Anti-inflammatory Effects of Schisandra chinensis (Turcz.) Baill Fruit Through the Inactivation of Nuclear Factor-κB and Mitogen-activated Protein Kinases Signaling Pathways in Lipopolysaccharide-stimulated Murine Macrophages

Young-Soon Kang1, Min-Ho Han1, Su-Hyun Hong1, Cheol Park2, Hye-Jin Hwang3,4, Byung Woo Kim3,5, Kim Ho Kyoung6, Young Whan Choi7, Cheol Min Kim8, Yung Hyun Choi1,3

1Department of Biochemistry, Dongeui University College of Korean Medicine, Busan, Korea, 2Department of Molecular Biology, 3Blue-Bio Industry RIC and Anti-Aging Research Center, 4Department of Food and Nutrition, 5Department of Life Science and Biotechnology, College of Natural Sciences and Human Ecology, Dongeui University, Busan, Korea, 6Herbal Medicine Resources Group, Herbal Medicine Research Division, Korea Institute of Oriental Medicine, Daejeon, Korea, 7Department of Horticultural Bioscience and Life and Industry Convergence Research Institute, College of Natural Resource and Life Sciences, Pusan National University, Miryang, Korea, 8Department of Biochemistry, Pusan National University School of Medicine, Yangsan, Korea

Background: Schisandraceae Fructus, the dried fruit of Schisandra chinensis (Turcz.) Baill. (Magnoliaceae), is widely used in traditional medicine for the treatment of a number of chronic inflammatory diseases. This study examined the anti-inflammatory effects of Schisandraceae Fructus ethanol extract (SF) on the production of pro-inflammatory substances in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

Methods: To measure the effects of SF on pro-inflammatory mediator and inflammatory cytokine’s expression and production in RAW 264.7 cells, we used the following methods: cell viability assay, Griess reagent assay, enzyme-linked immunosorbent assay, reverse transcriptase-polymerase chain reaction, Western blotting analysis and immunofluorescence staining.

Results: Stimulation of the RAW 264.7 cells with LPS caused an elevated production of nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, which was markedly inhibited by the pretreatment with SF without causing any cytotoxic effects. SF also inhibited the expression of inducible NO synthase, TNF-α, and IL-1β protein and their mRNAs in LPS-stimulated RAW 264.7 cells. Furthermore, SF attenuated LPS-induced nuclear translocation of nuclear factor-κB (NF-κB) by reducing inhibitory-κB degradation, and reduced the phosphorylation of mitogen-activated protein kinases (MAPKs), implying that SF regulated LPS-induced NF-κB-dependent inflammatory pathways through suppression of MAPKs activation.

Conclusions: SF may be useful for the treatment of various inflammatory diseases.

Key Words: Schisandra chinensis, Anti-inflammation, Nuclear factor-kappa B, Mitogen-activated protein kinases

INTRODUCTION

Inflammation refers to the pathological and physiological processes involved in numerous diseases and is a complex process that is modulated by an array of inflammatory factors released by activated macrophages.1,2 The activation of macrophages by inflammatory stimuli is an important part of initiating defensive reactions, and inflammatory mediators including nitric oxide (NO) and pro-inflammatory cytokines are released to enhance the defense capacity. Among them, NO is synthesized via the oxidation of the terminal guanidine nitrogen of L-arginine by nitric oxide synthases (NOSs). To date, three NOS
isoforms have been identified based on the cell type or the location and manner of expression, such as endothelial NOS (\(\text{eNOS}\)), neuronal NOS (\(\text{nNOS}\)), and inducible NOS (\(\text{iNOS}\)). Both \(\text{nNOS}\) and \(\text{eNOS}\) isoforms, but not \(\text{iNOS}\), are constitutively expressed.\(\text{iNOS}\) can be rapidly induced by inflammatory stimuli, including toxins such as lipopolysaccharide (\(\text{LPS}\)) and cytokines, so that dysregulated \(\text{iNOS}\) expression might be intimately involved in the development of various inflammatory diseases. Activated macrophages also have the capability of releasing a variety of soluble pro-inflammatory cytokines such as tumor necrosis factor (\(\text{TNF-}\alpha\)) and interleukin (\(\text{IL-}\)1\(\beta\)). Overproduction of those pro-inflammatory cytokines has been implicated in the pathogenesis of many inflammatory disease processes.\(^{6,7}\) Therefore, agents that can suppress high production of pro-inflammatory mediators and cytokines can be used as potential therapeutic tools in the development of anti-inflammatory drugs.

