Synedrella nodiflora (Linn.) Gaertn. inhibits inflammatory responses through the regulation of Syk in RAW 264.7 macrophages

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Received September 11, 2019; Accepted March 03, 2020

DOI: 10.3892/etm.2020.8750

Abstract. *Synedrella nodiflora* (Linn.) Gaertn. (*S. nodiflora*) has long been used for the treatment of inflammatory diseases, including liver disease, asthma, rheumatism and earache, in tropical countries throughout America, Asia and Africa. However, the biological effects of *S. nodiflora* have not been extensively studied at the molecular level. Notably, it remains unclear how *S. nodiflora* exerts anti-inflammatory activity. In the present study, the anti-inflammatory mechanism of a methanol extract of *S. nodiflora* (MSN) in RAW 264.7 macrophages activated by lipopolysaccharide (LPS) was investigated. Non-cytotoxic concentrations of MSN (≤400 μg/ml) decreased the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which resulted in a decrease in nitric oxide and prostaglandin E_2 (PGE_2) production. The mRNA expression of pro-inflammatory cytokines such as interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α was reduced upon MSN treatment. In addition, the activation of spleen tyrosine kinase (Syk) and Akt was suppressed by MSN. Taken together, these findings recommend the traditional medicinal application of *S. nodiflora* in the treatment of several inflammation-associated diseases and indicate the possibility of MSN as a novel therapeutic reagent of inflammation-related diseases.

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Key words: synedrella nodiflora, macrophages, lipopolysaccharide, inflammatory mediators, nuclear factor-κB, spleen associated tyrosine kinase

Introduction

A living organism continually faces intrusion by various pathogens such as bacteria, viruses and fungi. Inflammatory reactions occur to protect the organism from extracellular threats (1). The intensity and duration of inflammatory responses are dependent on the balance between pro-inflammatory and anti-inflammatory mediators (2). Therefore, an imbalance in these mediators causes severe cellular damage that can lead to chronic inflammatory disorders, including rheumatoid arthritis, autoimmune diseases, cancers and atherosclerosis (3). Macrophages function as a first alarm of the defense system against common pathogens and are activated in response to cytokines and pathogen-associated molecular patterns such as lipopolysaccharide (LPS), flagellin, and lipoteichoic acid (4-6). Toll-like receptor 4 (TLR4) at the surface of immune cells recognizes LPS, which is a cell-wall component of gram-negative bacteria (7). In the presence of LPS, activated macrophages induce a wide range of pro-inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, nitric oxide (NO), and prostaglandin E_2 (PGE_2), to stimulate other immune cells (8,9). Pro-inflammatory cytokines exert biological activities related to acute or chronic inflammatory diseases (10). TNF-α is a potent stimulator of inducible nitric oxide synthase (iNOS) expression in vascular smooth muscle cells (11). When activated, iNOS produces a high concentration of NO, a regulatory molecule of various physiological functions such as vasodilatation (12). Another major event during inflammation is the arachidonic acid cascade produced from membrane phospholipids (13). Arachidonic acid is degraded by cyclooxygenase (COX) to prostaglandins (PGs), which mediates inflammatory responses (13). COX-2 is induced by cytokines and is responsible for releasing a high concentration of PGE_2 (14,15). Both NO and PGE_2 are involved in pain-induction and -perception (16). These inflammatory mediators function to protect the host cells from infection. However, the unnatural expression or activation of inflammatory mediators is associated with inflammatory

disease (17,18). RAW 264.7 is a monocyte/macrophage-like Abelson leukemia virus transformed cell line derived from BALB/c mice. This cell line is a commonly used model of mouse macrophages for the study of cellular responses to anti-inflammatory extracts. Upon stimulation with LPS, RAW 264.7 cells increase nitric oxide (NO) production and enhance phagocytosis. Furthermore, these cells are able to kill target cells by antibody-dependent cytotoxicity (19). The RAW 264.7 cell line is designed to grow and differentiate more stably than other macrophage cells. These cells are suitable for monitoring anti-inflammatory mechanisms mediated by various cytokines or LPS (20).

In folk medicinal remedies, *Synedrella nodiflora* (Linn.) Gaertn. (*S. nodiflora*), which belongs to the Asteraceae family, has been used for the prevention and treatment of diverse diseases in Asia. It is a traditional plant of tropical America and is also found in Ghana, India, China, Malaysia, Japan, and other Indopacific countries (21). In Ghana, traditional medical practitioners have used aqueous extracts of *S. nodiflora* for epilepsy and pain management after boiling the whole plant (22). *S. nodiflora* has been treated as an external medicine to cure earache, headache, and inflammation in Malaysia (23). The leaves of *S. nodiflora* have also been used to treat stomachache and hiccup and in threatened abortion cases (24). Furthermore, a methanol extract of *S. nodiflora* leaves (MSN) proved to have insecticidal, sedative, anti-oxidative, anti-convulsant, and anti-inflammatory effects (25,26). The components of MSN are already well reported by many researchers (21,27,28). The phytochemical profile indicates that extracts of *S. nodiflora* contain alkaloids, flavonoids, triterpenes, saponins, simple phenolics, glycosides, and polyose (29,30). Glycosides suppress the expression of inflammatory mediators via TNF-α inhibition (31). Triterpenes inhibit nuclear factor (NF)-κB-regulated gene expression and transforming growth factor-β-activated kinase 1 (TAK1)-mediated NF-κB activation (32). In general, flavonoids regulate the inflammatory responses associated with activating protein-1 or NF-κB, thereby suppressing chronic inflammatory diseases (33,34). Several researchers have reported that total alkaloids show anti-inflammatory effects and regulate proto-oncogene tyrosine-protein kinase (Src)/spleen tyrosine kinase (Syk) of NF-κB signaling (35). Saponins have been reported to suppress the inflammatory response by inhibiting the PI3K/Akt signaling pathway in macrophages (36).

