Inhibition of Lipopolysaccharide-Induced Expression of Inducible Nitric Oxide and Cyclooxygenase-2 by Chiisanoside *via* Suppression of Nuclear Factor-κB Activation in RAW 264.7 Macrophage Cells

Jong-Heon Won,[a] Sang-Yong Park,[a] Sang-Gil Nam,[a] Hee-Juhn Park,[b] Jong-Won Choi,[c] and Kyung-Tae Lee*,[a]

[a] *Department of Biochemistry, College of Pharmacy, Kyung-Hee University; Seoul 130–701, South Korea:* [b] *Department of Botanical Resources, Sangji University; Wonju 220–702, South Korea: and* [c] *College of Pharmacy, Kyung-Sung University; Daeyeon-Dong, Pusan 608–736, South Korea.* Received May 2, 2005; accepted June 27, 2005

In the present study, the effects of several triterpenes isolated from the leaves of *Acanthopanax chiisanensis* (Araliaceae), namely, chiisanoside, isochiisanoside, 22-hydroxychiisanoside and chiisanogenin (the aglycone of chiisanoside) were evaluated on lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production by the RAW 264.7 macrophage cell line. Of the triterpenes tested, chiisanoside was found to most potently inhibit NO and PGE_2 production. In addition, chiisanoside significantly reduced the release of inflammatory cytokines like TNF-α and IL-1β. Consistent with these observations, the protein and mRNA expression levels of iNOS and COX-2 enzyme were found to be inhibited by chiisanoside in a concentration-dependent manner. Furthermore, chiisanoside inhibited the nuclear factor-κB (NF-κB) activation induced by LPS and this was associated with a reduction in p65 protein in the nucleus and with the phosphorylations of ERK1/2 and JNK MAP kinases. Taken together, our data indicate that the anti-inflammatory properties of chiisanoside might be the result from the inhibition of iNOS, COX-2, TNF-α and IL-1β expression through the down-regulation of NF-κB binding activity.

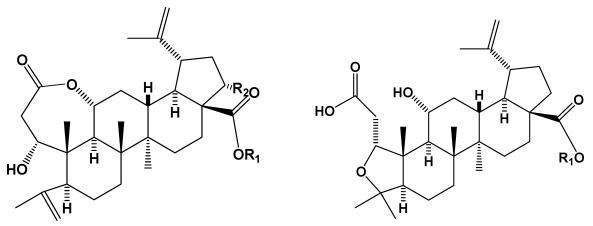
Key words chiisanoside; *Acanthopanax chiisanensis*; nitric oxide; prostaglandin E_2; NF-κB

The plants belonging to *Acanthopanax* species (Araliaceae) are traditionally used in Korea as anti-rheumatoid arthritis, anti-inflammatory and anti-diabetic drugs and are recognized to have ginseng-like activities.[1,2] 3,4-Secolupane-type triterpenoid family members, such as chiisanoside, chiisanogenin, 22-hydroxychiisanogenin, 24-hydroxychiisanogenin, *etc.* have been previously isolated from *Acanthopanax* species.[1,3] Chiisanoside, the main terpenoid component of this plant, has been reported to have anti-hepatotoxic, anti-diabetic and antiviral effects, the latter of which was found to be associated with the inhibition of mitogen-induced lymphocyte proliferation.[4—6] We previously described the anti-inflammatory effects of chiisanoside and chiisanogenin in the rats induced by carrageenan- and Freund's complete adjuvant.[7] In addition, we demonstrated that chiisanoside can be converted to its aglycon, chiisanogenin by human intestinal bacteria and that chiisanogenin showed more potent cytotoxicity and anti-rotaviral activities.[1]

During the inflammatory processes, large amounts of proinflammatory mediators, nitric oxide (NO) and prostaglandin E_2 (PGE_2) are generated by the inducible isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively.[8] In mammalian cells, NO is synthesized by three different isoforms of nitric oxide synthase (NOS), namely, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Although nNOS and eNOS are constitutively expressed, iNOS is expressed in response to interferon-γ, lipopolysccharide (LPS) and to a variety of proinflammatory cytokines.[9,10] Cyclooxygenase (COX) is the enzyme that converts arachidonic acid to PGs. Like NOS, COX existed in two isoforms, *i.e.*, COX-1 and COX-2.[11] COX-1 is expressed constitutively in most tissues and appears to be responsible for maintaining normal physiological functions. In contrast, COX-2 is detectable in only certain types of tissues and is induced transiently by growth factors, proinflammatory cytokines, tumor promoters and bacterial toxins.[12,13] Moreover, elevated levels of COX have been detected in different tumor types and this may account for the excessive production of inflammatory PGs.[14]

Nuclear transcription factor kappa-B (NF-κB) is one of the most ubiquitous transcription factors and regulates gene involved in cellular proliferation, inflammatory responses and cell adhesion. The activation of NF-κB has been reported to induce the transcriptions of many pro-inflammatory mediators, *e.g.*, iNOS, COX-2, TNF-α and interleukin (IL)-1β, -6 and -8.[15,16]

