Anti-inflammatory effects on murine macrophages of ethanol extracts of *Lygodium japonicum* spores via inhibition of NF-κB and p38

YOUNG-CHANG CHO, BA REUM KIM, HIEN THI THU LE and SAYEON CHO

Laboratory of Molecular Pharmacological Cell Biology, College of Pharmacy, Chung-Ang University, Seoul 06974, Republic of Korea

Received February 24, 2017; Accepted June 28, 2017

DOI: 10.3892/mmr.2017.7070

**Abstract.** The spores of *Lygodium japonicum* (Thunb.) Sw. (*L. japonicum*) have been used in traditional Chinese medicine for the treatment of various inflammatory diseases. However, the molecular mechanisms underlying their anti-inflammatory effects have yet to be elucidated. In the present study, we investigated the anti-inflammatory effects of ethanol extracts of *L. japonicum* spores (ELJ) by measuring the production of inflammatory mediators, and explored the molecular mechanisms underlying the effects of ELJ in murine macrophages *in vitro* using immunoblotting analyses. At non-cytotoxic concentrations of (50-300 µg/ml), ELJ was revealed to significantly suppress the production of nitric oxide (NO) and tumor necrosis factor (TNF)-α in lipopolysaccharide (LPS)-stimulated murine RAW 264.7 macrophages; ELJ repressed the production of interleukin (IL)-6 only at high concentrations (≥200 µg/ml). The ELJ-mediated decrease in NO production was demonstrated to depend on the down-regulation of inducible NO synthase mRNA and protein expression. Conversely, the mRNA and protein expression of cyclooxygenase-2 were not affected by ELJ. In addition, ELJ was revealed to inhibit the mRNA expression of IL-6, IL-1β, and TNF-α in LPS-stimulated RAW 264.7 macrophages. The effects of ELJ on proinflammatory mediators may have been due to the stabilization of inhibitor of κBα and the inhibition of p38 mitogen-activated protein kinase (MAPK). These results suggested that ELJ may suppress LPS-induced inflammatory responses in murine macrophages *in vitro*, through the negative regulation of p38 MAPK and nuclear factor (NF)-κB. Therefore, ELJ may have potential as a novel candidate for the development of therapeutic strategies aimed at alleviating inflammation.

**Introduction**

Inflammation is an important immune response that serves to protect the host against pathogen infections or tissue injury. During inflammatory responses, macrophages are the major immune cells that regulate innate or adaptive immunity (1). Macrophages are activated by infection and tissue injury, and produce proinflammatory mediators, including interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, nitric oxide (NO), and prostaglandin (PG) E₂ (2); proinflammatory mediators then induce inflammatory responses in adjacent tissues and activate neighboring immune cells (3). The production of inflammatory mediators is regulated through intracellular signaling pathways, including mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB pathways (4,5). However, the aberrant activation of these pathways, as a result of persistent infection and continuous exposure to chemical stimuli, may lead to chronic inflammation (6,7). Chronic inflammation is induced and maintained by increased levels of proinflammatory mediators and can result in several inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, asthma, Alzheimer's disease and chronic obstructive pulmonary disease (8-10).

The spores of *Lygodium japonicum* (Thunb.) Sw. (*L. japonicum*) are also called Lygodii Spora, and are widely distributed in East Asia and Australia. The *L. japonicum* plant, its spores or its roots have been used in traditional medicine in several Asian countries, including China, Korea, India, Nepal and Pakistan, for the treatment of various diseases, including inflammatory disorders, swelling, ulcers, *Herpes* infections, wounds, dysuria, skin eczema, nephritis, enteritis, diarrhea and urinary tract infections (11-14). Ethanol extracts of *L. japonicum* spores (ELJ) have been reported to inhibit testosterone 5α-reductase and may have potential for the treatment of benign prostatic hyperplasia (13). In addition, ELJ has been revealed to significantly reduce chronic inflammation in ethylene glycol-induced kidney calculi rat models (15), while methanol extracts have demonstrated neuroprotective effects against glutamate-induced neurotoxicity (16). Several active...
components, including fatty acids, flavones, phenolic acids and phenolic glycosides, have been identified in *L. japonicum* spores and may be responsible for its effects (17-19).