Although *S. nodiflora* has been evaluated for its pharmacological activities, there has been no systematic study of the mechanisms underlying the anti-inflammatory effects of MSN. Therefore, this study focused on the analysis of the potential anti-inflammatory effects of MSN at the protein level in macrophages activated by LPS.

Materials and methods

MSN preparation. S. nodiflora was collected from the Slamet Mountains, Central Java, Indonesia. Plant samples were collected and identified by staff at the Center for Pharmaceutical and Medical Technology (PTFM), and verified at the Herbarium Bogoriense (LIPI). Voucher specimens recorded as KRIB 0039477 and PMT 1171, were deposited in the herbarium (KRIB) of the Korean Research Institute of Bioscience and Biotechnology (Daejeon, Korea) as well as in the Center for Pharmaceutical and Medical Technology (PTFM) and the Herbarium Bogoriense. The extract was deliquesced in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) and added to the culture media to the final concentration as indicated. It was confirmed that *S. nodiflora* is not a protected or endangered species (37,38).

Cell culture and reagents. RAW 264.7 macrophages were purchased from ATCC. RAW 264.7 cells were cultured under the following condition: 10% fetal bovine serum (FBS, Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2. LPS for activation of RAW 264.7 cells was purchased from Sigma-Aldrich; Merck KGaA.

Cell viability assay. RAW 264.7 cells were seeded in 96-well plates ($4.5x10^4$ cells/well), pre-treated with MSN (100, 200, 300, 400 and 600 μg/ml) for 2 h, and then incubated with LPS (1 μg/ml) at 37˚C for 24 h. The cell viability was measured using the EZ-Cytox cell viability assay kit (Daeil Tech Co., Ltd.) according to the manufacturer's instructions. Cell viability was calculated following the absorbance for viable cells at 450 nm and reference absorbance at 650 nm (A_{450}-A_{650}) with the Synergy H1 Microplate Reader (BioTek Instruments, Inc.).

Nitrite assay. Cells ($4.5x10^4$ cells/well; 96-well plate) were incubated with MSN (100, 200, 300 and 400 μg/ml) for 2 h and then with LPS (1 μg/ml) at 37˚C for 24 h. Nitrite assay was performed as described in a previous study (39).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RAW 264.7 cells ($2x10^5$ cells/well; 12-well plate) were pre-treated with MSN (100, 200, 300 and 400 μg/ml) for 2 h and activated by LPS (1 μg/ml) for 3 h at 37°C. Total RNA preparation, cDNA synthesis, and quantification of mRNA were performed as previously described (39). Quantification of gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (40). Calculated gene expression was normalized to reference genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin. The sequences of the PCR primers are listed in a previous study (41).

Enzyme-linked immunosorbent assay (ELISA). RAW 264.7 macrophages were seeded in 96-well plates ($4.5x10^4$ cells/well) and incubated at 37˚C overnight. The cells were pre-treated with MSN (100, 200, 300 and 400 μg/ml) for 2 h and then incubated with LPS (1 μg/ml) at 37˚C for 24 h. Culture supernatants were collected by centrifugation at 1,500 x g for 1 min at room temperature (RT). ELISA kit for the detection of IL-6 (cat. no. 88-7064) and TNF-α (cat. no. 88-7324) were from eBioscience (Thermo Fisher Scientific, Inc.). TNF-α and IL-6 levels in cell supernatants were measured using sandwich ELISA with monoclonal antibodies specific to each mediator according to the manufacturer's instructions. Briefly, a 96-well-ELISA plate was pre-coated with the capture antibody at 4˚C for overnight. The plate was washed 4 times with

1X phosphate-buffered saline (PBS)/5% Tween 20 (PBST) and blocked with 1X assay diluent at RT for 1 h. Then, 100 μl of the sample was added to each well and incubated at RT for 2 h. Subsequently, a biotinylated detection antibody solution was added at RT for 1 h. After this, the plate was treated with HRP-streptavidin solution at RT for 30 min, and then 100 μl of 3,3',5,5'-tetramethylbenzidine was added for further incubation at RT for 10 min in the dark. The further reaction was blocked by adding 50 μl of 1N H_3PO_4. The absorbance was measured (450 nm) with a Synergy H1 Microplate Reader. The concentrations of the secreted cytokines were calculated based on a standard curve.

Preparation of total cell lysates. RAW 264.7 cells were treated with MSN (100, 200, 300 and 400 μg/ml) at 37˚C for 2 h and then stimulated with LPS (1 μg/ml) at 37˚C for 3 min (for IκBα, Src, Syk, Akt, TAK1), 15 min (for MAPKs), or 24 h (for iNOS and COX-2), and subsequently washed twice with cold PBS (pH 7.4). Cells were collected and lysed in lysis buffer containing 150 mM NaCl, 20 mM trisaminomethane hydrochloride (Tris-HCl) (pH 8.0), 0.5% IGEPAL CA-630 (NP-40), 1 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, 1% glycerol, 10 mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate. The lysates were centrifuged at 15,814 x g at 4˚C for 30 min. Supernatants were transferred to a new tube.

