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# Shikonin derivatives inhibited LPS-induced NOS in RAW 264.7 cells via downregulation of MAPK/NF-κB signaling

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ABSTRACT

*Aim of the study:* Shikonin/alkannin (SA) derivatives, analogs of naphthoquinone pigments, are the major components of root extracts of the Chinese medicinal herb (*Lithospermum erythrorhizon*; LE) and widely distributed in several folk medicines.

In the present study, the effect and the underline molecular mechanism of shikonin derivatives isolated from root extracts of *Lithospermum euchroma* on lipopolysaccharide (LPS)-induced inflammatory response were investigated.

*Materials and methods:* Effects of five SA derivatives, including SA, acetylshikonin, β,β-dimethylacrylshikonin, 5,8-dihydroxy-1.4-naphthoquinone, and 1,4-naphthoquinone on LPS-induced nitric oxide (NO) and prostaglandin E2 (PGE2) production in mouse macrophage RAW264.7 cells were examined.

*Results:* Data suggested that SA derivatives inhibited LPS-induced NO and $PGE_2$ production, and iNOS protein expression. RT-PCR analysis showed that SA derivatives diminished LPS-induced iNOS mRNA expression. Moreover, the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 in LPS-stimulated RAW 264.7 cells was concentration-dependently suppressed by SA derivatives. SA inhibited NF-κB activation by prevention of the degradation of inhibitory factor-κB and p65 level in nuclear fractions induced by LPS.

*Conclusions:* Taken together, these results suggest that the anti-inflammatory properties of SA derivatives might result from inhibition of iNOS protein expression through the downregulation of NF-κB activation via suppression of phosphorylation of ERK, in LPS-stimulated RAW 264.7 cells.



## 1. Introduction

Shikonin/alkannin (SA) derivatives, analogs of naphthoquinone pigments, the major components of root extracts of the Chinese medicinal herb (*Lithospermum erythrorhizon*; LE), are ingredients of several folk medicines. Treatment indications claimed for LE roots include burns, anal ulcers, hemorrhoids, infected crusts, bedsores, external wounds, and oozing dermatitis (Papageorgiou et al., 1999). Shikonin was first isolated as its acetate form from the roots of LE by the Japanese chemists Majima and Kuroda (1922), after several decades, such compounds have also been shown to exhibit significant antitumor, antibacterial, anti-HIV-1 (Ueba et al., 1993; Chen et al., 2003), anti-DNA topoisomerase, antiangiogenesis (Hisa et al., 1998), and anti-inflammatory activities (Papageorgiou et al., 1999). Studies suggest that the anti-inflammatory effects of shikonin derivatives may be attributable to several mechanisms of action, e.g., suppression of mast cell degranulation and protection of the vasculature from mediator challenge (Wang et al., 1995), inhibition of phosphatidylinositol signaling in neutrophils (Wang and Kuo, 1997), selective blocking of chemokine binding to CC chemokine receptor-1 (Chen et al., 2001), antioxidant and free radical-scavenging activities *in vivo* by interference with free radical processes (Kourounakis et al., 2002), and inhibition of phorbol 12-myristate 13-acetate-induced cyclooxygenase (COX)-2 expression (Subbaramaiah et al., 2001). Staniforth et al. (2004) reported that shikonin inhibits the transcriptional activity of the human tumor necrosis factor (TNF)-α promoter through inter-

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ference with the basal transcription machinery, suggesting that shikonin may have clinical potential as an anti-inflammatory therapeutic (Staniforth et al., 2004). Although a broad range of biological and pharmacological activities of shikonin have been reported, the exact mechanism of the inflammatory action of SA derivatives has not been fully elucidated.

The inflammatory process involves activation of monocytes and/or macrophages; among these, macrophage activation is crucial for the progression of multiple inflammatory diseases via the release of inflammatory mediators, such as cytokines, nitric oxide (NO), and prostaglandins (PGs) (Liang et al., 1999). Physiologically, over-induction of NO leads to various harmful responses including tissue injury, septic shock (Petros et al., 1991; Xie et al., 1992), apoptosis, and necrosis (Nagai et al., 2003). In some cases, the induction of NO by stimulating macrophages leads to organ destruction in some inflammatory (Heiss et al., 2001) and autoimmune diseases (Kleemann et al., 1993). Thus, the inhibition of NO production by blocking iNOS expression may be a useful strategy for treating various inflammatory diseases.

In addition to NO, macrophages also release prostaglandins, which are COX products. COX is an enzyme that converts arachidonic acid to PGs, and is found as two isoforms (COX-1 and -2). COX-1 is constitutively expressed while COX-2 is barely detectable under normal physiological conditions, and can be induced by proinflammatory cytokines and mediators by the same stimulus as iNOS (Chi et al., 2003; Aktan, 2004). NF-κB is one of the most ubiquitous transcription factors, regulates the expressions of genes involved in cellular proliferation, inflammatory responses, and cell adhesion, and is activated in response to various extracellular stimuli, including interferon γ (INF)-γ, LPS, and oxidative stress. NF-κB sites have been identified in the promoter region of the iNOS gene (Xie et al., 1993; Eberhardt et al., 1996) and COX-2 gene (Appleby et al., 1994).

LPS is a major inflammatory molecule that triggers the production of proinflammatory toxins, cytokines such as iNOS, COX-2, TNF-α, and IL-1β in various cell types (MacMicking et al., 1997). In the present study we demonstrate that SA derivatives (Fig. 1) inhibited NO generation in LPS-induced RAW-264.7 macrophages, as well as levels of iNOS messenger (m)RNA and protein. The inhibition of iNOS protein expression appeared to be due to downregulation of NF-κB activation by inhibition of ERK phosphorylation.

