**Atractylodes japonica** Suppresses Lipopolysaccharide-Stimulated Expressions of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 in RAW 264.7 Macrophages

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*Atractylodes japonica* has traditionally been used for the treatment of pain and arthritis. The effect of *Atractylodes japonica* against lipopolysaccharide-induced inflammation was investigated using reverse transcription-polymerase chain reaction (RT-PCR), nitric oxide detection, and prostaglandin E$_2$ (PGE$_2$) immunoassay in mouse RAW 264.7 macrophages. The aqueous extract of *Atractylodes japonica* suppressed nitric oxide production and PGE$_2$ synthesis by inhibition of the lipopolysaccharide-stimulated enhancement of inducible nitric oxide synthase and cyclooxygenase-2 mRNAs expressions in RAW 264.7 macrophages. These results suggest that *Atractylodes japonica* exerts anti-inflammatory and analgesic effects probably by suppression of the inducible nitric oxide synthase and cyclooxygenase-2 expressions.

Key words *Atractylodes japonica*; lipopolysaccharide; inducible nitric oxide synthase; cyclooxygenase-2

Lipopolysaccharide (LPS) initiates a number of major cellular responses which play a vital role in the pathogenesis of inflammatory responses including activation of inflammatory cells and production of cytokines and other mediators. Of these, nitric oxide (NO) is endogenously generated from L-arginine by nitric oxide synthase (NOS) and plays an important role in the regulation of many physiological processes.1) Several isoforms of NOS exist and fall into three major classes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). NO synthesized by iNOS has been implicated as a mediator of rheumatoid arthritis and other autoimmune diseases.2) In addition, prostaglandins (PGs) are key inflammatory mediators that are converted from arachidonic acid by cyclooxygenase. There are two isoforms of cyclooxygenase: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). While COX-1 is a constitutively expressed form in normal physiologic functions, COX-2 is expressed only in response to inflammatory signals such as cytokines and bacterial endotoxin LPS. COX-2 produces large amounts of PGE$_2$, which induce inflammatory reaction.3,4)

*Atractylodes japonica* has traditionally been used for the treatment of water retention in the body. Administration of the aqueous extract of *Atractylodes japonica* causes diuresis in mice.5,6) In addition, prostaglandins (PGs) are key inflammatory mediators that are converted from arachidonic acid by cyclooxygenase. There are two isoforms of cyclooxygenase: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). While COX-1 is a constitutively expressed form in normal physiologic functions, COX-2 is expressed only in response to inflammatory signals such as cytokines and bacterial endotoxin LPS. COX-2 produces large amounts of PGE$_2$, which induce inflammatory reaction.3,4)

**MATERIALS AND METHODS**

**Cell Culture** Murine macrophage RAW 264.7 cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) at 37 °C in 5% CO$_2$–95% O$_2$ in a humidified cell incubator. To obtain the water extract of *Atractylodes japonica*, 200 g of *Atractylodes japonica* was added to distilled water, and extraction was performed by heating at 80 °C, concentrating with a rotary evaporator, and lyophilization. The resulting powder, weighing 30 g, was dissolved in saline.

**RNA Isolation and RT-PCR** To identify the expressions of iNOS and COX-2 mRNAs, RT-PCR was performed. Total RNA was isolated from RAW 264.7 cells using RNAzol® (TEL-TEST, Friendswood, TX, U.S.A.). Two micrograms of RNA and 2 μl of random hexamers (Promega, Madison, WI, U.S.A.) were added together, and the mixture was heated at 65 °C for 10 min. One microliter of AMV reverse transcriptase (Promega), 5 μl of 10 mm dNTP (Promega), 1 μl of RNasin (Promega), and 5 μl of 10× AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 50 μl with diethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 °C for 1 h.

PCR amplification was performed in a reaction volume of 40 μl containing 1 μl of the appropriate cDNA, 1 μl of each set of primers at a concentration of 10 pm, 4 μl of 10×RT buffer, 1 μl of 2.5 mm dNTP, and 2 units of Taq DNA polymerase (TaKaRa, Shiga, Japan). For mouse iNOS, the primer sequences were 5’-GTGTTCCACCAGGAGATGTTG-3’ (a 21-mer sense oligonucleotide) and 5’-CTCCTGCCCACTGAGTTCGTC-3’ (a 21-mer anti-sense oligonucleotide). For mouse COX-2, the primer sequences were 5’-TGCATGGTGCTGGATGTCTCAA-3’ (a 25-mer sense oligonucleotide) and 5’-CACTAAGACAGACCGCTACCTCCA-3’ (a 25-mer anti-sense oligonucleotide). For cyclophilin, the in-
ternal control used in the study, the primer sequences were 5'-ACCCACGTTGTTTCTGAC-3' (a 20-mer sense oligonucleotide starting at position 52) and 5'-CATTGCG- CATGGACAAAGTG-3' (a 20-mer anti-sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 583 bp (for COX-2), 500 bp (for iNOS), and 299 bp (for cyclophilin).

