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6-Shogaol, a ginger product, modulates neuroinflammation: A new approach to neuroprotection

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ABSTRACT

Inflammatory processes in the central nervous system play an important role in a number of neurodegenerative diseases mediated by microglial activation, which results in neuronal cell death. Microglia act in immune surveillance and host defense while resting. When activated, they can be deleterious to neurons, even resulting in neurodegeneration. Therefore, the inhibition of microglial activation is considered a useful strategy in searching for neuroprotective agents. In this study, we investigated the effects of 6-shogaol, a pungent agent from *Zingiber officinale Roscoe*, on microglia activation in BV-2 and primary microglial cell cultures. 6-Shogaol significantly inhibited the release of nitric oxide (NO) and the expression of inducible nitric oxide synthase (iNOS) induced by lipopolysaccharide (LPS). The effect was better than that of 6-gingerol, wogonin, or N-monomethyl-L-arginine, agents previously reported to inhibit nitric oxide. 6-Shogaol exerted its anti-inflammatory effects by inhibiting the production of prostaglandin E_2 (PGE_2) and proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and by downregulating cyclooxygenase-2 (COX-2), p38 mitogen-activated protein kinase (MAPK), and nuclear factor kappa B (NF-κB) expression. In addition, 6-shogaol suppressed the microglial activation induced by LPS both in primary cortical neuron-glia culture and in an *in vivo* neuroinflammatory model. Moreover, 6-shogaol showed significant neuroprotective effects *in vivo* in transient global ischemia via the inhibition of microglia. These results suggest that 6-shogaol is an effective therapeutic agent for treating neurodegenerative diseases.

1. Introduction

Inflammation in the brain is closely associated with the pathogenesis of numerous neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease, and cerebral ischemia (Kim and Joh, 2006; McGeer and McGeer, 1995; Stoll et al., 1998). Neuroinflammation results primarily from the activation of astrocytes and microglia, which are the resident immune cells of the brain. Under normal conditions, microglia act in immune surveillance, and astrocytes serve to maintain neuron survival by secreting nerve growth factors and buffering the actions of neurotransmitters (Aloisi, 1999; Kreutzberg, 1996). Activated microglia secrete a variety of proinflammatory and cytotoxic factors, such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), arachidonic acid, eicosanoids, and reactive oxygen species (McGeer and McGeer, 1995; Minghetti and Levi, 1998). The accumulation of proinflammatory and cytotoxic factors is deleterious to neurons *in vitro*, and these factors are thought to participate actively in the progression of neurodegenerative diseases *in vivo* (Banati et al., 1993; Boje and Arora, 1992; Bronstein et al., 1995; Jeohn et al., 1998; Kreutzberg, 1996; Raine, 1994). Therefore, the inhibition of proinflammatory mediators secreted from activated microglia would be an effective therapeutic approach to regulate the progression of neurodegenerative diseases.

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Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is a potent activator of microglia. LPS induces intracellular signaling pathways involving nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), such as p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK). Both the NF-κB and MAPK pathways are capable of regulating the expression of numerous immune response genes, including proinflammatory cytokines and chemokines (Hanada and Yoshimura, 2002).

Ginger, the rhizome of the plant *Zingiber officinale* Roscoe in the family *Zingiberaceae*, has long been used widely as a spice for cooking and as a medicinal herb in traditional herbal medicine. Ginger is reported to have antioxidative, anti-inflammatory, antimicrobial, and anticarcinogenic properties (Ali et al., 2008; Shukla and Singh, 2007). Ginger contains 1.0–3.0% volatile oils and a number of pungent compounds (Chrubasik et al., 2005). Gingerols are the most abundant pungent compounds in fresh roots, and ginger contains several gingerols of various chain lengths (n6 to n10), with 6-gingerol being the most abundant. Shogaols, the dehydrated form of gingerols, are found in only small quantities in the fresh root and mainly in dried and thermally treated roots, with 6-shogaol being the most abundant (Jolad et al., 2004). 6-Gingerol and 6-shogaol have a number of pharmacological activities, including anti-inflammatory, antipyretic, analgesic, antitussive, and hypotensive effects (Pan et al., 2008; Suekawa et al., 1984). Although ginger is commonly used in foods and folk medicines and various activities of its constituents have been revealed, it is still not known whether the neuroprotective effects of it are derived from the inhibition of microglia or not.

This study evaluated the anti-inflammatory effects of 6-shogaol in primary microglia cells and in an *in vivo* systemic inflammatory model induced by LPS. Based on the anti-inflammatory activities of 6-shogaol *in vitro* and *in vivo*, we investigated the effects of 6-shogaol on neuroprotection via microglial inactivation in a primary cortical neuron-glia culture, in a transient global ischemia model.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle medium (DMEM), minimum essential medium (MEM), MEM-α, fetal bovine serum (FBS), horse serum (HS), and penicillin-streptomycin (PS) were purchased from Invitrogen (Carlsbad, CA, USA). LPS, N-monomethyl-L-arginine (NMMA), sodium nitroprusside and polyinosinic-polycytidylic acid [poly(I:C)] were obtained from Sigma Chemical Company (St. Louis, MO, USA). A synthetic lipopeptide (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH trihydrochloride (Pam3Cys-Ser-Lys4; PamCSK) was obtained from Calbiochem (San Diego, CA, USA). 6-Gingerol and 6-shogaol were purchased from Wako Pure Chemical (Osaka, Japan).

2.2. Animal

Animal maintenance and treatment were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use guidelines of Kyung Hee University, Seoul, Korea. Sprague-Dawley (SD) rats were purchased from Orient Bio (Kyunggido, Korea). Male C57BL/6 mice (7 weeks) were purchased from the Dae Han Biolink Company (Eumseong, Korea). Animals were housed 5 or 6 per cage at an ambient temperature of 23 ± 1 °C and a relative humidity of 60 ± 10% under a 12 h light/dark cycle, with free access to water and food.

2.3. Cell culture

The BV-2 mouse microglial cell line, originally developed by Dr. V. Bocchini at the University of Perugia (Perugia, Italy), has both the phenotypic and functional properties of reactive microglia cells (Blasi et al., 1990). BV-2 cells were generously provided by Dr. E. Choi at Korea University (Seoul, Korea). BV-2 cells were maintained in 10 ml of DMEM supplemented with 5% FBS and 1% PS. Primary microglia cells were cultured from the cerebral cortices of neonatal SD rats (1-day-old) which were purchased from Orient Bio (Kyunggido, Korea). Cortices were triturated into single cells in MEM-α containing 10% FBS and plated into a 75 cm^2 T-flask for 2 weeks. The microglia were detached by mild shaking and applied to a nylon mesh (70 μm, Spectrum, California, USA) to remove cell clumps. The purity of the microglial cultures was over 95%, as judged by immunostaining with anti-OX-42 antibody (Chemicon, Temecula, CA).

2.4. Measurement of NO production and cell viability

Nitrite, a soluble oxidation product of NO, was measured in the culture media using the Griess reaction. The supernatant (50 μl) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamine, 0.1% naphthylethylene diamine dihydrochloride, 2% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using a microplate reader. Sodium nitrite was used as a standard to calculate the NO_2^- concentration. Cell viability was assessed by a 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assay.

2.5. NO radical scavenging assay

NO generated from sodium nitroprusside (SNP) was measured using the method of Marcocci et al. (1994). Briefly, the reaction mixture (5.0 ml) containing SNP (5 mM) in phosphate buffered saline (PBS, pH 7.3), with or without 6-shogaol at different concentrations, was incubated at 25 °C for 180 min. The supernatant (50 μl) was harvested and mixed with an equal volume of Griess reagent. The absorbance at 540 nm was measured using a microplate reader.

2.6. iNOS activity assay

iNOS activity assay was performed using the method of Chen et al. (2001). BV-2 cells were cultured in a 100-mm plate and activated with LPS (1 μg/ml) for 12 h. Cells were collected and washed twice with PBS to remove LPS. Cells were plated at a concentration of 5×10^4 cells/ml into 96-well plates, and indicated 6-shogaol was added. NMMA, a classical inhibitor of iNOS enzyme activity, was used as a positive control. After 12 h, the amount of nitrite was measured by the Griess reaction as described above.

2.7. Measurement of prostaglandin E_2 (PGE_2), IL-1β and TNF-α

Media was collected and centrifuged 24 h after treatment with LPS (100 ng/ml) in the presence or absence of 6-shogaol. PGE_2, IL-1β and TNF-α were measured by a competitive enzyme immunoassay kit (PGE_2, Cayman Chemical, Ann Arbor, MI, USA), specific ELISA kit (IL-1β and TNF-α, R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

2.8. NF-κB assay

Nuclear extracts from treated microglia were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). NF-κB activity was measured by a NF-κB p65 assay kit (Active Motif) according to the manufacturer's protocol.