## 2. Materials and methods

### 2.1. Chemicals

Fetal bovine serum (FBS) was from Gibco BRL (Grand Island, NY), Dulbecco's modified Eagle's medium (DMEM), and medium supplement for cell culture were obtained from Invitrogen (Carlsbad, CA). LPS was obtained from Sigma (St. Louis, MO). 5,8-Dihydroxy-1,4-naphthoquinone and 1,4-naphthoquinone were purchased from ACROS (New Jersery, USA). The specific antibody for iNOS was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The NF-κB oligonucleotide for the electrophoretic mobility shift assay (EMSA) was synthesized by MDBio (Taipei, Taiwan). Reagents for the gel shift assay system (Promega, Madison, WI) and radioactive [γ-$^{32}$P]-ATP were purchased from Amersham International (Buckinghamshire, UK). All other chemicals were of the highest quality available.

### 2.2. Plant materials

Commercially available dried roots of *Lithospermum euchroma* were purchased from a local Chinese medicine drug store, and its authenticity was confirmed by Prof. Hui-Wen Cheng of Taipei Medical University. A voucher specimen was kept and stored at the Institute of Pharmacy, Taipei Medical University, Taipei, Taiwan.

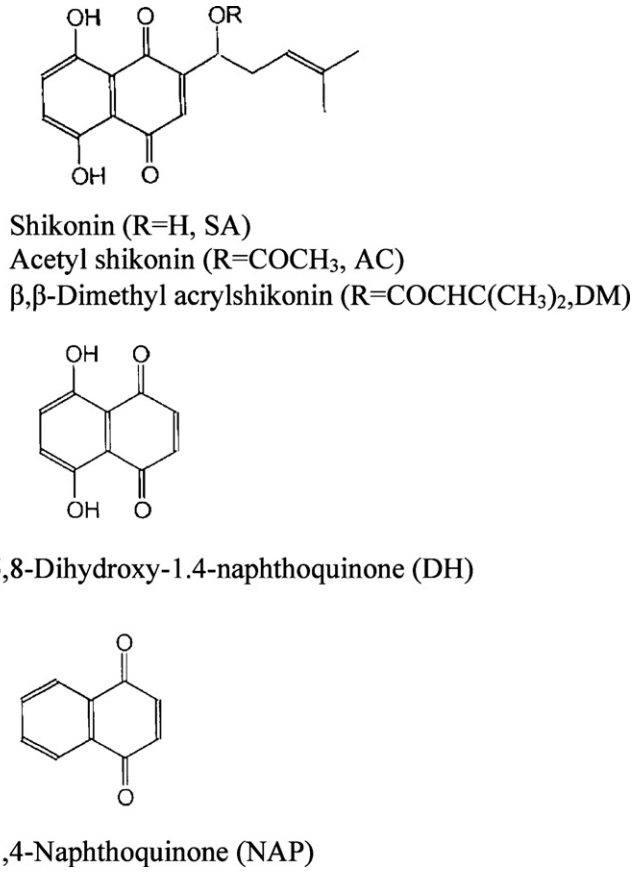


**Fig. 1.** Chemical structures of shikonin derivatives (SA, AC, and DM) and naphthoquinone pigments (DH and NAP).

### 2.3. Isolation of shikonin/alkannin and their derivatives

Shikonnin/alkannin (SA) was isolated following a procedure previously described by Cheng et al. (1995). Briefly, dried roots of *Lithospermum euchroma* were extracted twice with *n*-hexane. The *n*-hexane extract was evaporated, and then the crude extract was dissolved in NaOH. After filtration, the solution was acidified with $H_2SO_4$ to pH 6 and re-extracted with *n*-hexane. The *n*-hexane layer was concentrated and fractionated through an open column of silica gel 60 (15 mm × 15 mm). Alternatively, acetylshikonin (AC) and β,β-demethylacrylshikonin (DM) were isolated from the crude *n*-hexane extract of *Lithospermum euchroma* using an open column of silica gel 60 (15 mm × 15 mm). The chemical structures of SA, AC, DM, 5,8-dihydroxy-1.4-naphthoquinone (DH), and 1,4-naphthoquinone (NAP) (as shown in Fig. 1) were determined by chemical and physical means and by comparison with data from the literature. A red crystal of shikonin/alkannin with an enantiomeric ratio of 17:83 was used in this study.

### 2.4. Cell culture

The mouse macrophage-like cell line, RAW 264.7, was purchased from American Type Culture Collection (ATCC TIB71, Manassas, VA), RAW 264.7 macrophages were grown in DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotics under 5% $CO_2$ at 37 °C in humidified air. In brief, macrophages were plated at a den-

sity of $1 \times 10^6$ cells/60-mm plate. After pre-incubation for 24 h, the test compounds were added 30 min before co-treatment with LPS (1 μg/ml) for another 18 h. Test compounds were dissolved in DMSO on the day of the experiment and diluted with serum-free DMEM at appropriate concentrations. The final concentration of DMSO was adjusted to 0.1% (v/v). Control groups also received the same amount of DMSO. Before stimulation with LPS, the medium was refreshed with 3 ml DMEM and treated with shikonin derivatives or naphthoquinone pigments and then stimulated with 1 μg/ml LPS for the indicated times in the figure legends.

### 2.5. NO determination

For determining the NO concentration, the stable conversion product of NO, nitrite ($NO_2^-$), was measured using the Griess reagent (1.0% sulfanilic acid and 0.1% *N*-(1)-naphthylethylenediamine-dihydrochloride) incubated at room temperature for 5 min. $NaNO_2$ was used to generate a standard curve, and NO production was determined by measuring the optical density at 550 nm with a microplate reader (Spectra Max, Molecular Devices, Sunnyvale, CA). In order to determine the direct inhibitory effects of the test compounds of the iNOS enzyme on cell levels, RAW 264.7 cells were cultured in 96-well plates using DMEM without phenol red and were pretreated with different concentrations of SA derivatives before being incubated for 30 min. Cellular NO production was induced by adding 1 μg/ml LPS and incubating for 18 h. After incubation, 100 μl of conditioned media containing $NO_2^-$ (the primary stable oxidation product of NO) was mixed with the same volume of Griess reagent and incubated for 18 h. LPS alone was used as the positive control from the medium, and the NO concentration was measured by the optical density at 550 nm on an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices). Nitrite production was determined by comparing the optical density with the standard curve obtained with $NaNO_2$.

