Inhibition of Methanol Extract from the Fruits of *Kochia scoparia* on Lipopolysaccharide-Induced Nitric Oxide, Prostaglandin E₂, and Tumor Necrosis Factor- α Production from Murine Macrophage RAW 264.7 Cells

Kyung-Min SHIN,^a Yang-Hee KIM,^a Wan-Su PARK,^a Insug KANG,^b Joohun HA,^b Jong-Won CHOI,^c Hee-Juhn PARK,^d and Kyung-Tae LEE^{*a}

^a College of Pharmacy, Kyung-Hee University; ^b College of Medicine, Kyung-Hee University; Dongdaemun-ku, Hoegi-Dong, Seoul 130–701, Korea; ^c College of Pharmacy, Kyung-Sung University; Daeyeon-Dong, Pusan 608–736, Korea; and

^d Division of Applied Plant Sciences, Sang-Ji University; Woosan-Dong, Wonju 220–702, Korea.

Received October 15, 2003; accepted December 17, 2003

In an attempt to search for bioactive natural products exerting antiinflammatory activity, we have evaluated the effects of the methanol extract of the fruits of *Kochia scoparia* (L.) CHARD. (Chenopodiaceae) on lipopolysaccharide (LPS)-induced nitric oxide (NO), prostaglandin E₂ (PGE₂), and tumor necrosis factor (TNF)- α release by the macrophage cell line RAW 264.7. Our data indicate that this extract is a potent inhibitor of NO production and it also significantly decreased PGE₂ and TNF- α release. Consistent with these observations, the protein and mRNA expression level of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 was inhibited by MeOH extracts of *Kochia scoparia* (KSM) in a dose-dependent manner. Furthermore, KSM inhibited the LPS-induced DNA binding activity of nuclear factor- κ B (NF- κ B), which was associated with prevention of the inhibitor κ B degradation. These results suggest that the methanol extract of *K. scoparia* inhibits LPS-induced iNOS and COX-2 expression by blocking NF- κ B activation.

Key words *Kochia scoparia*; nitric oxide; prostaglandin E₂; tumor necrosis factor- α ; nuclear factor κ B

Chronic inflammation and infections lead to the up-regulation of a series of enzymes and signaling proteins in affected tissues and cells. These proinflammatory enzymes, including the inducible forms of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are responsible for elevated levels of NO and prostaglandins (PGs), respectively, are involved in the pathogenesis of many chronic diseases including multiple sclerosis, Parkinson's and Alzheimer's diseases, and colon cancer.¹⁾ The proinflammatory properties of NO are attributed to the excessive production of NO by iNOS and are influenced by factors such as interaction of NO with other oxidants (e.g., peroxynitrite formation with superoxide anion), duration of NO production, and substrate availability. A number of studies has shown that the chronic phase of inflammation particularly correlates with an increase in iNOS activity.²⁾ The most conclusive evidence for NO as a mediator of tissue injury has been obtained from studies on an animal arthritis model, human osteoarthritis, and rheumatoid arthritis.³⁾ In contrast to iNOS, the constitutive epithelial and neuronal forms of NOS are known to contribute relatively little to inflammation and carcinogenesis. COX is the enzyme that converts arachidonic acid to PGs. Like NOS, COX has been found in two isoforms, and COX-2 is an inducible form responsible for the production of large amounts of proinflammatory PGs at the inflammatory site.⁴⁾ Furthermore, tumor necrosis factor (TNF)- α is a potent proinflammatory cytokine that plays an important role in immunity and inflammation. TNF- α activates vascular endothelium and increases vascular permeability, which leads to increased entry of IgG, complement, and cells to tissues and increased fluid drainage to lymph nodes as local effects. It also has systemic effects, many of which are harmful, such as fever, systemic edema, and shock.⁵⁾

