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Original Research Article

Anti-inflammatory Effects of *Magnolia sieboldii* Extract in Lipopolysaccharide-Stimulated RAW264.7 Macrophages

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Abstract

Purpose: *To investigate the effect of Magnolia sieboldii extract (MSE) on the production of pro-inflammatory cytokines by macrophage.*
Methods: *The whole plant of M. sieboldii was extracted with methanol at room temperature. The in vitro anti-inflammatory activity of MSE was investigated on lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. LPS-induced nitric oxide(NO) production was determined by Griess method. Production of pro-inflammatory cytokines including Interleukin-1 beta (IL-1 β), Interleukin-6 (IL-6), tumor necrosis factor- alpha (TNF-α) and cyclooxygenase-2 (COX-2) was examined using reverse trancriptase - polymerase chain reaction (RT-PCR) and Western blot analysis.*
Results*: Under in vitro conditions, MSE in doses ranging from 25 - 100 µg/mL significantly inhibited lipopolysaccharide (0.5 µg/mL)-induced nitric oxide production ($p < 0.001$), and the production of pro-inflammatory mediators ($p < 0.05$).*
Conclusions*: The anti-inflammatory effect of MSE on pro-inflammatory cytokines seems to ameliorate inflammatory symptoms via immune regulation.*

Keywords: *Anti-inflammatory, Nitric oxide, Cytokines, Immune regulation, Macrophage, Lipopolysaccharide, Polymyxin B, Magnolia sieboldii*

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INTRODUCTION

M. sieboldii is a traditional medicinal plant in Korea and has been used to treat various inflammatory diseases such as rhinitis, pneumonia, endometritis etc. However, there have been limited reports on the function and action of *M. sieboldii* in inflammation. *Magnolia sieboldii* K. Koch (Magnoliaceae) is an important plant used in traditional Chinese medicine and is available in various forms such as Magnoliae Cortex and Magnoliae Flos. A number of biologically active substances such as magnolol and honokiol [1-3], are isolated from plants of the Magnoliaceae family. Previous investigations have reported that some constituents of syringin, a new phenylpropanoid glycoside, and sinapyl alcohol were isolated from the stem bark of *M sieboldii,* which exhibited nitric oxide synthase inhibitory activity in the endotoxin-activated murine macrophage [4].

Inflammation has an important role in the body's first line defense system against injury and infective microorganisms such as bacteria and viruses. Inflammation is a major process involved in the healing of damaged tissues. Macrophages play critical role in the modulation of immune inflammatory system [5, 6]. Inflammation increases the expression of cytokines or proteins such as Interleukin-1 beta (IL-1 β), Interleukin-6 (IL-6), tumor necrosis factor- alpha (TNF-α) and cyclooxygenase-2 (COX-2) in macrophages [7].

Although nitric oxide (NO) is indispensable to physiological cellular activities, uncontrolled overproduction of NO by inducible nitric oxide synthase (iNOS) results in a catastrophic breakdown of important physiological functions [8]. NO is also reported to modulate the activity of prostaglandin endoperoxide H synthase 2 (cyclooxygenase-2) in a dose-dependent manner [9]. Cyclooxygenase (COX), an enzyme also known as prostaglandin (PG) H synthase (EC 1.14.99.1), converts arachidonic acid to prostaglandin, plays a crucial role as a mediator in inflammatory responses [10]. The adverse effects of COX-2 are evident from various pathogeneses of chronic inflammatory diseases, and its selective antagonists have been favorably reported in diverse experiments and clinical treatments [11,12]. In the present study, we investigated the effect of MSE on anti-inflammatory activity in LPS-stimulated RAW264.7 macrophages.

EXPERIMENTAL

Chemicals

The antibodies against COX-2, IL-1β were obtained from (Santa Cruz Biotechnology, USA) or β-actin (Abcam UK). Lipopolysaccharide from *Escherichia coli* 0127:B8, indomethacin, phosphate buffer saline (PBS), polymyxin B (PMB), N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma–Aldrich (Sigma–Aldrich, MO, USA). Fetal bovine serum (FBS), RPMI-1640 medium, penicillin–streptomycinwere bought from Gibco (Invitrogen, CA, USA). ECL reagent (Amersham Biosciences) and Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan) were also purchased.