Immunoblot analysis. Briefly, the protein concentration was measured using Bradford protein assay (Bio-Rad) according to the manufacturer's instructions. Aliquots of cell lysates were mixed with 5X sodium dodecyl sulfate (SDS)-polyacrylamide sample buffer including 12 mM Tris-HCl (pH 6.8), 5% glycerol, 1% β-mercaptoethanol, 0.4% SDS, and 0.02% bromophenol blue and boiled at 95˚C for 5 min. Samples were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (GE Healthcare). The membranes were blocked with 5% nonfat-dried skim milk in 1X TBST solution, followed by incubation at 4˚C overnight with the following primary antibodies: Mouse polyclonal anti-p38 (cat no. sc-7972), mouse monoclonal anti-c-Jun N-terminal kinase (JNK; cat no. sc-7345), rabbit polyclonal phosphorylated (p)-anti-IκBα (Ser32/36; cat no. sc-101713), mouse polyclonal anti-spleen tyrosine kinase (Syk; cat no. sc-1240), mouse monoclonal anti-c-proto-oncogene tyrosine-protein kinase Src (c-Src; cat no. sc-19), rabbit polyclonal anti-p-c-Src (Tyr424; cat no. sc-81521), rabbit polyclonal anti-Akt1/2/3 (cat no. sc-8312), rabbit polyclonal anti-p-Akt1/2/3 (Ser473; cat no. sc-7985), goat polyclonal anti-cyclooxygenase 2 (COX-2; cat no. sc-1745), rabbit polyclonal anti-inducible NO synthase (iNOS; cat no. sc-651) and mouse monoclonal anti-α-tubulin (cat no. sc-5286) antibodies were purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal transforming growth factor-β-activated kinase 1 (TAK1; cat no. 4505), rabbit monoclonal anti-p-TAK1 (Thr184/187; cat no. 4508), rabbit polyclonal anti-p-p38 (Thr180/Tyr182; cat no. 9211), rabbit polyclonal anti-extracellular signal-regulated kinase (ERK; cat no. 9102), rabbit monoclonal anti-p-ERK (Thr202/Tyr204; cat no. 9106), rabbit polyclonal anti-p-JNK (Thr183/Tyr185; cat no. 9252) and rabbit polyclonal anti-p-Syk (Tyr525/526; cat no. 2711) antibodies were purchased from Cell Signaling Technology, Inc. All primary antibodies were diluted to 1:1,000 in 5% non-fat dried milk. Each membrane was washed 4 times with 1X TBST and incubated with the following secondary antibodies: Polyclonal anti-rabbit IgG-HRP (1:5,000; cat no. LF-SA8002) and polyclonal anti-mouse IgG Fc-HRP (1:5,000; cat no. LF-SA8001) were from AbFrontier; Young In Frontier Co., Ltd., at RT for 2 h. Each protein level was visualized using an enhanced chemiluminescence (ECL) immunoblotting detection reagent (Pierce; Thermo Fisher Scientific, Inc.). Protein levels were scanned and analyzed with LabWorks v.4.0 software (UVP Inc.).

Statistical analysis. All experiments were performed 3 times. The results are represented as means ± standard error of the mean (SEM). Differences between experimental conditions were assessed using one-way ANOVA followed by Tukey's test in Prism v.3.0 (GraphPad Software, Inc.). $P<0.05$ was concidered to indicate a statistically significant difference.

Results

Effect of MSN on cell viability. Since ethnopharmacological records indicate that *S. nodiflora* has been shown to exhibit potent anti-inflammatory effects (42), MSN was evaluated to identify its molecular mechanisms of action in the present study. To determine the maximal non-cytotoxic concentration of MSN, a cell viability assay was performed. RAW 264.7 cells were treated with MSN in the presence or absence of LPS. The cell viability was not altered until treatment with 400 μg/ml of MSN (Fig. 1A). When cells were treated with 600 $\mu\mu$g/ml of MSN, cell viability was significantly reduced. Therefore, concentrations <400 μg/ml of MSN were used in the subsequent experiments.

Inhibitory effect of MSN on the production of nitric oxide (NO) in LPS-stimulated macrophages. NO is an essential cellular signaling molecule involved in diverse physiological and pathological processes in mammals (43). In the inflammatory response, NO levels are increased due to induced iNOS in cells, and the produced NO plays a dual role in immune and inflammatory responses (44). To investigate the anti-inflammatory effects of MSN, the MSN-regulated NO expression was examined in LPS-stimulated macrophages that showed inflammatory responses. MSN effectively inhibited NO production in a dose-dependent manner (Fig. 1B). Since iNOS is the essential enzyme of NO production, iNOS expression at the mRNA and protein levels following MSN treatment was evaluated. RT-qPCR analysis showed that upregulated *iNOS* expression by LPS stimulation was suppressed following MSN treatment (Fig. 1C). In addition, immunoblot analysis showed that increased protein expression of iNOS by LPS treatment was dose-dependently reduced by MSN (Fig. 1D). These results suggest that MSN negatively regulates NO production by suppression of iNOS at the transcriptional level.

Inhibitory effect of MSN on the production of pro-inflammatory mediators. Since COX-2 catalyzes the production of PGE_2, which causes fever, diarrhea, and excessive uterine contraction (45), the anti-inflammatory effects of MSN were assessed by evaluating mRNA and protein expression of