2.9. Western blot analysis

BV-2 and primary microglia cells were seeded in a 6-well plate and exposed to LPS (100 ng/ml) in the presence or absence of 6-shogaol for various times. Protein samples from the cell extracts were separated by 8% or 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% skim milk and incubated with primary antibodies against iNOS (BD Transduction Laboratories, San Diego, CA, USA), cyclooxygenase (COX)-2 (Santa Cruz Biotechnology, CA, USA), p38, ERK, JNK, phospho-p38, phospho-ERK, phospho-JNK, inhibitory kappa B (IκB), and phospho-IκB (Cell Signaling, Beverly, MA, USA). After washing with TBST, HRP-conjugated secondary antibodies (goat anti-rabbit IgG, Amersham Pharmacia Biotech; donkey anti-goat IgG, Santa Cruz Biotechnology) were applied. The blots were developed using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech). Densitometry analysis of bands was performed with the ImageMaster™ 2D Elite software, version 3.1 (Amersham Pharmacia Biotech).

2.10. Cortical neuron-glia culture

Neuron-glia cultures were prepared from the cerebral cortices of embryonic day 16 (E16) SD rats according to a previously reported method with slight modifications (Qin et al., 2002). Briefly, the meninges were removed and dissociated by trituration in HBSS media. After trypsinization, cells were harvested and seeded at a density of 5×10^5 cells in 24-well plates coated with 20 μg/ml of poly-D-lysine (Sigma, St. Louis, MO, USA). The culture medium consisted of MEM supplemented with 10% FBS, 10% HS, 1 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, and 0.5% PS. Cultures were maintained in a humidified 5% CO_2 incubator at 37 °C. Seven-day-old cultures were used for the experiment. The composition of cortical neuron-glia cultures was determined by immunostaining with antibodies against MAP-2, Neu-N, and OX-42. Cortical neuron-glia cultures

contained 60% Neu-N-immunoreactive (IR) neurons and 3% OX-42-IR microglia. The remaining cells were presumed to be astrocytes.

2.11. Immunocytochemistry

Cortical neurons were stained with an antibody against MAP-2, a marker for both cell bodies and neuritis, and with an antibody against Neu-N, a marker for neuronal cell bodies. Microglial cells were visualized by staining with an OX-42 antibody. Briefly, cells were fixed for 20 min in 3.7% paraformaldehyde in phosphate buffered saline (PBS). After washing twice with PBS, the cultures were incubated with 1% hydrogen peroxide (H_2O_2) for 10 min. The cultures were blocked with the appropriate normal serum followed by incubation overnight at 4 °C with the primary antibody. Then, cells were washed three times for 10 min in PBS. Afterward, biotinylated anti-primary secondary antibody was incubated for 1 h. The cells were washed three times for 10 min and incubated in Vectastain ABC reagent (Vector Laboratory, Piscataway, NJ, USA) for 1 h. The color was developed with 3,3′-diaminobenzidine (DAB). Images were recorded using a Zeiss inverted microscope connected to a digital CCD camera (Axiocam, Zeiss, Oberko, Germany). For cell counting, nine representative areas per well were counted under the microscope at ×200 magnification.

2.12. Inflammatory model in mice

6-Shogaol and NMMA (positive control) were dissolved in 10% DMSO and administered orally at two doses of 5 mg/kg and 20 mg/kg once per day for 3 days before LPS treatment. Three hours after the last drug administration, LPS dissolved in normal saline was injected intraperitoneally at a dose of 5 mg/kg. An equal volume of vehicle was given to the control and LPS groups. Three hours after LPS injection, the mice were prepared for the histological analysis.

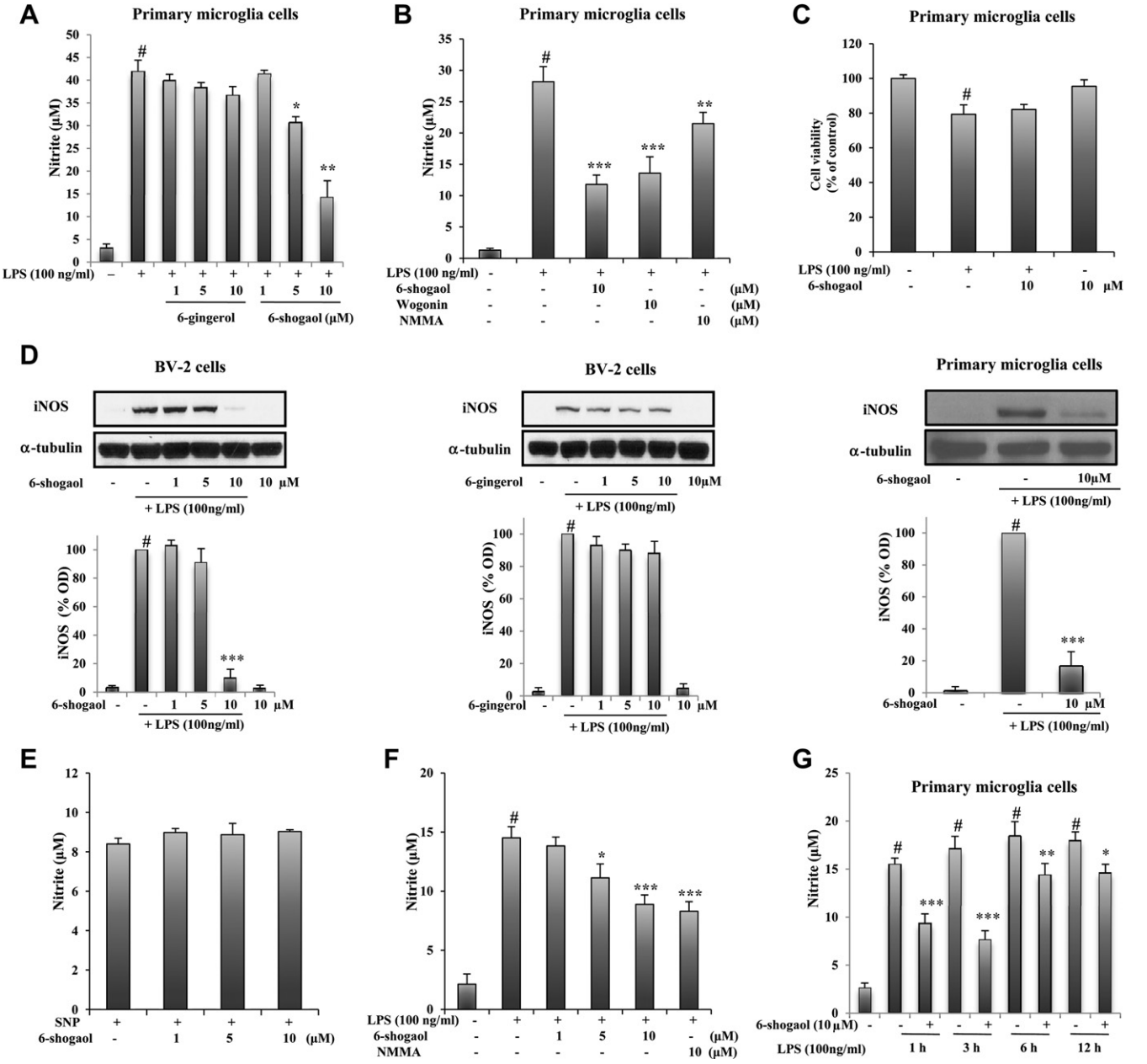

Fig. 1. Effect of 6-shogaol on LPS-induced NO production and iNOS expression in microglia cells. (A) Effect of 6-gingerol and 6-shogaol on LPS-induced NO production in primary microglia cells. (B) Effects of 6-shogaol, wogonin, and NMMA on LPS-induced NO production in primary microglia cells. (C) Effect of 6-shogaol on cell viability in LPS-stimulated primary microglia cells. (D) Effect of 6-gingerol and 6-shogaol on LPS-induced iNOS expression in microglia cells. (E) Effect of 6-shogaol on NO scavenging. (F) Effect of 6-shogaol on iNOS activity in BV-2 cells. (G) Effect of 6-shogaol post-treatment on NO production in LPS-stimulated primary microglia cells. All data are presented as the mean ± S.E.M of three independent experiments. #$p < 0.05$ indicates statistically significant difference between the control and LPS alone-treated groups. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate statistically significant differences compared to treatment with LPS alone.