### 2.6. $PGE_2$ determination

RAW264.7 cells ($1 \times 10^5$ cells/well) were cultured in 96-well plates using DMEM without phenol red and pretreated with different concentrations of SA derivatives before being incubated for 30 min. Cellular $PGE_2$ production was induced by adding 1 μg/ml LPS and incubating for 18 h. LPS alone was taken as the positive control. After incubated, culture supernatants were collected, and the $PGE_2$ concentration was measured by ELISA using a monoclonal antibody, as specified by the manufacturer. The $PGE_2$ content in the medium was assayed according to the protocol provided by the manufacturer ($PGE_2$ EIA kit, Cayman, Chemicals, Ann Arbor, MI).

### 2.7. Lactate dehydrogenase (LDH) assay

The LDH released into the culture medium was used as an index of cell death. Toxicity was quantitated by determining the percent leakage of the cytosolic enzyme, LDH, from cells into the medium. RAW 264.7 cells ($1 \times 10^5$ cells/ml) were cultured in 96-well plates using DMEM without phenol red, in an incubator at 37 °C for 24 h. After being pretreated with different concentrations of SA derivatives and incubated for 30 min, cellular LDH production was induced by adding 1 μg/ml LPS, and incubated for 18 h. Briefly, the substrate reaction buffer (0.5 mM lactic acid, 0.1 N NaOH, and 0.1 M glycine buffer) was added to the medium. Dinitrophenylhydrazine (0.02%) was added as a chromogenic agent, and the absorbance values at 460 nm were read in a spectrophotometer (Beckman DU 640).

### 2.8. Reverse-transcription (RT)-PCR

Total cellular RNA from treated cells was isolated using an NE-PERTM kit (Pierce, Rockford, IL) according to the manufacturer's instructions. From each sample, 3 μg of RNA was reverse-transcribed for 1 h at 37 °C in a reaction mixture containing 5 U RNase inhibitor (Invitrogen), 0.5 mM dNTP (Boeringer Mannheim, Indianapolis, IN), 2 mM random hexamer (Promega, Madison, WI), 1× RT buffer, and 5 U reverse transcriptase. A PCR analysis was then performed on aliquots of the complementary cDNA preparations to detect iNOS β-actin gene expression using a PTC-150 Minicycler MJ (MJ Research, Watertown, MA). The reaction was carried out in a volume of 25 ml containing 1 unit of Taq DNA polymerase, 0.2 mM dNTP, 10× reaction buffer, and 0.2 μmol of the 5′- and 3′-primers. After initial denaturation for 5 min at 95 °C, 30 amplification cycles were performed including 1 min of denaturation at 95 °C, 1 min of annealing at 56 (for iNOS) or 53 °C (for β-actin), and 2 min of extension at 72 °C. The PCR primers used in this study were as follows. Two specific probes: iNOS (5′-TGG-GAA-TGG-AGA-CTG-TCC-CAG/3′-GGG-ATC-TGA-ATG-TGA-TGT-TTG) and β-actin (5′-AAG-GTG- TGA-TGG-TGG-GAA-TG/3′-ATG-GCT-ACG-TAC-ATG-GCT-GG) were used, and these were then run on 2% agarose gels for the cDNA fragment determination. The condition of reverse transcription was based on protocols provided by the manufacturer. Analysis of the resulting PCR products on 1% agarose gels showed single-band amplification products with the expected sizes.

### 2.9. Western blotting

Cellular proteins were extracted from control and SA derivative-treated RAW264.7 cells. Cells were collected by centrifugation and washed twice with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaFl, and 0.5 mM sodium orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and then incubated at 4 °C for 20 min. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The concentration of cell lysates was determined using a Bio-Rad Protein Assay Kit (Bio-Red, Richmond, CA) according to the manufacturer's instructions. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 10% polyacrylamide gels and then transferred onto PVDF membranes by the Panther™ Semidry Electroblotter (Owl Scientific, Portsmouth, NH). The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4 °C, and then incubated with primary antibodies that recognize iNOS (BD transduction), β-actin (Sigma), COX-2, IκB-α, p65, MAPKs (ERK, JNK, p38, pERK, pJNK, and pp38), and Histone-1 (Santa Cruz). After washing with TBST, horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham, Piscataway, NJ) (1:3000 dilution in TBST) were applied, and the blots developed by an enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology).

### 2.10. Electrophoretic mobility shift assay (EMSA)

RAW264.7 macrophage cells were plated in 100-mm dishes ($1 \times 10^6$ cells/ml). Cells were preincubated with each of the shikonin derivatives for 30 min before the addition of 1 μg/ml LPS for 18 h. Cells were then washed twice, scraped off into cold PBS, and centrifuged. Pellets were resuspended in hypotonic extraction buffer containing 10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.2 mM PMSF,

4 μg/ml leupeptin, and 20 μg/ml aprotinin, and incubated on ice for 10 min. Then, cells were lysed by the addition of 0.5% Nonidet P-40 and vigorous vortexing for 10 s. The nuclei were pelleted by centrifugation at 12,000 × *g* for 10 min at 4 °C and resuspended in high-salt buffer (20 mM HEPES (pH 7.9), 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, and 1 mM sodium orthovanadate). Five micrograms of nuclear extract, poly(dI-dC), and biotin-labeled double-strain NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were end-labeled with [γ-32P]dATP (the underlining indicates a κB consensus sequence or a binding site for the NF-κB/cRel homodimeric or heterodimeric complex). Binding reactions were performed according to the manufacturer's instructions: mixing with the binding buffer (Lightshift Chemiluminescent EMSA kit; Pierce Chemical) to a final volume of 20 μM, and then incubation at room temperature for 30 min. An unlabeled double-stranded NF-κB oligonucleotide (5′-AGTTGAGGcGACTTTCCCAGGC-3′), and an AP-1 oligonucleotide (5′-CGCTTGATGAGTCAGCCGGAA-3′) were also used to confirm specific binding. The P65 antibody was used to induce a supershift, indicating the binding affinity of NF-κB. The nuclear protein–DNA complex was separated by 5% Tris/boric acid/EDTA-polyacrylamide gel at 100 V in 0.5× TBE buffer. The gels were vacuum-dried for 1 h at 80 °C and exposed to X-ray film at −70 °C for 24 h.

