Spilanthes acmella inhibits inflammatory responses via inhibition of NF-κB and MAPK signaling pathways in RAW 264.7 macrophages

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Received August 18, 2016; Accepted February 23, 2017

DOI: 10.3892/mmr.2017.6555

Abstract. *Spilanthes acmella* Murr. (*S. acmella*) has been used traditionally in India and Sri Lanka to treat various inflammatory diseases. However, the anti-inflammatory effects and underlying mechanism of action of *S. acmella* are unclear. The present study assessed the anti-inflammatory properties of methanol extracts of *S. acmella* (MSA) in murine macrophages. MSA (≤300 μg/ml) inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages through transcriptional inhibition of inducible nitric oxide synthase expression in a dose-dependent manner. Furthermore, the LPS-induced prostaglandin E2 production and cyclooxygenase-2 expression were inhibited by MSA (300 μg/ml). MSA treatment inhibited interleukin (IL)-6 production and decreased the mRNA expression levels of proinflammatory cytokines, including *IL-6* and *IL-1β*. In addition, no significant inhibition in tumor necrosis factor-α production was detected. Inhibitory effects of MSA on the production of inflammatory mediators were mediated by reduced activation of mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB. The LPS-induced phosphorylation of transforming growth factor beta-activated kinase 1, an upstream kinase of both MAPKs and NF-κB, was also inhibited by MSA treatment. Taken together, MSA inhibits the excessive inflammatory responses in LPS-stimulated murine macrophages by inhibiting the phosphorylation of MAPKs and NF-κB, implicating *S. acmella* in the treatment of severe inflammatory states based on its ethnopharmacological importance and its anti-inflammatory properties.

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Key words: *Spilanthes acmella* Murr., macrophages, lipopolysaccharide, inflammatory mediators, mitogen-activated protein kinases, nuclear factor-κB

Introduction

Inflammatory responses elicit a host defense for outer stimuli, including bacterial components and invasion of pathogens trigger sequential innate immune responses. The main immune cells that act in innate immune responses include neutrophils, dendritic cells and macrophages, whose inflammatory properties involve phagocytic action, antigen presentation and inflammatory mediator production (1). In particular, macrophages initiate and maintain inflammation through the production of inflammatory mediators, including nitric oxide (NO), prostaglandin E2 (PGE_2) and proinflammatory cytokines (2). The tight regulation of inflammatory responses is important because excess inflammatory responses cause severe inflammatory diseases, such as inflammatory bowel disease, atherosclerosis and rheumatoid arthritis. However, tightly regulated inflammation can rescue the human body from infectious diseases (3,4). Therefore, the discovery of candidates having anti-inflammatory properties is a valuable strategy for the treatment of severe inflammatory states.

Spilanthes acmella Murr. *(S. acmella)*, of the family Compositae, is a small plant found in India, Sri Lanka and other tropical countries (5). This plant has been traditionally used to treat many inflammatory diseases, including toothache, urinary calculi, wound, itching and psoriasis, and as a diuretic in India and Sri Lanka (5,6). Recent scientific approaches proved that the pharmacological effects of *S. acmella* noted its use of an anesthetic, antipyretic, anti-inflammatory, analgesic, antifungal, antimalarial, diuretic and antinociceptive, through the use of various animal models (7-10). Phytochemical analyses have revealed that *S. acmella* contains spilanthol, an isobutylamide, as a major component and secondary metabolites including β-sitosterol, stigmasterol, α-amyrin, limonene, β-caryophyllene, myrecene and vanillic acid as minor components (9,11-13). Of these, spilanthol has been reported to exhibit antimalarial and anti-inflammatory properties, however, the in-depth mechanism study was not pursued (2,14).

The ethnopharmacological anti-inflammatory effects of *S. acmella* remain unclear at the molecular level, as most studies

researched its pharmacological effects. A previous study with *S. acmella* extract was limited to the preliminary evaluation of the anti-inflammatory and analgesic effects in experimental animal models (15). In the present study, the authors investigated the anti-inflammatory effects of the methanol extract of *S. acmella* (MSA) and its precise regulatory molecular action on the inflammatory signaling pathways, mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB), in murine macrophages.

Materials and methods

MSA preparation. A methanol extract (cat. no. KRIB0043997) of the *S. acmella* from Lam Dong (Vietnam) was obtained from the International Biological Material Research Center [Korea Research Institute of Bioscience & Biotechnology (KRIBB), Daejeon, Korea]. The concentrated methanol extract was manufactured by standard protocol of KRIBB. Briefly, the leaves and stem of plants (>1 kg dry weight) were dried at room temperature (RT), treated with methanol (HPLC grade), and sonicated several times at 50˚C for 3 days. The extracts were filtrated to remove solid substances and concentrated with reduced pressure at 50˚C. To ensure dissolution of both polar and non-polar compounds in the extract and to avoid evaporation, dimethoxysulfoxide (DMSO) was used to make stock solution (200 mg/ml) of the extract. The stock extract was stored at -20˚C before use.

