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Shikonin Isolated from *Lithospermum erythrorhizon* Downregulates Proinflammatory Mediators in Lipopolysaccharide-Stimulated BV2 Microglial Cells by Suppressing Crosstalk between Reactive Oxygen Species and NF-κB

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Abstract

According to the expansion of lifespan, neuronal disorder based on inflammation has been social problem. Therefore, we isolated shikonin from *Lithospermum erythrorhizon* and evaluated anti-inflammatory effects of shikonin in lipopolysaccharide (LSP)-stimulated BV2 microglial cells. Shikonin dose-dependently inhibits the expression of the proinflammatory mediators, nitric oxide (NO), prostaglandin E_2 (PGE_2), and tumor necrosis factor-α (TNF-α) as well as their main regulatory genes and products such as inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and TNF-α in LPS-stimulated BV2 microglial cells. Additionally, shikonin suppressed the LPS-induced DNA-binding activity of nuclear factor-κB (NF-κB) to regulate the key regulatory genes of the proinflammatory mediators, such as *iNOS*, *COX-2*, and *TNF*-α, accompanied with downregulation of reactive oxygen species (ROS) generation. The results indicate that shikonin may downregulate the expression of proinflammatory genes involved in the synthesis of NO, PGE_2, and TNF-α in LPS-treated BV2 microglial cells by suppressing ROS and NF-κB. Taken together, our results revealed that shikonin exerts downregulation of proinflammatory mediators by interference the ROS and NF-κB signaling pathway.

Key Words: Shikonin, Proinflammatory mediators, Reactive oxygen species, Nuclear factor-κB

INTRODUCTION

Microglia are immune-surveillance phagocytic cells in the brain and spinal cord; they constitute approximately 10-20% of the total glial cell population in the brain (Duffield, 2003). In response to inflammatory stimuli, microglia release proinflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), and inflammatory cytokines such as tumor necrosis factor-α (TNF-α) via the activation of nuclear factor (NF)-κB, which normally triggers a protective response in the central nervous system (CNS) to clear pathogens and infected cells (González-Scarano and Baltuch, 1999). However, prolonged activation of microglia enhances excessive release of those proinflammatory mediators, which consequently lead to neuronal inflammation and neuronal cell death (González-Scarano and Baltuch, 1999). It is well known that NO and PGE_2 have beneficial immunomodulatory, microbicidal, antiviral, and antitumor effects; however, aberrant production of NO and PGE_2 leads to inflammatory destruction of target tissues at the inflammation sites (Block and Hong, 2005). Notably, high levels of nitrated proteins *in vivo* are detected in the tissue isolated from patients with neurodegenerative disorders, suggesting that NO is a symptom of neurodegenerative onset and is a good therapeutic target to prevent neuroinflammatory diseases (Zhao, 2005; Doherty, 2011). Emerging evidence also indicates that targeting the PGE_2 signal pathway has potent efficacy in neurological diseases via both immunosuppressive and proinflammatory actions (Cimino *et al*., 2008). Additionally, TNF-α is an important proinflammatory cytokine the involved in increased secretion of other inflammatory cytokines such as interleukin-1, which causes neuronal dysfunction and death (Tracey and Cerami, 1994). As mentioned above, uncontrolled production of these proinflammatory mediators might cause severe neuronal disorders, including Alzheimer's

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disease, Parkinson's disease, and multiple sclerosis (Block *et al*., 2007). Therefore, the downregulation of proinflammatory mediators in microglia is a potential strategy in the treatment of severe neuronal inflammatory diseases.

Excessive release of proinflammatory mediators might initiate neurodegenerative onset via many different signaling pathways. Notably, NF-κB, which is a main transcription factor controlling the immune system, translocates to the nucleus and promotes the expression of proinflammatory genes processing NF-κB-specific binding sites in their promoter regions, such as *inducible NO synthase* (*iNOS*), *cyclooxygenase-2* (*COX-2*), and *TNF-α*, which in turn promote the production of the main proinflammatory mediators, NO, PGE_2, and TNF-α (Surh *et al*., 2001). Therefore, Camandola and Mattson substantially discussed the role of NF-κB in the onset of pathogenesis in several neurodegenerative diseases as a potential pharmaceutical target (Camandola and Mattson, 2007). Alternatively, other research has been trying to find upstream molecules such as reactive oxygen species (ROS), to regulate NF-κB activity (Bonizzi *et al*., 1999); several phytocompounds inhibit the expression of iNOS, COX-2, and TNF-α in microglia by blocking improper NF-κB activation via inhibition of ROS generation (Surh *et al*., 2001; Camandola and Mattson, 2007), but the relevance of ROS in regulation of NF-κB is still controversial (Korn *et al*., 2001).

Shikonin is the main component of the red pigment from *Lithospermum erythrorhizon* occurring naphthoquinone compound. Both shikonin and its analogs are potential pharmaceutical agents with anticancer activities via inhibiting topoisomerase-I, inducing apoptosis, regulating the activities of phosphorylated extracellular regulated protein kinase (pERK) and c-Jun N-terminal kinase (JNK), and suppressing the expression of tumor necrosis factor receptor-associated protein 1 (TRAP1) (Xuan and Hu, 2009; Wu *et al*., 2013; Zhang *et al*., 2013). In the current study, we found that shikonin attenuates the levels of iNOS, COX-2, and TNF-α expression, as well as their corresponding proinflammatory mediators, NO, PGE_2, and TNF-α, *in vitro* in LPS-treated BV2 microglial cells by inhibiting crosstalk between ROS and NF-κB.