For iNOS and COX-2, the PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, U.S.A.) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 40 amplification cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s, with an additional extension step at the end of the procedure at 72 °C for 5 min. For cyclophilin, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular Analyst™ version 1.4.1 (Bio-Rad, Hercules, CA, U.S.A.).

**Western Blot Analysis** Cells were lysed in a lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid, 1% NP40, 0.1% sodium dodecyl sulfate (SDS), 1 mM PMSF, and 100 μg/ml leupeptin. Protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). Protein of 50 μg was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Rabbit iNOS antibody (1 : 500; Santa Cruz Biotech, Santa Cruz, CA, U.S.A.) and goat COX-2 antibody (1 : 500; Santa Cruz Biotech) were used as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit antibody for iNOS and anti-goat antibody for COX-2 were used as secondary antibodies. Band detection was performed using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech GmbH).

**Determination of Nitric Oxide Synthesis** In order to determine the effect of *Atractylodes japonica* on NO synthesis, the amount of nitrite in the supernatant was measured using a commercially available NO detection kit (iNtRON, Inc., Seoul, Korea). After collection of 100 μl of supernatant, 50 μl of N1 buffer was added to each well, and the plate was incubated at room temperature for 10 min. N2 buffer was then added, and the plate was incubated at room temperature for 10 min. The absorbance of the content of each well was then measured at 450 nm. The nitrite concentration was calculated from a nitrite standard curve.

**Measurement of Prostaglandin E2 Synthesis** Assessment of PGE2 synthesis was performed using a commercially available PGE2 competitive enzyme immunoassay kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, U.S.A.). Supernatants (100 μl) from culture medium and standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE2 antibody and peroxidase-conjugated PGE2 were added to each well, and the plate was incubated at room temperature with shaking for 1 h. The wells were drained and washed, and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution was added. The plate was incubated at room temperature with shaking, and the reaction was stopped after 30 min through the addition of H2SO4. The absorbance of the content of each well was then measured at 450 nm.

**Statistical Analysis** Results are expressed as mean ± standard error mean (S.E.M.). Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test using SPSS. Differences were considered statistically significant at \( p < 0.05 \).

**RESULTS**

**Effect of *Atractylodes japonica* on mRNA Expressions of iNOS and COX-2** From the results of MTT assay, *Atractylodes japonica* treatment is not indicative of any significant change in viability.

RT-PCR analysis of the mRNA levels of iNOS and COX-2 was performed in order to provide an estimate of the relative levels of expressions of these genes. In the present study, the mRNA levels of iNOS and COX-2 in the control cells were used as a control value 1.00. The level of iNOS mRNA was markedly increased to 4.61 ± 0.04 following treatment with 5 μg/ml LPS for 24 h, while it was decreased to 2.34 ± 0.03, 1.25 ± 0.05, and 0.94 ± 0.04 in cells treated with *Atractylodes japonica* at 0.1 mg/ml, 1 mg/ml, and acetylsalicylic acid (ASA) at a concentration of 100 μg/ml, respectively. The level of COX-2 mRNA was markedly increased to 10.21 ± 1.21 following treatment with 5 μg/ml LPS for 24 h, while it was decreased to 5.14 ± 0.97, 2.21 ± 0.12, and 0.94 ± 0.04 in cells treated with *Atractylodes japonica* at 0.1 mg/ml, 1 mg/ml, and ASA at a concentration of 100 μg/ml, respectively (Fig. 1).

**Western Blot Analysis of iNOS and COX-2** Increased levels of iNOS protein (130 kDa) and COX-2 protein (66 kDa) were detected in cells of the 5 μg/ml LPS-treated groups. Treatment with LPS and *Atractylodes japonica*, however, resulted in decreased levels of expression of iNOS and COX-2 (Fig. 2).

**Effect of *Atractylodes japonica* on NO Synthesis** From NO detection assay, after 24 h of exposure to LPS, the amount of nitrite was increased from 2.35 ± 0.51 μM to 21.50 ± 2.41 μM, while it was decreased to 12.43 ± 2.14 μM, 5.21 ± 0.89 μM, and 4.18 ± 1.20 μM by treatment with *Atractylodes japonica* at 0.1 mg/ml, 1 mg/ml, and ASA at a concentration of 100 μg/ml, respectively (Fig. 3).

**Effect of *Atractylodes japonica* on PGE2 Synthesis** From PGE2 immunoassay, after 24 h of exposure to LPS, the amount of PGE2 was increased from 210.00 ± 34.58 pg/well to 872.00 ± 41.25 pg/well, while it was decreased to 617.00 ± 36.94 pg/well, 486.00 ± 48.51 pg/well, and 441.00 ± 39.44 pg/well by the treatment with *Atractylodes japonica* at 0.1 mg/ml, 1 mg/ml, and ASA at a concentration of 100 μg/ml, respectively (Fig. 4).