Cell culture

RAW 264.7, a mouse macrophage-like cell line, was obtained from the American Type Culture Collection (Cryosite, Lane Cove, NSW, Australia). RAW 264.7 cells were grown in Dulbecco's Modified Eagle Medium **(** DMEM) with 10 % fetal bovine serum **(**FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were cultured at 37 °C in a humified incubator with an atmosphere of 5 % carbon dioxide (CO_2).

Extraction of plant material

M. sieboldii was obtained from the Plant Extract Bank of the Korean Research Institute of Bioscience and Biotechnology (111 Gwahangno Yuseong-gu, Deajeon, Korea). The whole plant parts of *M. sieboldii* (200 g) were extracted with methanol (1 L) at room temperature. The methanol extract was evaporated to obtain powdered sample. The extract was dissolved in dimethylsulfoxide (DMSO) to give 0.1 v/v concentration and used at appropriate concentrations (0, 25, 50, 75, 100 μg/mL).

Cell viability assay

The concentration of MSE affecting cell viability was evaluated using CCK-8 Kit. Briefly, RAW264.7 cells were plated at a density of 1 × 10^4 cells per well in a 96-well plate, and were incubated at 37°C for 24 h. The cells were treated with various concentrations of MSE or vehicle alone, and incubated at 37°C for an additional 24 h. After incubation, 10 μL of CCK-8 solution was added to each well and incubated under the same conditions for another 3 h and the resulting color was assayed at 450 nm using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA, USA). Each assay was carried out in triplicate. For control studies, 0.05 % DMSO was used.

Nitric oxide assay

Nitrite concentration in the medium was measured as an indicator of nitric oxide production according to the Griess reaction method. Each nitrite standard and sample were assayed in triplicate. A freshly prepared standard curve was used each time the assay was performed. In brief, 1 × 10^5 RAW264.7 cells were seeded in 24-well plates, incubated for 24 h and pre-treated with the indicated concentrations (0, 25, 50, 75, 100 μg/mL) of MSE for another 30 min, then challenged with LPS (0.5 μg/mL) for an additional 18 h. 100 μl of cultured medium and Griess reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphtylethyl-enediamine dihydrochloride in distilled water) were mixed and incubate the plate at room temperature for 10 min, the absorbance at 540 nm was determined with a microplate reader and the absorption coefficient was calibrated using a standard solution of sodium nitrite. For positive control studies, 10 μg/mL polymyxin B was used.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was prepared by disrupting the RAW264.7 cells in TRIZOL reagent (Life Technologies, USA). Complementary DNA was synthesized from 1 μg of total RNA in a 25 μl reverse transcription reaction mixture. For RT-PCR, aliquots of cDNA were amplified in a 20 μl PCR mixture according to the manufacturer's protocol (Promega, USA). The primers for each gene were as follows: forward primer for IL-1β (387 bp): 5′-TGCAGAGTTCCCCAACTGGTA CATC-3′ and its reverse primer: 5′-GTGCTGCCTAATGTCCCCTTGAATC-3′; forward primer for IL-6 (147 bp): 5′-GAGGAT ACCACTCCCAACAGACC-3′ and its reverse primer: 5′-AAGTGCATCATCGTTGTTCA TACA-3′; forward primer for COX-2 (721 bp): 5′-GGAGAGACTATCAAGATAGT-3′ and its reverse primer: 5′-ATGGTCAGTAGACTTTTA CA-3′; forward primer for TNF-α (351 bp): 5′-ATGAGCACAGAAAGCATGATC-3′ and its reverse primer: 5′-TACAGGCTT GTCACTCG AATT-3′; forward primer for β-actin (310 bp): 5′-TCATGAAGTGTGACGTTG ACATCCGT-3′ and its reverse primer: 5′-CCTAGAAGCATTTGCGG TGCACGATG-3′. The thermal cycling conditions were as follows: 24-32 cycles at 94°C for 1 min, 55-60°C for 45 sec and 72°C for 45 sec. PCR products were electrophoresed on 1.5% agarose gels.