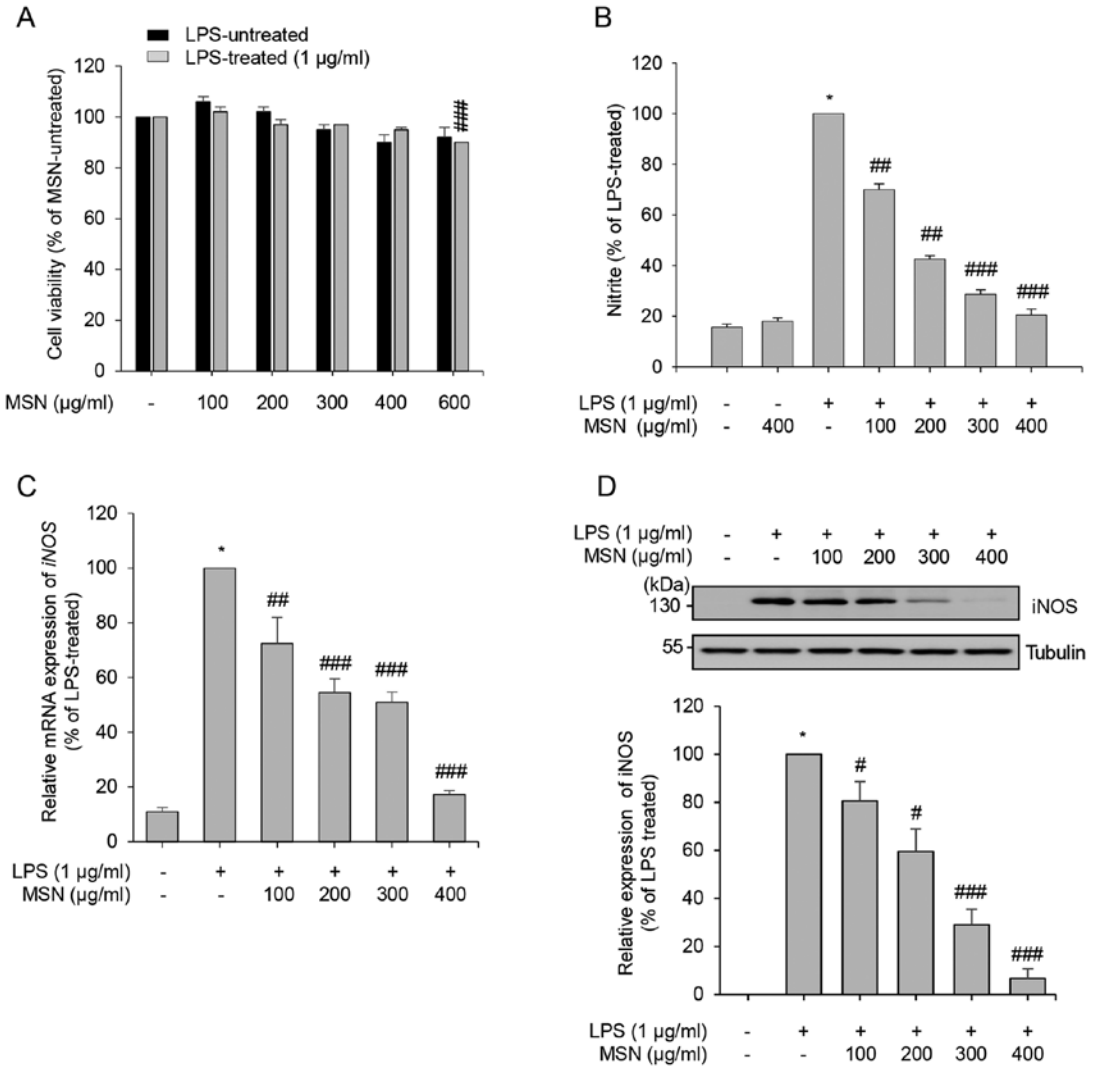

Figure 1. Effect of MSN on cell viability. (A) RAW 264.7 macrophages were treated with MSN (100, 200, 300, 400 and 600 μg/ml) at 37˚C for 2 h and incubated in the absence or presence of LPS (1 μg/ml) at 37˚C for an additional 24 h. The cell viability was examined using EZ-Cytox assay kit. Cell viability of each group was calculated based on the LPS-treated or LPS-untreated control group. (B) RAW 264.7 macrophages were pre-treated with MSN (100, 200, 300, and 400 μg/ml) at 37˚C for 2 h and then stimulated with LPS (1 μg/ml). After 24 h stimulation, the levels of NO secretion in the culture media were measured using Griess reagent. The amounts of NO secretion were calculated according to a standard curve according to a nitrite standard solution. (C) Total cell lysates were collected after 24 h LPS stimulation, and RT-qPCR analysis was performed. Relative expression levels of iNOS are represented as a bar graph. (D) After 3 h LPS stimulation, iNOS was analyzed by immunoblotting. The expression of *iNOS* was detected using ECL reagent and quantified by analysis with LabWorks software. All the protein levels were normalized to corresponding tubulin levels. Data are represented as the mean ± SEM. *P<0.01 vs. LPS-untreated control group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS-treated and MSN-untreated control group. MSN, methanol extract of *Synedrella nodiflora* (Linn.) Gaertn.; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase.

COX-2. LPS-induced expression of *COX-2* gene and COX-2 protein was suppressed by MSN in a dose-dependent manner (Fig. 2A and B). Therefore, these results suggest that MSN regulates COX-2 in LPS-activated macrophages through the inhibition of the *COX-2* gene and COX-2 protein expression.

To further investigate the anti-inflammatory effects of MSN, the production of pro-inflammatory cytokines, which are induced by LPS in macrophages, was measured in the presence or absence of MSN. As shown in Fig. 2C and D, pro-inflammatory cytokines including IL-6 and TNF-α were significantly increased upon stimulation with LPS and decreased by MSN treatment. The effect of MSN on the gene expression of pro-inflammatory cytokines, including IL-6, IL-1β, and TNF-α, was also analyzed using RT-qPCR. The mRNA expression of the pro-inflammatory cytokine genes was inhibited by MSN in LPS-activated RAW 264.7 macrophages (Fig. 2E). Taken together, these results suggest that MSN suppresses the LPS-induced production of pro-inflammatory cytokines by inhibition of *IL-6*, *IL-1β*, and *TNF-α* transcription.

Differential inhibitory role of MSN on MAPK and NF-κB in RAW 264.7 macrophages. NF-κB and mitogen-activated protein kinase (MAPK) regulate the genes involved in inflammatory responses of RAW 264.7 cells (46). Phosphorylation at Ser32/36 of IκBα was found to lead to the degradation of IκBα bound to NF-κB, and freed and activated NF-κB translocated to the nucleus, inducing transcription of target inflammatory genes (47). We, therefore, measured the phosphorylation levels at Ser32/36 of IκBα and the protein level of IκBα to examine whether MSN caused a change in IκBα