Multiple studies have shown that nuclear factor (NF)-\(\kappa\) \(\text{B}\) is one of the most ubiquitous transcription factors and regulates the expression of genes involved in inflammatory responses. In the resting state, NF-\(\kappa\) \(\text{B}\) proteins are sequestered in the cytoplasm by interaction with inhibitory proteins, like inhibitor \(\kappa\) \(\text{B}\) \(\text{Is} \; (I \; \kappa \; \text{B} \text{Is}).\) However, I \(\kappa\) \(\text{B}\) Is are rapidly phosphorylated by I \(\kappa\) \(\text{B}\) kinase respond to pro-inflammatory stimuli, with subsequent degradation by proteasome, which results in the release of free NF-\(\kappa\) \(\text{B}\) dimers (p50 and p65). These released dimers translocate to the nucleus, where they induce gene transcription through the cis-acting \(\kappa\) \(\text{B}\) element.\(^{6,9}\)

The mitogen-activated protein kinase (MAPK) pathway in macrophages is one of the most extensively investigated intracellular signaling cascades involved in pro-inflammatory responses.\(^{10,11}\) The MAPKs include extracellular signal-regulated kinase 1/2 (\(\text{ERK}\)1/2), p38, and c-Jun N-terminal kinase (\(\text{JNK}\)) as a group of serine/threonine-specific protein kinases. A major consequence of MAPK phosphorylation is activation of these transcription factors, which serve as immediate or downstream substrates of the kinases. Thus, NF-\(\kappa\) \(\text{B}\) and MAPKs activation pathways are commonly targeted with anti-inflammatory drugs.

**Schisandrae Fructus**, the dried fruit of *Schisandra chinensis* (Turcz.) Baill. (Magnoliaceae), is one of the most important traditional herbal medicines and has been extensively used in Asia (Korea, China, and Japan) as well as Russia.\(^{12,13}\) It has been traditionally used for protection, immunostimulating, and anti-cancer activities.\(^{14-24}\) Several studies have been conducted on the anti-inflammatory activity of *Schisandrae Fructus*, but the detailed molecular signaling pathway by which *Schisandrae Fructus* exerts its anti-inflammatory effects via the activation of NF-\(\kappa\) \(\text{B}\) and MAPKs pathways has not yet been well studied. Thus, as a part of our ongoing screening program to evaluate the anti-inflammatory potential of natural compounds, we have examined the effects of *Schisandrae Fructus* ethanol extract (SF) on the production of NO, TNF-\(\alpha\), IL-6, and IL-1\(\beta\), and associated signaling pathways involved in their regulation, in a murine RAW 264.7 macrophage cell line following stimulation with LPS. Our data demonstrate that SF suppresses the LPS-induced production of NO and pro-inflammatory cytokines (TNF-\(\alpha\) and IL-1\(\beta\)) in RAW 264.7 cells. SF also inhibits the LPS-induced \(\text{iNOS}\), TNF-\(\alpha\), and IL-1\(\beta\) mRNA and protein expression associated with the reduction of NF-\(\kappa\) \(\text{B}\) and MAPK activation.

**MATERIALS AND METHODS**

1. **Preparation of Schisandrae Fructus ethanol extract**

Schisandrae Fructus were collected around Mungyeong-city (Gyeongbuk, Korea) and washed three times with tap water before storage at \(\text{\(\sim\)}\)\text{20°C. The frozen samples were lyophilized and homogenized using a grinder before extraction. The materials were extracted with 20% ethanol (SF) at room temperature for 24 hours, filtered, and concentrated using a rotary vacuum evaporator (Buchi Rotavapor R-144; BUCHI Labortechnik, Flawil, Switzerland). The extract was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) as a 50 mg/mL stock solution. The stock solution was stored at \(\text{4°C and diluted with medium to the desired concentration prior to use.}

2. **Cell culture and cell viability assay**

RAW 264.7 murine macrophages were maintained in Dulbecco’s modified Eagle’s medium (DMEM; WelGENE Inc., Daegu, Korea) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 \(\mu\)g/mL) at \text{37°C in a humidified atmosphere containing 5% CO2. Cell viability was analyzed by incubating the cells (2 \times 10^5 cells/mL) with the indicated concentrations of SF 1 hour before treatment with LPS (1.0 \(\mu\)g/mL) for 24 hours. Cell viability was determined by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) assay. Briefly, after treatments, the medium was removed and cells were incubated with 0.5 mg/mL MTT solution. Following incubation for 2 hours at \text{37°C and 5% CO2, the**
MTT solution was removed, and the cells were dissolved in DMSO. Optical density of the samples was measured at 540 nm using a microplate reader (Dyneatech MR-7000; Dynatech Laboratories, Chantilly, VA, USA).

