-gingerol significantly suppressed iNOS protein and mRNA levels in a dose-dependent manner as shown in Fig. 1C and D.

TNF-α is the principal mediator of the response to LPS and may play a role in the innate immune response. Therefore, the effect of 6-gingerol on TNF-α levels and IL-10 gene expression was examined. The level of TNF-α inhibition after 6-gingerol treatment (Fig. 1G) was monitored in RAW 264.7 macrophages exposed to LPS. We further evaluated the effects of 6-gingerol on the LPS-induced expression of IL-10 in macrophages. LPS mediated expression of IL-10 was effectively suppressed by 6-gingerol (Fig. 1C).

To determine the intracellular Ca^{2+} concentration in RAW264.7 cells during LPS challenge, the fluorescence of the probe Fluo-4, which reacts with free intracellular Ca^{2+} to generate a green fluorescence, was measured. LPS induces Ca^{2+} overload in RAW264.7

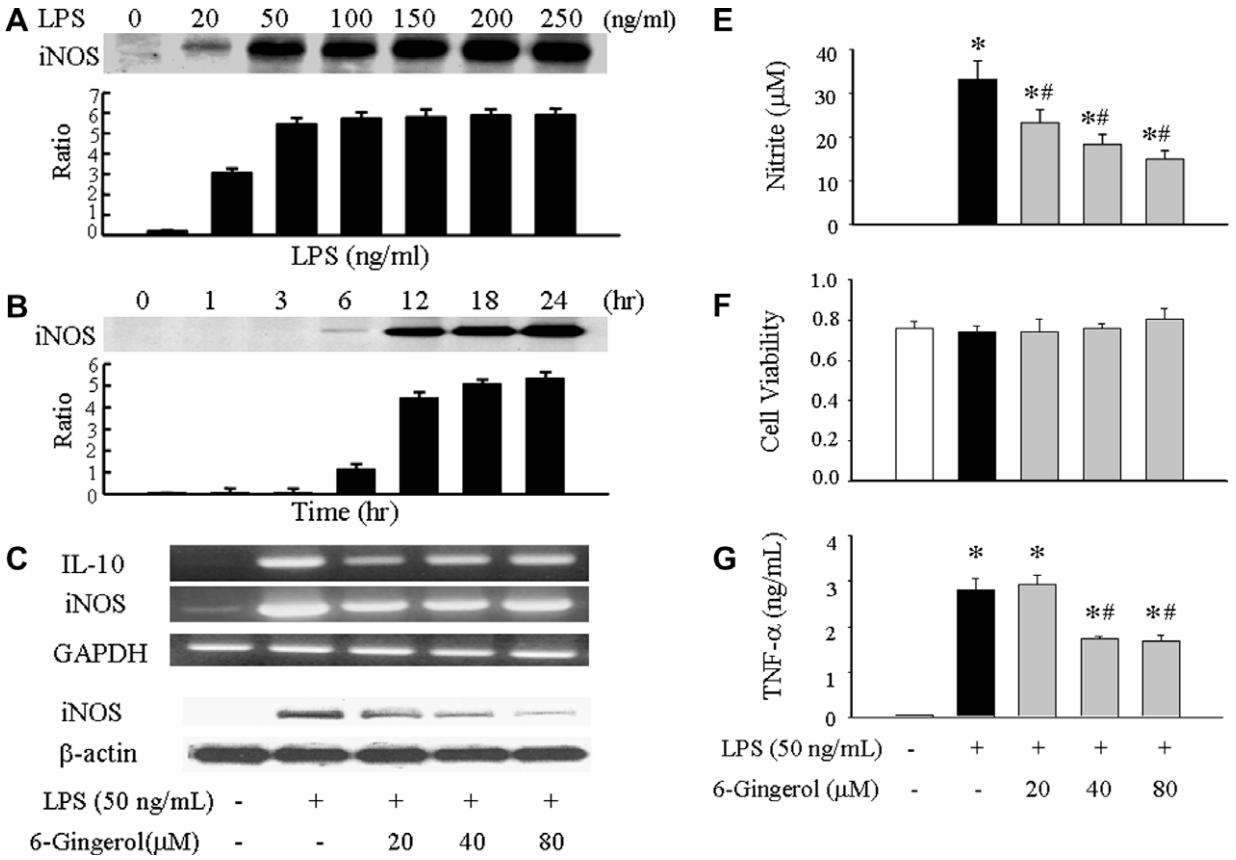

Fig. 1. Dose–response (A) and time course (B) effects of LPS-induced iNOS protein expression in RAW 264.7 cells. Effect of 6-gingerol on LPS-induced IL-10 and iNOS mRNA levels (C), iNOS protein expression (D) and nitric oxide production (E) in RAW 264.7 cells. Effect of 6-gingerol upon TNF-α production (G) in LPS-induced RAW264.7 cells. Collected supernatant was subjected to an ELISA for TNF-α. Cell viability (F) according to the MTT assay formula given in Materials and methods. Cells were induced and treated with 6-gingerol for 12 h. The data represents means ± SEM of three independent experiments. $^{*}p < 0.05$, for the comparison of LPS-stimulated cell treated with DMSO (0.1%, v/v) control. $^{\#}p < 0.05$ indicates the results statistically significant when compared with LPS-stimulated cells alone.

cells and subsequently induces ROS generation. Treatment with 6-gingerol prevented the intracellular Ca^{2+} overload induced by LPS (Fig. 2A). LPS-induced ROS production in RAW 264.7 was also inhibited by 6-gingerol treatment (Fig. 2C). This data suggests that the protective effect of 6-gingerol occurs by preventing the calcium overload induced by H_2O_2. Disruption of mitochondrial membrane potential (MMP) is one of the critical events in oxidative stress pathways. While MMP was disrupted in RAW264.7 cells treated with LPS, the population of cells with disrupted MMP decreased in the presence of 6-gingerol (Fig. 2B).