Cell culture and reagents. RAW 264.7 macrophages (ATCC, Manassas, VA, USA), a mouse monocytic cell line, were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (both GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in humidified air containing 5% CO_2. Rabbit anti-inhibitor of κBα (IκBα; sc-371) and anti-GAPDH (sc-25778) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit anti-inducible NO synthase (iNOS; 2982), anti-cyclooxygenase (COX)-2 (4842), anti-p-IκBα (Ser32/36; 9246), anti-p38 (9212), anti-p-p38 (Thr180/Tyr182; 9211), anti-extracellular signal-regulated kinase (ERK; 9102), anti-c-Jun N-terminal kinase (JNK; 9252), anti-p-JNK (Thr183/Tyr185; 9251), anti-transforming growth factor beta-activated kinase 1 (TAK1; 4505), anti-p-TAK1 (Thr184/187; 4508) antibodies, and mouse anti-p-ERK (Thr202/Tyr204; 9106) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Goat anti-rabbit IgG (LF-SA8002) and goat anti-mouse IgG (LF-SA8001) were purchased from AbFrontier Co., Ltd. (Seoul, Korea). DMSO was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Ez-cytox solution was purchased from Daeil Lab Service Co., Ltd. (Seoul, Korea). Ready-SET-Go! ELISA kits for the detection of IL-6 (88-7064) and tumor necrosis factor (TNF)-α (88-7324) were from eBioscience (San Diego, CA, USA). The PGE_2 ELISA kit (510410) was from Cayman Chemical Company (Ann Arbor, MI, USA). Accuzol reagent was from Bioneer Corporation (Daejeon, Korea) and TOPscript cDNA synthesis kit was from Enzynomics Co., Ltd. (Daejeon, Korea). The iTaq Universal SYBR-Green Supermix was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Measurement of cell viability. RAW 264.7 macrophages were pretreated with MSA (50, 100, 200, 300 and 400 µg/ml) for 2 h and further incubated for 24 h at 37˚C in the absence or presence of LPS (1 µg/ml). Following incubation, Ez-cytox solution (1/10 dilution of culture medium) was added to each well and incubated for 1 h. Supernatants were transferred to new 96-well plates and the absorbance was measured at 450 nm using Synergy H1 Microplate reader (BioTek Instruments, Inc., Winnoski, VT, USA).

Measurement of NO production. Cells were seeded at 96-well plates ($4.0x10^4$ cells/well) and incubated at 37˚C overnight. Cells were pretreated with various concentrations of MSA (50, 100, 200 and 300 µg/ml) for 2 h prior to LPS treatment. Following stimulation with LPS (1 µg/ml) for 24 h, the supernatants (100 µl) were transferred to new 96-well plate and 100 µl Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride and 2.5% phosphoric acid) was added to each well. $NaNO_2$ solution (2.5, 5, 10, 25, 50 and 100 M) was used to generate standard curve for calculating the quantity of NO in supernatants. The absorbance was measured at 540 nm using Synergy H1 Microplate reader (BioTek Instruments, Winooski, VT, USA).

ELISA. Cells were seeded at 96-well plates ($4.0x10^4$ cells/well) and incubated at 37˚C overnight. Cells were pretreated with various concentrations of MSA (50, 100, 200 and 300 µg/ml) for 2 h prior to LPS treatment. Following stimulation with LPS (1 µg/ml) for 24 h, the supernatants were collected and diluted according to predetermined dilution rate for each proinflammatory cytokines. The production of proinflammatory cytokines, including IL-6 and TNF-α, was measured using Ready-SET-Go! ELISA kits for each cytokines according to manufacturer's protocol. Briefly, the 96-well plate was coated with coating solution for overnight at 4˚C, washed with 1X phosphate-buffered saline/0.05% Tween 20 (PBST) for 3 times, and treated with 1X Assay Diluent (from the ELISA kit) for 1 h at room temperature. Following the emptying of the wells, diluted supernatants and standard solutions were added to each well. At 2 h after treatment at RT, the plate was washed with 1X PBST for 3 times and detection Ab solution (also from the ELISA kit) diluted in 1X Assay Diluent was added to plate. The plate was washed following a 1 h treatment, horseradish peroxidase-streptavidin solution was added for 30 min, and washed with 1X PBST 5 times. A solution of 3,3',5,5'-Tetramethylbenzidine was added to the plate and incubated for 10 min at the dark. An additional 1 N H_3PO_4 was added to the plate to stop the reaction and absorbance of each well was measured using Synergy H1 Microplate reader at 450 nm.

The production of PGE_2 was measured using PGE_2 ELISA kit according to manufacturer's protocol. Briefly, 96-well plate pre-coated with goat anti-mouse IgG was incubated with tracer, antibody and either standards or samples for 16 h. Then, the plate was washed with supplied washing buffer 5 times to remove unbound reagents and developed with Ellman's

reagent for 1 h. The absorbance of each well was measured at 405 nm and the obtained values were analyzed by performing 4-parameter logistic fit.