3. Nitrite quantification assay

Nitrite accumulation, an indicator of NO synthesis, was measured in culture media based on a diazotization reaction using the Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride: Sigma-Aldrich). The RAW 264.7 cells were seeded into a 96-well plate at the density of 5 × 10^4 cells/mL. After incubation, the cells were pretreated with various concentrations of SF with or without 100 ng/mL LPS (from Escherichia coli 0111:B4; Sigma-Aldrich) for 24 hours. An aliquot (100 μL) of the supernatant was mixed with an equal volume of Griess reagent, and incubated at room temperature for 10 minutes, and then the absorbance at 540 nm was measured in a microplate reader. Fresh culture media were used as blanks in all experiments. Nitrite concentration was determined using a dilution of sodium nitrite as a standard.

4. Enzyme-linked immunospecific assay assay for cytokines

RAW 264.7 macrophage cells were treated with SF in the absence or presence of LPS. After 24 hours incubation, the conditioned medium was collected. The production of TNF-α or IL-1β in the conditioned medium was determined by using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

5. Reverse transcriptase-polymerase chain reaction analysis

RAW 264.7 cells were treated with SF in the absence or presence of LPS for 6 hours. Total RNA was extracted from cells using a Trizol reagent kit (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer’s instructions. Aliquots of 2 μg of total RNA from each sample were reverse-transcribed into cDNA with 10,000 U of reverse transcriptase and 0.5 μg/μL oligo-(dT)15 primer (Promega, Madison, WI, USA). The cDNA was amplified by polymerase chain reaction (PCR) using the primer sequences. The conditions for PCR amplification were as follows: INOS, forward 5'-GCC AGT GAT GG-3', reverse 5'-CTT CTG AGT CAA GGC TGA GA-3'; TNF-α, forward 5'-TGG CAC AGT CAA GGC TGA CAT C-3' and reverse 5'-GTG CTG CCT AAT GTC CCC TTG TTG AAT C-3'; NF-κB, forward 5'-ACA AGC CTG CTA GGT TTC C-3' and reverse 5'-CTG CAC AGT CAA GCC TGA GA-3 and reverse 5'-CTT CTG AGT CAA GCC TTG G-3'.

6. Western blot analysis

RAW 264.7 cells were incubated with SF for 1 hour prior to LPS treatment. After 24 hours incubation, the cells were collected, washed twice with cold phosphate buffered saline (PBS), and then lysed in a lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM EGTA, 1 mM NaVO₃, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonfluoride, 25 μg/mL aprotinin, and 25 μg/mL leupeptin). In a parallel experiment, nuclear and cytosolic proteins were prepared using nuclear extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer’s protocol. Aliquots of the lysates (30 to 50 μg of protein) were separated on 10% to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Amersham Biosciences, Piscataway, NJ, USA) with a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl [pH 8.8], and 20% MeOH [v/v]). Non-specific sites on the membrane were blocked by incubating the membrane in the blocking solution containing 5% non-fat dry milk in Tris-buffered saline Tween (TBS-T). MTT solution was removed, and the cells were dissolved in DMSO. Optical density of the samples was measured at 540 nm using a microplate reader (Dyneatech MR-7000; Dynatech Laboratories, Chantilly, VA, USA).

7. Immunofluorescence staining

RAW 264.7 cells were plated directly on glass coverslips in 6-well plates for 24 hours to detect NF-κB p65 localization by immunofluorescence assay using a fluorescence microscope. After stimulation with LPS in the presence or absence of SF, the cells were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature and permeabilized with 100% MeOH for 10
minutes at 20°C. Polyclonal antibody against anti-NF-κB p65 was applied for 1 hour followed by 1 hour incubation with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit immunoglobulin G (IgG). After washing with PBS, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), and fluorescence was visualized under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

8. Statistical analysis

All results are presented as the mean ± the standard deviation and are representative of three or more independent experiments. Data were compared by using Student’s t-test and P-values less than 0.05 were considered statistically significant.