LPS treatment caused the PKC-α protein to translocate from the cytosol to the membrane in RAW 264.7 cells (Fig. 3). After exposure to LPS (50 ng/ml) for 12 h, the intensity of the PKC-α band significantly decreased in the cytosolic extract, while it increased in the membrane extract, compared to the control. However, confocal imagery shows that 6-gingerol treatment blocks PKC-α translocation in LPS-stimulated RAW 264.7 cells (Fig. 3B).

An EMSA was performed to examine whether the suppression of iNOS expression by 6-gingerol was dependent upon the inhibition of LPS-induced NF-κB activation. As shown in Fig. 4, nuclear extract from LPS-stimulated macrophages displayed a marked increase in NF-κB DNA-binding activity compared to nuclear extract from 6-gingerol treated macrophages. Moreover, 6-gingerol treatment dose-dependently suppressed LPS-induced cytoplasmic I-κBα phosphorylation in RAW264.7 cells when examined by Western blot analysis.

Discussion

Macrophage-derived NO is an important host defense, and microbial and tumor cell killing agent, as well as a regulator of pro-inflammatory genes in vivo [19]. Indeed, it is possible that modulation of iNOS expression could potentially control both chronic and acute inflammatory diseases [20–22].

Redox signaling can play a significant role in regulating the inflammatory response. The ROS play a critical role in the regulation of cell growth and differentiation, and in the control of cellular responses to cytokines and stresses [23], including inflammation [24]. ROS, including superoxides, are generated from different sources, including normal cell respiration and mitochondria electron flux [25,26]. ROS are also involved in the signaling pathway for LPS-induced iNOS expression [27,28]. However, treatment of cells with 6-gingerol significantly effected the ROS production in LPS-stimulated cells, suggesting that ROS are involved in the inhibitory effect of 6-gingerol on LPS-stimulated NO production in RAW 264.7 cells. Therefore, 6-gingerol can act as antioxidative compound and scavenge reactive radicals. The PKC sites are essential for LPS-mediated NO production [29,30]. To determine the specific signal transduction pathway involved in the inhibition of PKC by 6-gingerol, we examined the effects of 6-gingerol on the LPS-induced translocation of PKC-α. We found that the activity of LPS-induced PKC-α and PKC-dependent ROS signaling pathways are inhibited by 6-gingerol.