Our previous investigations of chiisanoside and chiisanogenin demonstrated their anti-inflammatory syndromes in the rat induced by carrageenan and Freund's complete adjuvant reagents.[7] Thus, as a prelude to reveal the underlying mechanisms for the anti-inflammatory effects of chiisanoside and chiisanogenin, we evaluated and compared the effects of various triterpenoid derivatives (Fig. 1) isolated from the leaves of *Acanthopanax chiisanensis* on LPS-induced NO



1 R_1=H, R_2=H
2 R_1= β-glc^6-β-glc^4-α-rha , R_2=H
3 R_1= β-glc^6-β-glc^4-α-rha , R_2=OH
4 R_1=β-glc^6-β-glc^4-α-rha

Fig. 1. Chemical Structures of Chiisanogenin (**1**), Chiisanoside (**2**), 22-Hydroxychiisanoside (**3**) and Isochiisanoside (**4**)

* To whom correspondence should be addressed. e-mail: ktlee@khu.ac.kr

and PGE_2 release and iNOS and COX-2 protein and mRNA expression in the RAW 264.7 macrophage cell line. We also investigated whether these triterpenoids influence the LPS-induced DNA binding activity of NF-κB and the protein level of its subunit, p65.

MATERIALS AND METHODS

Materials Chiisanoside, isochiisanoside, 22-hydroxychiisanoside, and chiisanogenin previously isolated from the leaves of *A. divaricatus* var. *albeofructus* was used.[6] Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.). iNOS, COX-2, p38, p-p38, JNK, p-JNK, ERK, p-ERK, p65, β-actin monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The enzyme immunoassay (EIA) kit for prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were obtained from R&D Systems (Minneapolis, MN, U.S.A.). NS-398, a COX-2 enzyme inhibitor, was from Calbiochem (San Diego, CA, U.S.A.). Luciferase assay kit was purchased from Promega (Madison, CA, U.S.A.). pNF-κB-Luc reporter plasmid was purchased from BD Biosciences (San Jose, CA, U.S.A.). Superfect reagent for transfection was purchased from Quiagen (Quiagen GmbH, Germany). RNA extraction kit was purchased from Intron Biotechnology. iNOS, COX-2, TNF-α, IL-1β and β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT), aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), dithiothreitol, caffeic acid, L-N^6-(1-iminoethyl)lysine (L-NIL), *Escherichia coli* lipopolysaccharide (LPS), triton X-100 and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Culture and Sample Treatment The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37 °C in DMEM medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO_2. Cells were incubated with the compounds being tested at various concentrations ot with the positive chemicals and stimulated with LPS 1 μg/ml for 24 h.

MTT Assay for Cell Viability RAW 264.7 viability after 24 h of continuous exposure to the tested compounds were measured with a colorimetric assay based on the ability of mitochondria in viable cells to reduce MTT as described previously.[17,18]

Nitrite Determination The nitrite accumulated in culture medium was measured as an indicator of NO production based on the Griess reaction. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine–HCl], incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured in a microplate reader (Perkin Elmer Cetus, Foster City, CA, U.S.A.). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was measured with the sodium nitrite serial dilution standard curve and nitrite production was measured.

Determination of PGE_2, TNF-α and IL-1β Production PGE_2, TNF-α and IL-1β level in macrophage culture medium were quantified by EIA kits according to the manufacture's instructions.

Western Blot Analysis Cellular proteins were extracted from control and chiisanoside-treated RAW 264.7 cells. The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride (NaF), 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated with 20 min at 4 °C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacture's instruction. Forty micrograms of cellular protein from treated and untreated cell extracts was electroblotted onto a nitrocellulose membrane following separation on a 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4 °C, followed by incubation for 4 h with a 1 : 500 dilution of monoclonal anti-iNOS, 1 : 1000 dilution of anti-COX-2 antibody, 1 : 1000 dilution of anti-p38, p-p38, JNK, p-JNK, ERK, p-ERK, p65, β-actin antibody and 1 : 500 dilution of anti-p65 antibody (Santa Cruz Biotechnology Inc.). Blots were washed two times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1 : 1000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Blots were again washed three times with and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).

RNA Preparation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology) according to the manufacturer's instructions. From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM dNTP, and oligo (dT_{12-18}) 0.5 μg/μl. Then PCR analyses were performed on the aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α, IL-1β and β-actin (as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA, U.S.A.). The reactions were carried out in a volume of 25 μl containing (final concentration) 1 units of Taq DNA polymerase, 0.2 mM dNTP, $\times$10 reaction buffer, and 100 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 95 °C, thirty amplification cycles were performed for iNOS (1 min of 95 °C denaturation, 1 min of 60 °C annealing, and 1.5 min 72 °C extension), COX-2 (1 min of 94 °C denaturation, 1 min of 60 °C annealing, and 1 min 72 °C extension), TNF-α (1 min of 95 °C denaturation, 1 min of 55 °C annealing, and 1 min 72 °C extension) and IL-1β (1 min of 94 °C denaturation, 1 min of 60 °C annealing, and 1 min 72 °C extension). PCR primers used in this study are listed below and were purchased from Bioneer (Seoul, Korea): sense strand iNOS, 5′-AAT GGC AAC ATC AGG-TCG GCC ATC ACT-3′, anti-sense strand iNOS, 5′-GCT GTG TGT CAC AGA AGT CTC GAA CTC-3′; sense strand COX-2, 5′-GGA GAG ACT ATC AAG ATA GT-3′, anti-