**DISCUSSION**

Inflammation is a complex process involving numerous mediators of cellular and plasma origin with elaborate and interrelated biological effects. NO and PGE2 are involved in various pathophysiological processes including inflammation and carcinogenesis, and iNOS and inducible COX-2 are mainly responsible for the production of large amounts of these mediators.10,11)

NO produced by the constitutive isof orm of NOS is a key
regulator of homeostasis, whereas the generation of NO by iNOS plays an important role in inflammation, host-defense responses, and tissue repair. Vane et al. indicated that NO is an important mediator of inflammation in animal models. Furthermore, because iNOS is up-regulated by endotoxin, interleukin-1, tumor necrosis factor (TNF)-α, and interferon γ, the increased synthesis of NO has been implicated in autoimmune disorders, allograft rejection, and systemic response to sepsis.

PGE₂, a major metabolite of the COX-2 pathway, has emerged as an important lipid mediator of inflammatory and immunoregulatory processes. PGE₂ is implicated in the pathogenesis of acute and chronic inflammatory states, and specific COX-2 inhibitors attenuate the symptoms of inflammation. COX activity and subsequent production of PGE₂ are closely related to the generation of NO radicals. Salvemini et al. reported that NO modulates the activity of COX-2.

**Fig. 1. Results of Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of the mRNA Levels of Inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2)**

Cells at a density of $2 \times 10^6$ were treated with *Atractylodes japonica* at concentrations of 0.1 mg/ml and 1 mg/ml with 5 μg/ml lipopolysaccharide (LPS) for 24 h. As the internal control, cyclophilin mRNA was also reverse-transcribed and amplified. *p < 0.05 compared to the control. *p < 0.05 compared to the LPS-treated group. M, Marker; A, Control; B, LPS-treated group; C, LPS- and 0.1 mg/ml *Atractylodes japonica*-treated group; D, LPS- and 1 mg/ml *Atractylodes japonica*-treated group; E, LPS- and 100 μg/ml acetylsalicylic acid (ASA)-treated group.

**Fig. 2. Results of Western Blot Analysis of the Protein Levels of Inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2)**

Cells at a density of $2 \times 10^5$ were treated with *Atractylodes japonica* at concentrations of 0.1 mg/ml and 1 mg/ml, with 5 μg/ml lipopolysaccharide (LPS) for 24 h. A, Control; B, LPS-treated group; C, LPS- and 0.1 mg/ml *Atractylodes japonica*-treated group; D, LPS- and 1 mg/ml *Atractylodes japonica*-treated group; E, LPS- and 100 μg/ml acetylsalicylic acid (ASA)-treated group. β-actin, used as the internal control, was detected at the position corresponding to a molecular weight of 46 kDa.

**Fig. 3. Measurement of Nitric Oxide (NO) Production in RAW264.7 Cells**

Cells at a density of $2 \times 10^4$ were treated with *Atractylodes japonica* at concentrations of 0.1 mg/ml and 1 mg/ml, with 5 μg/ml lipopolysaccharide (LPS) for 24 h. *p < 0.05 compared to the control. *p < 0.05 compared to the LPS-treated group. A, Control; B, LPS-treated group; C, LPS- and 0.1 mg/ml *Atractylodes japonica*-treated group; D, LPS- and 1 mg/ml *Atractylodes japonica*-treated group; E, LPS- and 100 μg/ml acetylsalicylic acid (ASA)-treated group.

**Fig. 4. Measurement of Prostaglandin E₂ (PGE₂) in RAW264.7 Cells**

Cells at a density of $2 \times 10^5$ were treated with *Atractylodes japonica* at concentrations of 0.1 mg/ml and 1 mg/ml, with 5 μg/ml lipopolysaccharide (LPS) for 24 h. *p < 0.05 compared to the control. *p < 0.05 compared to the LPS-treated group. A, Control; B, LPS-treated group; C, LPS- and 0.1 mg/ml *Atractylodes japonica*-treated group; D, LPS- and 1 mg/ml *Atractylodes japonica*-treated group; E, LPS- and 100 μg/ml acetylsalicylic acid (ASA)-treated group.
COX-2 as cGMP-independent fashion and plays a critical role in the release of PGE₂ by direct activation of COX-2. Inhibition of iNOS expression in murine macrophages may be another possible mechanism of nonsteroidal anti-inflammatory drugs. It has been reported that families of *Atractylodes* prevent stomach damage through an anti-ulcer effect and an inhibitory action on gastric secretion, and *Atractylodes japonica* consists of atractylon, hydroxyatractylon, 5αH,10β-selina-4(14),7(11)-diene-8-one. Of these, atractylon, the major active constituent of *Atractylodes japonica* extracts, was reported to inhibit Na⁺,K⁺-ATPase activity, and this Na⁺ transport is closely implicated with PGE₂ production. However, the molecular mechanisms on the actions of *Atractylodes japonica* have not been clarified to date. In the present study, the aqueous extract of *Atractylodes japonica* was shown to suppress PGE₂ production by inhibition of the LPS-stimulated enhancement of COX-2 enzyme activity and iNOS expression in RAW 264.7 macrophages. The present results suggest that *Atractylodes japonica* exerts anti-inflammatory and analgesic effects probably by suppressing of COX-2 and iNOS expressions, resulting in inhibition of PGE₂ synthesis.

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REFERENCES