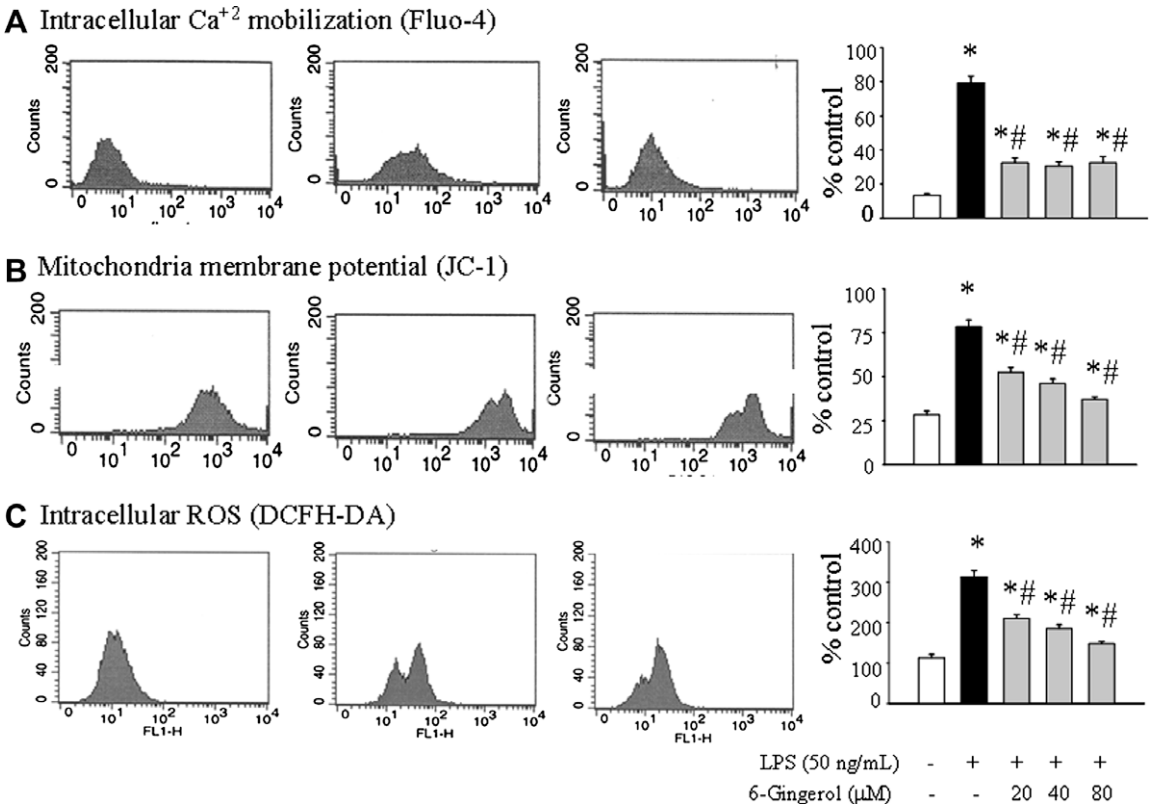

Fig. 2. Flow cytometric analysis of intracellular Ca^{+2} (A), mitochondrial membrane potential (B) and ROS (hydrogen peroxide, H_2O_2) (C) of RAW264.7 cells after incubation with LPS for 4 h without or with various dose of 6-gingerol. Cells were harvested and the percentage of cells were determined by FACS analysis using Fluo-4, JC1 or DCFH-DA staining as described in the materials and methods. The results are representative as means ± SEM of three independent experiments. $^{*}p < 0.05$, for the comparison of LPS-stimulated cell treated with DMSO (0.1%, v/v) control. $^{\#}p < 0.05$ indicates the results statistically significant when compared with LPS-stimulated cells alone.