One of the most ubiquitous transcription factors that regulate gene expressions involved in cellular proliferation, in-

flammatory responses, and cell adhesion is nuclear transcription factor kappa-B (NF- κ B). Functionally active NF- κ B exists mainly as a heterodimer consisting of subunits of the Rel family, which is normally sequestered in the cytosol as an inactive complex by binding to the inhibitors of κ B (I κ Bs) in unstimulated cells.⁶⁾ The activation mechanism of NF- κ B involves the phosphorylation of I κ Bs in two critical serine residues (Ser³², Ser³⁶) via the I κ B kinase (IKK) signalosome complex.^{7–9)} Once I κ Bs are phosphorylated, they are targeted for ubiquitination and subsequent degradation by the 26S proteasome.¹⁰⁾ The resulting free NF- κ B is translocated to the nucleus, where it binds to the κ B binding sites in the promoter regions of target genes, thereby controlling their expression.^{11,12)}

The fruits of *Kochia scoparia* (Chenopodiaceae) have been used for the treatment of skin disease in Chinese medicine,^{13,14)} as a remedy for diabetes mellitus, and for rheumatoid arthritis in the folk medicine of Korea. It has been reported that fruit of *K. scoparia* has a peripheral antinociceptive effect mediated by antiinflammatory action, and that its active component can be partially attributed to momordin Ic.¹³⁾ Our previous investigation of *K. scoparia* also demonstrated its antiinflammatory action in a rat induced by Freund's complete adjuvant reagent.¹⁵⁾ Thus, as a prelude to determining the underlying mechanisms of the antiinflammatory effect of *K. scoparia*, we evaluated the effects of the MeOH extracts of the fruits of *K. scoparia* L. (KSM) on lipopolysaccharide (LPS)-induced NO and PGE₂ release, and TNF- α production and then iNOS and COX-2 enzyme and mRNA expression levels in the macrophage cell line RAW 264.7. In the present study, we also examined whether KSM influences the LPS-induced DNA binding activity of NF- κ B and the protein level of its subunits p65 and p50.

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

MATERIALS AND METHODS

Plant Materials and Preparation of the Methanol Fraction The fruits of *K. scoparia* were purchased from the Chun-II Oriental Herbal Store in Wonju, Korea, and the plant origin was identified by S. Y. Yun (Division of Applied Plant Sciences, Sangji University, Korea). A voucher specimen (#NATCHEM-21) was deposited in the Laboratory of Natural Products Analysis, Division of Applied Plant Sciences, Sangji University, Korea. The plant material (2.5 kg) was extracted three times with MeOH under reflux. The extract was filtered and evaporated on a rotary evaporator under reduced pressure. The concentrated extract was again dried using a freeze-dryer to give a solid MeOH extract (243 g).

Chemicals 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.). COX-2, iNOS, p65, and I κ B- α 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 determination of prostaglandin E₂ and TNF- α was obtained from R&D Systems (Minneapolis, MN, U.S.A.). NS-398, a COX-2 enzyme inhibitor, was from Calbiochem (CA, U.S.A.). An RNA extraction kit was purchased from Intron Biotechnology (Seoul, Korea). iNOS, COX-2, TNF- α , and β -actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tertzolium bromide (MTT), sulfanilamide, aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), dithiothreitol, L-N⁶-(1-iminoethyl)lysine (L-NIL), *Escherichia coli* LPS, 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 Korean Cell Line Bank (Seoul, Korea). These cells were grown at 37 °C in DMEM containing 10% heat-inactivated FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μ g/ml) in a humidified atmosphere of 5% CO₂. Cells were incubated with KSM at increasing concentrations (7.5, 15, 30 μ g/ml) or positive chemical and stimulated with LPS 1 μ g/ml for the indicated time.

MTT Assay for Cell Viability Cytotoxicity studies were performed in 96-well plates. RAW 264.7 cells were mechanically scraped and plated at 1×10^5 /well in 96-well plates containing 100 μ l of DMEM with 10% heat-inactivated FBS and incubated overnight. KSM was dissolved in DMSO, and the DMSO concentrations in all assays did not exceed 0.1%. After overnight incubation, the test material was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 μ l of FBS-free medium containing MTT 5 mg/ml. After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue that formed in the cells was dissolved in DMSO 100 μ l. The optical density was measured at 540 nm.

Nitrite Determination The nitrite accumulated in culture medium was measured as an indicator of NO production based on the Griess reaction. Briefly, 100 μ l of cell culture medium was mixed with 100 μ l of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-HCl], incubated at

room temperature for 10 min, and then the absorbance at 550 nm was measured in a microplate reader. 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.