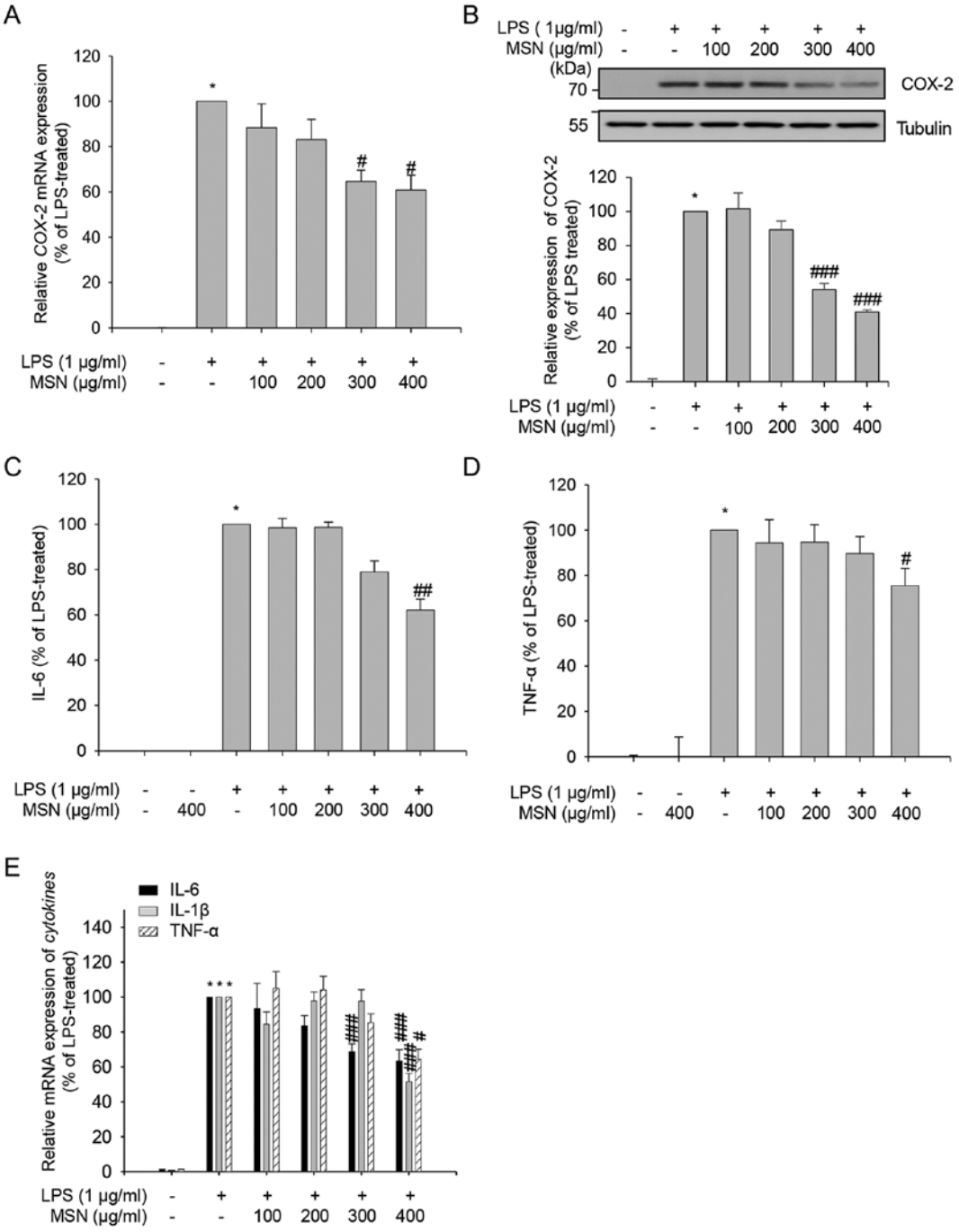

Figure 2. Inhibitory effects of MSN on pro-inflammatory mediators. RAW 264.7 macrophages were pre-treated with MSN (100, 200, 300, and 400 μg/ml) at 37˚C for 2 h and then stimulated with LPS (1 μg/ml) for an indicated time. (A) After 3 h LPS stimulation, COX-2 was amplified by RT-qPCR, and the expression of each group was compared to that in the LPS-treated group. (B) Total cell lysates were collected after 24 h stimulation, and immunoblot analyses were performed. The protein expression levels of COX-2 were detected using ECL reagent and quantified by analysis with LabWorks software. All the protein levels were normalized to corresponding tubulin levels. Relative expression levels of COX-2 are represented as a bar graph. After stimulation for 24 h, the levels of (C) IL-6 and (D) TNF-α in the cultured media were quantified using ELISA. The secretion of each cytokine was calculated using a standard curve. (E) After 24 h stimulation, IL-1β, IL-6 and TNF-α were amplified by RT-qPCR, and the expression of each group was compared to that in the LPS-treated and MSN-untreated control group. Data are represented as the mean ± SEM. *P<0.01 vs. LPS-untreated control group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS-treated and MSN-untreated control group. MSN, methanol extract of Synedrella nodiflora (Linn.) Gaertn.; LPS, lipopolysaccharide; COX-2, cyclooxygenase-2; IL-6, interleukin-6; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α.

phosphorylation and protein levels. As presented in Fig. 3A, LPS induced the phosphorylation and degradation of IκBα, while the phosphorylation of IκBα decreased and IκBα levels increased in the MSN-treated groups. These results suggest that MSN inhibits NF-κB activation through the change in IκBα phosphorylation.

Since MAPK signaling, another LPS-induced inflammatory signaling pathway, regulates the production of inflammatory cytokines and mediators (48), the regulation of MAPK signaling by MSN was investigated in the present study. Phosphorylation levels of p38, JNK, and ERK in their activation loop were increased in RAW 264.7 cells upon exposure to LPS (Fig. 3B).