sense strand COX-2, 5′-ATG GTC AGT AGA CTT TTA CA-3′; sense strand TNF-α, 5′-ATG AGC ACA GAA AGC ATG ATC-3′, anti-sense strand TNF-α, 5′-TAC AGG CTT GTC ACT CGA ATT-3′; sense strand IL-1β, 5′-TGC AGA GTT CCC CAA CTG GTA CAT C-3′, anti-sense strand IL-1β, 5′-GTG CTG CCT AAT GTC CCC TTG AAT C-3′; sense strand β-actin, 5′-TCA TGA AGT GTG ACG TTG ACA TCC GT-3′, anti-sense strand β-actin, 5′-CCT AGA AGC ATT TGC GGT GCA CGA TG-3′. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA) RAW 264.7 macrophages were plated in 100-mm dishes (1×10^6 cells/ml). The cells were treated with various chiisanoside concentrations (25, 50, 100 μM), stimulated with LPS for 1 h, washed once with PBS, scraped into 1 ml of cold PBS, and pelleted by centrifugation. Nuclear extracts were prepared as described previously with slight modification. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 μg/ml aprotinin) and incubated on ice for 15 min. Then the cells were lysed by the addition of 0.1% Nonidet P-40 and vigorous vortexing for 10 s. The nuclei were pelleted by centrifugation at 12000×***g*** for 1 min at 4 °C and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate). Nuclear extract 10 μg was mixed with the double-stranded NF-κB oligonucleotide. 5′-AGTTGAGGGGACTTTCC-CAGGC-3′ end-labeled by [γ-^{32}P] dATP (underlying indicates a κB consensus sequence or a binding site for NF-κB/cRel homodimeric and heterodimeric complex). Binding reactions were performed at 37 °C for 30 min in 30 μl of reaction buffer containing 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 μg of poly (dI-dC), and 1 mM DTT. The specificity of binding was examined by competition with the 80-fold unlabeled oligonucleotide. DNA-protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5× TBE buffer. The gels were vacuum dried for 1 h at 80 °C and exposed to X-ray film at −70 °C for 24 h.

Transient Transfection and Luciferase Assay (Reporter Gene Assay) Transfection of RAW 264.7 cells was performed according to the manufacturer's instructions using superfect reagent (Qiagen GmbH, Germany) and pNF-κB-Luc reporter plasmid (BD Biosciences, San Jose, CA, U.S.A.). Cells were incubated for 2 h before the addition of 5 ml of DMEM/10% FBS. Forty-eight hours after the start of transfection, cells were stimulated with LPS (1 μg/ml) and chiisanoside. Following 3 h activation, cells were lysed and the luciferase activity was determined using the Promega luciferase assay system (Promega, Madison, CA, U.S.A.) and luminometer (Perkin Elmer Cetus, Foster City, CA, U.S.A.). The luciferase activity was normalized to the protein concentration of the sample.

Statistical Analysis The results were expressed as the mean±S.D. of triplicate experiments. Statistically significant values were compared using Student's *t*-test and a *p*-values less than 0.05 were considered statistically significant.

RESULTS

Effects of Triterpenoids on Cell Viability and LPS-Induced NO and PGE_2 Production To assess the effects of chiisanoside, isochiisanoside, 22-hydroxychiisanoside and chiisanogenin on the LPS-induced NO and PGE_2 production in RAW 264.7 cells, cell culture media were harvested and nitrite and PGE_2 levels were measured. Chiisanogenin, chiisanoside and isochiisanoside reduced NO production dose-dependently with IC_{50} values of 12.34 μM, 47.58 μM and 64.25 μM, respectively (Fig. 2A). L-NIL (10 μM) was used as a positive NO production inhibitor. To examine whether these four compounds inhibit PGE_2 production, cells were pre-incubated with the chiisanoside, isochiisanoside, 22-hydroxychiisanoside and chiisanogenin for 1 h and then activated with 1 μg/ml of LPS for 24 h. As shown in Fig. 2B, chiisanoside or isochiisanoside inhibited the production of PGE_2 in a dose-dependent manner. Chiisanogenin significantly inhibited NO production but not PGE_2 accumulation, whereas 22-hydroxychiisanoside had no effect on NO or PGE_2 production. The cytotoxic effects of these four triterpenoids were evaluated in the absence or presence of LPS. None of these triterpenoids affected cell viability at the concentrations used (25, 50, 100 μM) to inhibit NO and PGE_2 in-