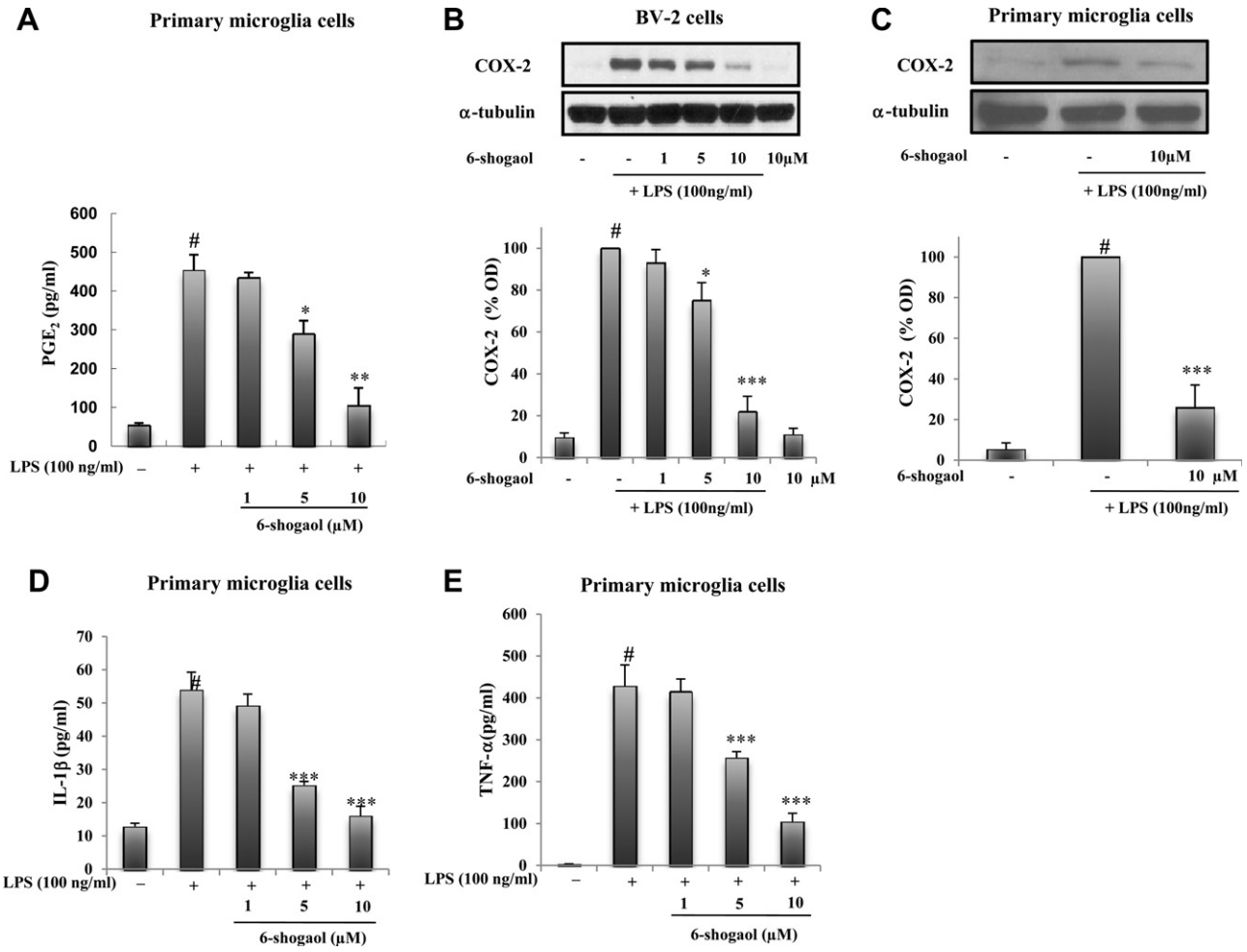

Fig. 2. Effect of 6-shogaol on PGE_2 production, COX-2 expression, IL-1β and TNF-α in LPS-treated microglia cells. (A) PGE_2 was assessed by using a competitive enzyme immunoassay kit after treatment with LPS (100 ng/ml) for 6 h in the presence or absence of 6-shogaol (1, 5, and 10 μM). (B, C) Expression of COX-2 was assessed by western blot analysis using COX-2 antibody. (D, E) Levels of IL-1β and TNF-α in the culture supernatants were determined by ELISA analysis. All data are presented as the mean ± S.E.M of three independent experiments. #$p < 0.05$ indicates statistically significant difference between the control and LPS alone-treated groups. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistically significant differences compared to treatment with LPS alone.

2.13. Transient global ischemia by bilateral common carotid arteries occlusion

Mice were anesthetized in a chamber with a mixture of N_2O and O_2 (70:30) containing 2% isoflurane. Bilateral common carotid arteries occlusion (2VO) was induced as described elsewhere (Cho et al., 2007), with minor modifications. Briefly, after making a median incision in the neck skin of a mouse, both common carotid arteries were exposed and occluded with aneurysm clips for 25 min. Body temperature was maintained at 37 ± 0.5 °C throughout surgery by a heating pad (Biomed S.L., Alicante, Spain). Circulation was restored by removing the clips. The mice which received the same surgical operation without clipping of the carotid arteries served as sham-operated controls. Right after 2VO induction, drug treatments were done once a day for three consecutive days. 6-Shogaol and NMMA were dissolved in 10% Tween 80 solution and administered orally. Seven days after reperfusion, the mice were prepared for the histological analysis.

2.14. Preparation brain tissues for the histology

The mice were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and perfused transcardially with 0.1 M phosphate buffer (pH 7.4) followed by ice-cold 4% paraformaldehyde. Brains were removed, post-fixed in the same fixative solution overnight, and then immersed in 30% sucrose solution (in 0.05 M PBS) for the cryoprotection at 4 °C until sectioned. Frozen brains were sectioned coronally into 30 μm sections on a cryostat (CM3000; Leica, Wetzlar, Germany) and then stored in storing solution containing glycerine, ethylene glycol, and phosphate buffer at 4 °C.

2.15. Cresyl violet staining and immunohistochemistry

For the cresyl violet staining, brain sections were mounted onto gelatin-coated slides, stained with 0.5% cresyl violet, dehydrated through graded alcohols (70, 80, 90, and 100% × 2), placed in xylene, and coverslipped using Histomount medium.

For the immunohistochemistry, free floating sections were rinsed in PBS at room temperature and pretreated with 1% hydrogen peroxide for 15 min. Then they were incubated overnight with anti-CD11b antibody (Mac-1), anti-IL-1β and anti-TNF-α antibody (each 1:1000 dilution, Santa Cruz Biotechnology) at 4 °C. Sections were then incubated for 90 min with biotinylated secondary antibody (1:200 dilution), treated with avidin-biotin-peroxidase complex (1:100 dilution) for 1 h at room temperature, and reacted with 0.02% DAB and 0.01% H_2O_2 for about 3 min. After each incubation step, sections were washed three times with PBS for 5 min. Finally, sections were mounted on gelatin-coated slides, dehydrated in an ascending alcohol series, and cleared in xylene.

2.16. Histological analyses

For the analysis in the inflammatory mice model, quantification of the microglia cells was performed by counting the number of these cells in the cortex and hippocampus at ×100 magnification using a Stereo Investigator (MicroBrightField, Williston, USA) and the images were photographed at ×200 magnification using a AxioSkop 2 microscope (CarlZeiss Inc., Göttingen, Germany); these values are presented as a percent of the control group values. Results were averaged for three sections per mouse for five or six mice per group.

For the analysis in the mouse model of transient global ischemia by 2VO, cell counts in CA1 were performed using a computerized image analysis system (Leica