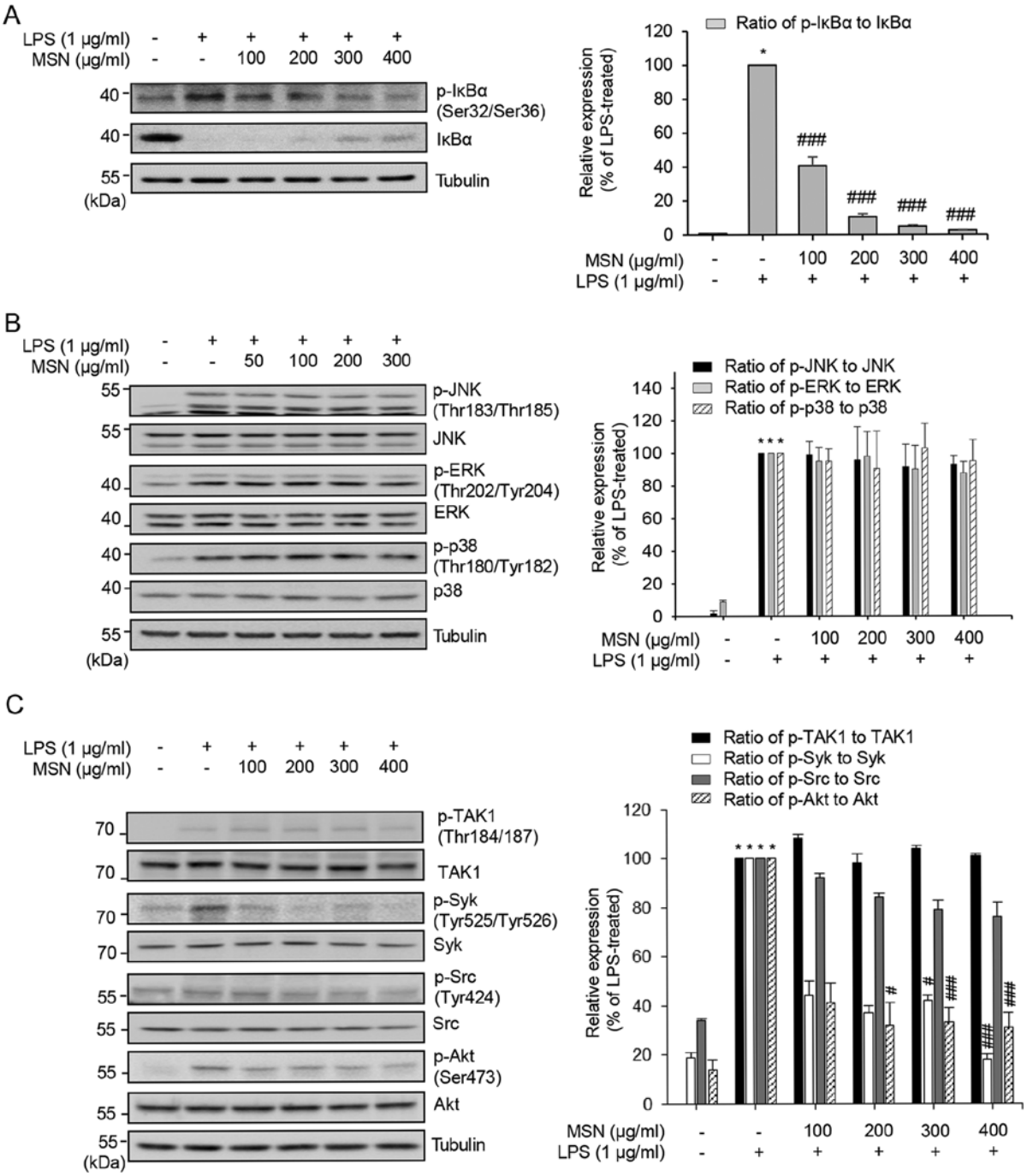

Figure 3. Inflammatory inhibition of MSN on MAPK phosphorylation and NF-κB activation in RAW 264.7 macrophages. RAW 264.7 macrophages were pre-treated with MSN (100, 200, 300, and 400 μg/ml) for 2 h and stimulated with LPS (1 μg/ml) at 37˚C for indicated times. (A) After stimulation for 3 min, total cell lysates were prepared and immunoblot analyses were performed. Tubulin was used as a loading control. Protein levels were quantified with LabWorks software. Relative expression levels of IκBα and p-IκBα were normalized to tubulin levels and then the ratio of phosphorylated (p)-IκBα vs. total IκBα protein level is shown as a bar graph. (B) Total cell lysates were collected after 15 min of LPS treatment and subjected to immunoblot analysis using appropriate antibodies. Tubulin was used as a loading control. Protein levels were measured using LabWorks software. Levels of phosphorylated MAPKs were normalized to corresponding total MAPK levels. The ratio of phosphorylated vs. total protein level for each MAPK is shown as a bar graph after normalization. (C) Cells were lysed after 3 min stimulation. Samples were subjected to immunoblot analysis. p-TAK1, p-Syk, p-Src, p-Akt, TAK1, Syk, Src, and Akt protein expression levels were determined. The phosphorylation levels of TAK1, Syk, Src, and Akt were normalized to the corresponding total protein levels. The ratios of phosphorylated vs. total protein levels are shown as a bar graph. *P<0.01 vs. LPS-untreated control group. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. LPS-treated and MSN-untreated control group. MSN, methanol extract of *Synedrella nodiflora* (Linn.) Gaertn.; LPS, lipopolysaccharide; IκBα, inhibitor of κBα; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; TAK1, transforming growth factor-β-activated kinase 1; Syk, spleen tyrosine kinase; Src, proto-oncogene tyrosine-protein kinase.

When the cells were treated with MSN prior to LPS stimulation, the levels of p-ERK, p-JNK, and p-p38 were not altered, suggesting that MSN has little effect on MAPK signaling.