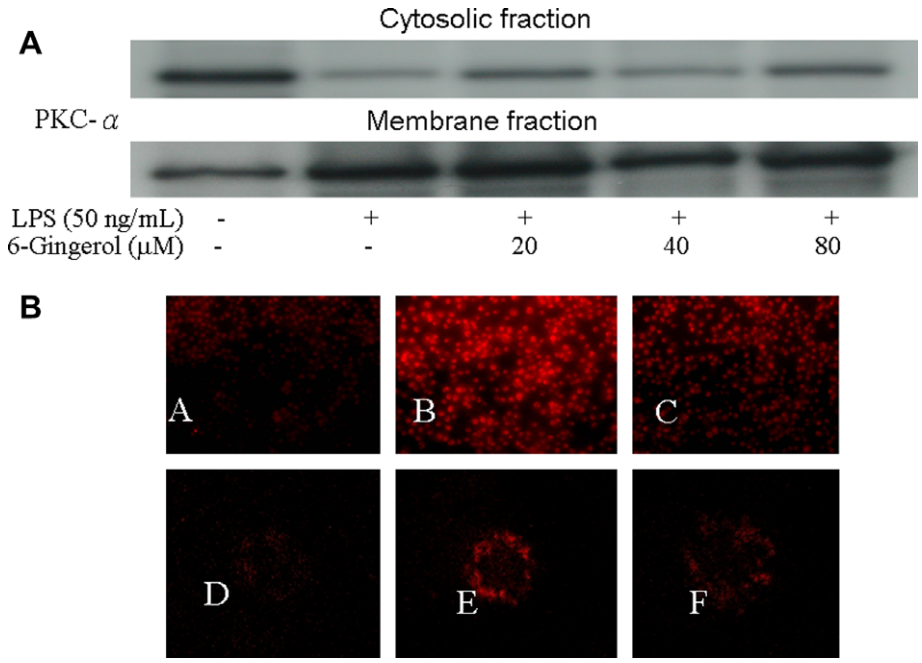

Fig. 3. (A) Effect of 6-gingerol upon LPS-induced membrane translocation of PKC-α in mouse macrophages. RAW 264.7 cells were treated with LPS without or with various doses of 6-gingerol for 12 h. Subsequently, cytosolic and membrane fractions of the cells were prepared to detect total PKC-α levels by Western blot analysis. The shown data was representative of three independent experiments with similar results. (B) For confocal microscopy images of PKC-α in RAW 264.7 cells, the cells were incubated with LPS alone (B,E) or with LPS and 6-gingerol (C,F). After the cells were washed with PBS and fixed with 4% paraformaldehyde, cell membranes were stained by orange-red fluorescence rhodamine conjugated antibodies.