RNA preparation and cDNA synthesis. RAW 264.7 macrophages were seeded in a 12-well plate ($8x10^5$ cells/well) and incubated at 37˚C overnight. Cells were pretreated with MSA (50, 100, 200 and 300 μg/ml) for 2 h and additionally stimulated with LPS (1 μg/ml) for 3 h. Total RNA was prepared from the cells using Accuzol (Bioneer Corporation) and reverse-transcribed into cDNA using a TOPscript cDNA synthesis kit, according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). PCR amplification of the cDNA was performed using iTaq Universal SYBR-Green Supermix according to the manufacturer's protocol. The PCR was run for 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 thermal cycler (Bio-Rad Laboratories, Inc.). Based on the $2^{-\Delta\Delta Cq}$ method (16), the results were normalized to multi reference genes, β-actin and GAPDH, and were expressed as the ratio of gene expressions to LPS treated group (100%). PCR primers used in these experiments were listed in a previous report of the authors (17). The sequences of PCR primers used in this study were: Mouse 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 TTG GAA CAG CAA GGA TTT-3'), TNF-α (sense, 5-CTG TAG CCC ACG TCG TAG C-3'; antisense, 5'-TTG AGA TCC ATG CCG TTG-3'), IL-6 (sense, 5'-TCT AAT TCA TAT CTT CAA CCA AGA GG-3'; antisense, 5'-TGG TCC TTA GCC ACT CCT TC-3'), IL-1β (sense, 5'-TTG ACG GAC CCC AAA AGA T-3'; antisense, 5'-GAT GTG CTG CTG CGA GAT T-3'), β-actin (sense, 5'-CGT CAT ACT CCT GCT TGC TG-3'; antisense, 5'-CCA GAT CAT TGC TCC TCC TGA-3') and GAPDH (sense, 5'-GCT CTC TGC TCC TCC TGT TC-3'; antisense, 5'-ACG ACC AAA TCC GTT GAC TC-3').

Semiquantitative reverse transcription (RT)-PCR. PCR primers used in these experiments were listed in a previous report of the authors (17). The sequences of PCR primers used in the study were as follows: Mouse iNOS (sense, 5'-GCA TGG AAC AGT ATA AGG CAA ACA-3'; antisense, 5'-GTT TCT GGT CGA TGT CAT GAG CAA-3'), COX-2 (sense, 5'-GCA TGG AAC AGT ATA AGG CAA ACA-3'; antisense, 5'-GTT TCT GGT CGA TGT CAT GAG CAA-3'), TNF-α (sense, 5'-GTG CCA GCC GAT GGG TTG TAC C-3'; antisense, 5-'AGG CCC ACA GTC CAG GTC ACT G-3'), IL-6 (sense, 5'-TCT TGG GAC TGA TGC TGG TGA C-3'; antisense, 5'-CAT AAC GCA CTA GGT TTG CCG A-3'), IL-1β (sense, 5'-AGC TGT GGC AGC TAC CTG TG-3'; antisense, 5'-GCT CTG CTT GTG AGG TGC TG-3') and GAPDH (sense, 5'-GTC TTC ACC ACC ATG GAG AAG G-3'; antisense, 5'-CCT GCT TCA CCA CCT TCT TGC C-3'). The PCR was run for 17-25 cycles of 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec by using a Bioer thermal cycler (Bioer Technology Co., Hangzhou, China). Following amplification, 10 μl of the PCR products were separated in 1.5% (w/v) agarose gels and stained with ethidium bromide.

Preparation of total cell lysates. RAW 264.7 macrophages pretreated with MSA were further stimulated with LPS (1 μg/ml) for the optimized time for the detection of target proteins (IκBα and TAK1 for 3 min; MAPKs for 15 min; iNOS and COX-2 for 24 h). Following stimulation for the indicated times, cells were washed 3 times with ice-cold PBS. Lysis buffer, containing 0.5% NP-40, 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 Na_3VO_4, was added to the washed wells and then collected in each microtube after 10 min. Following centrifugation at 15,814 x g for 30 min at 4˚C, the supernatants were prepared in new microtubes.

Immunoblotting analysis. Protein concentration was measured using the Bradford reagent (Bio-Rad Laboratories, Inc.). Briefly, optical density was measured at 595 nm and the concentration of each lysate was calculated by applying the values to the bovine serum albumin standard plot. After boiling the mixture of lysates and sample buffers, aliquots of the samples (20 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes with transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), and 20% methanol (v/v)]. Following blocking with 5% non-fat dried milk, each membrane was incubated overnight at 4˚C with primary antibodies (1:1,000 dilution for all primary antibodies). Each membrane was incubated for an additional 1 h with secondary peroxidase-conjugated IgG (1:5,000) at room temperature. After washing 5 times with 1X PBST, the target proteins were detected using Pierce ECL Western Blotting Substrate for enhanced chemiluminescence (Thermo Fisher Scientific, Inc.). Protein levels were quantified by scanning the immunoblots and analyzing them with LabWorks software version 4.6 (UVP, LLC; Analytik Jena AG, Upland, CA, USA).

Statistical analysis and experimental replicates. The data are represented as means ± standard error of the mean. Comparisons between multiple experimental groups were performed using one-way analysis of variance followed by Dunnett's post-hoc test using GraphPad Prism (version 3.0; GraphPad Software, Inc., La Jolla, CA, USA) and $P<0.01$ was considered statistically significant. The data from nine replicates were analyzed, including three independent experiments with three replicates in each.