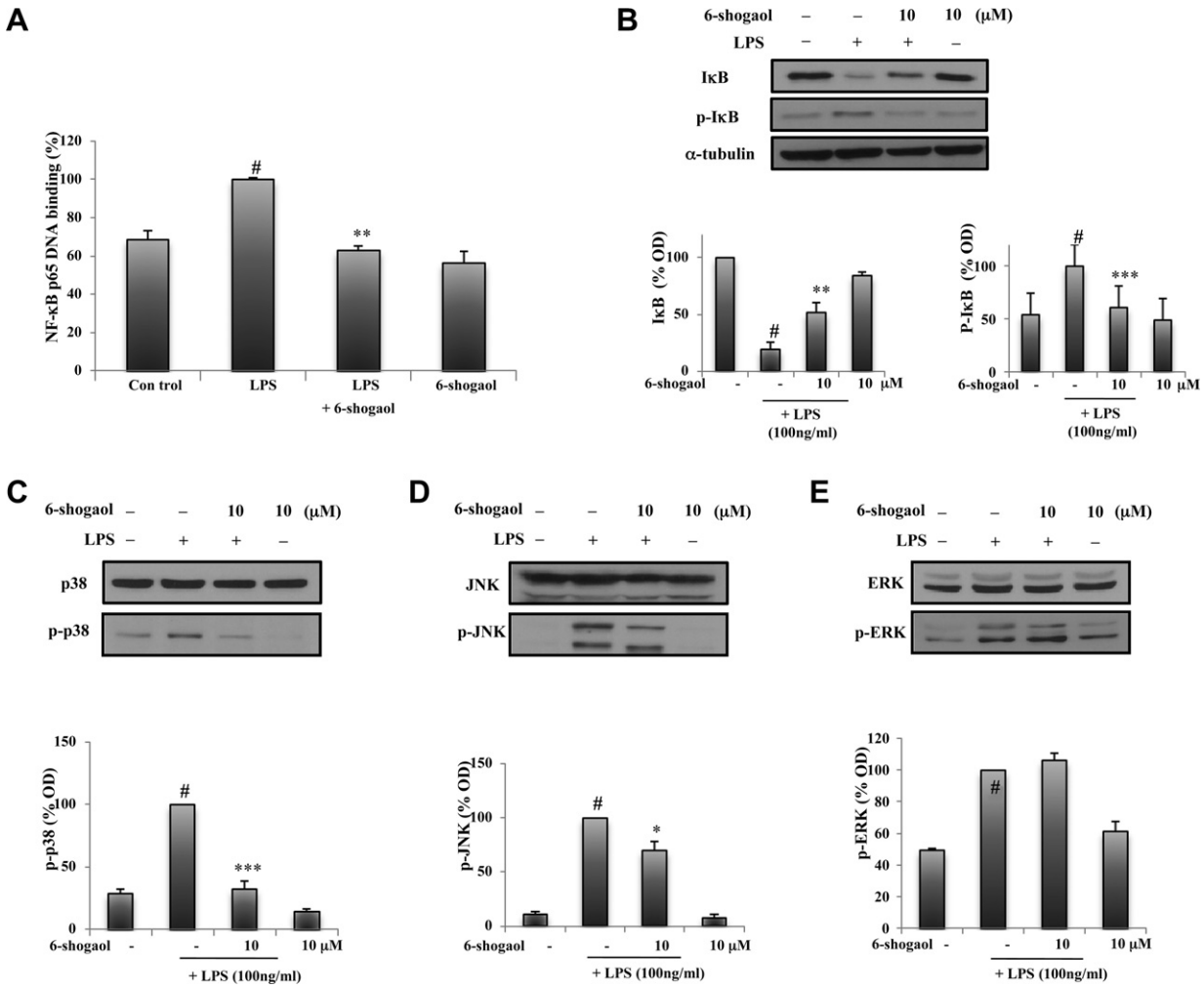

Fig. 3. Effect of 6-shogaol on LPS-induced NF-κB and MAPKs activation in primary cultured microglia cells. (A) Nuclear extracts were prepared by using a nuclear extract kit. NF-κB activity was measured using an ELISA kit. (B) Protein levels of IκB and p-IκB were evaluated by western blot analysis. (C–E) Activation of MAPKs was evaluated by western blot analysis using antibodies that recognize the phosphorylated or unphosphorylated forms of p38, JNK, and ERK. The data are expressed relative to percentage of control (IκB) or LPS stimulation (p-IκB, p-p38, p-JNK, p-ERK) and are presented as the mean ± S.E.M of three independent experiments. #$p < 0.05$ indicates statistically significant difference between the control and LPS alone-treated groups. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistically significant differences compared to treatment with LPS alone.

Microsystems AG, Wetzlar, Germany). Cells in the hippocampal CA1 region were counted in six sections per mouse for four mice per group by one person unaware of the treatment history. Cell counts were performed using a computerized image analysis system (Leica Microsystems AG).

2.17. Statistical analysis

The data were analyzed using Statistical Analysis System software (PRISM). All the data are expressed as mean ± S.E.M. Statistical comparisons between the different treatments were performed using one-way ANOVA with Tukey's multiple comparison post test. p values of <0.05 were considered to be statistically significant.

3. Results

3.1. Effect of 6-shogaol on NO production and iNOS regulation in microglia

The effects of pretreatment with 6-gingerol and shoagol on NO production were tested in BV-2 and primary microglia cells. 6-Shogaol effectively decreased LPS-induced NO production in primary microglia cells, while 6-gingerol did not inhibit NO production significantly (Fig. 1A). The inhibitory effects of 6-shogaol, wogonin, potent well-known anti-inflammatory agent from *Scutellaria baicalensis*, and NMMA (the iNOS inhibitor) on NO production were evaluated in primary microglia cells. Among these compounds, 6-shogaol showed the most potent NO inhibitory activity (Fig. 1B). The results of the MTT assay showed that the concentrations of 6-shogaol used in this study did not affect cell viability (Fig. 1C).

NO production in microglia is regulated primarily by the iNOS enzyme. We performed western blot to determine whether the NO inhibitory effect of 6-shogaol is related to the regulation of the expression of iNOS. As shown in Fig. 1D, pretreatment of cells with 6-shogaol led to a significant decrease in iNOS protein level at 10 μM. Similar to what was observed in BV-2 cells, 6-shogaol inhibited expression of iNOS protein in primary cultured microglia. In contrast, 6-gingerol did not have an inhibitory effect on LPS-induced iNOS expression.

To investigate the precise mechanisms of 6-shogaol on NO regulation, we also performed NO radical scavenging assay and iNOS activity assay. As shown in Fig. 1E and F, 6-shogaol did not influence on the accumulation of nitrite upon decomposition of NO· doner, sodium nitroprusside. However, 6-shogaol (5 and 10 μM) significantly reduced iNOS activity.

The effect of post-treatment with 6-shogaol on NO production in primary microglia was examined. As shown in Fig. 1G, while treatment

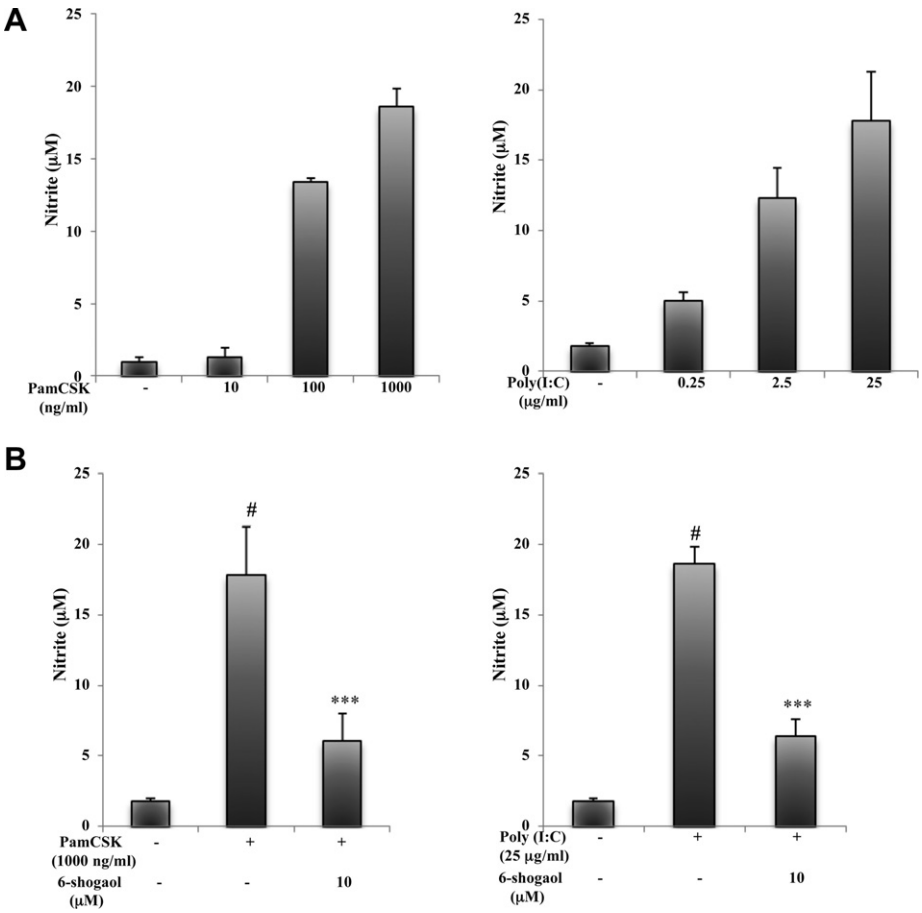

Fig. 4. Effect of 6-shogaol on NO production induced by TLRs agonist in primary microglia cells. (A) Primary microglia cells were stimulated with PamCSK (synthetic lipopeptide, TLR2 agonist, 0.01, 0.1, and 1 μg/ml) or poly(I:C) (double-stranded RNA, TLR3 agonist, 0.25, 2.5, and 25 μg/ml) for 24 h. (B) Primary microglia cells were pretreated with 6-shogaol (10 μM) for 30 min and then stimulated with PamCSK (1 μg/ml) or poly(I:C) (25 μg/ml) for another 24 h. The culture medium was then collected for a nitrite assay. Nitrate was measured using a Griess reaction. All data are presented as the mean ± S.E.M of three independent experiments. #$p < 0.05$ indicates statistically significant difference between the control and TLR agonists alone-treated groups. ***$p < 0.001$ indicates a statistically significant difference compared to treatment with LPS alone.

of microglia cells with LPS for 1, 3, 6, and 12 h increased NO production severely, post-treatment with 10 μM of 6-shogaol had a significant inhibitory effect on NO production, consistent with pretreatment result.