MATERIALS AND METHODS

Chemicals

The roots of *L. erythrorhizon* were purchased from an herb market (Jecheon, Republic of Korea). A voucher specimen has been deposited in Division of Wood Chemistry & Microbiology, Department of Forest Products, Korea Forest Research Institute (Seoul, Republic of Korea). The roots (1 kg) were extracted with acetone (4 L) by Ultrasonic (JAC 4020P, Republic of Korea) for 4 h at room temperature and repeated three times. After filtration, the solution was evaporated to remove $CHCl_3$. Purification was carried out on Sephadex LH-20 column (10× 400 mm) eluting with $CHCl_3$/EtOH (2:1, v/v) and separated into four fractions. Subfraction was separated by MPLC (EYERA system) with YMC-GEL ODS-A (S-75 μm, AA12S75, 30×100 mm, Kyoto, Japan) column chromatography and eluted with MeOH/H_2O (7:3, v/v). UV detected at 280 nm to yield shikonin (15 mg). The chemical structures of shikonin (as shown in Fig. 1A) were determined by ^{1}H-NMR and ^{13}C-NMR (Varian Unity-Inova 500 MHz, Palo Alto, CA, USA).

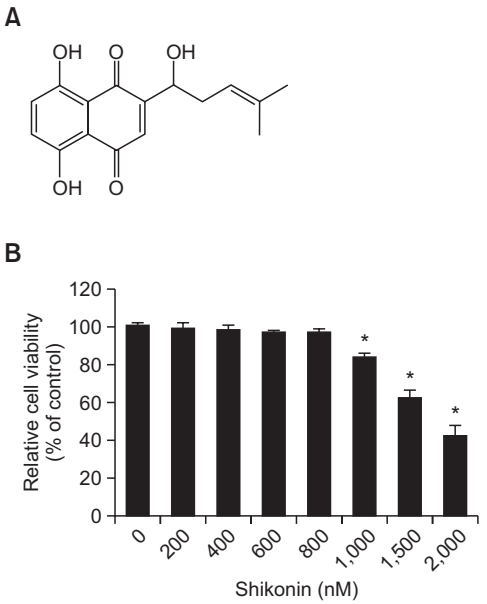

Fig. 1. Effects of shikonin on the viability of BV2 microglial cells. (A) Chemical structure of shikonin isolated from *L. erythrorhizon*. (B) Cells were seeded at 2×10^5 cells/ml and were incubated with the indicated concentrations of shikonin 1 h before treatment with LPS (500 ng/ml) for 24 h. Cell viability was determined by an MTT assay. Data from three independent experiments are expressed as overall mean ± S.E. Statistical significance was determined by one-way ANOVA (*p<0.05 vs. untreated control).

Shikonin

Red purple solid. ESI-MS (positive mode) *m/z*: 311 $[M+Na]^+$, ^{1}H-NMR ($CDCl_3$-d_1): δ 1.66 (3H, *s*, H-6′), 1.76 (3H, *s*, H-5′), 2.37 (1H, *m*, H-2′), 2.66 (1H, *m*, H-2′), 4.92 (1H, *dd*, *J*=4, 7 Hz, H-1′), 5.21 (1H, *t*, *J*=6.9, 14.5 Hz, H-3′), 7.18 (1H, *s*, H-3), 7.2 (2H, *d*, *J*=4. 7 Hz, H-7, H-6), 12.50 (1H, *s*, OH-5), and 12.61 (1H, *s*, OH-8). ^{13}C-NMR ($CDCl_3$-d_1): δ 18.08 (C-6′), 25.96 (C-5′), 35.64 (C-2′), 68.30 (C-1′), 111.51 (C-9), 111.99 (C-10), 118.40 (C-3′), 131.83 (C-3), 132.25 (C-7), 132.39 (C-6), 137.49 (C-4′), 151.43 (C-2), 164.76 (C-8), 165.37 (C-5), 179.91 (C-1), and 180.72 (C-4).

Reagents and antibodies

Rabbit anti-human antibodies against iNOS, COX-2, p50, p65, β-actin, and C23 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from KOMA Biotechnology (Seoul, Republic of Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), LPS, glutathione (GSH), and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma (St. Louis, CA, USA). Proteasome inhibitor (PSI) and pyrrolidine dithiocarbamate (PDTC) were purchased from Calbiochem (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotic mixtures were obtained from WelGENE Inc. (Daegu, Republic of Korea).

Cell culture and viability

BV2 microglial cells were cultured at 37°C in 5% CO_2 in DMEM in supplemented with 5% FBS and antibiotics. MTT assays were used to determine cell viability. Briefly, BV2 mi-

croglial cells (2×10^5 cells/ml) were plated onto 24 well plates and incubated overnight. The cells were treated with the various concentrations of shikonin for 1 h and then stimulated with LPS (500 ng/ml) for 24 h. Then, the cells were incubated with a solution of 0.5 mg/ml MTT and incubation for 45 min at 37°C and 5% CO_2. Supernatant was removed and the formation of formazan was observed by monitoring the signal at 540 nm using a microplate reader (Thermo Electron Corporation, Marietta, OH, USA).