Despite its widespread traditional use, the molecular mechanisms underlying the anti-inflammatory effects of *L. japonicum* have yet to be elucidated. In the present study, the anti-inflammatory effects of ELJ on the production of proinflammatory mediators were investigated in lipopolysaccharide (LPS)-stimulated murine macrophage cells, and the roles of inflammatory signal transduction pathways in the mechanisms underlying the effects of ELJ were explored in vitro.

Materials and methods

Cell culture. The murine RAW 264.7 macrophage cell line and the 293 cell line (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Little Chalfont, UK) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) throughout the study. Cells were maintained and treated at 37°C in a humidified 5% CO₂ atmosphere.

Reagents. A 95% ethanol extract of *L. japonicum* spores (cat no. PBC417A) was obtained from Korea Plant Extract Bank (KPEB, Daejeon, Korea). A stock solution (200 mg/ml) of the extract was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and stored at -20°C until use. Rabbit polyclonal anti-inhibitor of xB (IxBo; cat no. sc-371), anti-IkB kinase (IIK) α/β (cat no. sc-7607) and mouse monoclonal anti-α-tubulin (cat no. sc-5286) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit polyclonal anti-inducible NO synthase (iNOS; cat no. 2982), anti-cyclooxygenase (COX)-2 (cat no. 4842), monoclonal anti-phosphorylated (p)-IkBa (Ser32/36; cat no. 9246), monoclonal anti-p38 MAPK (cat no. 9212), anti-p-p38 (Thr182/Tyr182; cat no. 9211), anti-extra-cellular signal-regulated kinase (ERK) 1/2 (cat no. 9102), anti-c-Jun N-terminal kinase (JNK; cat no. 9252), anti-p-JNK (Thr183/Tyr185; cat no. 9251), monoclonal anti-p-IKKα/β (Ser176/180; cat no. 2697), polyclonal anti-p-MAPK kinase (MKK) 3/6 (Ser189/207; cat no. 9231), anti-MKK6 (cat no. 9264), anti-transforming growth factor β-activated kinase 1 (TAK1; cat no. 4505), monoclonal anti-p-TAK1 (Thr184/187; cat no. 4508) and mouse monoclonal anti-p-ERK1/2 (Thr202/Tyr204; cat no. 9106) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). EZ-Cytox Cell Viability, Proliferation & Cytotoxicity Assay kit was purchased from Daeil Lab Service, Co., Ltd. (Seoul, Korea). Ready-SET-Go! ELISA kits for the detection of IL-6 (cat no. 88-7064) and TNF-α (cat no. 88-7324) were obtained from eBioscience (Thermo Fisher Scientific, Inc.). Accuzol Total RNA Extraction solution was from Bioneer Corporation (Daejeon, Korea) and TOPscript cDNA Synthesis kit was from Enzymomics, Co., Ltd. (Daejeon, Korea). iTaq Universal SYBR Green Supermix was from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

RNA preparation and cDNA synthesis. RAW 264.7 macrophages were seeded into a 12-well plate at a density of 8x10⁵ cells/well and incubated at 37°C overnight. Cells were

**Enzyme-linked immunosorbent assay (ELISA).** RAW 264.7 macrophages were treated with ELJ (50, 100, 200, 300 and 400 µg/ml) for 2 h, followed by an additional 24 h of incubation at 37°C in the absence or presence of LPS (1 µg/ml). Subsequently, 25 µl EZ-Cytox solution was added to each well in 250 µl medium and cells were incubated for 1 h at 37°C. Supernatants were transferred to new 96-well plates and cell viability was measured by calculating the absorbance at 450 and 650 nm (A₄₅₀/A₆₅₀) using Synergy H1 Hybrid Microplate Reader.

