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Eleutherococcus senticosus extract attenuates LPS-induced iNOS expression through the inhibition of Akt and JNK pathways in murine macrophage

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Abstract

Eleutherococcus senticosus (*Araliaceae*) is immunological modulator which has been successfully used for anti-inflammatory effectors on anti-rheumatic diseases in oriental medicine. Mitogen-activated protein kinases (MAPKs) and Akt modulate the transcription of many genes involved in the inflammatory process. In this study, we investigated the inhibitory effects of *Eleutherococcus senticosus* on the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharides (LPS)-activated macrophages. Finally, we studied the involvement of MAPKs and Akt signaling in the protective effect of *Eleutherococcus senticosus* in LPS-activated macrophages. *Eleutherococcus senticosus* significantly attenuated LPS-induced iNOS expression but not COX-2 expression. In using the standard inhibitors (MAPKs and Akt), our results show that *Eleutherococcus senticosus* downregulates inflammatory iNOS expression by blocking JNK and Akt activation.
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Keywords: *Eleutherococcus senticosus*; MAPKs; Akt; iNOS; COX-2

1. Introduction

Eleutherococcus senticosus (Rupr. & Maxim.) Maxim. (*Araliaceae*), also called the "Siberian Ginseng", has been used as a crude drug to treat stress-induced physiological changes (Nishibe et al., 1990; Fujikawa et al., 1996; Fujikawa et al., 2005). *Eleutherococcus senticosus* is known to include acanthosides, eleutherosides, chiisanoside, senticoside, triterpenic saponin, syringin, flavone, vitamins and minerals, and they are responsible for its diverse biological activities (Davydov and Krikorian, 2000). The herb has also been used clinically for treatment of various allergic diseases in Korea. But, it is still unclear how it inhibits allergic responses and how effective it is in experimental models. Especially, only a few studies reported the anti-inflammatory effects of *Eleutherococcus senticosus* extract using *in vitro* (Jung et al., 2003; Tokiwa et al., 2006). Consequently, detailed action mechanism and pharmacological target are mostly unknown.

During inflammatory disease the macrophage produces excess amounts of mediators such as nitric oxide (NO), prostanoids and pro-inflammatory cytokines (Ahn et al., 2005; Fujihara et al., 2003). Probably the most pivotal enzymes involved in maintaining inflammation are the inducible enzymes; inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are responsible for the

Abbreviations: LPS, lipopolysaccharides; MAPKs, mitogen-activated protein kinases; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; JNK, c-Jun N-terminal kinase; Akt, anti-apoptosis mediator protein kinase B; ERK, extracellular signal-regulated kinases

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catalysis of NO and prostaglandin E_2 (PGE_2), respectively (Surh et al., 2001). Expression of the COX-2 and iNOS in macrophages is regulated mainly at the induction of transcription factors through mitogen-activated protein kinases (MAPKs) and PI3K/Akt pathways (Chan and Riches, 2001; Uto et al., 2005). MAPKs important to macrophage cells include p38, c-jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). LPS stimulation of RAW264.7 cells rapidly activates the MAPKs (Chen and Wang, 1999, Ho et al., 2004; Nick et al., 2000; Nieminen et al., 2006; Kim et al., 2006). Thus, p38, ERK1/2, and JNK activation is used as a hallmark of LPS-induced signal transduction in RAW 264.7 cells. Akt signals were thought to be MAPKs independent component of LSP-induced NF-κB activation (Hattori et al., 2003).

In this study, we investigated the inhibition on COX-2 and iNOS expression of the 80% ethanol extract of *Eleutherococcus senticosus* with respect to the inhibition of pro-inflammation of RAW 264.7 cells.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco Life Technologies (MD, USA). COX-2, iNOS and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (CA, USA). Phospho-ERK1/2, phospho-p38 MAPK, phospho-JNK1/2 and phospho-Akt monoclonal antibodies were purchased from Cell Signaling Technology Inc. (MA, USA). Akt inhibitor (LY294002) and MAPKs inhibitors (SB203580, PD98059 and SP600125) were purchased from Calbiochem (CA, USA). 2′,7′-dichlorodihydrofluorescein (DCHF-DA) was purchased from Molecular Probes Inc. (OR, USA). The ECL detection agents were purchased from Amersham Biosciences (Piscataway, NJ). Phenyl-methylsulfonylfluoride (PMSF), 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), LPS and all other chemicals were purchased from Sigma–Aldrich Co. (MO, USA).