Results

MSA inhibits the production of proinflammatory mediators in RAW 264.7 macrophages. Since the inhibitory effect of an anti-inflammatory reagent should be assessed under non-cytotoxic concentrations, the authors determined the maximal effective and non-cytotoxic concentration with a cell viability assay. MSA did not induce cytotoxicity in concentrations up to 300 μg/ml. However, clear cytotoxicity was observed at 400 μg/ml for both groups with LPS (1 μg/ml) and without LPS, when compared to their respective 0 μg/ml MSA groups ($P<0.05$; Fig. 1A). Thereafter, the authors used 300 μg/ml and lower concentrations of MSA throughout the research. To evaluate the anti-inflammatory properties of MSA, the effect of MSA on the productions of

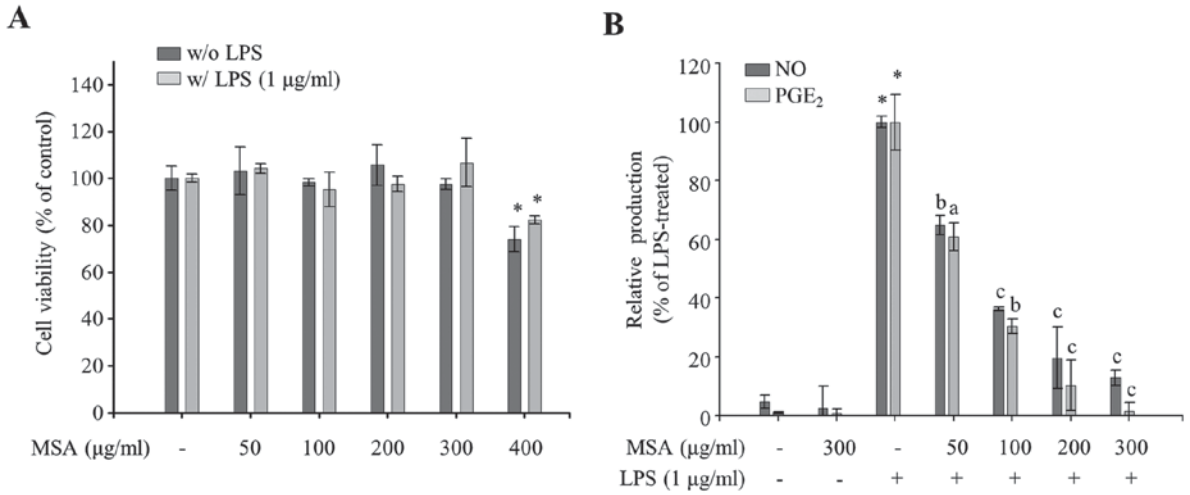

Figure 1. Effects of MSA on cell viability and the production of NO. (A) MSA-pretreated (50, 100, 200, 300 and 400 μg/ml) RAW 264.7 macrophages were incubated for 24 h in the presence or absence of LPS. Cell viability of each group was compared with that of the LPS-treated or untreated control group. Data were represented as mean ± standard error of the mean and analyzed using one-way analysis of variance. *P<0.01 vs. LPS-untreated or-treated control groups. (B) RAW 264.7 macrophages were pretreated with MSA (50, 100, 200 and 300 μg/ml) and then incubated with LPS (1 μg/ml). Following 24 h stimulation, NO and PGE_2 levels in the supernatants were measured. Relative production of NO and PGE_2 levels to LPS-treated group (100%) by MSA was represented as bar graphs. Data were represented as mean ± standard error of the mean and analyzed using one-way analysis of variance. *P<0.0001 vs. LPS-untreated control groups. ^{a}P<0.01, ^{b}P<0.001 and ^{c}P<0.0001 vs. LPS-treated groups. MSA, *S. Acmella*; NO, nitric oxide; LPS, lipopolysaccharide; PGE_2, prostaglandin E2.