**Cell viability assay.** RAW 264.7 macrophages were pre-treated with ELJ (50, 100, 200, 300 and 400 µg/ml) for 2 h, followed by stimulation with LPS (1 µg/ml) for 24 h. Subsequently, the supernatants were collected after centrifugation at 1,500 x g for 1 min at room temperature and diluted according to the predetermined dilution ratio for each proinflammatory cytokine. The production of the proinflammatory cytokines IL-6 and TNF-α was measured using commercially available ELISA kits according to the manufacturer's protocol. Briefly, a 96-well plate was coated with the coating solution at 4°C overnight, washed 3 times with 1X PBS containing 0.05% Tween-20 (PBST), and treated with 1X Assay Diluent for 1 h at room temperature. The solution was removed, and the supernatants (diluted as appropriate) and standard solutions were added to the wells. Following 2 h of treatment at room temperature, the plate was washed 3 times with 1X PBST and the absorbance was measured using a Synergy H1 Hybrid Microplate Reader.
pre-treated with ELJ (50, 100, 200 and 300 µg/ml) for 2 h and then incubated with LPS (1 µg/ml) for 3 h. Total RNA was extracted from cells using AccuZol Total RNA Extraction solution and was reverse transcribed into cDNA for 1 h at 37°C using the TOPOscript cDNA Synthesis kit, according to the manufacturer’s protocol.

Quantitative polymerase chain reaction (qPCR). PCR amplification of cDNA was performed using iTaq Universal SYBR Green Supermix according to the manufacturer’s protocol. Thermocycling conditions were as follows: Initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 5 sec and annealing/extension at 60°C for 30 sec using a CFX Connect Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). Using the 2^△△Ct method (20), gene expression was quantified and normalized to the reference genes β-actin and GAPDH; gene expression was expressed as a ratio over the expression in the LPS treated group (defined as 100%). The primers used for PCR are as follows, in accordance with our previous study (21): iNOS, sense 5'-TGG CCA CCA AGC TGA ACT-3', antisense 5'-TCA TGA TAA CGT TTC TGG CTC TT-3'; COX-2, sense 5'-GAT GCT CTT CCG AGC TGT G-3', antisense 5'-GGA TGG TTG GAA CAG CAA GGA TTT-3'; TNF-α, sense 5'-CTG TAG CCC AGC TCG TAG C-3', antisense 5'-TTG AGA TCC ATG CCG CTT-3'; IL-6, sense 5'-CTC TAT CTA TTG GAA CCA AGA G-3', antisense 5'-TGG TCC TTA GCC ACT CCT TC-3'; IL-1β, sense 5'-TTG AGC GAC CCC AAA AGA T-3'; antisense 5'-GAT GTG CTG CGT CCA GAT T-3'; β-actin, sense 5'-CGT CAT ACT CTT GCT TGG TC-3', antisense 5'-CCA CAT GTC TCC TCC TGA-3'; and GAPDH, sense 5'-GCT CTC CTC TCC TCC TGT TC-3' and antisense 5'-ACG ACC AAA TTC GTC GAT TC-3'.

Luciferase reporter assay. HEK 293 cells were seeded into a 100-mm dish. When 70% confluent, they were transfected using polyethylenimine (Polysciences, Inc., Warrington, PA, USA) as the transfection reagent for 6 h at 37°C with 4.5 µg pNF-κB-luc or pAP-1-luc reporter plasmids (Agilent Technologies, Inc., Santa Clara, CA, USA), which contained the NF-κB or activator protein 1 (AP-1) promoter, respectively and the luciferase reporter gene. The gWIZ-green fluorescent protein (GFP) plasmid was used as an internal control for transfection efficiency. Transfected cells were seeded into 12-well plates, incubated overnight at 37°C, and treated with various concentrations of ELJ (50, 100, 200 and 300 µg/ml) in the presence of phorbol 12-myristate 13-acetate (PMA), as an activator of NF-κB. Following incubation for 24 h, cells were lysed with Cell Culture Lysis Reagent (Promega Corporation, Madison, WI, USA). For determination of luciferase activity, the Luciferase Assay System (cat no. E1500; Promega Corporation) was used. GFP expression was determined by measuring the fluorescence at 525 nm following excitation at 485 nm and was used as a control for luciferase activity. The luminescence and fluorescence were measured using Synergy H1 Hybrid Microplate Reader and analyzed using Gen5 software version 1.11.5 (BioTek Instruments, Inc.).