### 2.11. Statistical analysis

Results are expressed as the mean ± S.D. of at least three experiments performed using different *in vitro* cell preparations. Statistically significant values were compared using Student's *t*-test followed by Dunn's test. Statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. Effects of SA derivatives on LPS-induced NO and $PGE_2$ production by LPS-treated RAW 264.7 cells

To assess the effect of SA derivatives on LPS-induced NO production in RAW 264.7 macrophages, cells were treated with various concentrations of SA derivatives for 30 min, and NO and $PGE_2$ were induced by co-treatment with LPS (1 μg/ml) for another 18 h. Neither LPS nor samples were added to the control group. Cell culture medium was harvested before treatment, and NO levels were quantified using the Griess reaction. All SA derivatives, including SA, DH, AC, and DM (0.3–3 μg/ml), and NAP (3–10 μg/ml) showed dose-dependent inhibition of LPS-induced NO production (Fig. 2A). To determine whether SA derivatives inhibited $PGE_2$ production, cells were treated with various concentrations of SA derivative for 30 min, and $PGE_2$ was induced by co-treatment with LPS (1 μg/ml) for another 18 h. As shown in Fig. 2B, only higher concentration of SA derivatives, including 3 μg/ml SA, DH, AC, and DM, and 10 μg/ml NAP, significantly inhibited the production of $PGE_2$. Among these, SA was found to have the most-potent effect on inhibition of $PGE_2$ accumulation. The cytotoxic effects of these five compounds were also evaluated with the LDH assay, and only higher concentrations of 3 μg/ml SA, DH, AC, and DM, and 10 μg/ml NAP showed an effect on cell viability (data not shown). Thus, the inhibitory effects of SA derivatives on NO production at 0.3–1 μg/ml were not attributable to any cytotoxic effect.

### 3.2. Effects of SA derivatives on iNOS and COX-2 protein expressions by LPS-treated RAW 264.7 cells

Since SA derivatives were found to inhibit the production of NO and $PGE_2$ induced by LPS-treated RAW 264.7 cells, we further investigated their effects on LPS-induced iNOS and COX-2 protein expression levels by Western blotting. The immunoblot assay showed that iNOS and COX-2 proteins were undetectable in resting RAW264.7 macrophages and were highly induced in the presence of LPS. SA derivatives significantly inhibited this iNOS production in a dose-dependent manner (Fig. 3B). A similar pattern was observed when the effect of SA was examined on LPS-induced COX-2 expression levels but not on DH, AC, DM, or NAP. However, none of these treatments affected the expression of the housekeeping gene, β-actin (Fig. 3A).

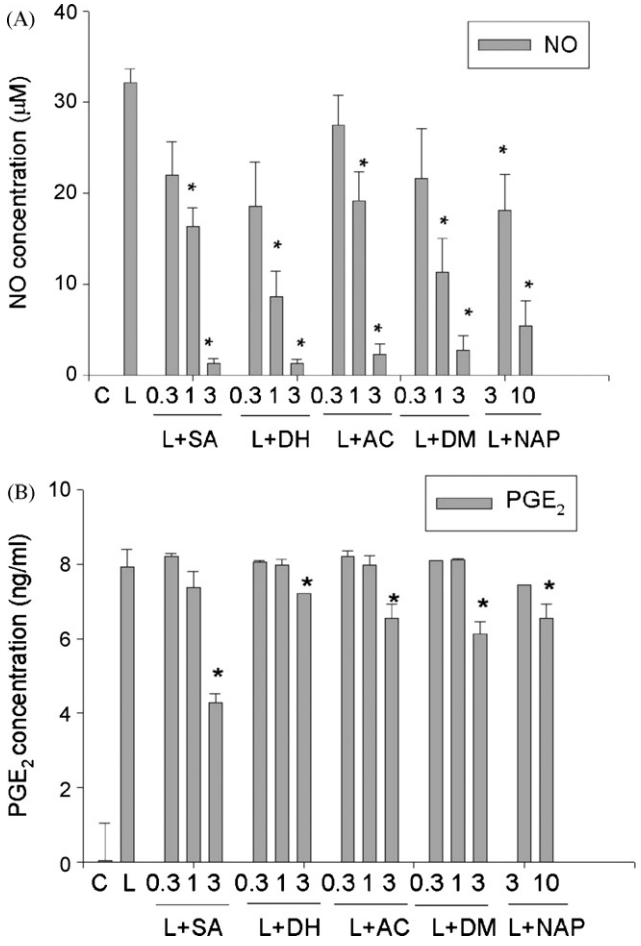


**Fig. 2.** Shikonin derivatives and naphthoquinone pigments dose-dependently inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO) but partially inhibited prostaglandin $E_2$ ($PGE_2$) production in RAW 264.7 macrophages. Cells were treated with 0.3, 1, or 3 μM of SAs or NAP at 3 or 10 μM at 37 °C for 30 min before the addition of 1 μg/ml LPS for 18 h. At the end of incubation, 100 and 500 μl of medium were collected for the NO (A) and $PGE_2$ (B) assays, respectively. C, control; L, LPS (1 μg/ml) treated. Data were obtained from three independent experiments and are expressed as the mean ± S.D. *Statistically significant difference compared to 18-h positive control at the $p < 0.05$ level using Student's *t*-test.