RESULTS

1. Effects of Schisandrae Fructus ethanol extract on cell viability of RAW 264.7 macrophages

Concentrations of SF that would not induce cell toxicity were determined by treating RAW 264.7 cells with various concentrations of SF in the presence or absence of LPS (100 ng/mL) for 24 hours, and monitoring the cell viability with the MTT assay. The assay data showed no significant changes in cell viability, indicating that SF was not cytotoxic at dosage up to 500 μg/mL (Fig. 1). Therefore, 500 μg/mL SF was selected as the optimal dose for studying the anti-inflammatory effects of SF in LPS-stimulated RAW 264.7 cells.
2. SF inhibits lipopolysaccharide-induced nitric oxide production in RAW 264.7 macrophages

The possibility that SF has anti-inflammatory properties was investigated by determining the effects of SF on the level of NO in the culture media of RAW 264.7 cells after a 24 hours treatment with 100 ng/mL LPS and SF. Treatment of RAW 264.7 cells with LPS resulted in a dramatic increase in NO production. Treatment with 500 μg/mL SF significantly inhibited this production of NO (Fig. 2A). The possibility that this inhibitory effect of SF on NO production occurred via inhibition of corresponding gene expression was investigated by determining the mRNA and protein expressions of iNOS by reverse transcriptase (RT)-PCR and Western blot analyses. Figure 2B and 2C show that mRNA and protein expression of iNOS was undetectable in RAW 264.7 cells without LPS stimulation. Treatment with LPS alone markedly increased iNOS mRNA and protein levels, while pretreatment with SF significantly suppressed these levels. The reduced expressions of iNOS mRNA and protein were consistent with the reductions in NO production in the culture media.

3. Schisandrae Fructus ethanol extract prevents lipopolysaccharide-induced tumor necrosis factor-α and interleukin-1β release in RAW 264.7 macrophages

The effects of SF on LPS-induced inflammatory related
cytokine production in RAW 264.7 cells were studied by evaluating the production of TNF-α and IL-1β induced by LPS by ELISA. Figure 3A shows that incubation of RAW 264.7 cells with LPS caused a marked enhancement of TNF-α and IL-1β release. Treatment with 500 μg/mL SF significantly blocked the production of IL-1β and showed non-significant decreases of TNF-α production when compared with LPS-treated cells. The effects of SF on the expression of pro-inflammatory cytokines following LPS treatment were examined by RT-PCR and Western blot analyses. Figure 3B and 3C show that LPS alone significantly elevated the expression of TNF-α and IL-1β. The expression of TNF-α and IL-1β at both mRNA and protein levels was significantly reduced by the pretreatment with SF.

4. Schisandrae Fructus ethanol extract attenuates lipopolysaccharide-induced nuclear translocation of nuclear factor-κB in RAW 264.7 macrophages

NF-κB plays a pivotal role in regulation of the expression of iNOS and pro-inflammatory cytokines; therefore, we examined the effects of SF on the activation of NF-κB using Western blot and immunofluorescence microscopy analyses. Figure 4A and 4B, show that the levels of p65, a subunit of NF-κB, were decreased in the cytoplasm and increased in nucleus after LPS treatment. Pretreatment with SF effectively reversed these trends.

We also used western blotting to determine whether SF would block LPS-stimulated degradation of IκB-α. Figure 4B shows that IκB-α was markedly degraded after LPS treatment. This LPS-induced IκB-α degradation was significantly inhibited by SF. The fluorescence images also revealed that NF-κ B p65 was normally sequestered in the cytoplasm, and that nuclear accumulation of NF-κ B p65 was strongly induced after LPS stimulation (Fig. 4C). This LPS-induced translocation of NF-κ B p65 was completely abolished after pre-treatment of cells with SF. Nuclear translocation of NF-κ B p65 was not induced in cells after pre-treatment with SF alone in the absence of LPS stimulation. Taken together, these findings demonstrated that SF suppressed the expression of iNOS and pro-inflammatory cytokines at least in part via an NF-κB-dependent mechanism.

5. Schisandrae Fructus ethanol extract inhibits the lipopolysaccharide-induced activation of itogen-activated protein kinases

We explored the molecular mechanism underlying the anti-inflammatory effects of SF by determining its effects on the inflammatory response in RAW 264.7 macrophages.
activation of MAPKs by Western blot analysis using phospho-specific MAPK antibodies. Stimulation of RAW 264.7 cells with LPS led to rapid activation of ERK, JNK, and p38, with peak levels of each phosho-MAPK occurring 1 hour after addition of LPS. SF pretreatment significantly inhibited phosphorylation of MAPKs in LPS-stimulated RAW 264.7 cells (Fig. 5). These results indicated that the inhibitory effects of SF on iNOS, TNF-α, and IL-1β expressions were possibly mediated via the inactivation of the MAPK pathway, which subsequently prevented production of pro-inflammatory mediators and cytokines.

