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 tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) as well as their main regulatory genes and products such as inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and TNF-\(\alpha\) in LPS-stimulated BV2 microglial cells. Additionally, microglia release proinflammatory mediators such as nitric oxide (NO), prostaglandin E\(_2\) (PGE\(_2\)), and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) via the activation of nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) to regulate the key regulatory genes of the proinflammatory mediators, such as iNOS, COX-2, and TNF-\(\alpha\), accompanied with downregulation of reactive oxygen species (ROS) generation. The results indicate that microglia may downregulate the expression of proinflammatory genes involved in the synthesis of NO, PGE\(_2\), and TNF-\(\alpha\) in LPS-treated BV2 microglial cells by suppressing ROS and NF-\(\kappa\)B. Taken together, our results revealed that microglia exerts downregulation of proinflammatory mediators by interference the ROS and NF-\(\kappa\)B signaling pathway.

Key Words: Shikonin, Proinflammatory mediators, Reactive oxygen species, Nuclear factor-\(\kappa\)B

Shikonin Isolated from Lithospermum erythrorhizon Downregulates Proinflammatory Mediators in Lipopolysaccharide-Stimulated BV2 Microglial Cells by Suppressing Crosstalk between Reactive Oxygen Species and NF-\(\kappa\)B

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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-\(\alpha\) (TNF-\(\alpha\)) as well as their main regulatory genes and products such as inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and TNF-\(\alpha\) in LPS-stimulated BV2 microglial cells. Additionally, shikonin suppressed the LPS-induced DNA-binding activity of nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) to regulate the key regulatory genes of the proinflammatory mediators, such as iNOS, COX-2, and TNF-\(\alpha\), 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-\(\alpha\) in LPS-treated BV2 microglial cells by suppressing ROS and NF-\(\kappa\)B. Taken together, our results revealed that shikonin exerts downregulation of proinflammatory mediators by interference the ROS and NF-\(\kappa\)B signaling pathway.
After filtration, the solution was evaporated to remove CHCl₃. The roots (1 kg) were extracted with 70% MeOH/H₂O (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 ¹H-NMR and ¹³C-NMR (Varian Unity-Inova 500 MHz, Palo Alto, CA, USA).

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 Ultrasound (JAC 4020P, Republic of Korea) for 4 h at room temperature and repeated three times. After filtration, the solution was evaporated to remove CHCl₃. Purification was carried out on Sephadex LH-20 column (10× 400 mm) eluting with CHCl₃/EtOH (2:1, v/v) and separated into four fractions. Subfraction was separated by MPLC (EYERA system) with YMC-GEL ODS-A (S-75 m, AA12S75, 30×100 mm, Kyoto, Japan) column chromatography and eluted with MeOH/H₂O (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 ¹H-NMR and ¹³C-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 2×10⁵ 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]*, ¹H-NMR (CDCl₃-d₆): 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). ¹³C-NMR (CDCl₃-d₆): 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-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 Biotech (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 pyrroldine 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% CO₂ in DMEM in supplemented with 5% FBS and antibiotics. MTT assays were used to determine cell viability. Briefly, BV2 mi-
croglial cells (2×10^6 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% CO₂. 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 instructions. 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 cca ccc tac cca gt-3′ and reverse 5′-cac cca aag tgt ctc agt ca-3′), COX-2 (forward 5′-aag act tgc cag gct gaa ct-3′ and reverse 5′-cct ctg cag tcc agg ttc aa-3′), TNF-α (forward 5′-gcc agc tgt aag cgg cag-3′ and reverse 5′-ccc atg gtc agc gac gtt tgt gat gat ggt ggg gat-3′), and β-actin (forward 5′-tct ggt ggt ctt ctc t-3′ and reverse 5′-ttt gat gtc acg cac gat gtt-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 cytoplasmic 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 (2×10^6 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 PGE₂ and TNF-α
The expression levels of PGE₂ and TNF-α were measured by an enzyme immunoassortent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Briefly, BV2 microglial cells (2×10^6 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 PGE₂ 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 (H₂DCFDA, Molecular Probes, Eugene, OR, USA) for 1 h and then 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 (Hybond-TM-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/cm². 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
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-
mRNA expression of shikonin attenuates LPS-induced PGE2 release via suppression of its presence in shikonin (Fig. 3C). These results 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, 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 ROStreated BV2 microglial cells. 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 ROS-mediated 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. Consistent with the data from the EMSA, shikonin attenuated LPS-stimulated NF-κB activity at 24 h (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 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 decreased the LPS-induced binding capacity of NF-κB (Fig. 6A). In addition, because the LPS-induced transcriptional activity of 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 p65 and p50. As seen in western blot analysis, shikonin attenuated the LPS-induced nuclear translocation of 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...
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, PGE₂, 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 PGE₂ during inflammation (Lipsky, 1999). Interestingly, COX-2-derived PGE₂ 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, 2010).
Thus, an approach that focuses on the inhibition of NO and PGE₂ overproduction by restricting iNOS and COX-2 expression could have potential for the development of anti-inflammatory 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, PGE₂, 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 down-regulation of NO, PGE₂, 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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