### 3.3. Effects of SA derivatives on iNOS mRNA expression by LPS-treated RAW 264.7 cells

Since iNOS expression decreased at the protein level, we investigated whether the decrease in NO production was due to downregulation of iNOS gene expression at the mRNA level using RT-PCR. In unstimulated RAW264.7 cells, the iNOS mRNA level was almost undetectable. However, in response to LPS, the expression of iNOS was markedly augmented, and SA derivatives significantly inhibited iNOS mRNA induction in a concentration-dependent manner (Fig. 4A and B).

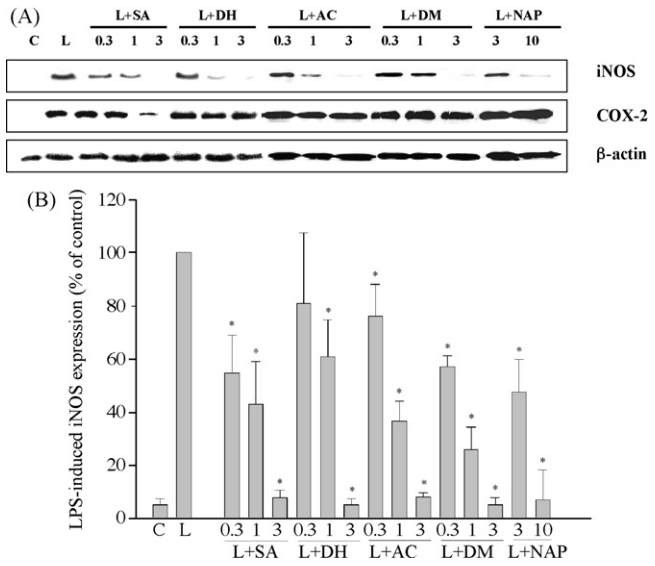


**Fig. 3.** Shikonin derivatives and naphthoquinone pigments dose-dependently inhibited lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) protein expression in RAW 264.7 macrophages. (A) Cells were treated with 0.3, 1, or 3 μM of SAs or 3 or 10 μM NAP at 37 °C for 30 min before the addition of 1 μg/ml LPS for 18 h. Equal amounts of total proteins (60 μg/lane) were subjected to 10% SDS–PAGE, and expressions of iNOS, COX-2, and β-actin protein were detected by Western blotting using specific anti-iNOS, COX-2, and anti-β-actin antibodies. (B) Quantified data. Data were obtained from three independent experiments and are expressed as the mean ± S.D. *Statistically significant difference compared to the 18-h positive control at the $p < 0.05$ level using Student's *t*-test.

### 3.4. Effect of SA on LPS-induced NF-κB activation

To further investigate the mechanism of the SA derivative-mediated inhibition of iNOS transcription, we focused on NF-κB, which is activated in cells challenged by LPS and other inflammatory stimuli, and is involved in the transcriptional activation of the responsive genes of iNOS and COX-2 (Surh et al., 2001; Lappas et al., 2002). Among these five chemicals, SA is a well-known chemical for anti-inflammation and was the only one which could inhibit COX-2 protein expression in our study. To elucidate the mechanism, we next determined whether SA inhibited the DNA-binding activity of NF-κB in LPS-stimulated RAW 264.7 macrophages. As shown in Fig. 5A, treatment with LPS caused a significant increase in the DNA binding activity of NF-κB. In the presence of SA (0.3–1 μg/ml), however, LPS-induced NF-κB–DNA binding was markedly suppressed in a dose-dependent manner (Fig. 5A and B). In addition, co-treatment with an unlabeled NF-κB oligonucleotide caused a significant decrease in the DNA-binding activity of NF-κB in the absence of SA, and when co-treated with the AP-1 oligonucleotide, the inhibition was markedly reversed. The addition of the p65 antibody caused a significant supershift of the NF-κB-DNA complex, further confirming the binding affinity of p65 and NF-κB (Fig. 5A).

Because p65 is the major component of NF-κB activated by LPS in macrophages, the translocation of p65 into the nucleus was determined using immunocytochemistry. Western blot analysis showed that the LPS-induced p65 level in nuclear fractions was reduced by SA (Fig. 5C).

### 3.5. Effect of SA on LPS-induced IκB degradation

The nuclear translocation and DNA binding of the NF-κB transcription factor are preceded by degradation of the inhibitory factor-κBα (I-κBα) (Rice and Ernst, 1993). The cytoplasmic levels of I-κBα were examined by a Western blot analysis at the indicated concentrations of SA derivatives to determine whether inhibition of NF-κB-DNA-binding by SA is related to I-κBα degradation. I-κBα protein expression was barely detected at 15 min after LPS treatment. However, SA caused a significant recovery of the level of I-κBα protein (Fig. 6). A similar pattern was observed when the effects on the recovery of LPS-treated I-κBα degradation by DH, AC, DM, and NAP.

### 3.6. Effects of SA derivatives on MAPK phosphorylation of LPS-treated RAW 264.7 cells

We investigated the molecular mechanism of NF-κB inhibition by its inhibitory effect on the activations of MAPKs (ERK1/2, p38, and JNK). Maximum phosphorylation of p38, ERK1/2, and JNK expression levels are known to occur 30 min after LPS (1 μg/ml) treatment in RAW 264.7 macrophages. However, the SA derivatives, SA, DH, AC, and DM (3 μg/ml), and NAP (10 μg/ml) significantly suppressed the LPS-induced activations of ERK 1/2 but not p38 MAPK or SAPK/JNK (Fig. 7A). The concentration-dependent inhibition of ERK phosphorylation is shown in Fig. 7B.