Western blot analysis

RAW264.7 macrophages were pre-treated with the indicated concentrations (0, 25, 50, 75, 100 μg/mL) of MSE for 30 min and stimulated with LPS (0.5 μg/mL) and incubated for 24 h. Adherent cells were scraped out from the culture plates and boiled with the lysis buffer containing 50 mM Tris (pH 7.4), 1500 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 % NP-40, 0.25 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS) and protease inhibitor cocktail. Proteins were separated by 10 – 12 % SDS polyacrylamide gel electrophoresis (SDS-PAGE), at 100 V for 90 min. Separated proteins were then transferred onto nitrocellulose membrane. After blocking non-specific binding sites with 5 % non-fat dry milk, the membranes were incubated with anti-COX-2 or anti-IL-1β (both diluted 1:1000), and anti-β-actin monoclonal primary antibody for 2 h at room temprature. After removal of the primary antibody, the membranes were washed, and then incubated with HRP-conjugated secondary antibody (1:2000 dilution) for 1 h at room temperature. The membranes were washed again with phosphate buffer saline with tween-20 (PBST) buffer on the rocker (N-Biotech Inc.) and the immunoreactive bands were visualized using ECL reagent (Amersham Biosciences). β-actin protein was used as an internal control.

Statistical analysis

Results were pooled from three independent experiments. Data from cell viability assay and flow cytometric analysis are expressed as mean ± SEM (standard error of mean) and analysis of variance (ANOVA) followed by the Tukey's test and Dunn's test performed on GraphPad Prism 5 (San Diego, CA, USA) were used to determine significant differences ($p \leq 0.05$) between experimental groups.

RESULTS

Cell viability assay

To determine the effect of MSE on cell viability, MSE-treated RAW264.7 cells grown in serum-free media were used for the CCK-8 assay. The cytotoxic effects of MSE are shown in Figure 1. No cytotoxic effect was observed for up to 100 μg/mL.

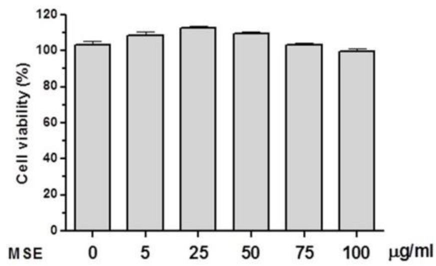

Figure 1: Effect of MSE on cell viability of RAW264.7 macrophages Values are expressed as mean ± SEM (n = 3).

MSE inhibits nitrite production in RAW264.7 macrophages

The effect of MSE on LPS-induced NO production in RAW 264.7 cells was investigated by measuring the amount of nitrite released into the culture medium using the Griess reaction. The amount of NO produced was determined by the amount of nitrite, a stable metabolite of NO. During incubation time of 18 h, RAW264.7 macrophages produced 3.04 ± 0.13 μM nitrite in the resting state. After LPS (0.5 μg/mL) stimulation, NO production increased dramatically to 61.3 ± 0.049 μM after 18 h. MSE significantly inhibited nitrite production 18 h after LPS stimulation in a dose-dependent manner corresponding to 16.6% at 25 μg/mL and 66.1% inhibition at 50 μg/mL (Figure 2). The iNOS inhibitor, Polymyxin B (PMB) significantly inhibited LPS-induced NO production (Figure 2).

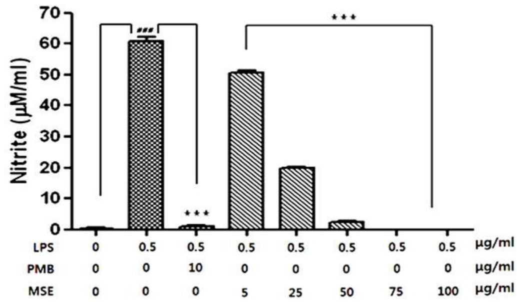

Figure 2: Dose-dependent inhibition of nitric oxide production in LPS-challenged RAW264.7 macrophages treated with 0, 5, 25, 50, 75, 100 μg/mL of MSE in the presence of 0.5 μg/mL LPS or with LPS alone for 18 h; ****p* < 0.001 indicates significant difference from the LPS-treated group; ### *p* < 0.001 indicates significant difference from the unstimulated control group.