Preparation of total cell lysates. RAW 264.7 macrophages were seeded into a 6-well plate (2x10^5 cells/well). Then, cells were pre-treated with ELJ (50, 100, 200 and 300 µg/ml) for 2 h at 37°C and stimulated with LPS (1 µg/ml) for the detection of the target proteins: For IκBα and TAK1 for 3 min; for MAPKs for 15 min; and for iNOS and COX-2 for 24 h. Following stimulation for the indicated times, cells were washed 3 times with ice-cold PBS and lysed with lysis buffer, containing 0.5% octylphenoxypolyethoxyethanol, 0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF and 1 mM Na3VO4, for 10 min at 4°C. Following centrifugation at 15,814 x g for 30 min at 4°C, the supernatants were collected.

Western blot analysis. Total protein was quantified using a Bradford protein assay, and the mixture of lysates and 5X Laemmli sample buffer (Bio-Rad Laboratories, Inc.) was boiled for 5 min at 100°C. Then, equal amounts of extracted protein samples (20 µg) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes using transfer buffer (192 mM glycine, 25 mM Tris-HCl pH 8.8 and 20% v/v methanol). Membranes were blocked for 1 h at room temperature with 5% non-fat dried milk, followed by incubation overnight at 4°C with the following primary antibodies (1:1,000 dilution): anti-IκBα, anti-IKKα/β, anti-α-tubulin, anti-iNOS, anti-COX-2, anti-p-IκBα (Ser32/36), anti-p38 MAPK, anti-p-p38 (Thr180/Tyr182), anti-ERK1/2, anti-JNK, anti-p-JNK (Thr183/Tyr185), anti-p- IKKα/β (Ser176/180), anti-p-MAPK kinase (MKK) 3/6 (Seri189/207), anti-MKK6, anti-TAK, anti-p-TAK1 (Thr184/187) and anti-p-ERK1/2 (Thr202/Tyr204). Subsequently, membranes were incubated for an additional 1 h at room temperature with HRP-conjugated goat anti-mouse (1:5,000; cat no. LF-SA8001A; Abfrontier; Young In Frontier Co., Ltd., Seoul, Korea) or goat anti-rabbit (1:5,000; cat no. LF-SA8002A; Abfrontier; Young In Frontier Co., Ltd.) secondary antibodies. After washing 5 times with 1X PBS containing 0.5% Tween-20, the immunoreactive bands were visualized using enhanced chemiluminescence. Blots were semi-quantified by densitometry using VisionWorksLS Analysis software (UVP, LLC, Upland, CA, USA).

Statistical analysis. Data are presented as the mean ± standard error of the mean of 3 independent experiments with 3 replicates each. The statistical significance of the differences between groups was assessed using one-way analysis of variance followed by a post hoc Dunnett’s test for multiple comparisons. Statistical analysis was performed using GraphPad Prism software version 3.0 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of ELJ treatment on cell viability. Since the cell-based evaluation of potential anti-inflammatory agents should be performed under non-cytotoxic conditions, the maximal non-cytotoxic concentration of ELJ was initially determined in murine RAW 264.7 macrophages. Macrophages were treated with various concentrations of ELJ in the presence or absence of stimulation with LPS. Cell viability was determined based on the ability of cells to metabolically reduce a tetrazolium salt.
to a formazan dye: As presented in Fig. 1A, ELJ did not exert cytotoxic effects at concentrations <300 µg/ml, regardless of LPS stimulation. However, cell viability was significantly reduced at a high concentration of ELJ (400 µg/ml; Fig. 1A). Therefore, ELJ concentrations <300 µg/ml were selected for subsequent experiments.