2.2. Plant material and extraction

The root bark of *Eleutherococcus Senticosus* Maxim. (*Araliaceae*) was purchased from Omniherb Co., Ltd. (Yeongcheon, Korea) and was identified by Professor, Hyung-Min Kim, Department of Pharmacology, College of Oriental Medicine, Kyunghee University where a voucher specimen (No. 014) is deposited. The root barks of *Eleutherococcus Senticosus* were extracted twice by repeat sonication (30 min with 80% ethanol). The extract was filtered and dried using a vacuum evaporator. The filtrate was combined and concentrated in a vacuum evaporator. The concentrate was freeze-dried and its yield was 7.3%.

2.3. Cell culture

Murine macrophage RAW 264.7 cells were obtained from the Korea Cell Line Bank (Seoul, Korea), and cultured in DMEM medium supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin A and 100 μg/ml of streptomycin), and 10% heat-inactivated fetal bovine serum and maintained at 37 °C in a 5% CO_2 humidified incubator.

2.4. Western blot analysis

The cells were treated with *Eleutherococcus senticosus* and LPS, then washed out with phosphate-buffered saline (PBS) and lysed with lysis buffer (40 mM Tris–HCl, pH 7.4, 10 mM EDTA, 120 mM NaCl, 0.1% Nonidet p-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitors) on ice for 20 min. The cell lysates were obtained by centrifugation at $13{,}000 \times g$ for 15 min at 4 °C. Protein concentrations were determined by the Bio-Rad protein assay kit using bovine serum albumin (BSA) as standard. Equal amounts of protein (30 μg) from the cell lysates were dissolved in LaemmLi's sample buffer and boiled for 5 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The membranes were then blocked in PBS-0.1% Tween-20 (PBST) containing 1% skim milk and 1% BSA for 1 h at room temperature. Thereafter, the membranes were incubated overnight at 4 °C with a 1:1000 dilution of monoclonal anti-iNOS, COX-2, phosphor-Akt, phospho-ERK1/2, phospho-p38 MAPK, phospho-JNK1/2, β-actin and α-tubulin antibodies. The membranes were then washed three times with PBST and further incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Subsequently, membranes were washed three more times with PBST and the immunobands visualized with an enhanced chemiluminescence detection system (Amersham-Pharmacia Biotech, Buckinghamshire, England).

2.5. RNA extraction and reverse transcriptase polymerase chain reactions (RT-PCR)

Both unstimulated and stimulated cells were washed, and RNA was extracted from these cells using TRIzol reagent (Invitrogen, Australia) according to the manufacturer's instructions. The purity of the RNA preparation was checked by measuring the absorbance ratio at 260/280 nm. Primers were designed for reverse transcriptase-polymerase chain reactions (RT-PCR) for iNOS and COX-2. The sense and antisense primers for iNOS were 5′-AATGGCAACATCAGGTCGGCCATCACT-3′ and 5′-GCTGTGTGTCACAGAAGTCTCGAACTC-3′, respectively (Shin et al., 2004), and the sense and antisense primers for COX-2 were 5′-GGAGAGACTATCAAGATAGT-3′and5′-ATGGTCAGTAGACTTTTACA-3′, respectively. Sense and antisense primers for rat GAPDH mRNA expression (used as a control for total RNA content for each sample) were 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′, respectively (Pan et al., 2006). The 1 μg of cDNA was amplified by PCR for 20 cycles of denaturation (95 °C for 1 min), annealing (iNOS, 40 °C for 1 min or COX-2, 60 °C for 1 min), polymerization (72 °C for

1 min), and a final elongation step of 5 min at 72 °C. The PCR products were separated by 2.5% agarose gel electrophoresis and visualized by ethidium bromide staining and UV irradiation.

2.6. Data analysis

All experimental data was examined by analysis of the variance (ANOVA) and significant differences among the means from triplicate determinations assumed at $P < 0.05$. Further statistical analyses were performed using Duncan's multiple range tests and the Statistical Analysis System (SAS institute Inc., Cary, NC, USA).