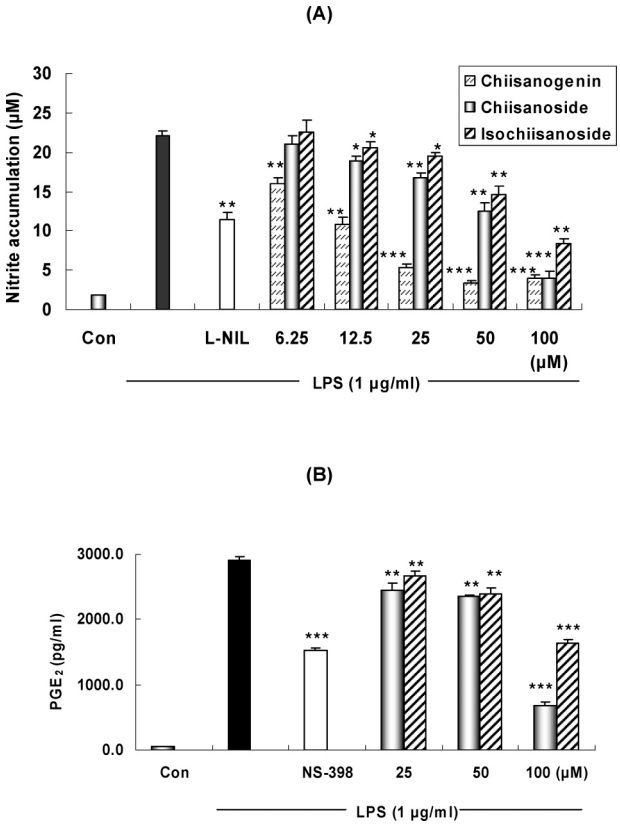


Fig. 2. The Effects of Chiisanoside and Its Derivatives on LPS-Induced NO and PGE_2 in RAW 264.7 Cells

(A) Cells were treated with LPS 1 μg/ml with/without different concentrations (6.25, 12.5, 25, 50, 100 μM) of chiisanogenin, chiisanoside, or isochiisanoside for 24 h. Control (Con) values were obtained in the absence of LPS, chiisanogenin, chiisanoside, or isochiisanoside. L-N^6-(1-iminoethyl) lysine (L-NIL) was used as an assay positive control at a concentration of 10 μM. (B) The conditions of sample treatment were identical to those described for Fig. 2A. NS-398 was used as a positive control in the assay. The values shown represent the means±S.D. of three independent experiments. $*p<0.05$, $**p<0.01$, $***p<0.001$ *vs.* the LPS-treated group; significances between treated groups were determined using the Student's *t*-test.

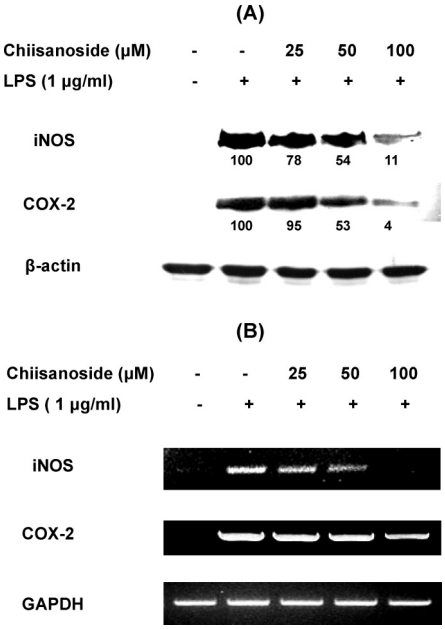


Fig. 3. The Effects of Chiisanoside on LPS-Induced iNOS and COX-2 Protein (A) and mRNA (B) Expressions in RAW 264.7 Cells

(A) Lysates were prepared from control or 24 h LPS (1 μg/ml)-stimulated cells alone or LPS plus with different concentrations (25, 50, 100 μM) of chiisanoside. All lanes contained 50 μg of total protein. An immunoblot representative of three separate experiments is shown. (B) Total RNA was prepared for the RT-PCR analysis of iNOS and COX-2 gene expression from RAW 264.7 macrophages stimulated with LPS (1 μg/ml) with/without different concentrations (25, 50, 100 μM) of chiisanoside for 4 h. iNOS-specific sequences (807 bp) and COX-2-specific sequences (721 bp) were detected by agarose gel electrophoresis. PCR of β-actin was performed to verify that the initial cDNA contents of the samples were similar. The experiment was repeated three times and similar results were obtained.