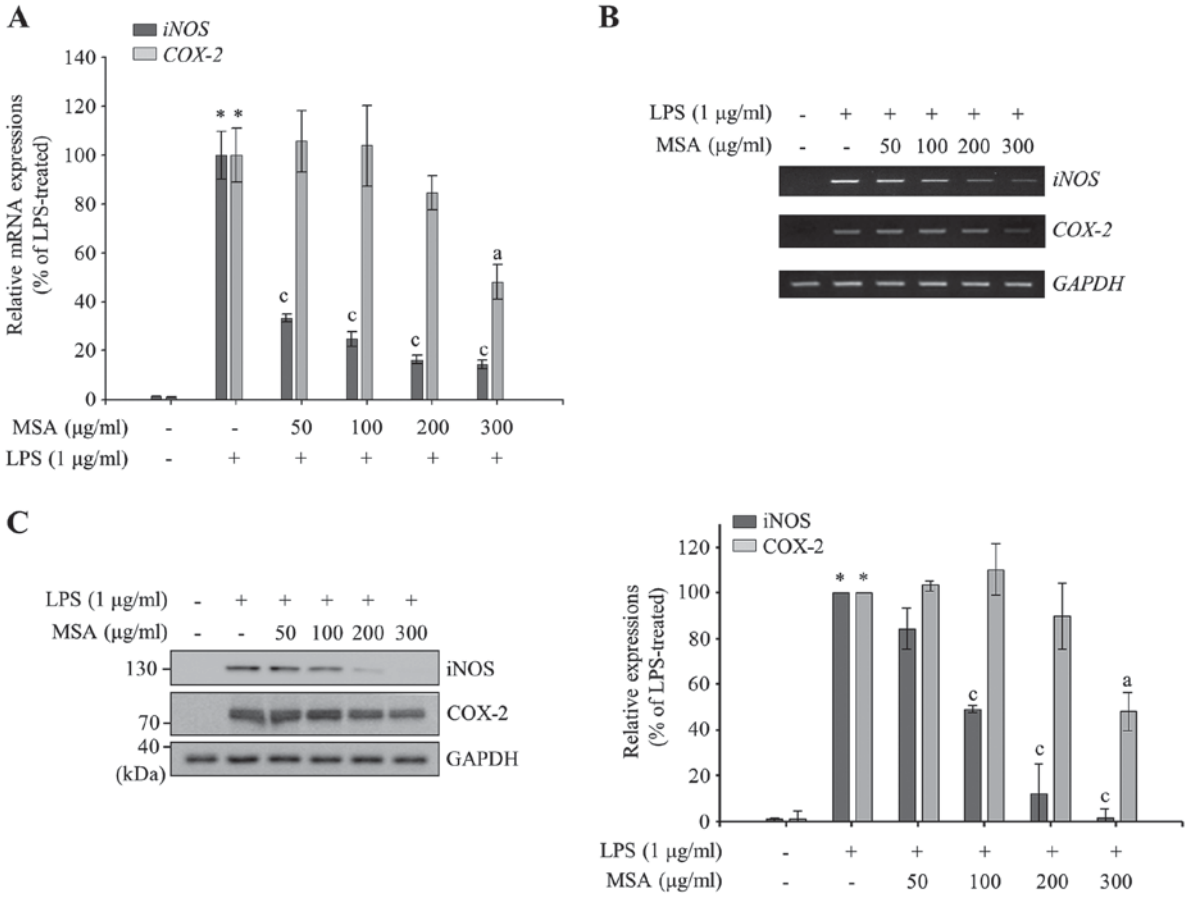

Figure 2. Inhibitory effects of MSA on the production of mRNA and protein expression levels of iNOS and COX-2. RAW 264.7 macrophages were pretreated with MSA (50, 100, 200 and 300 μg/ml) and then incubated with LPS (1 μg/ml) for the indicated times. (A) *iNOS* and *COX-2* were amplified, and the expression of *iNOS* and *COX-2* in each group was compared with that of the LPS-treated control group. Data were represented as mean ± standard error of the mean and analyzed using one-way analysis of variance. *P<0.0001 vs. LPS-untreated control groups. ^{a}P<0.01 and ^{c}P<0.0001 vs. LPS-treated groups. (B) *iNOS* and *COX-2* were amplified and detected using a gel documentation system. GAPDH served as an internal control. (C) The iNOS and COX-2 protein expressions were detected using an enhanced chemiluminescence reagent (left panel). Expression levels were quantified and normalized to the corresponding GAPDH levels. Relative expression levels of iNOS and COX-2 are represented as bar graphs (right panel). Data were represented as mean ± standard error of the mean and analyzed using one-way analysis of variance. *P<0.0001 vs. LPS-untreated control groups. ^{a}P<0.01 and ^{c}P<0.0001 vs. LPS-treated groups. MSA, *S. Acmella*; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide.