MSE suppresses IL-1β, IL-6, TNF-α and COX-2 mRNA expression

In Figure 3, LPS-activated macrophages expressed increased levels of IL-1β, IL-6, TNF-α and COX-2 mRNA. MSE induced a dose-dependent inhibition of the production of pro-inflammatory cytokines by LPS activated macrophages. The extract significantly inhibited the production of IL-1β at the concentration 50-100 μg/mL (Figure 3A), the production of IL-6 at the concentration 75-100 μg/mL (Figure 3B) and the production of TNF-α at the concentration 100 μg/mL (Figure 3C) in LPS-activated macrophages. In addition, MSE suppressed LPS-induced COX-2 expression at the concentration 75-100 μg/mL (Figure 3D). MSE slightly stimulated IL-1β, IL-6, TNF-α and COX-2 mRNA expression at low (5- 25 μg/mL).

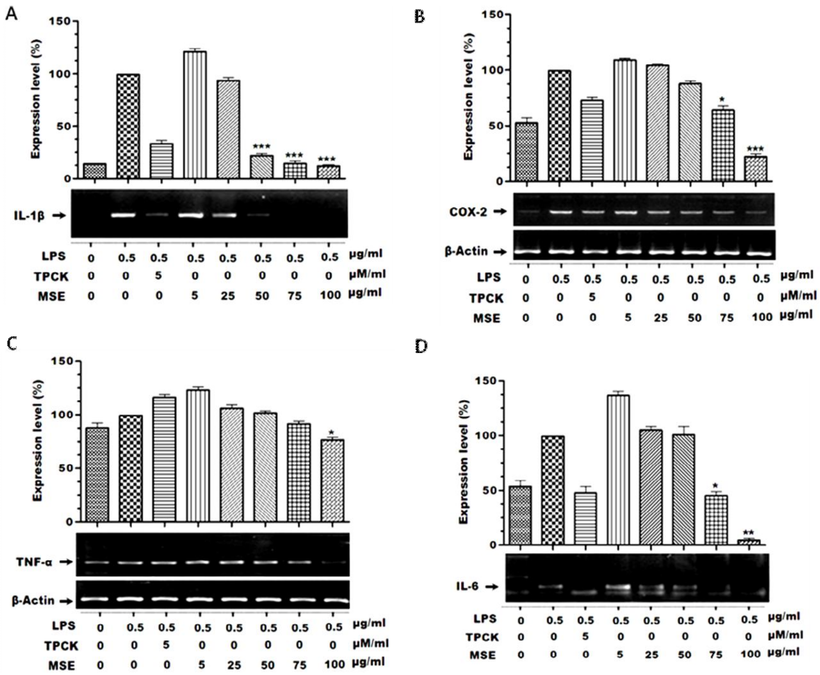

Figure 3: Down-regulation of COX-2, iNOS, IL-1β, and IL-6 mRNA expression by MSE in LPS-stimulated RAW264.7 macrophages; cDNA-based gene amplification of IL-1β (A), IL-6 (B), COX-2 (C) and TNF-α (D) were performed as described in Experimental section; data are expressed as mean ± SEM; (n = 3); **p* < 0.05 and ****p* < 0.001 indicate significant difference from the LPS-treated group.

MSE suppresses COX-2 and IL-1β protein expression in RAW 264.7 cells

To confirm the anti-inflammatory activity of MSE on IL-1β and COX-2 protein expression, we tested the effects of MSE on LPS induced COX-2 protein up-regulation in RAW 264.7 cells by western blotting. Cells pretreated with MSE (50 μg/mL) showed a inhibition in IL-1β protein expression following LPS stimulation for 24 h (Figure 4A). COX-2 protein expression was detected in cells not treated with LPS and increased markedly after treatment with 0.5 μg/mL LPS for 24 h compared with the negative control (NC). Cells pretreated with MSE showed a dose dependent inhibition of IL-1β and COX-2 protein expression following LPS stimulation for 24 h (Figure 4A, 4B).