3.2. Effect of 6-shogaol on PGE_2 production and COX-2 expression in microglia

Pretreatment of primary microglia cells with 6-shogaol has decreased LPS-induced PGE_2 production significantly in a dose-dependent manner (Fig. 2A).

It is known that COX-2 mediates PGE_2 production in response to proinflammatory stimulation. Therefore, the effect of 6-shogaol on the expression of the COX-2, a key enzyme responsible for PGE_2 production, was determined using western blot analysis in BV-2 and primary microglia cells. 6-Shogaol reduced LPS-induced expression of COX-2 significantly in a concentration-dependent manner (Fig. 2B and C). Furthermore, 6-shogaol at 10 μM similarly decreased COX-2 expression in primary microglia cells. These results indicate that 6-shogaol suppresses LPS-induced PGE_2 synthesis through downregulation of COX-2.

3.3. Effect of 6-shogaol on proinflammatory cytokines in microglia

To investigate whether 6-shogaol inhibits the production of proinflammatory cytokines such as IL-1β and TNF-α, primary microglia cells were treated with LPS alone or with 6-shogaol (1, 5, and 10 μM) for 24 h. Stimulation of microglia cells with LPS led to the increased production of IL-1β and TNF-α. The production of these cytokines was significantly decreased in a dose-dependent manner by pretreatment with 6-shogaol (Fig. 2D and F).

3.4. Effect of 6-shogaol on NF-κB activation in microglia

Because NF-κB is an important upstream modulator of proinflammatory cytokines, iNOS, and COX-2 expression (Baeuerle and Henkel, 1994; Nomura, 2001), the effects of 6-shogaol on NF-κB activity were investigated using an NF-κB ELISA kit and western blot analysis. As shown in Fig. 3A, LPS significantly enhanced the DNA binding activity of nuclear NF-κB p65 in primary microglia. The increase in NF-κB activity was decreased significantly by pretreating cells with 10 μM 6-shogaol.

NF-κB is inactivated in the cytosol by binding to IκB, and becomes active through translocation to the nucleus preceded by LPS-induced proteolytic degradation of IκB (Baeuerle and Baltimore, 1988; Zandi et al., 1997). As shown in Fig. 3B, IκB was phosphorylated and degraded 1 h after LPS treatment. Pretreatment of primary microglia cells with 6-shogaol (10 μM) decreased to phosphorylation and degradation of IκB in response to LPS, indicating that the subsequent NF-κB inactivation induced by 6-shogaol.

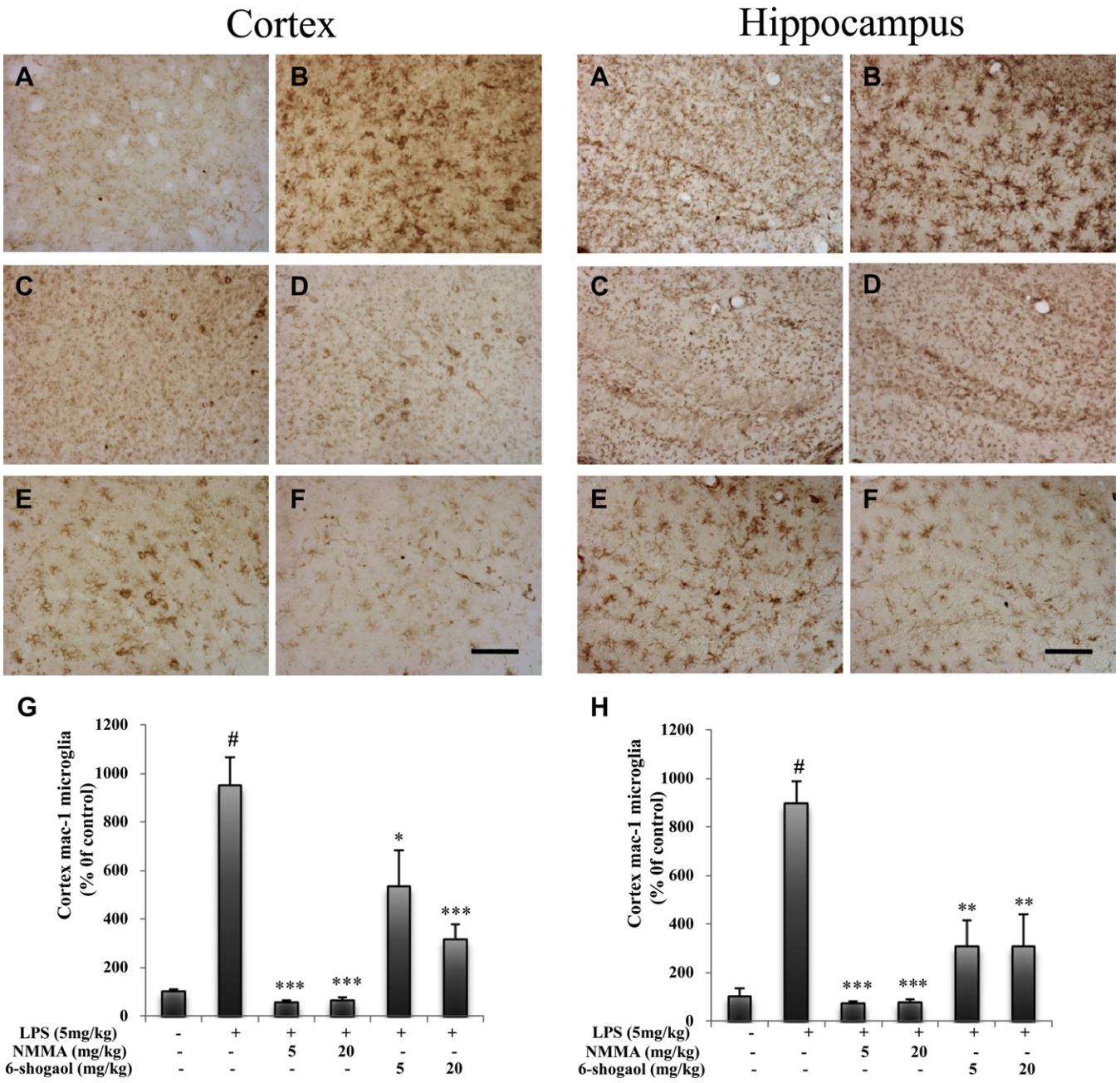

Fig. 5. Effect of 6-shogaol on microglial activation in mice models of LPS-induced neuroinflammation. (A–F) Representative photomicrographs of mac-1-IR microglia in the cortex (left column) and hippocampus (right column) of each group at magnification of ×200. The scale bar = 100 μm. Quantification of the microglia cells was performed by counting the number of microglial cells in (G) cortex and (H) hippocampus at ×100 magnification using a Stereo Investigator (MicroBrightField, U.S.A.) and was presented as a percent of the control group values. (A) control group; (B) LPS group; (C) LPS + NMMA 5 mg/kg group; (D) LPS + NMMA 20 mg/kg group; (E) LPS + 6-shogaol 5 mg/kg group; (F) LPS + 6-shogaol 20 mg/kg group. The data are represented as the mean ± S.E.M. #$p < 0.05$ indicates statistically significant difference between the control and LPS alone-treated groups. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistically significant differences compared with the LPS-only treated group.

3.5. Effect of 6-shogaol on LPS-induced MAPKs activation

We evaluated the effect of 6-shogaol on MAPK signaling which plays an important role in the regulation of inflammatory responses and coordinates the induction of many genes encoding inflammatory mediators (Kaminska, 2005). As shown in Fig. 3C–E, treatment with LPS (100 ng/ml) for 30 min stimulated the phosphorylation of p38, ERK, and JNK in primary microglia cells. The amount of non-phosphorylated p38, ERK, and JNK was unaffected by LPS or 6-shogaol treatment. p38 and MAPK phosphorylation in response to LPS was strongly suppressed by 6-shogaol. Furthermore, 6-shogaol inhibited the activation of JNK, but not that of ERK.