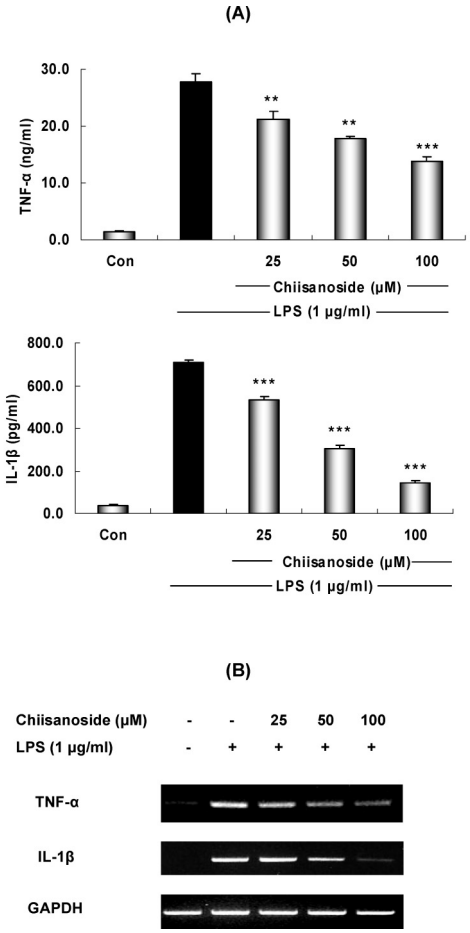


Fig. 4. The Effect of Chiisanoside on LPS-Induced TNF-α and IL-1β Release in RAW 264.7 Cells

(A) Cells were treated with LPS 1 μg/ml alone or LPS plus with different concentrations (25, 50, 100 μM) of chiisanoside for 24 h. Control (Con) values were obtained in the absence of LPS or chiisanoside. The experiment was repeated three times and similar results were obtained. Values represent means±S.D. of three independent experiments. ** $p<0.01$, *** $p<0.001$ *vs.* LPS-treated group; the significances of the difference between the treated groups was evaluated using the Student's *t*-test. (B) Total RNA was prepared under conditions identical to those described for Fig. 3B and RT-PCR was performed to compare the mRNA levels of TNF-α and IL-1β. PCR of β-actin was performed to verify that the initial cDNA contents of the samples were similar. The experiment was repeated twice with similar results.

hibition. Thus, the inhibitory effects observed were not attributable to cytotoxic effects.

Effects of Chiisanoside on LPS-Induced iNOS and COX-2 Protein and mRNA Expressions Western blot and RT-PCR analyses were performed to determine whether the inhibitory effects of chiisanoside on the pro-inflammatory mediators NO and PGE_2 are related to the modulation of the expressions of iNOS and COX-2. In unstimulated RAW 264.7 cells, iNOS protein and mRNA were undetectable. However, in response to LPS iNOS and COX-2 were strongly expressed and chiisanoside significantly inhibited this iNOS and COX-2 expression in a concentration-dependent manner (Fig. 3A). RT-PCR analysis showed that iNOS and COX-2 mRNA expressions related with their protein levels (Fig. 3B). In addition, we found that chiisanogenin did not have any inhibitory effect on iNOS or COX-2 protein expression (data not shown).

Effects of Chiisanoside on LPS-Induced TNF-α and IL-1β Production and mRNA Expression Since chiisanoside was found to most potently inhibit the pro-inflammatory mediators, we further investigated the effect of chiisanoside on LPS-induced TNF-α and IL-1β release by enzyme immunoassay and RT-PCR. Pretreatment of cells with chiisanoside reduced TNF-α and IL-1β production (Fig. 4A) and mRNA expression in a concentration-dependent manner (Fig. 4B), whereas chiisanogenin was practically inactive at these concentrations.

Effects of Chiisanoside on LPS-Induced NF-κB Translocation and p65-DNA Binding Since the activation of NF-κB is critically required for the activations of iNOS, COX-2, TNF-α and IL-1β by LPS, an electrophoretic mobility shift assay (EMSA) was performed in order to examine whether chiisanoside suppresses NF-κB activation. Accordingly, a DNA binding assay of NF-κB was carried out using nuclear extracts obtained from RAW 264.7 cells stimulated with LPS in the presence or absence of chiisanoside. Treatment with LPS (1 μg/ml) was found to increase NF-κB/DNA binding and pretreating cells with chiisanoside prior to LPS decreased NF-κB/DNA binding in a concentration-dependent manner (Fig. 5A). In an additional study conducted to confirm whether chiisanoside inhibits the activation of NF-κB in LPS-induced macrophages, we transiently transfected RAW 264.7 macrophage cells with an NF-κB-dependent luciferase reporter plasmid (pNF-κB-luc). As shown in Fig. 5B, LPS-induced NF-κB transcription activity increased 2-fold in these transfected cells but this was reduced dose-dependently by chiisanoside treatment at 25, 50, 100 μM.