**ELJ inhibits LPS-induced iNOS expression and NO production.** Since NO serves a key role in inflammatory responses (22), the present study investigated the effects of ELJ on LPS-induced NO production. The levels of NO in RAW 264.7 macrophages were significantly decreased following treatment with ELJ in a dose-dependent manner (Fig. 1B). In addition, the mRNA and protein expression levels of iNOS in ELJ-treated macrophages were analyzed using RT-qPCR and western blot analysis, respectively, since iNOS is a key regulator of NO production during inflammatory responses (23). As presented in Fig. 1C and D, treatment with ELJ resulted in a dose-dependent downregulation in iNOS mRNA and protein expression levels in LPS-stimulated macrophages. These findings suggested that ELJ may counteract the LPS-induced increase in NO production through suppressing iNOS expression in macrophages in vitro.

**ELJ suppresses the LPS-induced production of proinflammatory mediators.** COX-2 is a proinflammatory enzyme that regulates the production of PGE$_2$ (24). RT-qPCR and western blot analysis revealed that treatment with ELJ did not affect the mRNA and protein expression levels of COX-2 in LPS-stimulated macrophages (Fig. 2A and B). These results suggested that ELJ may selectively suppress the expression of iNOS but not COX-2 in macrophages in vitro.

Proinflammatory cytokines, including IL-1β, IL-6 and TNF-α, are induced following LPS stimulation and serve pivotal roles during LPS-mediated inflammatory responses (3). Therefore, the effects of ELJ on the expression of proinflammatory cytokines were investigated at the mRNA and protein level. As presented in Fig. 2C, the production of TNF-α was significantly inhibited by ELJ in LPS-stimulated RAW 264.7 macrophages in a dose-dependent manner. In addition, treatment with high concentrations of ELJ (200-300 µg/ml) was revealed to suppress the production of IL-6 (Fig. 2D). Furthermore, RT-qPCR demonstrated that ELJ downregulated the mRNA expression of proinflammatory cytokines (Fig. 2E). These findings suggested that ELJ may exert anti-inflammatory effects, via inhibiting the production of proinflammatory cytokines in vitro.
ELJ inhibits NF-κB and p38 signaling pathways. To investigate whether the regulation of transcription may be involved in the inhibitory effects of ELJ on proinflammatory mediator production, the transcriptional activity of NF-κB and AP-1 was assessed in vitro. NF-κB and AP-1 are major transcription factors implicated in the inflammatory response; AP-1 is phosphorylated and thus activated by MAPKs (25). A luciferase reporter assay was used in HEK 293 cells treated with PMA, which induces the transcriptional activation of NF-κB- and AP-1-dependent genes (5); the luciferase reporter gene was placed under the control of NF-κB or AP-1 transcriptional activity. As presented in Fig. 3A and B, the NF-κB- and AP-1-regulated luciferase activity was significantly induced following treatment with PMA. However, luciferase activity was revealed to be suppressed following ELJ treatment in PMA-stimulated cells. These findings suggested that ELJ may exert anti-inflammatory effects through the inhibition of NF-κB and AP-1 transcriptional activity.