**DISCUSSION**

During the progress of inflammation, macrophages actively participate in inflammatory responses by releasing pro-inflammatory mediators and cytokines, which play a key role in the pathogenesis of many acute and chronic inflammatory diseases. NO, a major iNOS-derived product at inflammatory sites, is induced during the response to various stimulants, and can trigger the development of inflammatory diseases.3-5

Pro-inflammatory cytokines are also produced in response to inflammatory stimuli and play a key role in the inflammatory cascade. Among them, IL-1β is mainly released from macrophages after LPS treatment and is an important component in the initiation and enhancement of inflammatory response. TNF-α is also a pivotal pro-inflammatory cytokine, and excessive production of NO by LPS also up-regulates TNF-α synthesis in macrophages. Therefore, we analyzed the effect of SF on the accumulation of NO and pro-inflammatory cytokines, including TNF-α, and IL-1β, in LPS-stimulated RAW 264.7 macrophages. Our results support a significant inhibition of LPS-induced NO production by SF via attenuation of the mRNA and protein expression of iNOS, without notable cytotoxicity (Fig. 2). We also confirmed that SF could suppress LPS-induced TNF-α and IL-1β production and that this suppression was correlated with down-regulation of LPS-induced mRNA and protein expressions of these cytokines (Fig. 3). Thus, SF appears to inhibit NO, TNF-α, and IL-1β production by regulating their transcription, which otherwise would be activated by LPS treatment.

Much evidence implicates the transcription factor NF-κB in the pathogenesis of inflammatory diseases, as it may play key regulatory roles in the transcription of pro-inflammatory mediators and cytokines in activated macrophages. Activation of NF-κB proceeds following the phosphorylation and degradation of the inhibitory subunit IκB. This allows NF-κB to translocate into the nucleus, where it specifically binds to target DNA elements and activates the transcription of genes that encode proteins involved in inflammation.8-9 Thus, the inhibition of the NF-κB signaling pathway may explain the potent activity of SF as a suppressor of inflammatory mediators and cytokines. The present study showed that LPS treatment induces translocation of NF-κB p65 from the cytoplasm to the nucleus and the degradation of IκB-α (Fig. 4), which is consistent with results of our previous studies in RAW 264.7 macrophages.27,30 SF also has ability to inhibit the LPS-induced nuclear translocation of NF-κB p65 and the degradation of IκB-α. These effects might arise through suppression of the phosphorylation and proteasome-mediated degradation of its inhibitor IκB-α, resulting in lower levels of NF-κB transactivation.

In addition to the NF-κB pathway, multiple lines of evidence have demonstrated that MAPKs play a critical role in regulating expression of inflammatory mediators and cytokines induced by inflammatory products such as LPS, leading to autoimmune and inflammatory diseases.10-11 Therefore, the MAPK signaling pathway is also regarded as an important molecular target for the development of potential anti-inflammatory drugs, as it has been
implicated in the regulation of various inflammatory processes.
In this regard, we investigated whether SF exerts an inhibitory effect on the phosphorylation level of ERK, JNK, and p38 in RAW 264.7 macrophages (Fig. 5). SF pretreatment markedly suppressed the LPS-induced phosphorylation of the three MAPKs, suggesting that suppression of the MAPK signal pathway might be involved in the anti-inflammatory effects of SF in the LPS-induced inflammatory response of RAW 264.7 cells.

In conclusion, our findings indicate that SF acted as an anti-inflammatory agent in RAW 264.7 macrophages via inhibition of NO/INOS pathway, as well as via inhibition of the production of pro-inflammatory cytokines, including TNF-α and IL-1β. These effects might be mediated through the inhibition of NF-κB activity and blocking of the MAPK signaling pathway in RAW 264.7 macrophages. Therefore, SF supplementation may be useful for preventing inflammatory diseases.

ACKNOWLEDGEMENTS

This work was supported by the R&D program of MOTIE/KEIT (10040991, Development of Functional Food Materials and Device for Prevention of Aging-associated Muscle Function Decrease) and High Value-added Food Technology Development Program (314043-3), Ministry of Agriculture, Food and Rural Affairs.

CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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