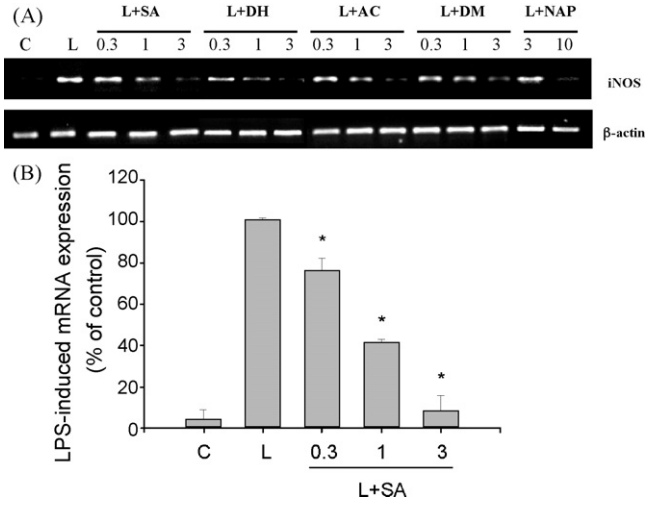


**Fig. 4.** Shikonin derivatives and naphthoquinone pigments dose-dependently inhibited lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) mRNA expression in RAW 264.7 macrophages. (A) Cells were treated with 0.3, 1, or 3 μM SAs of 3 or 10 μM NAP at 37 °C for 30 min before the addition of 1 μg/ml LPS for 18 h. Reverse transcription was performed on total RNA (3 μg) as described in Section 2. Subsequently, PCR was conducted on cDNA using specific primers to iNOS and β-actin and then run on 2% agarose gels to examine the cDNA fragments. C, control; L, LPS (1 μg/ml) treated. (B) Quantified data of SA on LPS-induced iNOS mRNA expression. Data were obtained from three independent experiments and are expressed as the mean ± S.D. *Statistically significant difference compared to the 18-h positive control at the $p < 0.05$ level using Student's *t*-test.

## 4. Discussion and conclusions

Shikonin is an enantiomer of alkannin, which has historical references for treatment of ulcers as early as the 4th century BC and is found in the roots of European dye-plant *Alkana tinctoria* (Hazra et al., 2004). In addition, shikonin is currently used in various medicinal and cosmetics and dyestuffs in Japan, while alkannin is mainly used for food coloring and cosmetics in Europe and the US (Papageorgiou et al., 1999). Although the efficacy of alkannin and shikonin derivatives has been demonstrated *in vivo*, the precise model of their anti-inflammatory actions remains undetermined. The purpose of this study was to elucidate the molecular mechanism of the anti-inflammatory action of shikonin (SA) and its derivatives (AC and DM), and the naphthoquinone pigments (DH

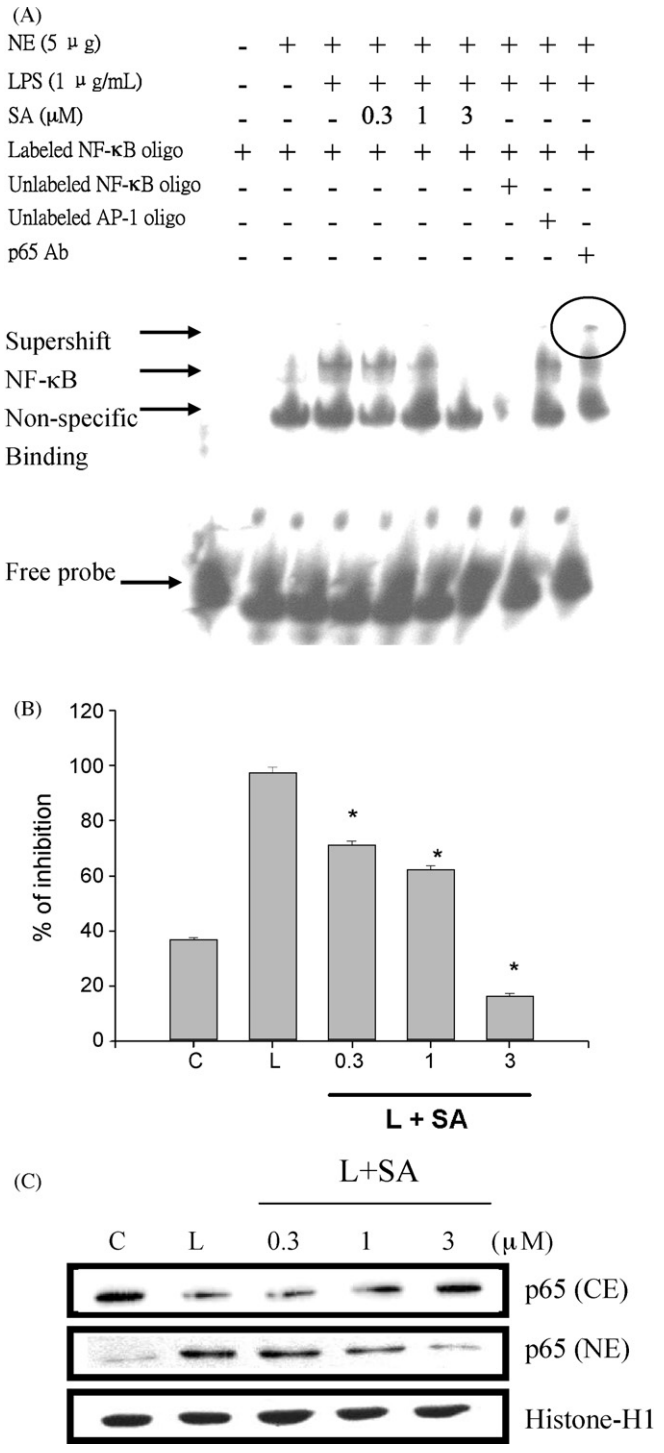


**Fig. 5.** Shikonin dose-dependently inhibits lipopolysaccharide (LPS)-induced NF-κB activation. Cells were seeded $1 \times 10^6$ cells/ml 10 ml in 100-mm plates. After 24 h of incubation, the medium was refreshed and treated with 0.3, 1, or 3 μM of SAs at 37 °C for 30 min before the addition of 1 μg/ml LPS for 18 h. (A) Nuclear extracts (5 μg) were used for the electrophoretic mobility gel shift assay (EMSA). (B) Quantified data. (C) Effects of shikonin derivatives on LPS-induced p65 translocation. Equal amounts of total proteins (30 μg/lane) were subjected to 10% SDS–PAGE, and expressions of p65 and histone-1 protein were detected by Western blotting using specific anti-p65 and anti-histone-1 antibodies. Histone-1 protein was used as an internal control. C, control; L, LPS (1 μg/ml) treated; CE, cytosolic extracts; NE, nuclear extracts. Data were obtained from three independent experiments and are expressed as the mean ± S.D. * Indicates a statistically significant difference compared to the 18-h positive control at the $^*p < 0.05$ level using Student's *t*-test.

and NAP) using the LPS-induced inflammatory responses in murine RAW 264.7 macrophage cells.