PGE₂ and TNF- α Assay PGE₂ and TNF- α levels in macrophage culture medium were quantified using EIA kits according to the manufacturer's instructions.

Western Blot Analysis Cellular proteins were extracted from control and KSM-treated RAW 264.7 cells. Cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). 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 PMSF, 0.5 mM dithiothreitol (DTT), 5 mM Na fluoride (NaF), 0.5 mM Na orthovanadate) containing 5 μ g/ml each of leupeptin and aprotinin and incubated for 30 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 manufacturer's instruction. Fifty micrograms of cellular protein from treated and untreated cell extracts as electrophoresed onto a nitrocellulose membrane following separation on 8–12% 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, COX-2, and NF- κ B (p65) antibody, and 1 : 1000 dilution of anti-I κ B- α 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 goat-anti-mouse IgG secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Blots were again washed three times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).

RNA Preparation and Polymerase Chain Reaction

Total cellular RNA was isolated using Easy Blue[®] kits (Intron Biotechnology) according to the manufacturer's instructions. From each sample, 1 μ g of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM dNTP, and oligo (dT_{12–18}) 0.5 μ g/ μ l. Then PCR analyses were performed on the aliquots of the cDNA preparations to detect iNOS, COX-2, TNF- α , 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 μ l containing (final concentration) 1 unit 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, 30 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), and TNF- α (1 min of 95 °C denaturation, 1 min of 55 °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'-AATGGCAACATC-AGGTCGGCCATCACT-3'; anti-sense strand iNOS, 5'-G-CTGTGTGTACAGAAAGTCTCGAACTC-3'; sense strand COX-2, 5'-GGAGAGACTATCAAGATAGT-3'; anti-sense

strand COX-2, 5'-ATGGTCAGTAGACTTTTACA-3'; sense strand TNF- α , 5'-ATGAGCACAGAAAGCATGATC-3'; anti-sense strand TNF- α , 5'-TACAGGCTTGTCACCTCGAATT-3'; sense strand β -actin, 5'-TCATGAAGTGTGACGTTGACATCCGT-3'; and anti-sense strand β -actin, 5'-CCTAGAAGCATTTGCGGTGCACGATG-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 RAW 264.7 macrophages were plated in 100-mm dishes (5×10^6 cells). The cells were treated with various KSM concentrations (7.5, 15, 30 $\mu\text{g/ml}$), 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.¹⁶⁾ 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 $\mu\text{g/ml}$ aprotinin) and incubated on ice for 15 min. Then the cells were lysed by the addition of 0.1% Noidet P-40 and vigorous vortexing for 10 s. The nuclei were pelleted by centrifugation at $12000 \times g$ 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'-AGTTGAGGGGACTTCCCA-GGC-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 $\times$ 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.

Statistical Analysis Data are reported as mean $\pm$ S.D. values of three independent determinations. All experiments were performed at least three times, each time with three or more independent observations. Statistical analysis was performed using Student's *t*-test with one-way analysis of variance.

RESULTS

Effect of KSM on Cell Viability and LPS-Induced NO Production The cytotoxic effect of KSM was evaluated in the presence or absence of LPS using the MTT assay. KSM in the presence or absence of LPS did not affect the cell viability of RAW 264.7 cells even at 100 $\mu\text{g/ml}$ for 24 h (data not shown). To assess the effects of KSM on NO production in RAW 264.7 cells, cell culture medium was harvested, and the production of nitrite was measured using the method of Griess. KSM reduced NO production in a dose-dependent manner with an IC_{50} of 15.85 $\mu\text{g/ml}$ as an approximated value from one of the three separate experiments (Fig. 1). L- N^6 -(1-iminoethyl)lysine (10 μM) was used as a positive inhibitor.

Fig. 1. Effect of KSM on LPS-Induced NO Production in RAW 264.7 Cells

The cells were treated with LPS 1 $\mu\text{g/ml}$ alone or plus various concentrations (7.5, 15, 30 $\mu\text{g/ml}$) of KSM for 24 h. Control (Con) values were obtained in the absence of LPS or KSM. L- N^6 -(1-iminoethyl)lysine (L-NIL) 10 μM was present in the assay as a positive control. The values are the mean $\pm$ S.D. from three independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs. LPS-treated group; significance of difference between treated groups in Student's *t*-test.