Since p65 is the major component of the NF-κB activated by LPS in macrophages, we examined p65 translocation to the nucleus by immunoblotting (Fig. 5C). RAW 264.7 cells

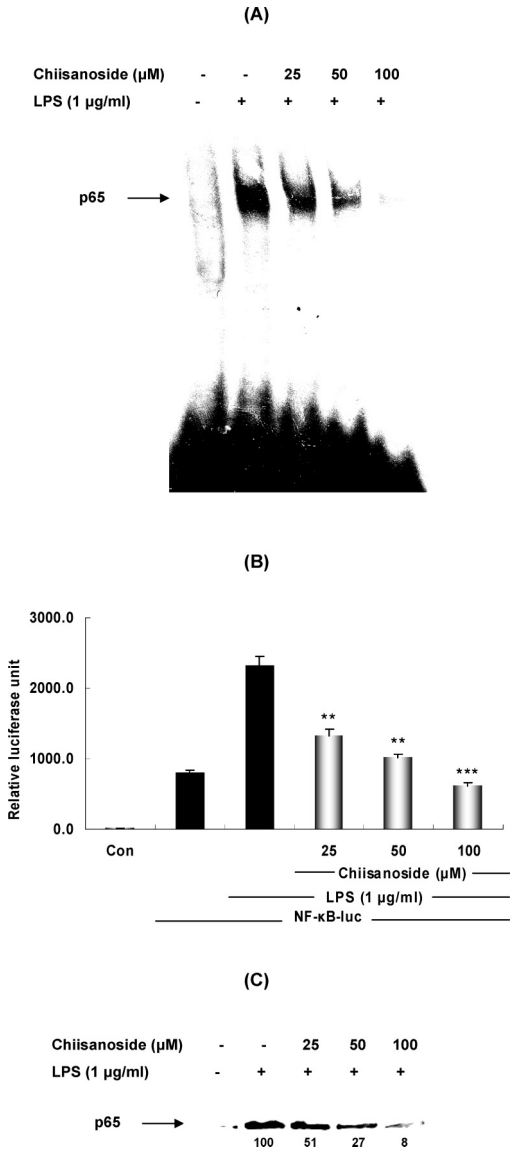


Fig. 5. The Inhibition of NF-κB-DNA Binding and p65 Nuclear Translocation by Chiisanoside

(A) Raw 264.7 cells were incubated for 60 min with different concentrations of chiisanoside. The arrow indicates the position of the NF-κB band. The data shown are representative of three independent experiments. (B) Cells were transiently co-transfected with pNF-κB-Luc reporter and then treated with alone or LPS plus with different concentrations (25, 50, 100 μM) of chiisanoside for 3 h. Cells were then harvested and luciferase activities determined using a Promega luciferase assay system and a luminometer. The values shown represent the means±S.D. of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$ *vs.* the LPS-treated group; the significances of differences between the treated groups were evaluated using the Student's *t*-test. (C) Raw 264.7 macrophages were treated with LPS (1 μg/ml) alone or in combination with increasing concentrations (25, 50, 100 μM) of chiisanoside. p65 levels in nuclear protein were determined by Western blotting.

had been incubated with LPS in the presence or absence of chiisanoside for 1 h. Negligible levels of p65 protein were detected in control cell nuclei but in contrast, p65 protein translocated into the nucleus 1 h after LPS treatment. Moreover, p65 protein levels were reduced in the cell nuclei cells exposed to LPS in combination with chiisanoside in a concentration-dependent manner, thus verifying that chiisanoside inhibits the nuclear localization of p65 protein.

Effects of Chiisanoside on LPS-Induced MAP Kinase Phosphorylation The mitogen-activated protein (MAP) kinases play critical roles in the regulation of cell growth and

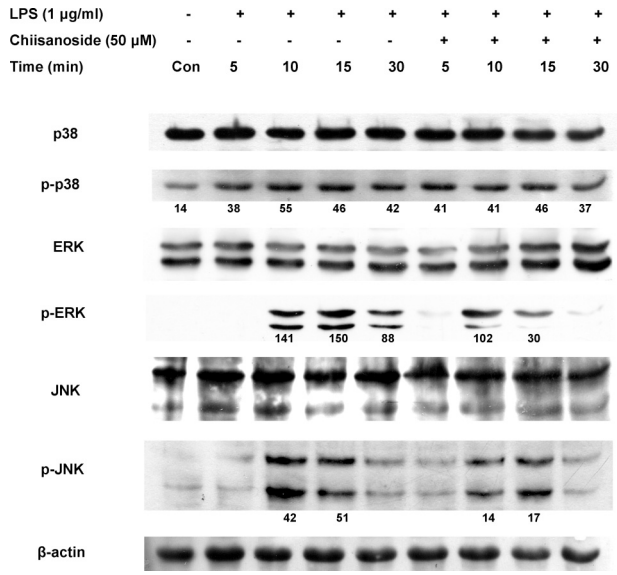


Fig. 6. Effects of Chiisanoside on the LPS-Induced Phosphorylation of MAP Kinase in RAW 264.7 Cells

RAW 264.7 macrophages were treated with LPS (1 μg/ml) and 50 μM of chiisanoside for the indicated times. All lanes contained 50 μg of total proteins. Western blot analysis using specific p38, p-p38, JNK, p-JNK, ERK, p-ERK, p65 and β-actin antibodies was repeated three times with similar results.

differentiation and in the control of cellular responses to cytokines and stresses. Moreover, they are also known to be important for the activation of NF-κB.[19] To investigate whether the inhibition of NF-κB activation by chiisanoside is mediated through the MAP kinase pathway, we examined the effects of chiisanoside on the LPS-stimulated phosphorylations of ERK1/2, SAPK/JNK and p38 MAP kinases in RAW 264.7 cells by western blotting. As shown in Fig. 6, chiisanoside suppressed the LPS-induced activations of ERK1/2 and SAPK/JNK MAP kinases in a time-dependent manner. However, non-phosphorylated ERK, JNK and P38 kinase expressions were unaffected by LPS or LPS plus chiisanoside. These results suggest that the phosphorylations of ERK1/2 and JNK kinases may be involved in the inhibitory effect of chiisanoside on LPS-stimulated NF-κB binding in RAW 264.7 cells.