Isolation of total RNA and RT-PCR

Total RNA was extracted using an easy-BLUE kit (iNtRON Biotechnology, Sungnam, Republic of Korea) according to the manufacturer's instruction. One microgram RNA was reverse-transcribed using moloney murine leukemia virus (MMLV) reverse transcriptase (Bioneer, Daejeon, Republic of Korea). cDNA was amplified by PCR using specific primer, *iNOS* (forward 5′-cct cct cca ccc tac caa gt-3′ and reverse 5′-cac cca aag tgc ttc agt ca-3′), *COX-2* (forward 5′-aag act tgc cag gct gaa ct-3′ and reverse 5′-ctt ctg cag tcc agg ttc aa-3′), *TNF-*α (forward 5′-gcg acg tgg aac tgg cag aa-3′ and reverse 5′-tcc atg ccg ttg gcc agg ag-3′), and β-*actin* (forward 5′-tgt gat ggt ggg aatggg tc-3′ and reverse 5′-ttt gat gtc acg cac gat tt-3′). The following PCR conditions were applied: *iNOS*, *COX-2*, and *TNF-*α, 25 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and extended at 72°C for 30 s; β-*actin*, 23 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extended at 72°C for 30 s.

Western blot analysis

Total cell extracts were prepared using PROPREP protein extraction kit (iNtRON Biotechnology). Cytoplasmic and nuclear extracts were prepared using NE-PER nuclear and cytosolic extraction reagents (Pierce, Rockford, IL, USA). Briefly, lysates were centrifuged at 14,000×*g* and 4°C for 10 min to obtain the supernatants. The supernatants were collected and protein concentrations determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The samples were stored at -80°C or immediately used for western blot analysis. The proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Proteins were detected using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA).

NO assay

BV2 microglial cells (2×10^5 cells/ml) were plated onto 24-well plates and pretreated with the indicated concentrations of shikonin 1 h prior to stimulation with 500 ng/ml LPS for 24 h. Supernatants were collected and assayed for NO production using Griess reagent. Briefly, the samples were mixed with equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) and then incubated at room temperature for 10 min. The absorbance was measured at 540 nm on a microplate reader (Thermo Electron Corporation). Sodium nitrite dilution series were used as a standard to determine the nitrite concentration in the supernatants.

Measurement of PGE_2 and TNF-α

The expression levels of PGE_2 and TNF-α were measured by an enzyme immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, BV2 microglial cells (2×10^5 cells/ml) were plated in 24-well plates and pretreated with the indicated concentrations of shikonin 2 h prior to stimulation with 500 ng/ml LPS for 24 h. One hundred microliters of culture medium supernatant was collected for determination of PGE_2 and TNF-α concentration by ELISA.

ROS analysis

BV2 microglial cells were seeded on 24-well plate at a density of 2×10^5 cells/ml and preincubated with florescence dye 6-carboxy-2′,7′-dichlorofluorescein diacetate (H_2DCFDA, Molecular Probes, Eugene, OR, USA) for 1 h and then treated the indicated concentrations of shikonin, NAC, and GSH 1 h before stimulation with LPS (500 ng/ml) for 24 h. The cells were lysed with triton and the sample was centrifuged and supernatant was analyzed for ROS production using GLOMAX luminometer (Promega).

Electrophoretic mobility assay (EMSA)

EMSA was performed with the nuclear extract. Synthetic complementary NF-κB (5′-agt tga ggg gac ttt ccc agg c-3′) binding oligonucleotides (Santa Cruz Biotechnology) were 3′-biotinylated using the biotin 3′-end DNA labeling kit (Pierce) according to the manufacturer's instructions, and annealed for 30 min at room temperature. Assays were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5× Tris borate/EDTA before being transferred onto a positively charged nylon membrane (HybondTM-N^+) in 0.5×Tris borate/EDTA at 100 V for 30 min. The transferred DNAs were cross-linked to the membrane at 120 mJ/cm^2. Horseradish peroxidase-conjugated streptavidin was used according to the manufacturer's instructions to detect the transferred DNA.

Statistical analysis

The images were visualized with Chemi-Smart 2000 (Vilber Lourmat, Marine, Cedex, France). Images were captured using Chemi-Capt (Vilber Lourmat) and transported into Photoshop. Statistical analyses were conducted using SigmaPlot software (version 12.0). Values were presented as mean ± S.E. Significant differences between the groups were determined using the one-way ANOVA. Statistical significance was regarded at $*p<0.05$.

RESULTS

Effect of shikonin on the viability of BV2 microglial cells

To determine the effect of shikonin on cell viability, BV2 microglial cells were treated with the indicated concentrations of shikonin for 24 h in the presence or absence of LPS. Cell viability was determined based on metabolic reduction of a tetrazolium salt to a formazan dye (MTT assay). Shikonin had little influence on cell viability at a dose of ≤800 nM (Fig. 1B). However, a statistically significant level of cytotoxicity was found at concentrations of ≥1000 nM shikonin. Therefore, sub-toxic concentrations (≤800 nM) were used in subsequent experiments.