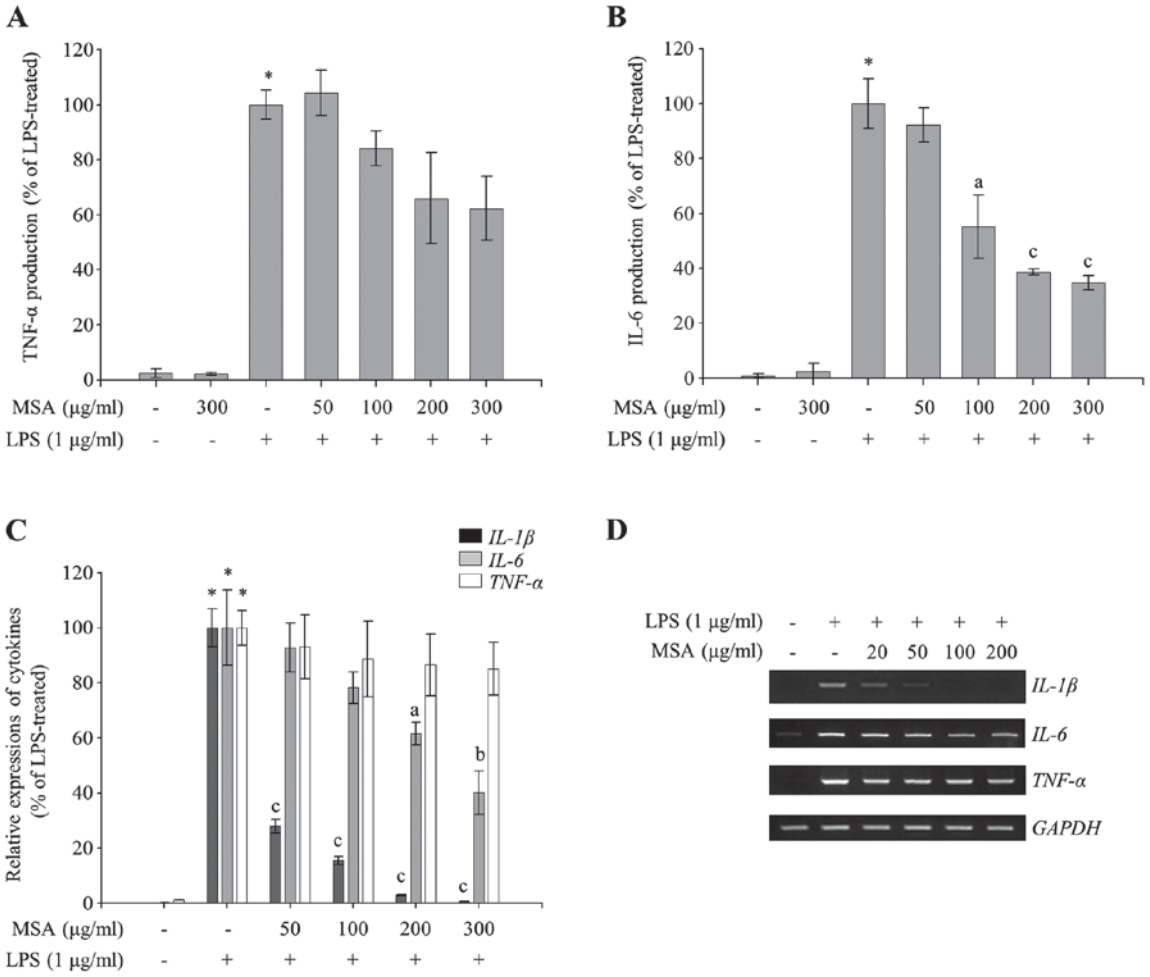

Figure 3. Inhibitory effect of MSA on the production of proinflammatory cytokines. RAW 264.7 macrophages were pretreated with MSA (50, 100, 200 and 300 μg/ml) and then incubated with LPS (1 μg/ml) for the indicated times. Following 24 h stimulation, an ELISA was used to measure levels of (A) TNF-α and (B) IL-6. The production of each cytokine was determined using a standard curve. Data were represented as mean ± standard error of the mean and analyzed using one-way analysis of variance. *P<0.0001 vs. LPS-untreated control groups. [a]P<0.01 and [c]P<0.0001 vs. LPS-treated groups. (C and D) At 3 h following stimulation, total RNA was extracted and reverse transcribed to cDNA. (C) *IL-1β*, *TNF-α* and *IL-6* were amplified and the expressions of *IL-1β*, *TNF-α* and *IL-6* in each group were compared with those of the LPS-treated group. Data were represented as mean ± standard error of the mean and analyzed using one-way analysis of variance. *P<0.0001 vs. LPS-untreated control groups. [a]P<0.01, [b]P<0.001 and [c]P<0.0001 vs. LPS-treated groups. (D) *IL-1β*, *TNF-α* and *IL-6* were amplified by PCR and detected using a gel documentation system. GAPDH was used as a loading control. MSA, *S. Acmella*; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL, interleukin.

NO and PGE_2, well-known proinflammatory mediators, were measured in LPS-treated RAW 264.7 cells. As demonstrated in Fig. 1B, MSA inhibited LPS-induced NO production in a dose-dependent manner and PGE_2 production when compared with LPS-untreated control groups. The demonstrated results imply that MSA inhibits LPS-induced NO and PGE_2 production in macrophages.

Following this, the effect of MSA was measured on the mRNA and protein expression levels of iNOS, an NO-synthesizing enzyme, and COX-2, a responsible enzyme for the production of PGE_2, to investigate the transcriptional regulation of proinflammatory mediators. As presented in Fig. 2A and B, RT-qPCR and semiquantitative RT-PCR data reveal that MSA inhibits LPS-induced *iNOS* and *COX-2* mRNA expression in a dose-dependent manner. The immunoblotting analysis with iNOS and COX-2 specific antibodies indicated an inhibitory effect of MSA on the LPS-induced iNOS and COX-2 expression similar to PCR results (Fig. 2C), indicating that NO and PGE_2 productions are tightly regulated at the level of transcription by MSA. It is of interest to note that iNOS production is regulated more strongly than COX-2 by MSA.

MSA selectively inhibits production of proinflammatory cytokines in LPS-treated RAW 264.7 macrophages. Since excessive production of inflammatory mediators, including IL-1β, IL-6, and TNF-α as well as NO and PGE_2, in macrophages is accompanied by severe inflammation (18-20), the effect of MSA on the production of proinflammatory cytokines in LPS-treated RAW 264.7 macrophages was investigated to evaluate other anti-inflammatory properties of MSA. As demonstrated in Fig. 3B, MSA inhibited LPS-induced production of IL-6 in a dose-dependent manner. The mRNA expression levels of LPS-induced proinflammatory cytokines resulting from MSA treatment were evaluated by RT-qPCR and semiquantitative RT-PCR to investigate whether the production of inflammatory cytokines was tightly regulated at the transcription level. As demonstrated in Fig. 3C and D, the mRNA expressions