Fig. 2. Effect of KSM on LPS-Induced PGE₂ Production in RAW 264.7 Cells

The cells were treated with LPS 1 $\mu\text{g/ml}$ alone or LPS plus different concentrations (7.5, 15, 30 $\mu\text{g/ml}$) of KSM for 24 h. Control (Con) values were obtained in the absence of LPS or KSM. NS-398 10 μM was used as a positive control in the assay. The values are the mean $\pm$ S.D. from three independent experiments. * $p < 0.05$, *** $p < 0.001$ vs. LPS-treated group; significance of difference between treated groups in Student's *t*-test.

Inhibition of LPS-Induced PGE₂ and TNF- α Production by KSM To examine whether the KSM could inhibit PGE₂, cells were preincubated with the KSM for 1 h, and then activated with 1 $\mu\text{g/ml}$ LPS for 24 h. As shown in Fig. 2, the production of PGE₂ was significantly inhibited by KSM in a dose-dependent manner. In the same manipulation, we also investigated the effect of KSM on LPS-induced TNF- α release using EIA and RT-PCR. Pretreatment of cells with KSM decreased TNF- α production and mRNA expression in a dose-dependent manner (Figs. 3A, B).

Effect of KSM LPS-Induced iNOS and COX-2 Protein and mRNA Expression Western blot and RT-PCR analyses were performed to determine whether the inhibitory effect of KSM on these inflammatory mediators (NO, PGE₂) was related to a modulation of iNOS and COX-2 enzymes. In unstimulated RAW 264.7 cells, iNOS and COX-2 protein and mRNA were not detectable. In response to LPS, the expression level of iNOS was markedly augmented, and KSM significantly inhibited iNOS protein induction in a dose-dependent manner (Fig. 4). RT-PCR analysis showed that the content of iNOS mRNA was correlated with its protein level (Fig. 5). A similar pattern was observed when the effect of

A

B.

Fig. 3. Effect of KSM on LPS-Induced TNF- α Release in RAW 264.7 Cells

(A) The cells were treated with LPS 1 μ g/ml alone or LPS plus different concentrations (7.5, 15, 30 μ g/ml) of KSM for 24 h. Control (Con) values were obtained in the absence of LPS or KSM. (B) Cells were lysed and total RNA was prepared for the RT-PCR analysis of gene expression. cDNA (10 ng) of RAW 264.7 macrophages either not treated, stimulated with LPS (1 μ g/ml), or cotreated with LPS (1 μ g/ml) and various concentrations (7.5, 15, 30 μ g/ml) of KSM for 4 h were subjected to PCR. TNF- α -specific sequences (351 bp) were detected by agarose gel electrophoresis. PCR of β -actin was performed to control similar initial cDNA content of samples. The values are mean $\pm$ S.D. from three independent experiments. (B) ** p < 0.01, *** p < 0.001 vs. LPS-treated group; significance of difference between treated groups in Student's t -test.

Fig. 4. Effect of KSM on LPS-Induced iNOS and COX-2 Protein Expression in RAW 264.7 Cells

Lysates were prepared from control or 24 h LPS (1 μ g/ml)-stimulated cells alone or in combination with increasing concentrations (7.5, 15, 30 μ g/ml) of KSM. All lanes contained 50 μ g of total proteins. The experiment was repeated three times with similar results.

KSM was examined on the LPS-induced COX-2 expression level; the densitometric analysis of three different experiments demonstrated that the COX-2 protein expression induced by LPS was inhibited by 85.7% in cells treated with KSM 30 μ g/ml (data not shown). Under the same conditions, COX-2 mRNA levels were also significantly decreased in a similar pattern (Fig. 5). KSM did not affect the expression of the housekeeping gene β -actin. In general, these results are consistent with the profile of the inhibitory effect of the KSM on NO (Fig. 1) and PGE₂ release (Fig. 2).