Effect of shikonin on LPS-induced iNOS expression and NO production

To evaluate the effects of shikonin on NO production in

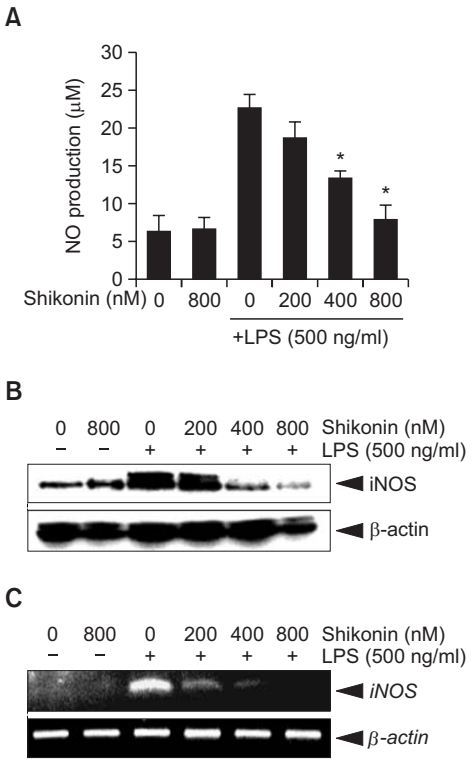

Fig. 2. Effects of shikonin on LPS-induced NO and iNOS expression in BV2 microglial cells. Cells were seeded at a density of 2×10^5 cells/ml and were incubated with the indicated concentrations of shikonin 1 h before treatment with LPS (500 ng/ml) for 24 h (A and B) or 6 h (C). (A) Culture supernatants were analyzed for NO production at 24 h. NO was quantified using Griess reagent, and a standard curve was constructed using $NaNO_2$ as the control. (B) Cell lysates were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed using antibodies against iNOS. (C) After treatment with LPS for 6 h, total cellular RNA was subjected to RT-PCR, and the final PCR product was resolved on 1% agarose gel by electrophoresis. β-Actin was used as an internal control for both western blot analysis and RT-PCR. Each value indicates the mean ± S.E. and is representative of results obtained from three independent experiments. *p<0.05 denotes a significant difference from the value obtained from cells treated with LPS alone.

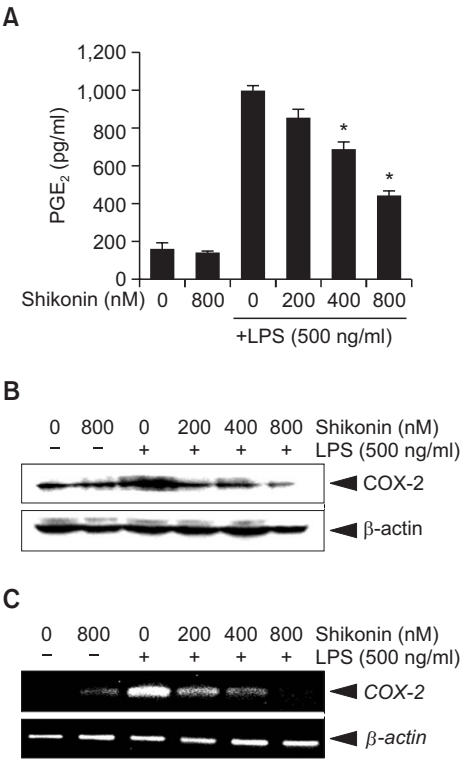

Fig. 3. Effects of shikonin on LPS-induced PGE_2 and COX-2 expression in BV2 microlgial cells. Cells were seeded at 2×10^5 cells/ml and were incubated with the indicated concentrations of shikonin 1 h before treatment with LPS (500 ng/ml) for 24 h (A) and (B) or 6 h (C). (A) Culture supernatants were analyzed for PGE_2 production at 24 h. The levels of PGE_2 in the media were detected using a specific enzyme immunoassay according to the manufacturer's instructions. (B) Cell lysates were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against COX-2. (C) After treatment with LPS for 6 h, total cellular RNA was subjected to RT-PCR and the final PCR product was resolved using 1% agarose gel electrophoresis. The relative amounts of COX-2 protein and mRNA were normalized with β-actin. Each value indicates the mean ± S.E. and is representative of results obtained from three independent experiments. *p<0.05 denotes a significant difference from the value obtained in cells treated with LPS alone.

LPS-treated BV2 microglial cells, we investigated the levels of nitrite released into the culture medium using Griess reagent. The untreated control group and high dosage (800 nM) of shikonin alone released low levels of NO, 6.2 ± 2.3 μM and 6.5 ± 1.8 μM, respectively; however, LPS alone significantly enhanced the levels of NO production (22.6 ± 1.9 μM). LPS-induced NO elevation in the medium decreased with shikonin treatment in a dose-dependent manner, 18.6 ± 2.2 μM, 13.2 ± 1.2 μM, and 7.8 ± 2.1 μM at 200 nM, 400 nM, and 800 nM shikonin, respectively (Fig. 2A). Additionally, western blot analysis also showed a significant increase in the expression of iNOS 24 h after LPS treatment; however, pretreatment with shikonin significantly attenuated LPS-induced iNOS expression (Fig. 2B). To assess whether the downregulation of iNOS protein is regulated at the transcriptional level, we performed RT-PCR. RT-PCR analysis showed that the decreasing pattern of *iNOS* mRNA expression was similar to that seen in iNOS protein expression (Fig. 2C). These data indicate that shikonin suppresses LPS-stimulated NO production by inhibiting *iNOS* expression at the transcriptional level.