3. Result and discussion

To examine whether 80% ethanol extract of *Eleutherococcus senticosus* could inhibit inflammation, mouse macrophage cell line RAW264.7 was chosen to evaluate the effects. Western blotting analysis demonstrated that iNOS and COX-2 proteins were absolutely undetectable in inactive cells but appeared in high amounts following induction (Fig. 1). *Eleutherococcus senticosus* extract displayed a significant dose-dependent downregulatory effect upon iNOS protein expression but did not inhibit COX-2 protein expression (Fig. 1). Changes in amounts of iNOS enzyme could reflect altered protein synthesis or degradation. RT-PCR was done to investigate whether *Eleutherococcus senticosus* suppressed LPS-mediated induction of iNOS and COX-2 via a pretranslational mechanism. *Eleutherococcus senticosus* was the potent inhibitor of expression of iNOS in LPS-activated macrophages, as measured by densitometer scans (Fig. 2). These data suggest that *Eleutherococcus senticosus* may inhibit the expression of iNOS at the transcription levels. Inhibition of COX-2 mRNA was not apparent by *Eleutherococcus senticosus* of up to 50 μg/ml. According to previous reports (Jung et al., 2003; Tokiwa et al., 2006), the isolated compounds from *Eleutherococcus senticosus* inhibited the iNOS as well as COX-2 expression. Dissimilar results may be due to efficacy according to concentration between whole extract and its derived compounds.

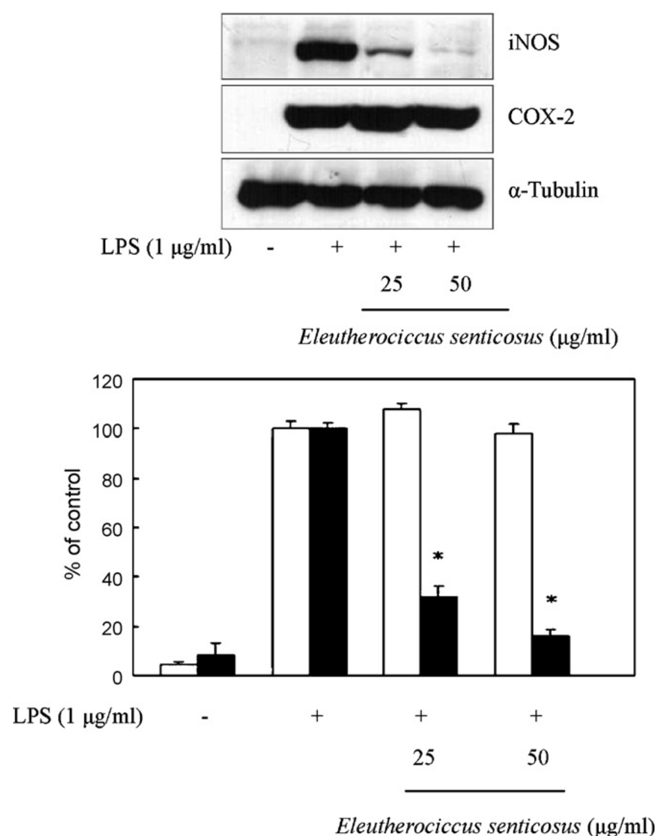

Fig. 1. Effects of 80% ethanol extract from *Eleutherococcus senticosus* on iNOS and COX-2 protein expression in LPS-induced macrophage RAW 264.7 cells. Cells were pretreated with different concentrations of sample for 1 h before LPS treatment (1 μg/ml), and the cells further incubated for 18 h. The protein levels of iNOS and α-tubulin were determined by Western blot analysis and the ratio of immunointensity between the iNOS and the α-tubulin calculated. Each bar (open bar, COX-2; closed bar, iNOS) represents the means ± S.D. from three independent experiments. $^{*}P < 0.05$ vs. LPS alone.