In murine RAW 264.7 macrophage cells, LPS induces iNOS transcription and translation, which is followed by NO production; therefore, suppression of the production of NO using drugs might be useful for treating inflammatory diseases and ectotoxin shock. Herein, we found that SA derivatives inhibited LPS-induced proinflammatory molecules including NO and $PGE_2$ in dose-dependent manners. To further investigate the mechanisms underlying these inhibitions by SA derivatives, expression levels of iNOS and COX-2 protein and the iNOS mRNA level were respectively examined by Western blotting and RT-PCR. Inhibition of iNOS gene expression was evidenced by a reduction in its mRNA levels, and this occurred simultaneously and in concentration-dependent manners (Fig. 4), suggesting that the inhibition of NO release might be attributable to the suppression of iNOS mRNA transcription and thus protein expression. In addition, we studied another important mediator of inflammation, COX-2, which acts on arachidonic acid to release PGs that further orchestrate the process of inflammation (Willoughby et al., 2000). Among these five SA derivatives, all inhibited $PGE_2$ induction at the higher concentration (Fig. 2), however, only SA showed an inhibitory effect on COX-2 protein expression (Fig. 3). Seto and co-workers studied the effects of shikonin and several derivatives on various aspects of wound healing, and their anti-inflammatory effects (Seto et al., 1992). Shikonin was shown to be more active than acetylshikonin and the other SA derivatives. The anti-inflammatory effects of extracts of *Arnebia euchroma* (alkanin esters), and β,β-dimethylalkannin in particular (Lin et al., 1980), have been studied and showed similar results (Seto et al., 1992). In our study, SA showed a more-potent effect than the other derivatives of inhibiting NO and $PGE_2$ induced by LPS-treated RAW

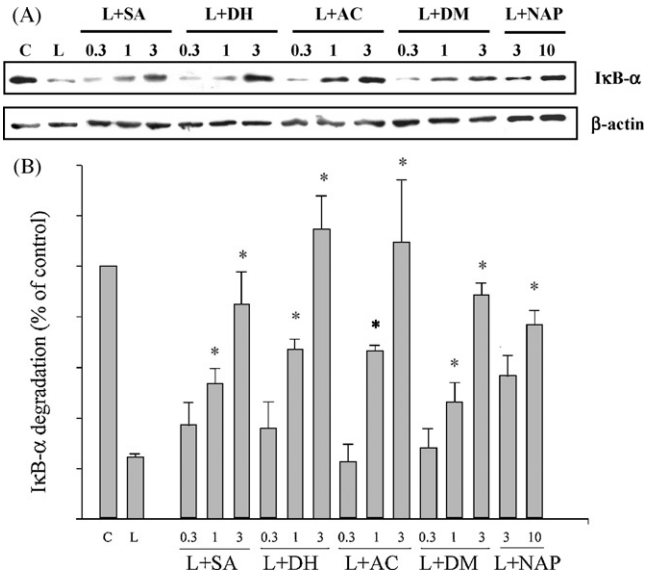


**Fig. 6.** Shikonin derivatives and naphthoquinone pigments block lipopolysaccharide (LPS)-induced NF-κB activation via abrogation of IκB-α degradation in RAW 264.7 macrophages. (A) Cells were treated with 0.3, 1, or 3 μM SAs or NAP at 3 or 10 μM at 37 °C for 30 min before the addition of 1 μg/ml LPS for 18 h. Equal amounts of total proteins (30 μg/lane) were subjected to 10% SDS–PAGE, and the expressions of IκB-α and β-actin proteins were detected by Western blotting using specific anti-IκB-α and anti-β-actin antibodies. β-Actin protein was used as the internal control. C, control; L, LPS (1 μg/ml) treated. (B) Effects of SA derivatives on LPS-induced IκB-α degradation in dose-dependent manners. Cells were treated with 1 μg/ml of LPS only or LPS plus 0.3, 1, or 3 μM of shikonin for the indicated times. Equal amounts of total proteins (30 μg/lane) were subjected to 10% SDS–PAGE, and the expressions of IκB-α and β-actin proteins were detected by Western blotting using specific anti-IκB-α and anti-β-actin antibodies. β-Actin protein was used as the internal control. C, control, no treatment; L, LPS at 1 μg/ml.