Effect of shikonin on LPS-induced COX-2 expression and PGE_2 production

To examine the effect of shikonin on PGE_2 release, PGE_2 levels in the culture medium were measured by ELISA at 24 h. Compared with the untreated group (151 ± 41 pg/ml), LPS-stimulated BV2 microglial cells showed significantly increased PGE_2 release (987 ± 34 pg/ml). In contrast, pretreatment with shikonin substantially reduced the LPS-induced PGE_2 release in a dose-dependent manner; PGE_2 concentrations were 846 ± 52 pg/ml, 678 ± 48 pg/ml, and 435 ± 32 pg/ml at 200 nM, 400 nM, and 800 nM, respectively (Fig. 3A). We next determined whether the inhibitory effect of shikonin on PGE_2 production was caused by a decrease in the expression of COX-2 pro-

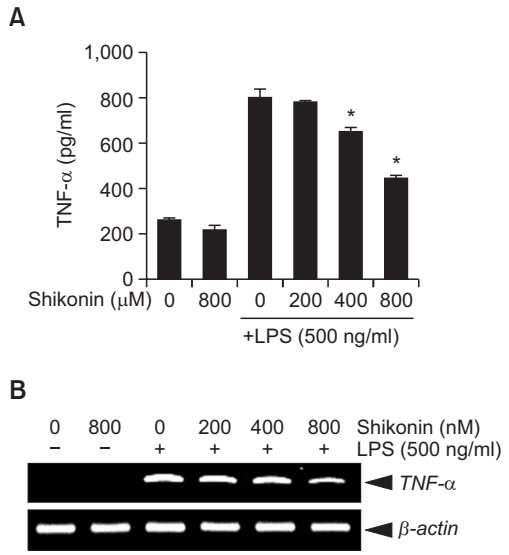

Fig. 4. Effects of shikonin on LPS-induced TNF-α production and mRNA expression in BV2 microglial cells. Cells were seeded at a density of 2×10^5 cells/ml and were incubated with the indicated concentrations of shikonin 1 h before treatment with LPS (500 ng/ml). (A) After incubation for 24 h, the culture supernatants were collected, and the amount of TNF-α was measured by ELISA. (B) After treatment with LPS for 6 h, the level of *TNF-α* mRNA expression was determined by RT-PCR analysis. β-Actin was used as an internal control for RT-PCR. Each value indicates the mean ± S.E. and is representative of results obtained from three independent experiments. **p*<0.05 denotes a significant difference from the value obtained from cells treated with LPS alone.

tein. To investigate whether shikonin regulates the expression levels of COX-2 protein and mRNA, we treated cells with the indicated concentrations of shikonin in the presence of LPS; COX-2 protein and mRNA expression levels were determined by western blot analysis and RT-PCR, respectively. Western blot analysis showed that COX-2 protein expression was weak in untreated cells and that LPS remarkably increased expression of COX-2 protein; however, LPS-induced COX-2 protein was downregulated in a dose-dependent manner after pretreatment with shikonin (Fig. 3B). Additionally, RT-PCR analysis showed a decrease in *COX-2* mRNA expression, similar to the changes observed for protein expression in the presence of shikonin (Fig. 3C). These results indicate that shikonin attenuates LPS-induced PGE_2 release via suppression of *COX-2* expression at the transcriptional level.

Effect of shikonin on LPS-induced TNF-α production and mRNA expression

We investigated the potential effect of shikonin on the production of proinflammatory cytokine TNF-α in LPS-stimulated BV2 microglial cells. TNF-α was weakly expressed (224 ± 32 pg/ml) in the untreated control group; however, LPS stimulation remarkably increased TNF-α release (813 ± 63 pg/ml) at 24 h. Pretreatment with shikonin dose-dependently prevented LPS-induced TNF-α release (789 ± 14 pg/ml, 678 ± 24 pg/ml, and 468 ± 21 pg/ml at 200 nM, 400 nM, and 800 nM, respectively) (Fig. 4A). To review whether the shikonin-induced inhibitory effect on TNF-α release was due to modulation of *TNF-α* gene expression, we performed RT-PCR analysis 6 h after LPS treatment. As seen from RT-PCR data, shikonin reduced the expression of *TNF-α* mRNA in LPS-stimulated BV2 microglial cells in a dose-dependent manner (Fig. 4B). These data indicate that shikonin regulates LPS-stimulated TNF-α release at the transcriptional level.

Effect of shikonin on LPS-induced ROS generation

Since LPS-induced intracellular ROS generation triggers many cellular signaling pathways related to neuroinflammation, the regulation of ROS is a key therapeutic strategy in LPS-induced neuroinflammation. Therefore, we examined the effect of shikonin on ROS generation in LPS-stimulated BV2 microglial cells. Fluorometric data showed that treatment with shikonin significantly decreased the LPS-induced high level of ROS generation (Fig. 5A). In order to confirm whether ROS inhibition regulates proinflammatory genes, such as *iNOS*, *COX-2*, and *TNF-α*, by suppressing NF-κB activity, we monitored ROS generation in the presence of ROS inhibitors. Presumably, pretreatment with ROS inhibitors, NAC and GSH, substantially downregulated the level of ROS generation (Fig. 5B). Furthermore, we conducted RT-PCR to evaluate mRNA expression of *iNOS, COX-2*, and *TNF-α* in the presence of ROS inhibitors, NAC and GSH. These inhibitors significantly decreased the LPS-induced expression of *iNOS, COX-2*, and *TNF-α* at 6 h (Fig. 5C). In order to evaluate the effect of ROS inhibitors on LPS-induced DNA-binding activity of NF-κB, EMSA was performed. EMSA data showed that treatment with LPS resulted in significantly increased DNA-binding activity of NF-κB as shown previously, while NAC and GSH potently affected downregulation of LPS-stimulated NF-κB activity (Fig. 5D). These data indicate that ROS is the first target molecule to regulate NF-κB activity in shikonin-mediated anti-inflammatory action in BV2 microglial cells.