Inhibition of LPS-Induced NF- κ B Activation by KSM

Fig. 5. Modulation by KSM of LPS-Induced iNOS and COX-2 mRNA Expression in RAW 264.7 Cells

Cells were lysed and total RNA was prepared for the RT-PCR analysis of gene expression. cDNA (10 ng) of RAW264.7 macrophages either not treated, stimulated with LPS (1 μ g/ml), or cotreated with LPS (1 μ g/ml) and various concentrations (7.5, 15, 30 μ g/ml) of KSM for 4 h were subjected to PCR. iNOS-specific sequences (807 bp) or COX-2-specific sequences (721 bp) were detected by agarose gel electrophoresis. PCR of β -actin was performed to control similar initial cDNA content of samples. The experiment was repeated three times with similar results.

Fig. 6. Inhibition of NF- κ B DNA Binding by KSM

Nuclear extract, prepared from control or 1 h LPS (1 μ g/ml)-stimulated cells alone or in combination with increasing concentrations (7.5, 15, 30 μ g/ml) of KSM, were prepared and analyzed for NF- κ B binding for EMSA. The arrow indicates the position of the NF- κ B and nonspecific (N.S.) bands. C.P. represents 80-fold cold probe only. Data represent one of three similar results.

To investigate further the mechanism of KSM-mediated inhibition of iNOS, COX-2, and TNF- α transcription, we focused on the NF- κ B, which is known to transactivate iNOS, COX-2, and TNF- α .¹¹ The electrophoretic mobility shift assay (EMSA) analyses demonstrated a reduction in NF- κ B DNA binding activity in nuclear extracts obtained from LPS-activated RAW 264.7 macrophages treated with KSM (7.5, 15, 30 μ g/ml) (Fig. 6). The extent of reduction was in a similar range (around 80%) as those of iNOS and COX-2 protein and iNOS, COX-2, and TNF- α mRNA expression.

Degradation of I κ B- α and Nuclear Translocation of p65 In unstimulated cells, NF- κ B is sequestered in the cytosol by its inhibitor I κ B, which upon LPS stimulation is phosphorylated by its inhibitor I κ B kinases, ubiquitinated, and rapidly degraded via the 26 S proteasome, thus releasing NF- κ B.⁶ KSM (30 μ g/ml) blocked LPS-induced degradation of I κ B- α , constituting the I κ B complex in cytosol until 60 min (Fig. 7A). We further investigated whether KSM prevented the translocation of the p65 subunit of NF- κ B, from the cytosol to the nucleus after release from I κ B. Western blot analyses showed that the LPS-induced p65 level in the nuclear fraction was decreased by KSM at 60 min (Fig. 7B).

Fig. 7. Western Blot Analyses of I κ B Degradation (A) and Nuclear Translocation of p65 (B)

(A) RAW 264.7 macrophages were treated with LPS (1 μ g/ml) and 30 μ g/ml of KSM for the indicated periods. Then cytosol protein fractions (C) were subjected to Western blot analysis for I κ B- α . (B) RAW 264.7 cells were treated with LPS (1 μ g/ml) and 30 μ g/ml of KSM for the indicated time, and nuclear proteins (N) were also subjected to Western blot analysis of p65.

DISCUSSION

Because the mechanism of the antiinflammatory effects of KSM has not been reported, we examined the effects of KSM on the release of several inflammatory mediators (NO, PGE₂, and TNF- α), the expression levels of iNOS and COX-2, and NF- κ B DNA binding activity on LPS-activated macrophages. The results of the present study indicate that KSM inhibits the induction of iNOS in LPS-activated murine macrophages at the transcriptional level. This is based on the following lines of evidence. 1) LPS-induced NO production was reduced by KSM in a concentration-dependent manner. 2) Western blot analysis demonstrated markedly reduced levels of iNOS protein in LPS-activated cells treated with KSM as compared with untreated cells. 3) RT-PCR showed a lower level of expression of iNOS mRNA in RAW 264.7 macrophages exposed to KSM.

In contrast to continuously expressed NO synthesis, iNOS is regulated primarily at the transcriptional level.¹⁷⁾ Thus substances that interfere with the induction of iNOS are not likely to affect the constitutively expressed NOS. This is of considerable importance since iNOS-dependent overproduction of NO is considered as proinflammatory whereas the protective and antiinflammatory properties of NO are mainly attributed to the constitutively expressed NOS system.¹⁸⁾ In this respect, NO synthase inhibitors for the treatment of NO-mediated inflammatory processes require high specificity for iNOS, and thus inhibitors of iNOS induction may function as relatively safe modulators of NO for various pathologic conditions.