DISCUSSION

We previously examined the anti-inflammatory activities of chiisanoside and chiisanogenin by activity-guided fractionation in carrageenan and Freund's complete adjuvant (FCA) induced rat models.[7] In the present study, we screened the effects of chiisanoside and of its derivatives on LPS-induced pro-inflammatory molecules, including NO and PGE_2. Chiisanoside was found to be the most potent inhibitor of these mediators among the tested compounds. To further explore the possible mechanism of these inhibitions by chiisanoside, the expression levels of iNOS and COX-2 proteins and iNOS, COX-2, TNF-α and IL-1β mRNA levels were examined. The inhibition by chiisanoside of the LPS-stimulated expressions of these molecules in RAW 264.7 cells was not attributable to chiisanoside cytotoxicity, as assessed by MTT assay and the expression of the housekeeping gene β-actin. The inhibition of iNOS and COX-2 gene expression was evidenced by reductions in their mRNA levels in a parallel con-

centration-dependent manner. Thus, the inhibition of NO and PGE_2 release may be attributed to the suppression of iNOS and COX-2 mRNA transcription followed by protein expression.

It has been reported that cytokines, such as IL-1β, TNF-α and IL-6 are proinflammatory *in vitro* and *in vivo*.[20—22] In the present study, we found that chiisanoside also significantly inhibits TNF-α and IL-1β release and mRNA expressions (Fig. 4).

NF-κB is known to play a critical role in the regulation of cell survival genes and to coordinate the expressions of proinflammatory enzymes and cytokines, such as iNOS, COX-2, TNF-α and IL-1β.[23—27] Since the expressions of these pro-inflammatory mediators are known to be modulated by NF-κB, we examined the possibility that chiisanoside inhibits NF-κB activity. Initially, we found that chiisanoside inhibits the expressions of iNOS and COX-2 proteins and iNOS, COX-2, TNF-α and IL-1β mRNA, which suggests that chiisanoside suppresses NF-κB. These findings are consistent with those of previous studies, which found that NF-κB response elements are present on the promoters of the iNOS, COX-2, TNF-α and IL-1β genes.[28—31] NF-κB is essentially composed of two proteins, p50 and p65, which are also referred to as RelA and cRel, respectively.[32] In the resting state, NF-κB is bound in the cytosol with an inhibitory protein, IκB. However, following its induction by a variety of agents such as LPS, TNF-α and tissue plasminogen activator, IκB is phosphorylated and this process triggers its proteolytic degradation *via* 26 s proteosome, which releases NF-κB from IκB and allows its translocated to the nucleus, where it binds to κB binding sites in the promoter regions of its target genes.[33] In the present study, we found that chiisanoside blocked the LPS-induced activation of NF-κB by inhibiting its translocation to the nucleus.

The MAP kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses.[19,34] Moreover, MAP kinases are involved in the LPS-induced iNOS expression signaling pathway.[35] Recently, it was found that JNK may associate with the cRel subunit of NF-κB and directly enhance NF-κB activation in the yeast two-hybrid system.[36] In the present study, we also investigated the effects of chiisanoside on the LPS-induced phosphorylation of MAP kinases in Raw 264.7 cells. Treatment with chiisanoside was found to significantly inhibit LPS-induced JNK and ERK phosphorylation but not p38 MAP kinase phosphorylation, suggesting that JNK and ERK are involved the inhibition of LPS-stimulated NF-κB binding by chiisanoside in Raw 264.7 cells.

The results of the present study indicate that chiisanoside is a potent inhibitor of the LPS-induced NO, PGE_2, TNF-α and IL-1β production *via* gene expression and that this inhibition is caused by blocking NF-κB activation and by ERK and JNK phosphorylation in RAW 264.7 macrophages. These findings suggest that chiisanoside is a potential therapeutic for the treatment of inflammatory pain syndrome.

Acknowledgments This research was supported by the Kyung Hee University Research Fund in 2005 (KHU-20050431).