Since the production of proinflammatory mediators is regulated by NF-κB and MAPK signaling pathways in LPS-stimulated macrophages (4,5), the effects of ELJ on NF-κB and MAPK signaling were evaluated in the
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IκBα binds NF-κB and stabilizes it in its inactive state, whereas the phosphorylation of IκBα at Ser32/36 is the prerequisite for its dissociation from NF-κB and its subsequent recognition by ubiquitin conjugating enzyme E2 D3, leading to IκBα polyubiquitination and degradation (26,27). Western blot analysis demonstrated that ELJ suppressed the LPS-induced phosphorylation and degradation of IκBα in a dose-dependent manner (Fig. 3C). In addition, the effects of ELJ on the phosphorylation of p38, ERK and JNK, which are major mediators of MAPK signaling, were assessed. MAPK phosphorylation at the phosphorylation sites in their activation loops results in their activation, and the subsequent activation of AP-1, which binds to the promoter of inflammatory mediator genes and induces their transcription (28,29). As presented in Fig. 3D, treatment with ELJ inhibited the phosphorylation of p38 in LPS-stimulated macrophages in vitro; however, it exerted no effects on the phosphorylation of ERK and JNK, as indicated by western blot analysis. The phosphorylation status of MAPKs is directly related to their kinase activity; therefore, these results suggested that ELJ may suppress the production of proinflammatory mediators via inhibiting NF-κB and p38 signaling pathways.

To further investigate the roles of ELJ in the regulation of NF-κB and p38 signaling, its effects on the phosphorylation of IKKα/β and MKK3/6 were explored; IKKα/β and MKK3/6 are upstream kinases of IκBα and p38, respectively (30,31). As demonstrated in Fig. 4A, treatment with ELJ inhibited the LPS-induced phosphorylation of IKKα/β and MKK3/6,
without affecting the total expression levels of the proteins. However, the LPS-induced phosphorylation of TAK1, which is an upstream kinase of NF-κB and MAPKs, did not appear to be affected following ELJ treatment (Fig. 4B). These findings suggested that ELJ may target factors located downstream of TAK1 and upstream of IKKα/β and MKK3/6 in the regulation of NF-κB and p38 signaling (Fig. 5).

Discussion

During inflammation, iNOS is activated and catalyzes the synthesis of NO, which is a free radical synthesized from L-arginine that can cause cellular damage at sites of inflammation (32). In addition, activated macrophages produce proinflammatory cytokines, including TNF-α, IL-1β and IL-6 (33). Proinflammatory cytokines stimulate an increase in blood flow and capillary permeability, which can lead to immune cell infiltration, and further potentiate the inflammatory response (33). However, the aberrant excessive production of iNOS and proinflammatory cytokines, as a result of external stimuli, has been associated with the pathophysiology of severe inflammatory disorders, including sepsis and arthritis (34). Previous studies have reported that natural products which inhibit the production of proinflammatory mediators may have potential as phytomedicines for

![Figure 4](image-url) Regulatory effects of ELJ on the phosphorylation of signaling molecules upstream of nuclear factor-κB and activator protein-1. (A) Total cell lysates from RAW 264.7 macrophages pre-treated with ELJ and incubated with LPS were used for western blot analysis. p-IKKα/β, IKKα/β, p-MKK3/6 and MKK6 protein expression levels were detected. Data are presented as the mean ± SEM. **P<0.01 vs. LPS-untreated control groups; ***P<0.001 vs. LPS-untreated control groups; &P<0.05, &&P<0.01 and &&&P<0.001 vs. LPS-treated groups. (B) RAW 264.7 macrophages were treated with ELJ and further incubated with LPS for 3 and 15 min. Total cell lysates were used for western blot analysis to detect the protein expression levels of TAK1, IkBα and p38. ELJ, ethanol extract of *Lygodium japonicum* spores; LPS, lipopolysaccharide; p-, phosphorylated; IKK, inhibitor of κB kinase; MKK, mitogen-activated protein kinase kinase; SEM, standard error of the mean; TAK, transforming growth factor β-activated kinase; IkBα, inhibitor of κB.