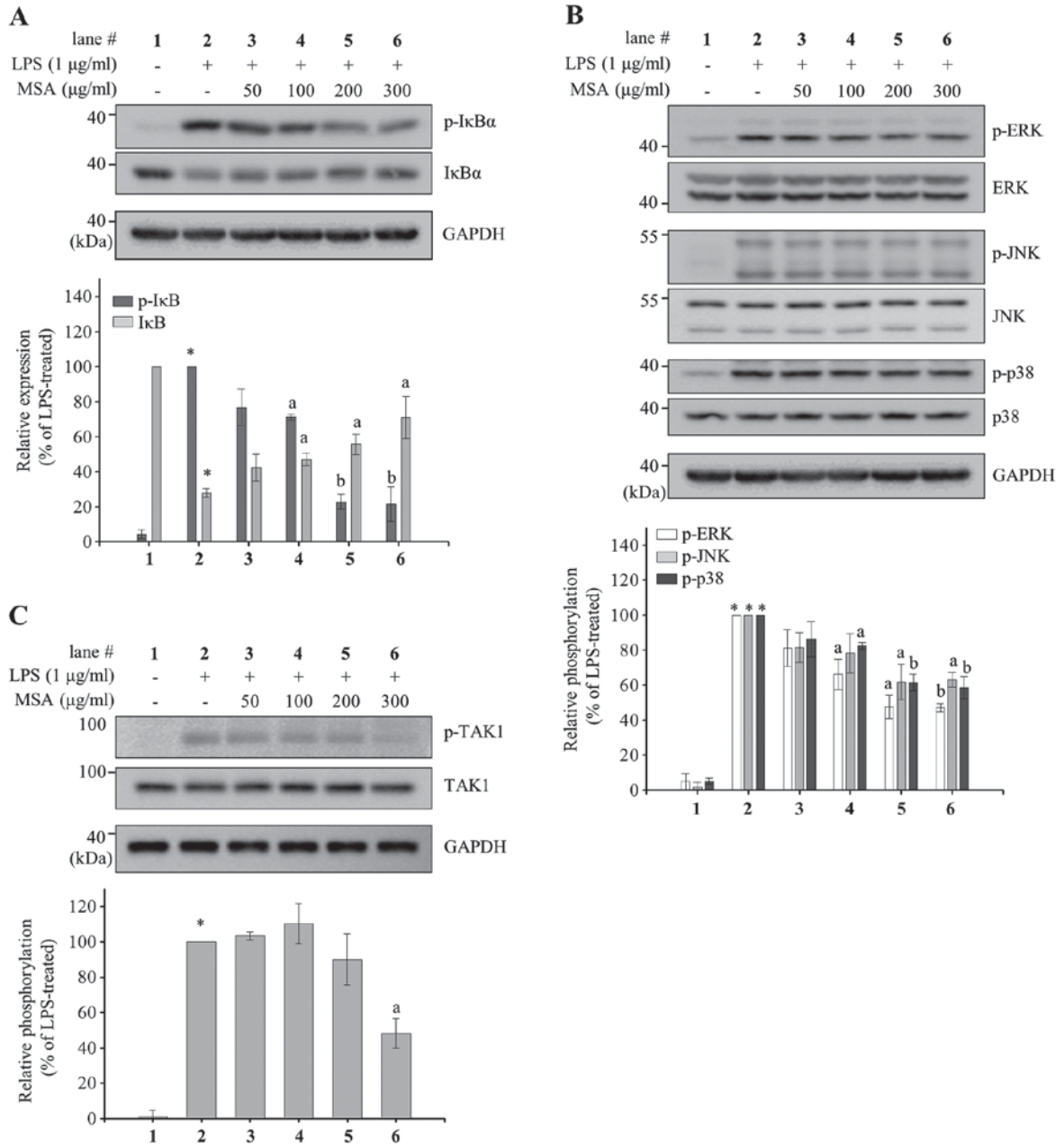

Figure 4. Inhibitory effects of MSA on NF-κB and MAPK. RAW 264.7 macrophages were pretreated with various concentrations of MSA (50, 100, 200 and 300 μg/ml) for 2 h and then incubated with LPS for 3 min (for detection of IκBα and TAK1) or 15 min (MAPKs). Total cell lysates were prepared and subjected to immunoblot analyses. The expression levels of (A) p-IκBα, IκBα, (B) p-JNK, JNK, p-ERK, ERK, p-p38, p38, (C) p-TAK1 and TAK1 were detected using specific antibodies. Relative expression levels of IκBα and p-IκBα were normalized to GAPDH levels. Levels of phosphorylated MAPKs and TAK1 were normalized to the corresponding MAPK and TAK1 levels. Quantitative analyses of phosphorylation and protein levels are shown as bar graphs following normalization. Data are represented as mean ± standard error of the mean and analyzed using one-way analysis of variance. *P<0.0001 vs. LPS-untreated control groups. ^{a}P<0.01 and ^{b}P<0.001 vs. LPS-treated groups. MSA, *S. Acmella*; NF-κB; nuclear factor-κB; MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; IκBα, inhibitor of κBα; TAK1, transforming growth factor beta-activated kinase 1; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.

of IL-6 and IL-1β were decreased with MSA treatment. LPS-induced TNF-α production (Fig. 3A) and its mRNA expression (Fig. 3C and D) were only slightly alleviated by MSA (P>0.05). These results indicated that MSA inhibits the production of the proinflammatory cytokines, including IL-1β and IL-6, whereas TNF-α production was weakly regulated by MSA in LPS-treated macrophages.