REFERENCES

1) Bae E. A., Yook C. S., Oh O. J., Nohara T., Kim D. H., *Biol. Pharm. Bull.*, **24**, 582—585 (2001).
2) Lee S. H., Kim B. K., Cho S. H., Shin K. H., *Arch. Pharm. Res.*, **25**, 280—284 (2002).
3) Kasai R., Matsumoto K., Taniyasu S., Tanaka O., Kim J. H., Hahn D. R., *Chem. Pharm. Bull.*, **34**, 3284—3289 (1986).
4) Hahn D. R., Kasai R., Kim J. H., Taniyasu S., Tanaka O., *Chem. Pharm. Bull.*, **32**, 1244—1247 (1984).
5) Kim Y. O., Cho D. H., Chung H. J., Kim J. H., Chang S. Y., Yook C. S., Yang K. S., Oh O. J., *Yakhak Hoeji*, **43**, 208—213 (1999).
6) Oh O. J., Chang S. Y., Yook C. S., Yang K. S., Park S. Y., Nohara T., *Chem. Pharm. Bull.*, **48**, 879—881 (2000).
7) Jung H. J., Nam J. H., Choi J. W., Lee K. T., Park H. J., *J. Ethnopharmacol.*, **97**, 359—367 (2005).
8) Vane J. R., Mitchell J. A., Appleton I., Tomlinson A., Bishop-Bailey D., Croxtall J., Willoughby D. A., *Pharmacology*, **91**, 2046—2050 (1994).
9) Moncada S., Palmer R. M., Higgs E. A., *Pharmacol. Rev.*, **43**, 109—142 (1991).
10) Yun H. Y., Dawson T. T., Dawson T. M., *Crit. Rev. Neurobiol.*, **10**, 291—316 (1996).
11) Funk C. D., Funk L. B., Kennedy M. E., Pong A. S., Fitzgerald G. A., *FASEB J.*, **5**, 2304—2312 (1991).
12) Hinz B., Brune K., *J. Pharmacol. Exp. Ther.*, **300**, 367—375 (2002).
13) Prescott S. M., Ritzpatrick F. A., *Biochim. Biophys. Acta*, **1470**, M69—M78 (2000).
14) Subbaramaish K., Dannenberg A. J., *Trends Pharmacol. Sci.*, **24**, 96—102 (2003).
15) Lappas M., Permezel M., Georgiou H. M., Rice G. E., *Biol. Reprod.*, **67**, 668—673 (2002).
16) Surh Y. J., Chun K. S., Cha H. H., Han S. S., Keum Y. S., Park K. K., Lee S. S., *Mutat. Res.*, **480**—**481**, 243—268 (2001).
17) Seo B. R., Lee K. W., Ha J., Park H. J., Choi J. W., Lee K. T., *Carcinogenesis*, **25**, 1387—1394 (2004).
18) Shin K. M., Kim I. T., Park Y. M., Ha J., Choi J. W., Park H. J., Lee Y. S., Lee K. T., *Biochem. Pharmacol.*, **68**, 2327—2336 (2004).
19) Vandenberghe W., Plaisance S., Boone E., Debosscher K., Schmitz M. L., Fiers W., Haegeman G., *J. Biol. Chem.*, **273**, 3285—3290 (1998).
20) Dinarello C. A., *J. Infect. Dis.*, **179**, 294—304 (1999).
21) Feldmann M., Brennan F. M., Maini R., *Int. Rev. Immunol.*, **17**, 217—228 (1998).
22) Mannel D. N., Echtenacher B., *Chem. Immunol.*, **74**, 141—161 (2000).
23) Chen F., Kuhn D. C., Sun S. C., Gaydos L. J., Demers L. M., *Biochem. Biophys. Res. Commun.*, **214**, 839—846 (1995).
24) Roshak A. K., Jackson J. R., McGough K., Chabot-Fletcher M., Mochan E., Marshall L. A., *J. Biol. Chem.*, **271**, 31496—31501 (1996).
25) Schmedtje J. F., Jr., Ji Y. S., Liu W. L., DuBois R. N., Runge M. S., *J. Biol. Chem.*, **272**, 601—608 (1997).
26) Xie Q. W., Kashiwabara Y., Nathan C., *J. Biol. Chem.*, **269**, 4705—4708 (1994).
27) Xie W., Merrill J. R., Bradshaw W. S., Simmons D. L., *Arch. Biochem. Biophys.*, **300**, 247—252 (1993).
28) Ahn K. S., Noh E. J., Zhao H. L., Jung S. H., Kang S. M., Kim Y. S., *Life Sci.*, **76**, 2315—2328 (2005).
29) Barnes P. J., Karin M., *N. Engl. J. Med.*, **336**, 1066—1071 (1997).
30) Chen Y., Yang L., Lee T. J., *Biochem. Pharmacol.*, **59**, 1445—1457 (2000).
31) Kim Y. M., Lee B. S., Yi K. Y., Paik S. G., *Biochem. Biophys. Res. Commun.*, **236**, 655—660 (1997).
32) Kracht M., Saklatvala J., *Cytokine*, **20**, 91—106 (2002).
33) Delfino F., Walker W. H., *Mol. Cell. Endocrinol.*, **157**, 1—9 (1999).
34) Johnson G. L., Lapadat R., *Science*, **298**, 1911—1912 (2002).
35) Caivano M., *FEBS Lett.*, **429**, 249—253 (1998).
36) Meyer C. F., Wang X., Chang C., Templeton D., Tan T. H., *J. Biol. Chem.*, **271**, 8971—8976 (1996).