3.6. Effect of 6-shogaol on NO production induced by TLRs agonist in primary microglia cells

We investigated the effect of 6-shogaol on NO production by TLRs agonist in microglia cells. While exposure to PamCSK (a synthetic lipopeptide TLR2 agonist) and poly (I:C) (a double-stranded RNA TLR3 agonist) stimulated NO production in a dose-

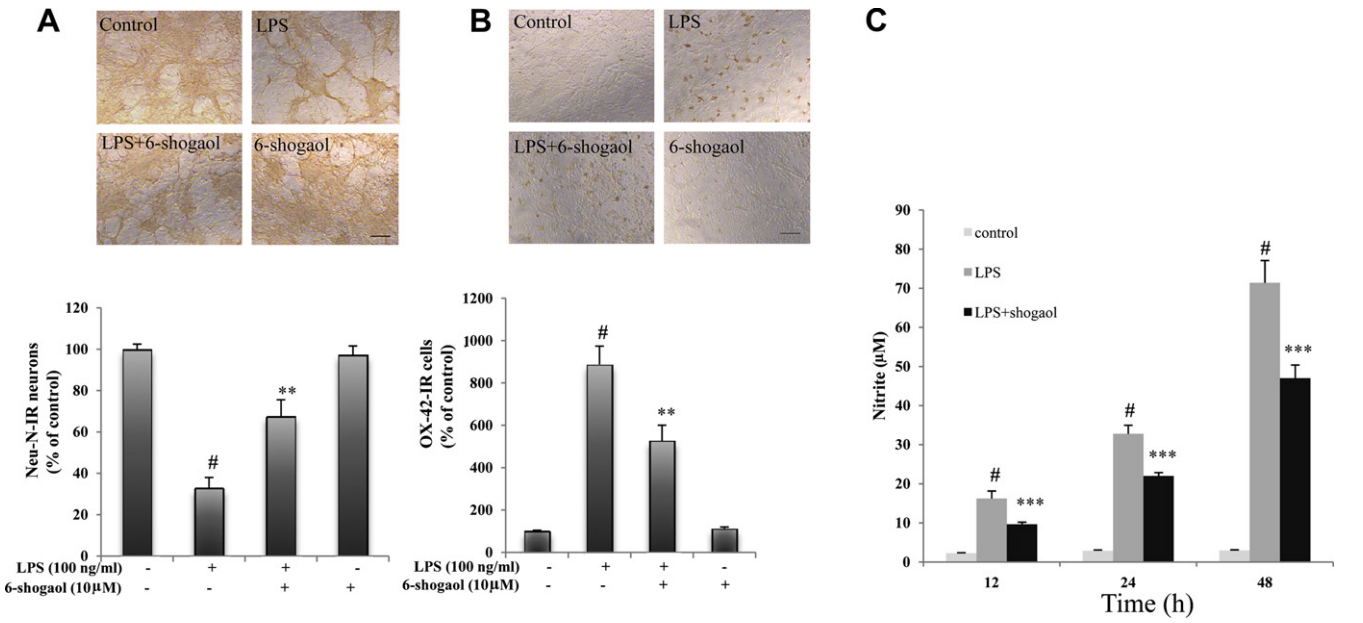

Fig. 6. Effect of 6-shogaol on LPS-induced neurotoxicity and -microglial activation in cortical neuron-glia cultures. (A) Cells were fixed and stained for MAP-2 as described in the methods section. After cell treatment, the number of Neu-N positive cortical neurons was counted. (B) Cells were stained for OX-42 as described in the methods section. Quantification of the microglia cells was performed by counting the number of microglial cells in cortical neuron-glia cultures. (C) Nitrate was measured using a Griess reaction. The data is represented as a mean ± S.E.M of the three independent experiments. The scale bar is 50 μm #$p < 0.05$ indicates statistically significant difference between the control and LPS alone-treated groups. **$p < 0.01$ and ***$p < 0.001$ indicate a statistically significant difference from treatment with LPS alone.

dependent manner (Fig. 4A), pretreatment with 6-shogaol for 30 min inhibited it significantly (Fig. 4B). TLR2 activated only the MyD88-dependent pathway, whereas TLR3 led to activation of the Toll/IL-1R (TIR) domain-containing adaptor inducing IFN-β (TRIF)-dependent pathway and not the MyD88-dependent pathway. These results suggest that 6-shogaol inhibits NO production induced by both the MyD88-dependent and TRIF-dependent pathways in primary microglia cells.

3.7. Effect of 6-shogaol on microglial activation in the neuroinflammatory mouse model

To evaluate the potential anti-inflammatory effect of 6-shogaol *in vivo*, neuroinflammation was induced by systemic administration of LPS (5.0 mg/kg, i.p.). 6-Shogaol was administered orally at 5.0 mg/kg and 20.0 mg/kg once per day for 3 days before LPS treatment (Fig. 5). Pretreatment of mice with 6-shogaol significantly reduced to microglial activation in the brain cortex by 43.9% and 66.8% at 5.0 mg/kg and 20.0 mg/kg, respectively. 6-Shogaol suppressed microglial activation in the hippocampus by 65.9% and 65.5% at 5.0 mg/kg and 20.0 mg/kg, respectively. NMMA, an iNOS inhibitor, was used as the positive control. These results suggest that 6-shogaol can regulate inflammation due to microglial activation *in vivo* as well as *in vitro*.

3.8. Effect of 6-shogaol on LPS-induced neurotoxicity and microglial activation in primary cortical neuron-glia culture

As shown in Fig. 6A, pretreatment with 10 μM 6-shogaol significantly attenuated the LPS-induced reduction in the number of neurons (34.7%). The addition of 6-shogaol alone did not result in any significant changes in the number of Neu-N positive neurons compared to the control group. Also, it is reported that LPS did not cause neuronal cell death in the absence of microglia (Chao et al., 1992; Xie et al., 2004, 2002). These results suggest that 6-shogaol can protect cortical neurons from LPS-induced neurotoxicity.

To examine the effects of 6-shogaol on LPS-induced microglial activation, cortical neuron-glia cultures were pretreated with 6-shogaol followed by treatment with LPS. Pretreatment with 6-shogaol (10 μM) have suppressed LPS-induced microglial activation by 41% (Fig. 6B). The effect of 6-shogaol on LPS-induced NO production was also evaluated. As shown in Fig. 6C, treatment of cortical neuron-glia cultures with 100 ng/ml LPS increased the production of NO markedly. 6-Shogaol inhibited the NO production induced by LPS at 12, 24 and, 48 h time points by 40.6%, 32.9%, and 34.2%, respectively.

3.9. Effect of 6-shogaol on neuronal cell death induced by the ischemic injury mouse model

To determine the neuroprotective effect of 6-shgaol *in vivo*, transient global ischemia was induced in mice by 2VO. Compared with vehicle-treated ischemic animals, oral administration of 6-shogaol at a dose of 10.0 mg/kg showed 30.0% inhibition of CA1 cell death (Fig. 7A and B). Moreover, treatment of 6-shogaol (10.0 mg/kg) suppressed active caspase-3-positive cells in the hippocampal CA1 region after 2VO (Fig. 7C and D).

Based on the anti-inflammatory effects of 6-shgaol *in vitro* and *in vivo*, we hypothesize that its neuroprotective effect is due to inhibition of neuroinflammation. Microglia plays an active role in brain inflammation. Thus, the effect of 6-shogaol on microglial activation was assessed through immunohistochemistry using an antibody against Mac-1. Ischemic injury strongly induced microglial activation, and the number of Mac-1 positive microglia was significantly reduced by 6-shogaol (Fig. 8A and B). 6-Shogaol at 10.0 mg/kg had inhibited microglial activation by 48.0%. Moreover, we found the expression of neuroinflammatory markers including IL-1β (Fig. 8C and D) and TNF-α (Fig. 8E and F) in the hippocampus. These markers were attenuated by 6-shogaol