![Figure 5](image-url) The putative molecular pathway underlying the regulatory effects of ELJ on inflammatory mediator production and activation in macrophages. ELJ may inhibit inflammatory responses via suppressing the activation of NF-κB and p38 signaling pathways in LPS-stimulated macrophages. ELJ, ethanol extract of *Lygodium japonicum* spores; NF, nuclear factor; LPS, lipopolysaccharide; TLR, Toll-like receptor; TAK, transforming growth factor β-activated kinase; MAP3K8, mitogen-activated protein kinase kinase kinase 8; IKK, inhibitor of κB kinase; MKK, mitogen-activated protein kinase kinase; IkBα, inhibitor of κB; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; p-, phosphorylated; NO, nitric oxide; IL-, interleukin; TNF, tumor necrosis factor.
the treatment of severe inflammation (35,36). In the present study, ELJ was demonstrated to attenuate the LPS-stimulated production of proinflammatory mediators in RAW264.7 macrophages in vitro, without exerting cytotoxic effects at low concentrations. These findings suggested that ELJ may have potential as an anti-inflammatory phytomedicine for the treatment of inflammatory disorders.

The results of the present study demonstrated that ELJ suppressed the expression of iNOS and the production of proinflammatory cytokines; however, it failed to inhibit COX-2 expression. The differential inhibitory effects of ELJ on proinflammatory mediators may be attributed to the selective regulation of proinflammatory signal transduction pathways. These findings are in accordance with our previous studies, which have reported that anti-inflammatory extracts, including the ethanol extract of Crataeva nurvala and the methanol extract of Xanthium sibiricum root, selectively regulated major proinflammatory signaling pathways and differentially modulated the production of proinflammatory mediators (21,37). Furthermore, previous studies have reported that the anti-inflammatory properties of several agents, including roscovitine and paenol, were associated with the selective inhibition of particular inflammatory mediators due to the differential modulation of specific inflammatory signaling pathways (38,39). However, the molecular mechanisms underlying the selective regulatory effects of ELJ on the production of proinflammatory mediators have yet to be elucidated. It may be hypothesized that the components of ELJ exhibit selectivity toward specific inflammatory signaling pathways. Previous studies have demonstrated that natural extracts regulated the production of proinflammatory mediators through the selective inhibition of NF-κB and MAPK-mediated signaling pathways, due to the different actions of the various bioactive components in the extracts (40,41). Based on the component analysis and bioassay with the extracts of interest, it may be hypothesized that the selective regulation of inflammatory responses may be attributed to the specific effects of each component in the extract. Furthermore, ELJ contains several anti-inflammatory components, including fatty acids and flavonoids (42-44), thus suggesting that the differential effects of ELJ on proinflammatory mediator production and signal transduction may be due to the selective actions of its various components.

ELJ contains fatty acids, flavonoids, phenolic acids, and phenolic glycosides (17-19). Among them, oleic acid has been reported to inhibit saturated fatty acid-induced inflammatory responses in human aortic endothelial cells through the inhibition of NF-κB signaling (45). Furthermore, linoleic acid has been revealed to exhibit anti-inflammatory effects, via inhibiting NF-κB and MAPK pathways in endothelial cells or Helicobacter pylori-infected gastric epithelial cells in vitro (46,47). The results of the present study demonstrated that NF-κB and p38 signal transduction pathways were involved in the anti-inflammatory effects of ELJ, thus suggesting that the anti-inflammatory properties of ELJ may be attributed to the oleic and linoleic acid components of the extract.

In conclusion, in the present study, ELJ was revealed to suppress the production of proinflammatory mediators, including iNOS, IL-1β, IL-6 and TNF-α, possibly through the inhibition of NF-κB and p38 signaling pathways in LPS-stimulated macrophages in vitro. Based on the ethnomedical and pharmacological use of ELJ for the treatment of inflammatory disorders and the presently demonstrated in vitro anti-inflammatory properties, it may be hypothesized that ELJ has potential as an alternative therapeutic strategy for the treatment of inflammatory conditions.

Acknowledgements

The present study was supported by the National Research Foundation of Korea funded by the Ministry of Science, ICT & Future Planning (grant nos. NRF-2015R1A2A2A11001446 and NRF-2015R1A5A1008958) and Ministry of Education, Science and Technology (grant no. NRF-2016R1A6A3A11931134).

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