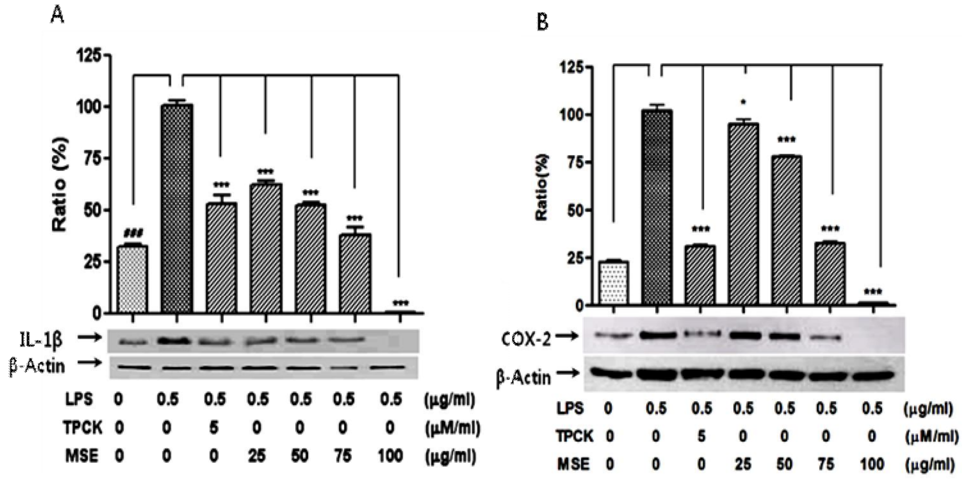

Figure 4: MSE-mediated inhibition of IL-1β protein and COX-2 expression in LPS-stimulated RAW264.7 macrophages. The values of relative ratio (%) are expressed as the mean ± SEM (n = 3); both the IL-1β (A) and COX-2 (B) signals were normalized to the β-actin signal; *$p < 0.05$ and ***$p < 0.001$ indicate significant difference from the LPS-treated group; $^{###}$ $p < 0.001$ indicate a significant difference from the unstimulated control group.

DISCUSSION

In this study we investigated whether *M. sieboldii* can inhibit the production of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) in LPS-activated macrophages and if *M. sieboldii* could decrease the expression of COX-2 in LPS-activated macrophages.

In addition to its a pivotal role in many body functions, NO has also been implicated in the pathology of many inflammatory diseases, including arthritis, myocarditis, colitis, and nephritis [14-18]. Therefore, NO inhibitors are essential for prevention of inflammatory diseases. In this study, we showed that MSE showed dose-dependent inhibitory effects on NO production in RAW264.7 cells (Figure 1). COX enzymes are responsible for the formation of important biological mediators known as prostanoids, which include prostaglandins (PGs), prostacyclin, and thromboxanes. Recent evidence suggests that PGs are involved in inflammatory processes, and that COX-2, an inducible isoform of COX, is mainly responsible for the production of large amounts of these mediators [26]. Our results indicate that the inhibition of NO production in LPS stimulated RAW264.7 cells by MSE occurred via the inhibition of pro-inflammatory cytokines. RT-PCR revealed that MSE treatment down-regulates mRNA levels of IL-1β, COX-2, TNF-α and IL-6. (Figure 3A, 3B, 3C and 4D). Next, we examined protein level of IL-1β and COX-2. MSE decreased protein levels of IL-1β and COX-2, as pro-imflammatory mediators in a dose-dependent manner (Figure 4A, 4B). Our results in this study showed that the extract of *Magnolia sieboldii* suppressed the production of pro-inflammatory cytokines and mediator including IL-1β, IL-6, TNF-α and COX-2 in LPS-activated macrophages in a dose dependent manner. These data suggest that *M. sieboldii* extract may be potentially beneficial in the treatment of inflammatory diseases through the inhibition of NO production.

CONCLUSION

We observed that *M. sieboldii* extract suppressed the pro-inflammatory cytokine production in LPS-stimulated RAW264.7 macrophages. Hence, *M. sieboldii* extract is a potential candidate for the development of pharmacological agents useful in the treatment of inflammatory diseases. Further research on the effects and molecular mechanisms of the active compound in the extract is needed to precisely define the structure–activity relationship in various molecular regulatory mechanisms.

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