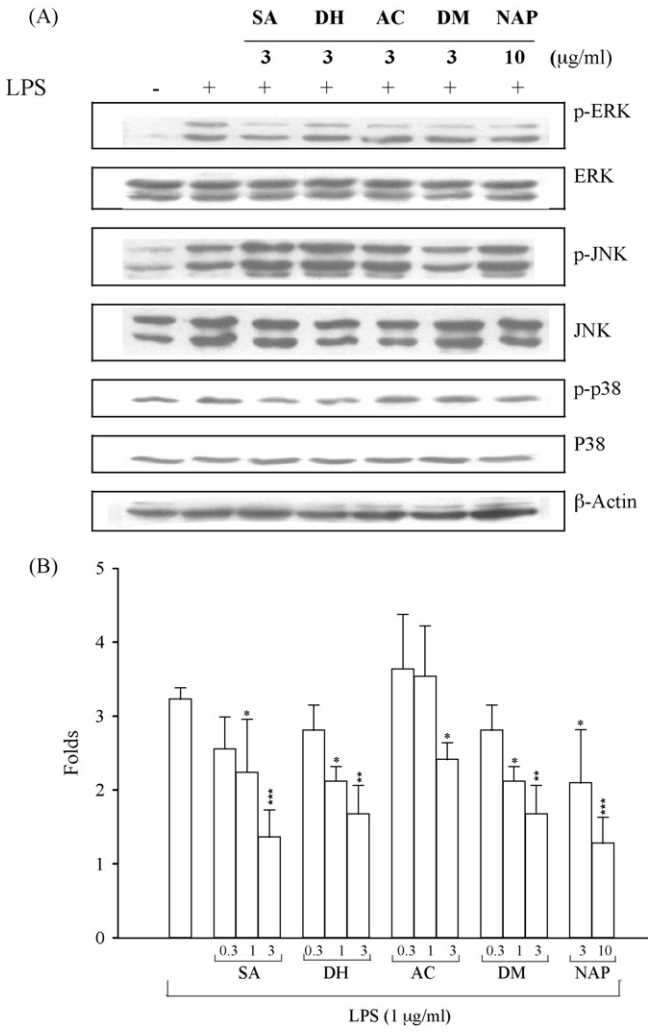


**Fig. 7.** Effect of shikonin derivatives and naphthoquinone pigments on the lipopolysaccharide (LPS)-induced phosphorylation of MAPKs in RAW 264.7 macrophages. (A) RAW 264.7 macrophages were treated with 0.3, 1, or 3 μM of SAs or 3 or 10 μM NAP at 37 °C for 30 min before the addition of 1 μg/ml LPS for 18 h. (B) Concentration-dependent effect of SA derivatives on ERK phosphorylation. Whole-cell lysates were analyzed by Western blotting using various antibodies against activated MAPKs. Equal amounts of total proteins (60 μg/lane) were subjected to 10% SDS–PAGE, and the expressions of p-ERK, p-JNK, p-P38, ERK, JNK, P38 and β-actin protein were detected by Western blotting using specific anti-p-ERK, p-JNK, p-p38, ERK, JNK, P38 and β-actin antibodies. Data were obtained from three independent experiments and are expressed as the mean ± S.D. *Statistically significant difference compared to the 18-h positive control at the $p < 0.05$ level using Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the positive control.

264.7 macrophages, as well as iNOS and COX-2 protein expressions, especially on mRNA induction of iNOS. In order to further investigate the mechanism involved in the anti-inflammatory effect of SA derivatives, we used SA to study the involvement of the NF-κB signaling pathway. The promoter of the iNOS gene is known to contain two transcriptional regions: an enhancer and a basal promoter (Lowenstein et al., 1993). There are a number of binding sites for transcription factors including κB sites, which are located in both the enhancer and basal promoter (Xie et al., 1994), and NF-κB, which is essential for LPS-mediated NO production. Therefore, we examined NF-κB-DNA binding activity to confirm that the inhibition of the expression of iNOS and COX-2 are influenced by the NF-κB signaling pathway. Our results indicated that DNA binding activity was inhibited in a concentration-dependent manner by SA (Fig. 5A and B) and these results corresponded with its inhibition of the expressions of iNOS. However, when cytoplasmic levels of I-κBα were examined by a Western blot analysis, we found that SA caused a significant recovery in the level of the I-κBα protein (Fig. 6). A similar pattern was observed when the effects of DH, AC, DM, and NAP were examined on the recovery of LPS-treated I-κBα degradation. Our results indicated that the inhibition of iNOS and COX-2 levels by SA was due to the inhibition of NF-κB activation. Downregulation of the NF-κB gene was further confirmed by inhibiting the respective whole-cell proteins p65 by SA. Furthermore, the protein level of p65 in the nucleus confirmed its downregulated expressions following treatment with SA (Fig. 5C).

It has been reported that activation of MAP kinase phosphorylation transduces signals to activate the transcription of NF-κB-mediated proinflammatory cytokines (Ren and Chung, 2007; Rajapakse et al., 2008). Ajizian et al. (1999) suggested that activation of ERK is thought to be involved in LPS-induced macrophage responses; in addition, JNK and p38 are activated by LPS stimulation and have been postulated to play important roles in controlling iNOS gene expression (Hambleton et al., 1996; Bhat et al., 1998). In our study, we showed that ERK, JNK, and p38 were phosphorylated with LPS stimulation. Furthermore, phosphorylation of ERK was inhibited by SA derivatives at 30 min of LPS stimulation, whereas there was no effect of SA derivatives on pp38 or pJNK (Fig. 7). MAPKs are also likely targets for the development of novel anti-inflammatory drugs; however, signaling from MAPKs to transcription factors mediating iNOS and COX-2 expression is not fully understood.

The anti-inflammatory effect of shikonin was first reported from success in treating burns, leprous ulcers, and acute fissures (Michaelides et al., 1993), dating back to the 1600 s. More recently, shikonin was shown to inhibit the biosynthesis of leukotriene B4 and 5-hydroxyeicosatetranoic acid (Wang et al., 1994). Chiu and Yang (2007), demonstrated that shikonin downregulates the splicing process of TNF-α pre-mRNA through inactivation of the PKR pathway in LPS-stimulated THP-1 monocytes. Altogether, although several research groups have investigated the biological and pharmacological mechanisms of shikonin derivatives, their precise mode of action remains undetermined. Our findings are consistent with the fact that SA derivatives are potentially potent anti-inflammatory agents, and this is the first investigation showing that SA markedly inhibits LPS-induced NO production and iNOS expression by blocking NF-κB activation and inhibits the phosphorylation of ERK in RAW 264.7 macrophages. The effect of SA on anti-inflammation not only comes from the iNOS/NO but also the COX-2/$PGE_2$ pathway. Our findings provide useful and additional mechanistic explanations for the anti-inflammatory effect of shikonin and highlight its pharmaceutical value.

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