MSA inhibits phosphorylation of IκBα and MAPKs in LPS-treated RAW 264.7 macrophages. The major regulatory signaling pathways for the production of inflammatory mediators are NF-κB and MAPK. To assess whether MSA regulates NF-κB activation, MSA-mediated IκBα phosphorylation levels and total IκBα levels were measured in LPS-treated RAW 264.7 cells. The p-IκBα levels were reduced by MSA in a dose-dependent manner and IκBα levels was increased by the MSA treatment (Fig. 4A), suggesting that MSA inhibits IκBα phosphorylation at Ser-32/36 and thus causes degradation of IκBα. In addition, the phosphorylation levels in the phosphorylation loops of three MAPKs (ERK, JNK, and p38)

were measured; these are involved in another major inflammatory signaling pathway by MSA in LPS-treated RAW 264.7 cells. This is because the phosphorylation in the phosphorylation loops of MAPKs leads to the activation of MAPKs. As indicated in Fig. 4B, MSA inhibited the phosphorylation of all MAPKs without changing total MAPK levels, although its effects on MAPKs are weaker than on IκBα.

Further investigations were conducted to elucidate the action point of MSA in LPS-treated RAW 264.7 cells. Since MSA inhibits both NF-κB and MAPKs, the authors investigated the regulation of TAK1 phosphorylation level by MSA treatment in LPS-stimulated RAW 264.7 cells. As demonstrated in Fig. 4C, LPS-induced phosphorylation of TAK1 was inhibited by MSA treatment without changing total TAK1 protein levels. As presented in Fig. 4C, LPS-induced phosphorylation of TAK1 was inhibited by MSA treatment without changing total TAK1 protein levels.

Discussion

The production of all of inflammatory mediators measured in the current study was inhibited by MSA treatment whereas TNF-α was not significant. Therefore, the authors assumed that the major inflammatory signaling pathways, including NF-κB and MAPKs, are involved in the MSA-mediated regulation of the production of inflammatory mediators, although each inflammatory mediator has different regulatory factors for those activation. LPS-induced NF-κB activation in macrophages is primarily mediated by IκBα phosphorylation at Ser-32/36, followed by IκBα degradation and the translocation of released cytoplasmic p50 and p65 complex to the nucleus (21-23). The phosphorylation levels in the phosphorylation loops of three MAPKs (ERK, JNK and p38) that are involved in another major inflammatory signaling pathway (24) in LPS-treated macrophages leads to the activation of MAPKs, which results in the transcriptional activation of activator protein-1, a transcription factor that binds to the promoter of inflammatory mediators (25). As indicated in Fig. 4A and B, both NF-κB and MAPK activation were tightly regulated by MSA treatment, indicating that MSA exhibits its anti-inflammatory properties in macrophages through inhibiting the activation of major inflammatory signaling pathways, NF-κB and MAPKs.

LPS binding to toll-like receptor 4 leads to the recruitment of accessory molecules including myeloid differentiation primary response 88, interleukin-1 receptor-associated kinase 1 and TNF receptor associated factor 6, and this complex formation induces phosphorylation and activation of transforming growth factor β-activated kinase 1 (TAK1) (26,27). Activated TAK1 through phosphorylation at its activation loop induces the cascades for the activation of both NF-κB and MAPKs (28). MSA treatment inhibited LPS-induced phosphorylation of TAK1 (Fig. 4C). This result implies that the inhibitory properties of MSA on NF-κB and MAPKs are due to the suppression of TAK1 or its upstream signaling molecules.

Regulatory mechanisms of many natural extracts for their anti-inflammatory effects are primarily focused on the regulation of NF-κB and MAPK signaling pathways. However, the targeted inflammatory mediators and action points of each extract are relatively selective when compared to the inhibitory properties of single compounds. Based on many studies for the anti-inflammatory properties by natural extracts, the selective inhibitory effects are due to multiple components that show anti-inflammatory effects. A recent report has revealed that mulberry fruit extract inhibits acute colitis by selective inhibition of the NF-κB and ERK pathways (29). The authors reported that the anti-inflammatory effects of mulberry fruit extract are due to its specific compounds, including linoleic acid and ethyl linolenate. Another study revealed that the analgesic and anti-inflammatory effects of *Litsea japonica* fruit are regulated by the inhibition of NF-κB, p38 and JNK (30). Hamabiwalactone A and Hamabiwalactone B were demonstrated to be the major components of the analgesic and anti-inflammatory effects. In the current study, MSA inhibits the production of inflammatory mediators by suppressing both NF-κB and MAPKs through the inhibition of the upstream kinase, TAK1. Taken together, the presented results suggested that the anti-inflammatory effects of MSA are primarily mediated by a single compound, since the regulation was conducted at a specific action point, TAK1.

In conclusion, MSA exhibits anti-inflammatory properties by inhibiting the production of various inflammatory mediators through the regulation of both NF-κB and MAPK signaling pathways. Although more studies are required to elucidate concise action mechanism for the anti-inflammatory effects of MSA, these results suggested that MSA may be a valuable candidate as an alternative medicine for the treatment of severe inflammation states.

Acknowledgements

The present research was supported by the National Research Foundation of Korea grant funded by the Ministry of Science, ICT & Future Planning (grant no. NRF-2015R1A2A2A11001446) and by a Chung-Ang University Research Scholarship grant.

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