TAK1 is an upstream signaling kinase of NF-κB and MAPK signaling transduction (49). In addition, the TAK1-independent Syk/Src/Akt signaling pathway plays a vital role in NF-κB

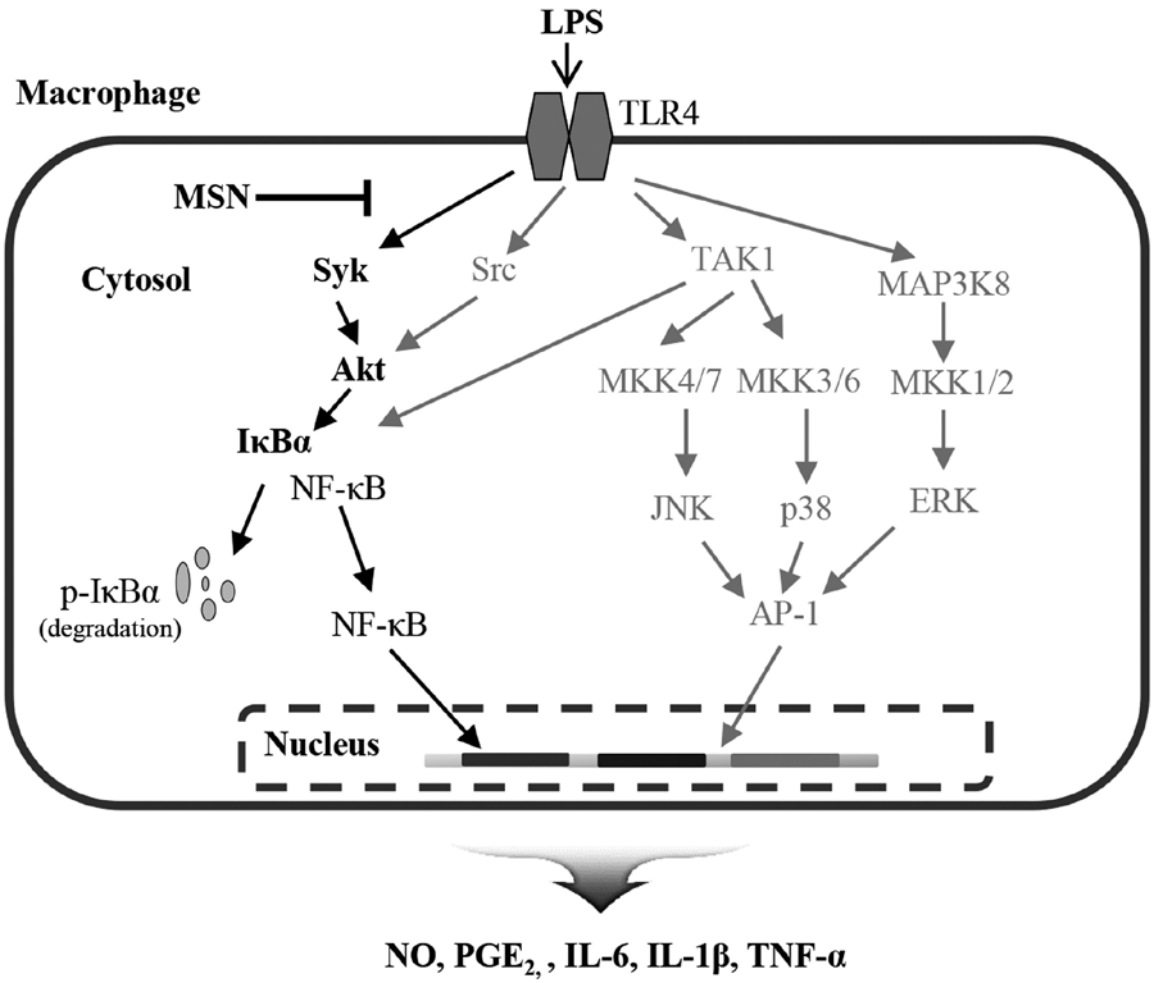

Figure 4. The molecular pathway of the inflammatory effects of MSN in LPS-stimulated RAW 264.7 cells. MSN, methanol extract of *Synedrella nodiflora* (Linn.) Gaertn.; LPS, lipopolysaccharide; IL-6, interleukin-6; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; NO, nitric oxide; PGE_2, prostaglandin E_2; COX-2, cyclooxygenase-2; IκBα, inhibitor of κBα; NF-κB, nuclear factor κB; Akt, protein kinase B; Src, proto-oncogene tyrosine-protein kinase; Syk, spleen tyrosine kinase; TLR4, toll-like receptor 4; TAK1, transforming growth factor-β-activated kinase 1; MAP3K8, mitogen-activated protein kinase kinase kinase 8; MKK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; AP-1, activator protein 1.

activation (50). The effect of MSN on the phosphorylation of TAK1/Syk/Src/Akt at their activation sites was investigated by immunoblot analysis to identify the targets of MSN in the regulation of NF-κB activation. The LPS-induced phosphorylation of Syk and Akt was reduced by MSN without changing the protein levels, whereas the phosphorylation of TAK1 and Src was not affected by MSN (Fig. 3C). These results suggest that MSN suppresses the phosphorylation of Syk and its downstream factors of the NF-κB pathway independently of TAK1 and Src.

Discussion

Despite the swift and proven efficacy of non-steroidal anti-inflammatory drugs, these drugs have the drawbacks of adverse side effects (51). Therefore, there have been efforts to develop anti-inflammatory drugs with fewer side effects and excellent healing effects (52). Among them, traditional and natural medicines have great benefits and potential (53,54). Several traditional medicines such as *Euphorbia cooperi* and *Thunbergia alata* have been reported to exert anti-inflammatory effects in folk remedies (24,29,55,56).