Effect of shikonin on LPS-induced NF-κB activity

NF-κB is an important factor in transcriptional regulation of proinflammatory genes such as *iNOS, COX-2*, and *TNF-α*. To determine whether shikonin inhibits the expression of *iNOS*, *COX-2*, and *TNF-α* by suppressing DNA-binding activity of NF-κB, an EMSA was conducted. Stimulation with LPS induced a significant increase in the DNA-binding activity of NF-κB at 30 min; however, pretreatment with shikonin strongly reduced the LPS-induced binding capacity of NF-κB (Fig. 6A). In addition, because the nuclear translocation of the NF-κB subunits p65 and p50 is essential for NF-κB activation, we investigated the inhibitory effects of shikonin on the LPS-induced nuclear translocation of NF-κB subunits. As seen in western blot analysis, shikonin attenuated the LPS-induced nuclear translocation of the NF-κB subunits, p65 and p50, and sustained their expression in the cytosol, suggesting that shikonin inhibits the DNA-binding activity of NF-κB by suppressing the nuclear translocation of p65 and p50 (Fig. 6B). Additionally, we found that shikonin decreases the phosphorylation of IκBα (Fig. 6B). Next, to investigate the functional effect of NF-κB activity, we used RT-PCR to evaluate the expression of *iNOS*, *COX-2*, and *TNF-α* mRNA in response to proteasome-mediated NF-κB inhibitors, PSI and PDTC. Consistent with the data from the shikonin treatment, PSI and PDTC significantly decreased the LPS-induced expression of *iNOS*, *COX-2*, and *TNF-α* (Fig. 6C). Further, fluorometric data showed that treatment with PSI and PDTC substantially downregulated the level of ROS generation (Fig. 6D). Taken together, these results indicate that

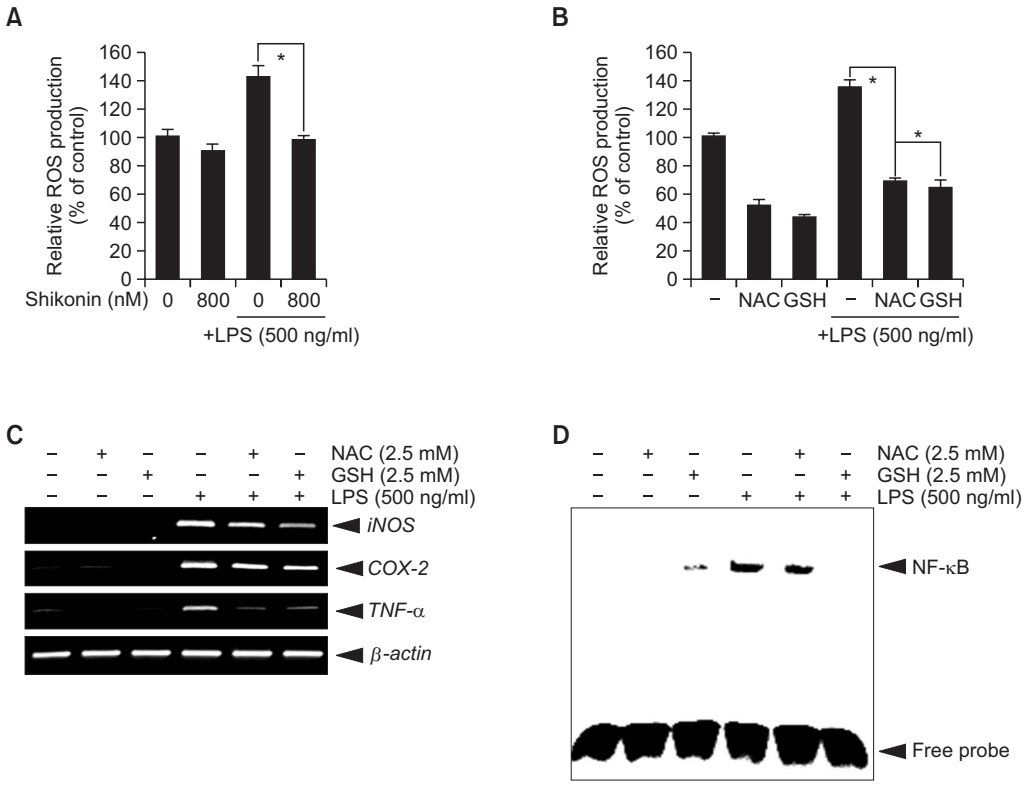

Fig. 5. Effects of shikonin on the generation of ROS in LPS-simulated BV2 microglial cells. Cells were pretreated with DCFDA (20 μM) for 1 h and then were incubated with shikonin (800 nM), NAC (2.5 mM), and GSH (2.5 mM) 1 h before stimulation with LPS (500 ng/ml). (A and B) Cells were lysed with Triton-X 100 and were centrifuged at 16,000 rpm for 10 min. Then, cell supernatant was collected. Generation of ROS was analyzed by fluorometry, and the relative ROS generation is presented. (C) In a parallel experiment, total RNA was isolated at 6 h, and RT-PCR analysis of *iNOS, COX-2* and *TNF-α* was performed. β-Actin was used as an internal control for RT-PCR. (D) The cells were incubated with NAC and GSH 1 h before LPS (500 ng/ml) treatment for 30 min. Nuclear extracts were prepared and were analyzed for DNA-binding activity of NF-κB using EMSA. Data from three independent experiments are expressed as overall mean ± S.E. Statistical significance was determined by one-way ANOVA (**p*<0.05 vs. LPS-treated group).

shikonin suppresses the expression of *iNOS*, *COX-2*, and *TNF-α* by inhibiting NF-κB activity, following suppression of ROS generation, in LPS-stimulated BV2 microglial cells.