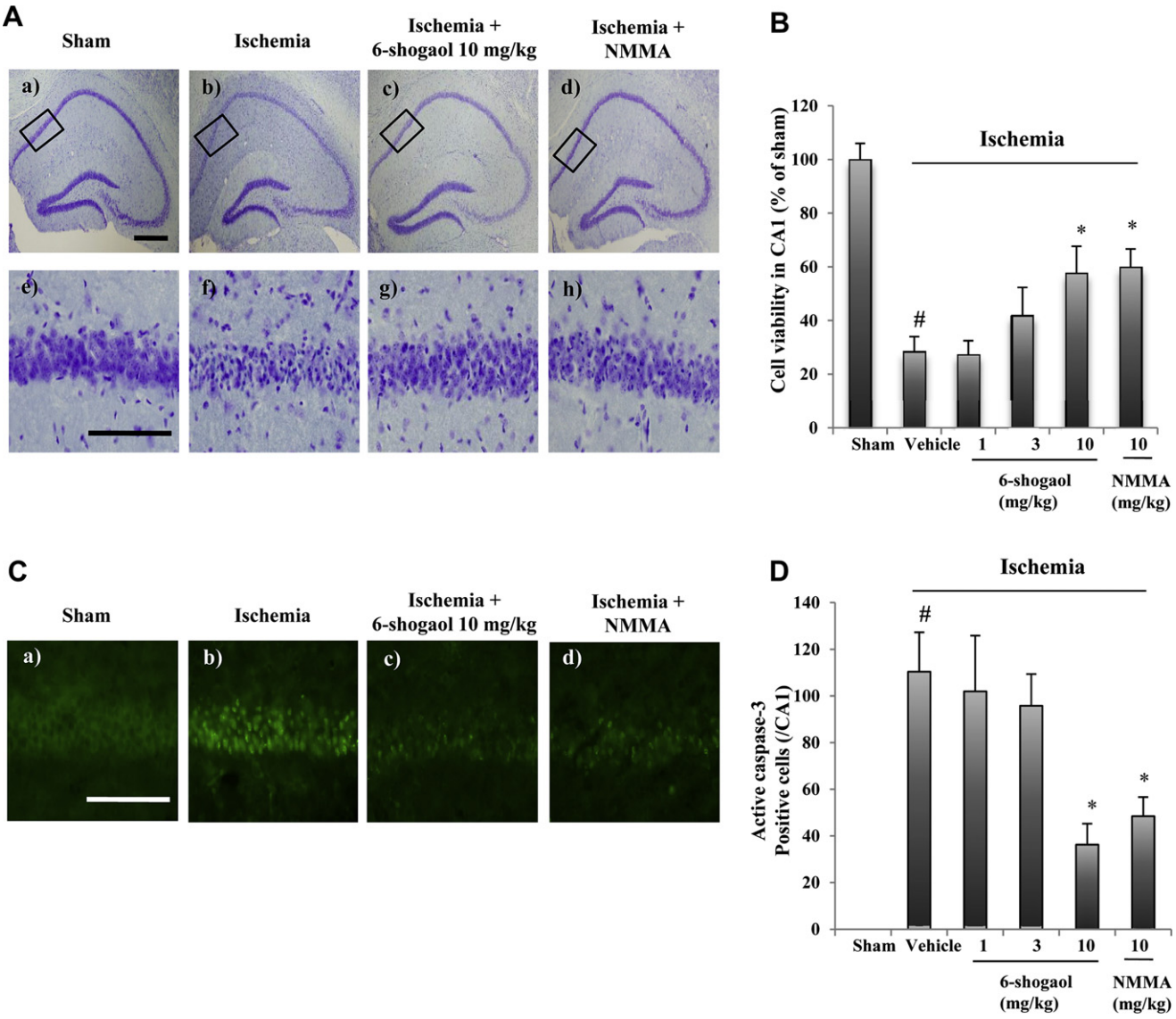

Fig. 7. Effect of 6-shogaol on neuronal cell death induced by ischemic injury. (A) Representative photomicrographs of cresyl violet-stained hippocampal regions of each group. Boxed regions in a, b, c, and d (×100) are shown in e, f, g, and h (×400), respectively. Scale bar in a and e is 200 and 50 μm, respectively. (B) Neuronal cell density in hippocampus was measured by cresyl violet staining. Viable cells in CA1 region of hippocampus were numbered at ×400 magnification. (C) Representative photomicrographs showed cleaved caspase-3-positive cells in CA1 region of hippocampus of each group (×400, Scale bar = 50 μm). (D) Quantification of active caspase-3-positive cells was performed by counting the number of hippocampus at ×400 magnification. The data are represented as the mean ± S.E.M of three independent experiments. #$p < 0.05$ indicates statistically significant difference between the sham-operated and ischemia-induced groups. *$p < 0.05$ indicates statistically significant differences compared to vehicle-treated ischemic group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

treatment, too. These results indicate that the neuroprotective effects of 6-shogaol are likely due to their anti-inflammatory properties.

4. Discussion

This study focused on the anti-inflammatory and neuroprotective effects of 6-shogaol, a main active ingredient of ginger, at the molecular, cellular, and *in vivo* levels. Ginger is used in Asian traditional medicine to treat many inflammatory conditions and associated pain (Grzanna et al., 2005). In 2009, Jung et al. reported that ginger extract inhibited the production of NO and proinflammatory cytokines in LPS-stimulated BV-2 microglial cells via the NF-κB pathway (Jung et al., 2009). 6-Gingerol had an inhibitory effect on the production of proinflammatory cytokines in murine peritoneal macrophages (Tripathi et al., 2007). Furthermore, 6-shogaol has been shown to inhibit LPS-induced iNOS and COX gene expression in macrophages (Pan et al., 2008). Thus, we hypothesized that 6-gingerol and 6-shogaol might regulate neuroinflammation by inhibiting microglial activation in brain. We found that 6-shogaol had anti-inflammatory effects, while 10 μM of 6-gingerol did not show significant inhibitory effects on NO production and iNOS expression in microglia cells. 6-Shogaol did down-regulate the microglial activation both in cortical neuron-glia culture and in a systemic inflammatory model. Moreover, 6-shogaol showed significant neuroprotective effects in the cortical neuron-glia culture, in the ischemia model. It might be due to anti-inflammatory effects via inhibition of microglial activation.

Inflammatory processes in the central nervous system (CNS) are believed to play an important role in neuronal cell death in neurodegenerative diseases. Brain inflammation itself does not