In the present study, a methanol extract of *S. nodiflora* (MSN) strongly suppressed the expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells in a dose-dependent manner. However, the 100 μg/ml MSN-treated group showed a prominent reduction on mRNA level compared that on protein level. This difference may be due to a variety of reasons, including transcriptional, post-transcriptional, translational and post-translational regulation, mRNA stability and protein stability (57-60). Moderate inhibitory activity on the production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and cyclooxygenase-2 (COX-2) was observed in the resent study. These results indicate that MSN may contain small amounts of anti-inflammatory components, inhibitors, or selective anti-inflammatory drugs. Analysis of the inflammation-regulated signaling pathways following treatment with MSN showed that only the NF-κB pathway was affected by MSN. The MAPK signaling pathway, the other major signaling pathway regulating inflammation, was not inhibited by MSN. Since both the NF-κB and MAPK pathways function in the regulation of inflammation (46,48), these data indicate why MSN showed a mild effect on the expression of TNF-α, IL-6, IL-1β, and COX-2. Furthermore, analysis of

spleen tyrosine kinase (Syk), proto-oncogene tyrosine-protein kinase (Src), and transforming growth factor-β-activated kinase 1 (TAK1), which are upstream regulators of the NF-κB signaling pathway, showed that Syk was inhibited by MSN while Src and TAK1 were not regulated by MSN. The specific inhibition of Syk by MSN suggests that the anti-inflammatory role of MSN may be dependent on highly selective anti-inflammatory components constituting MSN, such as alkaloids, flavonoids, triterpenes, saponins, simple phenolics, glycosides, and polyose. Even though the constituents of MSN have been previously reported (29,30), High-performance liquid chromatography (HPLC) analysis of MSN is still needed for further study of MSN and its constituents together with the anti-inflammatory mechanisms, which is the limitation of the present study. In the present study, these anti-inflammatory components were interpreted as responsible for the selective inhibition of Syk, Akt, and NF-κB pathways. Thus, further studies are needed to identify the specific functions of each anti-inflammatory component comprising MSN on selective inhibition.

One of the most prominent steps of inflammation is NO production by iNOS (61). Several studies have attempted to identify candidates for anti-inflammatory drugs and elucidate their mechanisms of action (62). In LPS-activated macrophages, induced NO serves as a pro-inflammatory mediator due to overproduction by iNOS. MSN inhibited the production of NO via decreased expression of both iNOS protein and mRNA in the present study. Furthermore, it is well known that the excessive production of prostaglandin E_2 (PGE_2) is related to inflammatory states (63), and COX-2 mediates the synthesis of PGE_2 (64). MSN negatively regulated COX-2 expression at both the mRNA and protein levels in the LPS-stimulated RAW 264.7 macrophages. Several studies have attempted to confirm novel anti-inflammatory agents that inhibit iNOS and COX-2 expression and have demonstrated them as novel drugs for inflammatory treatment (64,65). Therefore, data in the present study suggest that MSN may be used as a drug candidate to treat inflammatory disease.

In response to LPS stimulation, RAW 264.7 macrophages produce several cytokines, including IL-6, IL-1β, and TNF-α (66). Previous studies have shown that *IL-1β* and *IL-6* genes have both NF-κB and STAT binding domain whereas the *TNF-α* promoter has NF-κB binding region but not STAT binding region (67,68). In the present study, MSN inhibited LPS-induced IL-6 and TNF-α production in macrophages. In addition, mRNA expression of *IL-6*, *IL-1β*, and *TNF-α* was also reduced. This suggests that MSN is possibly involved in the regulation of the STAT3 signaling pathway and that both NF-κB and STAT3 function in collaboration to regulate *IL-6* and *IL-1β* genes, resulting in potent inhibition of these genes.

Binding of LPS to Toll-like receptors induces the activation of both MAPKs and NF-κB (64). However, the activities of MAPKs and NF-κB are regulated by different upstream molecules, such as specific MAP3Ks and MAP2Ks for MAPKs and IκBα kinases for NF-κB (69). Following the LPS stimulation, IκBα is phosphorylated and degraded (69,70). With the degradation of IκBα, NF-κB is unconstrained to enter the nucleus, where it chains to and triggers the transcription of target genes (71,72). In the present study, MSN treatment suppressed IκBα phosphorylation dose-dependently, which in turn reduced IκBα degradation. When the degradation of IκBα is suppressed, NF-κB nuclear translocation is inhibited, which fails to transcribe its downstream target genes (72). Since the activity of NF-κB is mostly regulated by IκBα binding, NF-κB target genes are potential targets of MSN treatment. In addition, upstream signaling of IκBα includes the TAK1/Syk/Src/Akt signaling pathway (73). MSN showed inhibitory effects by reducing the phosphorylation of Syk and Akt. Therefore, these findings support the hypothesis that MSN shows an anti-inflammatory effect by inhibiting NF-κB activation.

Anti-oxidant, anti-bacterial, and even anti-psychotic effects as well as anti-inflammatory effects of MSN have been previously reported (74,75). However, previous studies have focused on the chemical analysis of the constituents of MSN or the physiological effects of MSN in mouse models (76). In the present study, the anti-inflammatory effects and the underlying anti-inflammatory mechanisms of MSN were evaluated. The Syk/Akt/IκBα pathway that is significantly suppressed by MSN provides insight for the further application of MSN in the development of anti-inflammatory agents. In addition, the mode of action needs to be investigated to reveal the target pathway of the extract or to avoid unexpected side effects.

In conclusion, the present study demonstrates that MSN exhibits anti-inflammatory properties via inhibition of the production of various inflammatory mediators such as NO, PGE_2, TNF-α, IL-6, and IL-1β. The inhibitory effects of MSN are dependent on the suppression of the Syk/Akt/IκBα signaling pathway and NF-κB activation (Fig. 4). Therefore, these results support the traditional use of MSN in the treatment of several inflammation-associated diseases and suggest that the novelty of the present study lies in the revealed pathway. Thus, MSN is a candidate anti-inflammatory agent for the treatment of inflammation.

Acknowledgements

The authors would like to thank the KRIBB Initiative Program of the Republic of Korea for information on plant extract list and MSN.

Funding

The present study was financially supported by the Chung-Ang University Research Scholarship Grants in 2018 and by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Science and ICT (grant no. NRF-2018R1A2B6005084).

Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

HTTL and SC designed the study. HTTL and JP performed the data acquisition. JP performed the cell viability and immunoblot assays. HTTL, JP, JH, SK, JHP, and SC analyzed and

interpreted the data. SK and JHP provided extraction methods and MSN. HTTL wrote the draft. JP, JH, SK, JHP, and SC revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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