DISCUSSION

Previous studies confirmed that shikonin inhibits tumorigenesis and proliferation of various cancer cells by suppressing the NF-κB signaling pathway (Xuan and Hu, 2009; Wu *et al*. 2013; Zhang *et al*., 2013) and that an oxidative mechanism of shikonin is required for the induction of cell death (Huang *et al*., 2014; Yang *et al*., 2014b). Nevertheless, it is not known whether NF-κB activity is regulated by oxidative stress mechanisms of shikonin in inflammatory responses. Recently, noteworthy results were published on the anti-inflammatory effects of shikonin in various LPS-treated animal models of asthma, lung injury, and ischemic stroke (Lee *et al*., 2010; Liang *et al*., 2013; Wang *et al*., 2014); however, little is known about the molecular mechanism of the anti-inflammatory effects of shikonin. Therefore, in the present study, we report that shikonin attenuates the production of NO, PGE_2, and TNF-α, as well as the expression of their respective regulatory genes *iNOS*, *COX-2*, and *TNF-α*, by suppressing crosstalk between NF-κB activity and ROS generation. Nevertheless, to confirm the possibility of shikonin being used to treat inflammatory disease, further study is needed to determine whether shikonin possesses anti-inflammatory effects *in vivo*.

In response to oxidative and proinflammatory stimuli, improper upregulation of iNOS and COX-2 has been associated with the pathophysiology of certain types of inflammatory disorders (Block and Hong, 2005). Because high output of NO induced by iNOS exacerbates deleterious effects, such as inflammatory injury, modulation of iNOS-mediated NO release is a major contributing factor during the inflammatory process (Nathan and Xie, 1994; MacMicking *et al*., 1997). Prostaglandins also play a major role as mediators of the inflammatory response and are synthesized by COXs. In particular, inducible COX-2 is responsible for the production of large amounts of proinflammatory PGE_2 during inflammation (Lipsky, 1999). Interestingly, COX-2-derived PGE_2 may play a pivotal role in the pathogenesis of many inflammatory diseases and chronic inflammation (Höcherl *et al*., 2002). Since the upregulation of iNOS or COX-2 has been observed during various inflammatory diseases, such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Block *et al*., 2007), inhibition of these processes presents unique opportunities for the chemoprevention of neuroinflammation (Murakami and Ohigashi,