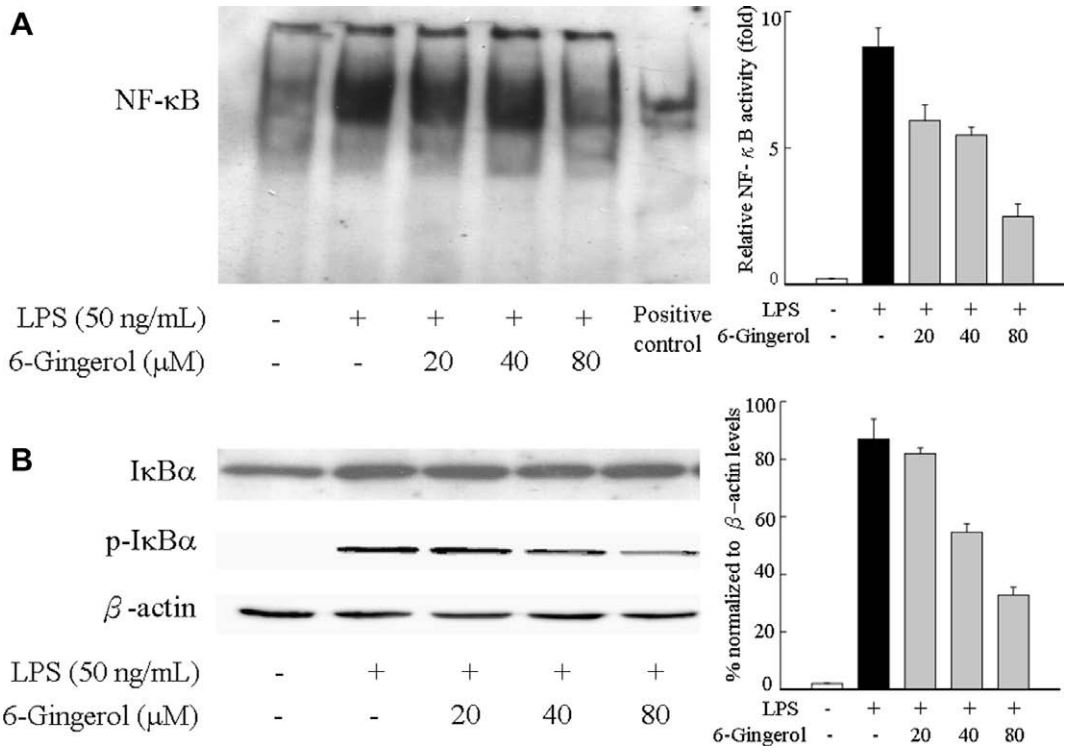

Fig. 4. Effect of 6-gingerol on NF-κB activation after stimulation with LPS and 6-gingerol for 1 h. Nuclear extracts from RAW 264.7 macrophages were used for electrophoretic mobility shift assays (EMSA) (A). Effect of 6-gingerol on the phosphorylation of IκBα in LPS-stimulated RAW 264.7 cells. The obtained cytosolic extracts obtained were subjected to Western blot analysis (B). Results were means ± SEM of three independent experiments.

The expression of iNOS is mainly regulated at the transcriptional level, with NF-κB acting as the major transcriptional regulator. NF-κB is also a key regulator of a variety of other genes involved in the immune and inflammatory responses [9,31,32]. In this study, we have shown that 6-gingerol-mediated down-regulation of NO production was effected through transcriptional regulation, based on the suppression of both iNOS mRNA and protein levels (Fig. 1). The promoter region of the murine iNOS gene contains two transcriptional regulatory regions; an enhancer and a basal promoter [10,33]. The inhibition of NF-κB-dependent expression of inflammatory genes, such as TNF-α, IL-10, and iNOS, and production of NO in LPS-stimulated macrophages by 6-gingerol may contribute to the above anti-inflammatory activities of 6-gingerol. Thus, the suppression of NF-κB-dependent inflammatory gene expression may be an effective therapeutic strategy for preventing inflammatory processes and diseases. Treatment with 6-gingerol inhibits the LPS-induced increase in the NF-κB DNA-binding activities and I-κBα phosphorylation (Fig. 4). These observations suggest that 6-gingerol may act as a functional molecule in immune cells stimulated with LPS.

Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) is among the most healthy and frequently consumed spices throughout the world. 6-Gingerol is the principal pungent and pharmacologically active ingredient of ginger, can act as a superoxide chelator and suppress both TNF-α production and AP-1 activation [34–36]. A recent study by Ippoushi et al., demonstrated that 6-gingerol inhibited iNOS and nitration in J774.1 macrophages [16]. In contrast, PKC and NF-κB appear to be critical for LPS-mediated induction of iNOS in RAW 264.7 cells, since LPS-induced PKC activation and NF-κB binding was markedly suppressed by 6-gingerol (Figs. 3 and 4). In summary, 6-gingerol inhibits LPS-induced NO production and the expression of iNOS mRNA and protein in macrophages. These effects are mediated, at least in part, by the inhibition of NF-κB DNA-binding activity and PKC-α activation. The fact that PKC is negatively regulated by 6-gingerol is important because this signaling factor plays a critical role in the regulation of a variety of inflammatory response genes involved in oxidative stress. In view of the fact that NO plays an important role in mediating inflammatory responses, and that 6-gingerol may be an important determinant in clinical responses to inflammatory diseases, further efforts to explore this therapeutic strategy appear to be warranted.

Acknowledgments

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