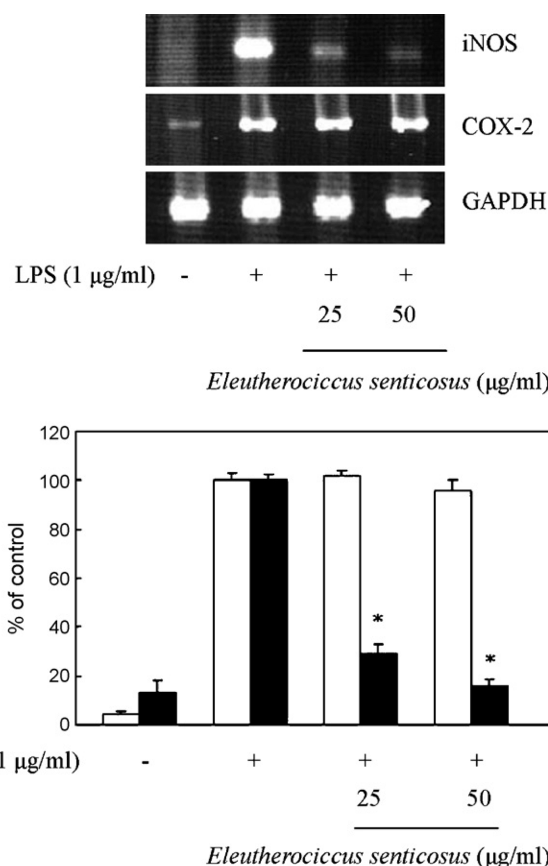

Fig. 2. Effects of 80% ethanol extract from *Eleutherococcus senticosus* on iNOS and COX-2 mRNA expression in LPS-induced macrophage RAW 264.7 cells. Cells were pretreated with different concentrations of the sample for 1 h before LPS treatment (1 μg/ml), and the cells further incubated for 18 h. Total mRNAs were prepared from the cell pellets using TRIzol. The relative levels of mRNA were assessed by RT-PCR. Each bar (open bar, COX-2; closed bar, iNOS) represents the mean ± S.D. from three independent experiments. $^{*}P < 0.05$ vs. LPS alone.

Because JNK, ERK and p38 MAPK have been shown to be involved in the LPS-mediated induction of iNOS and COX-2 in mouse macrophages (Nick et al., 2000; Ho et al., 2004) and cytokine activation of PI3K/Akt pathway leads to the

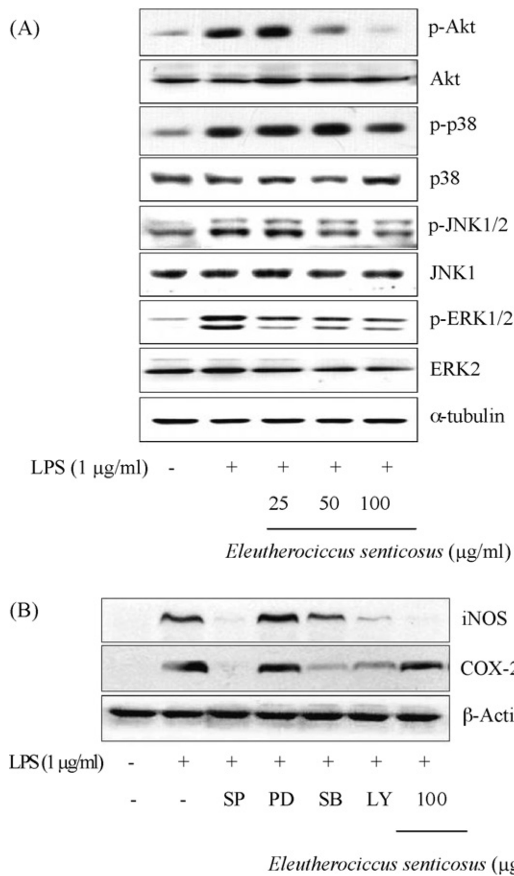

Fig. 3. Effects of 80% ethanol extract from *Eleutherococcus senticosus* on LPS-induced activation of Akt and MAPK kinases. (A) The expression of phospho-ERK1/2 (p-ERK), phospho-p38 (p-p38), phospho-JNK1/2 (p-JNK) and phospho-Akt (p-Akt) were analyzed by Western blot. Cells were treated with LPS (1 μg/ml) for 30 min. β-actin was used as a "loading" control. (B) Cells treated with LPS (1 μg/ml) for 30 min in the presence of JNK (SP600125, 40 μM), ERK (PD98059, 40 μM), p38 MAPK (SB203580, 10 μM) and Akt (LY294002, 10 μM) inhibitors.

phosphorylation and activation of the NF-κB (Sizemore et al., 1999), we investigated the effects of *Eleutherococcus senticosus* on the activation of JNK, ERK, p38 MAPK and Akt in LPS-stimulated macrophages. Using immunoblot analysis with anti-phospho-specific antibody, we found activation of MAPKs and Akt to peak after 10–30 min of treatment with LPS (data not shown). When the cells were co-treated with both *Eleutherococcus senticosus* and LPS for 30 min, *Eleutherococcus senticosus* was found to attenuate the LPS-stimulated activation of JNK, ERK and Akt, but did not suppress the expression of p38 MAPK (Fig. 3A). The LPS-stimulated activation of iNOS and COX-2 were attenuated by JNK (SP600125), p38 MAPK (SB203580) and Akt inhibitors (LY294002), but not affected by ERK inhibitor (Fig. 3B). The present data suggest that *Eleutherococcus senticosus* might block LPS-induced iNOS expression by inhibiting JNK and Akt pathway. Nevertheless, no changes in the expression of COX-2 could be observed.

The data suggest that *Eleutherococcus senticosus* inhibits pro-inflammatory action on activated macropahges through blocking signal pathway (JNK and Akt), and, therefore, that it may have anti-inflammation properties valuable for application in food and drug products.

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