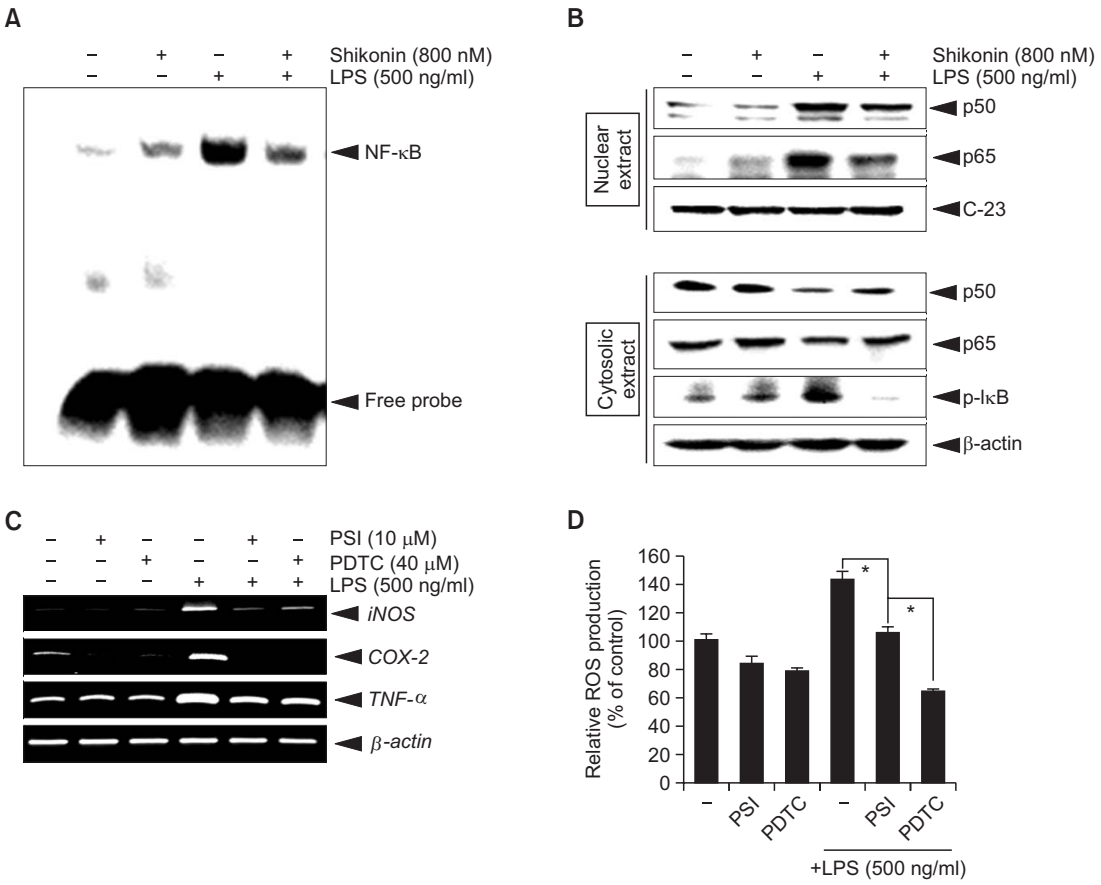

Fig. 6. Effects of shikonin on LPS-induced NF-κB activity. (A) BV2 microglial cells were preincubated with 800 nM shikonin 1 h before stimulation with 500 ng/ml LPS for 30 min and then the nuclear extracts were assayed for NF-κB activity by EMSA. (B) The levels of p50, p65, and p-IκBα in the nuclear and cytosolic compartments were assessed by western blot analysis at 30 min. (C) Cells were incubated with 10 μM PSI and 40 μM PDTC 1 h before treatment with 500 ng/ml LPS for 6 h; then, total RNA was isolated and RT-PCR analyses of *iNOS*, *COX-2*, and *TNF-α* expression were performed. (D) Cells were pretreated with DCFDA (20 μM) for 1 h and then were incubated with 10 μM PSI and 40 μM PDTC 1 h before stimulation with LPS (500 ng/ml). Cells were lysed with Triton-X 100 and were centrifuged at 16,000 rpm for 10 min. Then, cell supernatant was collected. Generation of ROS was analyzed by fluorometry, and the relative ROS generation is presented. **p*<0.05 vs. untreated control.

2007). Thus, an approach that focuses on the inhibition of NO and PGE_2 overproduction by restricting iNOS and COX-2 expression could have potential for the development of anti-neuroinflammatory drugs. Because TNF-α is major inflammatory cytokine involved in inflammation, immunity, and both tissue destruction and recovery, anti-TNF-α therapy has recently been broadly introduced to treat various inflammatory diseases (Silva *et al*., 2010). Here, we found that shikonin attenuates LPS-induced NO, PGE_2, and TNF-α production in BV2 microglia cells, suggesting that shikonin may be a nutraceutical agent to regulate LPS-mediated BV2 agitation. Besides our data, the data from many other studies have proved the anti-inflammatory effects of shikonin via suppression of T cells and dendritic cells (Lee *et al*., 2010; Li *et al*., 2013), enhancement of TGF-β (Andújar *et al*., 2013), and downregulation of high mobility group box 1 involved in nucleosome stabilization (Yang *et al*., 2014b). These accumulated data significantly support therapeutic effects of shikonin in various inflammatory responses.

NF-κB plays a key role in regulating immune responses; however, aberrant or incorrect regulation of NF-κB has been linked to improper neuroinflammation by upregulating release of proinflammatory mediators (Maqbool *et al*., 2013) because NF-κB-binding sequences have been identified in proinflammatory genes such as *iNOS*, *COX-2*, and *TNF-α* (Kempe *et al*., 2005). Therefore, NF-κB has been considered as a therapeutic target in neuroinflammation (Camandola and Mattson, 2007), and many researchers have attempted to develop nutraceutical compounds that suppress NF-κB activity to attenuate excessively activated microglial cells (Surh *et al*., 2001). It is well-known that shikonin inhibits NF-κB activity in various experimental models (Lee *et al*., 2010; Liang *et al*., 2013; Wang *et al*., 2014), and our data also showed that shikonin attenuates LPS-induced proinflammatory mediators by suppressing NF-κB activity, which indicate that shikonin may be a good compound in LPS-triggered inflammation. In addition to NF-κB, LPS-induced inflammation is mediated by various transcription factors, including signal transducer and activator of transcription-2, activation protein-1, and cyclic AMP-responsive element-binding protein, which may regulate the expression of proinflammatory genes such as *iNOS*, *COX-2*, and *TNF-α* (Cho *et al*., 2003; Lee *et al*., 2003). Therefore, fur-

ther studies are needed to evaluate the relationships among the various signaling pathways and the transcription factors involved in the shikonin-induced inhibition of proinflammatory gene expression.

ROS is an essential messenger to stimulate NF-κB activity in switching extracellular stimuli to the intracellular signaling pathway (Siomek, 2012). Especially, some researchers confirmed the importance of NF-κB-mediated *iNOS* mRNA induction in microglial cells by generating ROS, suggesting that ROS contribute to neurodegeneration via NF-κB-mediated microglial activation (Qin and Crews, 2012; Kim *et al*., 2013). In previous studies, high dosage of shikonin considerably triggered ROS generation to induce death of cancer cells by activating caspase-dependent mechanisms, implying that the oxidative function of shikonin also is essential to trigger cancer cell death (Huang *et al*., 2014; Yang *et al*., 2014a); in contrast, low or sub-toxic dosages of shikonin attenuated NF-κB activity, which may be the anti-oxidant effect of shikonin (Liang *et al*. 2013; Wang *et al*. 2014). This discrepancy could be explained by the dose-dependent, dual role of shikonin, similar to the results of a previous study on the dual effects of curcumin (Kawanishi *et al*., 2013; Zikaki *et al*., 2014). In the present study, our data illustrate that shikonin diminishes ROS generation and, consequently, inhibits NF-κB activity. Nevertheless, the inhibition of NF-κB, which has been regarded as a downstream molecule of ROS generation, reciprocally controls ROS generation. Although many previous data showed that ROS is an upstream molecule to activate NF-κB in the inflammatory responses (Qin and Crews, 2012; Siomek, 2012; Kim *et al*., 2013), each inhibitor of ROS and NF-κB in this study mutually interferences the signaling pathway in downregulating the expression of proinflammatory genes. These data confirm that the inhibition of crosstalk between NF-κB and ROS generation modulates shikonin-mediated downregulation of proinflammatory mediators in microglial cells.

In summary, this study demonstrated that shikonin possesses anti-inflammatory activity, which involved the downregulation of NO, PGE_2, and TNF-α, as well as their major regulatory genes, via the suppression of crosstalk between LPS-induced NF-κB activation and ROS production.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests to disclose.

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