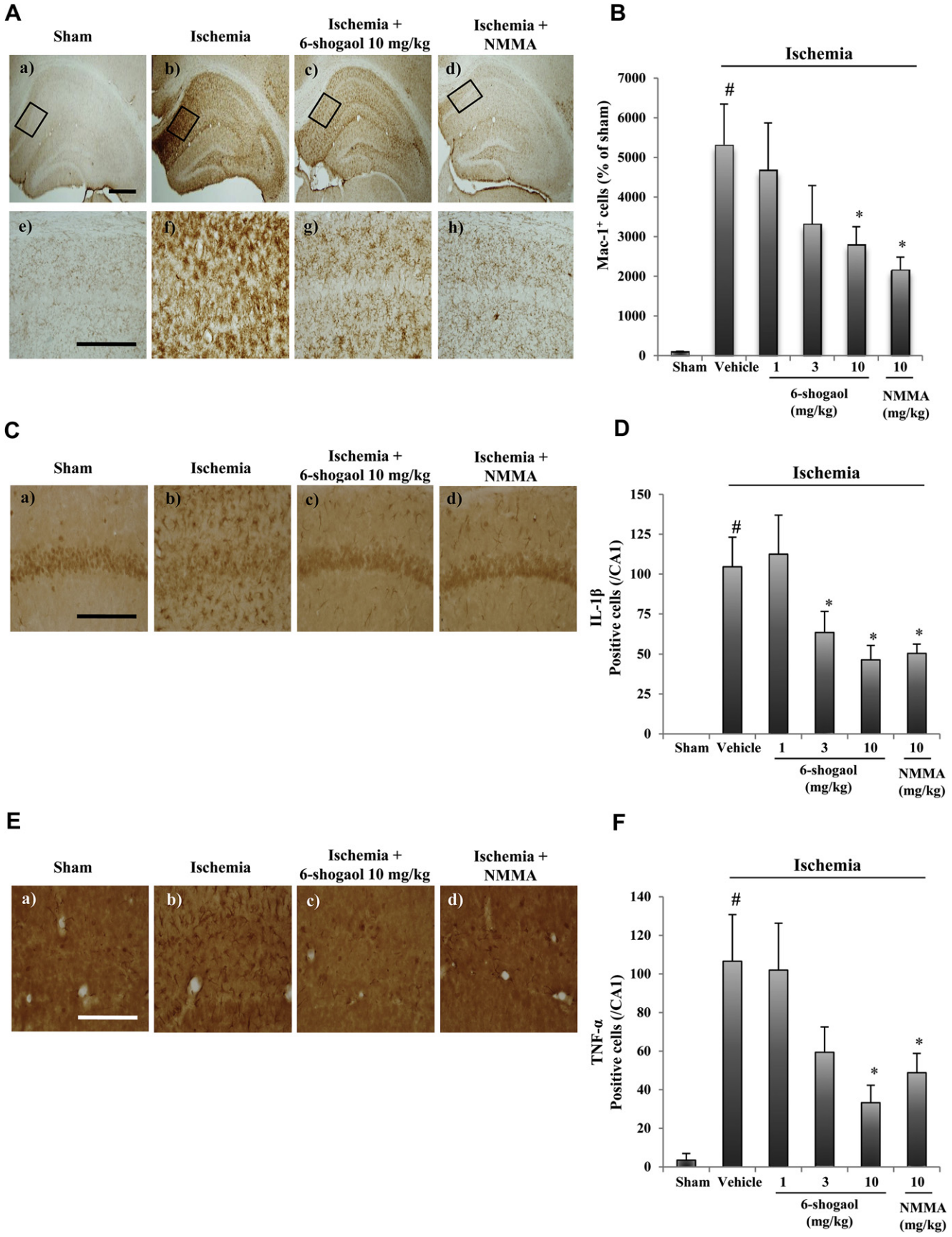

Fig. 8. Effect of 6-shogaol on microglial activation induced by ischemic injury. (A) Representative photomicrographs showed mac-1-IR microglia in hippocampus of each group. Boxed regions in a, b, c, and d (×100) are shown in e, f, g, and h (×400), respectively. Scale bar in a and e is 200 and 50 μm, respectively. (B) Quantification of the microglia cells was performed by counting the number of hippocampus at ×400 magnification. (C and E) Representative photomicrographs showed IL-1β (C) and TNF-α positive cells (E) in CA1 region of hippocampus of each group (×400, Scale bar = 50 μm). (D and F) Quantification of IL-1β (D) and TNF-α positive cells (F) was performed by counting the number of hippocampus at ×400 magnification. The data are represented as the mean ± S.E.M of three independent experiments. #$p < 0.05$ indicates statistically significant difference between the sham-operated and ischemia-induced groups. *$p < 0.05$ indicates statistically significant differences compared to vehicle-treated ischemic group.

cause neuronal cell death. When uncontrolled, however, it may lead to potentially damaging consequences as seen in several inflammatory diseases. Inflammation in the brain is mediated primarily by activated microglia. Chronic neurodegeneration is accompanied by an inflammatory response characterized by the selective activation of the microglial cells in the CNS (Campbell, 2004; Liu and Hong, 2003; McGeer and McGeer, 2004). Activation of microglia has been observed during the development of neurodegenerative diseases such as Alzheimer's and PD (Dickson et al., 1993; McGeer et al., 1988).

Several studies have suggested that inhibition of microglial activation is a promising therapeutic strategy for neurodegenerative diseases. Minocycline, a derivative of tetracycline, has shown neuroprotective effects in several neurodegenerative disease models by inhibiting microglial activation (Fan et al., 2007; Henry et al., 2008; Yrjanheikki et al., 1998, 1999). Recently, natural products such as non-steroidal anti-inflammatory drugs (NSAID) have received significant attention due to their ability to regulate the inflammatory response. A number of studies have provided scientific proof that anti-inflammatory herbal medicine and its constituents are effective at slowing-down the neurodegenerative process. For example, curcumin exerted a neuroprotective effect by reducing microglial activation in neuron-glia cultures (Lee et al., 2007; Yang et al., 2008). In the previous studies in our lab, we showed that apigenin and wogonin suppress activation of microglia which results in neuroprotection (Ha et al., 2008; Lee et al., 2003). These results suggest that regulation of microglial activation is a promising treatment strategy for neurodegenerative diseases. Thus, anti-neuroinflammatory effect of 6-shogaol *in vitro* and *in vivo* was evaluated.

It has been reported that iNOS and COX-2 were highly expressed in CNS-related diseases (Hunot et al., 1996; Teismann et al., 2003), even though iNOS is not expressed in the normal brain. However, inflammatory mediators such as LPS and cytokines induce expression of iNOS in microglia and astrocytes (Murphy, 2000), and possibly in neurons (Heneka and Feinstein, 2001). iNOS continuously produces high levels of NO. Whereas NO at low concentrations functions as a signaling molecule (Murphy, 2000), at high levels it induces neuronal cell death. Neuronal stress, such as ischemia and excitotoxicity, is associated with strong upregulation of neuronal COX-2 expression, which suggests that COX-2 is involved in neurotoxic mechanisms (Planas et al., 1995; Tocco et al., 1997). COX-2, a rate-limiting enzyme for PGE_2 synthesis, is induced during inflammation and participates in inflammation-mediated cytotoxicity. Furthermore, PGE_2 is an important mediator of inflammation. Recently, it has been reported that selective COX-2 inhibitors such as celecoxib and rofecoxib can slow-down the development of some neurological diseases (Aisen et al., 2003; Candelario-Jalil et al., 2003; Klivenyi et al., 2003). In this study, 6-shogaol inhibited the release of NO and PGE_2 by suppressing iNOS and COX-2 protein expression, respectively. This study also shows that 6-shogaol suppresses the activation of NF-κB and MAPK.

Additional experiments were carried out to examine the effect of 6-shogaol on the activation of NF-κB. It has been reported that NF-κB is related to inflammatory responses and other chronic diseases (Karin et al., 2004). The transcription factor NF-κB plays an important role in the production of proinflammatory cytokines and is believed to be a promising target for the treatment of inflammatory diseases. Recently, 6-shogaol was found to inhibit iNOS and COX-2 expression by blocking LPS-induced NF-κB activation in macrophages (Pan et al., 2008). The effect of 6-shogaol on the NF-κB pathway was also evaluated. After activating NF-κB in primary microglia, 6-shogaol reduced activation of NF-κB by blocking phosphorylation of IκB and the subsequent degradation of IκB. Therefore, 6-shogaol may exert anti-neuroinflammatory effects by inhibiting NF-κB in primary microglia.

Upon exposure to LPS, multiple signaling pathways are known to be activated in microglia. Especially, MAPKs are known to play important roles in inflammatory processes. Several studies have shown that MAPKs are required for NF-κB-dependent gene expression (Carter et al., 1999; Meyer et al., 1996). Furthermore, a previous study reported that 6-shogaol inhibited ERK and Akt activation in LPS-stimulated macrophages, but did not affect activation of p38 MAPK (Pan et al., 2008). In the current study, the effects of 6-shogaol on MAPKs (p38, ERK, and JNK) were investigated. Exposure to LPS in primary microglia cells strongly activated all MAPKs. The phosphorylation of p38 and JNK in response to LPS was reduced by 6-shogaol treatment. However, 6-shogaol had no effect on ERK. Our results are consistent with a previous report that p38 MAPK mediated inflammatory responses in microglia (Bhat et al., 1998) and that inhibition of JNK reduced the induction of several genes regulated by AP-1, including COX-2, TNF-α, and IL-6 in LPS-stimulated primary microglia (Waetzig et al., 2005). Our study indicates that 6-shogaol has anti-inflammatory effects through inhibition of p38 MAPK and JNK in microglia cells.

LPS triggers innate immune responses through TLR4, a member of the TLR family that participates in pathogen recognition. Cellular response to LPS occurs through the interaction of LPS with a circulating LPS-binding protein and CD14, and subsequently activates TLR4. LPS-induced dimerization of TLR4 is required for the activation of downstream signaling pathways including NF-κB. Ahn et al. reported 6-shogaol inhibited LPS-induced TLR4 dimerization (Ahn et al., 2009). 6-Shogaol may regulate TLR activity via modulation of receptor dimerization. It can lead to decrease inflammatory gene expression.

Post-ischemic inflammation and the formation of oxygen-derived free radicals are thought to be pivotal for reperfusion-induced delayed neurodegeneration (Giulian and Vaca, 1993; Kitagawa et al., 1990; Yamamoto et al., 1997). Cerebral ischemia evokes secondary inflammation in the brain that contributes to ischemic insults (Barone and Feuerstein, 1999). During the delayed progression of ischemic stroke, post-ischemic inflammation may play an important role in brain damage (Dirnagl et al., 1999). iNOS is in part responsible for ischemic injury (del Zoppo et al., 2000). COX-2 inhibition prevents delayed death of CA1 hippocampal neurons in global ischemia (Nakayama et al., 1998; Nogawa et al., 1997). In the present study, 6-shogaol significantly inhibited the delayed death of CA1 hippocampal neurons and the activation of microglia in transient global ischemia model. Therefore, the anti-inflammatory effects of 6-shogaol may provide neuroprotection against ischemic brain injury.

Ginger is widely used in foods as a spice all over the world and it has been one of the most frequently used medicinal plants, for a wide array of unrelated diseases for a long time. And so numerous research and review articles have reported various pharmacological actions of ginger (Ali et al., 2008). In this study, we discovered the neuroprotective effects of 6-shogaol, one of the major components of ginger, which were mediated by microglia inactivation related with regulations of various pathways. Natural products are still proving to be the source that leads most consistently to successful development of new drugs (Rollinger et al., 2006), and in the future, they will continue to play a major role as active substances, and model molecules for the discovery and validation of drug targets. 6-Shogaol is a promising ingredient for new drugs because of its strengths in the simplicity of structure and richness in resources. Thus, 6-shogaol could be a good candidate for neurodegenerative disease which related with neuroinflammation.

Acknowledgments

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