The mechanism of various antiinflammatory drug actions is at least shared by the inhibition of PG synthesis, which is mediated by COX.¹⁹⁾ COX exists in two isoforms, COX-1 and COX-2, each with a distinct expression pattern in various

cell types. COX-1 has been suggested to provide a physiologic level of PGs for normal platelet, stomach, and kidney function. In contrast, COX-2 has been found to be highly induced at inflammatory sites in animals as well as patients with inflammatory diseases^{20,21)} and thus it is considered to be responsible for proinflammatory PG production. We demonstrated that KSM also significantly inhibits PGE₂ production and COX-2 protein and mRNA expression.

The expression of iNOS and COX-2 in murine macrophages has been shown to be dependent on NF- κ B activation.¹¹⁾ The possibility that KSM may inhibit the activity of NF- κ B was examined. The results indicate that KSM inhibition of expression of iNOS and COX-2 proteins and iNOS, COX-2, and TNF- α mRNA was most likely due to suppression of NF- κ B. This is consistent with the reports that NF- κ B response elements are present on the promoters for the iNOS, COX-2, and TNF- α genes.^{22–26)} NF- κ B is composed mainly of two proteins, p50 and p65, also called RelA and cRel, respectively.¹¹⁾ Under resting states, NF- κ B is present in the cytosol and is bound to the inhibitory protein I κ B. After induction by a variety of agents such as LPS, TNF- α , and tissue plasminogen activator, I κ B is phosphorylated to trigger proteolytic degradation *via* the 26 S proteasome. NF- κ B is then released from I κ B and is translocated into the nucleus where it binds to the κ B binding sites in the promoter regions of target genes. In the present study, we found that KSM blocked LPS-induced activation of NF- κ B *via* inhibition of the degradation of I κ B. These results suggest that KSM inhibition of LPS-induced expression of the iNOS and COX-2 genes occurs through blocking NF- κ B activation, although inhibition of other factors such as AP-1, the interferon response element, and γ -activated site may be involved.

In conclusion, the results of the present study indicate that KSM is a potent inhibitor of LPS-induced NO, PGE₂, and TNF- α production *via* gene expression by blocking NF- κ B activation in RAW 264.7 macrophages. KSM appears to be a potential therapeutic agent for treating LPS-induced sepsis syndrome.

Acknowledgments This research was supported by a grant (PF0320301-00) from the Plant Diversity Research Center of the 21st Frontier Research Program funded by the Ministry of Science and Technology of the Korean government.

REFERENCES

- 1) Heiss E., Herhaus C., Klimo K., Bartsch H., Gerhauser C., *J. Biol. Chem.*, **276**, 32008–32015 (2001).
- 2) Miller M. J., Grisham M. B., *Mediators Inflamm.*, **4**, 387–396 (1995).
- 3) Cochran F. R., Selph J., Sherman P., *Med. Rev. Res.*, **16**, 547–563 (1996).
- 4) Weisz A., Cicatiello I., Esumi H., *Biochem. J.*, **316**, 209–215 (1996).
- 5) Janeway C. A., Travers P., Walpot M., Shlomchik M., "Immunobiology," 5th ed., Garland Publishing, New York, 2001.
- 6) Baeuerle P. A., *Cell*, **95**, 729–731 (1998).
- 7) Brown K., Gerberger S., Carlson L., Franzoso G., Siebenlist U., *Science*, **267**, 1485–1488 (1995).
- 8) DiDonato J., Mercurio F., Rosette C., Wu-Li J., Suyang H., Ghosh S., Karin M., *Mol. Cell. Biol.*, **4**, 1295–1304 (1996).
- 9) O'Connell M. A., Bennett B. L., Mercurio F., Manning A. M., Makman N., *J. Biol. Chem.*, **273**, 30410–30414 (1998).
- 10) Sanchez-Perez I., Benitah S. A., Martinez-Gomariz M., Lacal J. C.,

- Perona R., *Mol. Biol. Cell*, **13**, 2933—2945 (2002).
- 11) Baeuerle P. A., Baltimore D., *Cell*, **87**, 13—20 (1996).
- 12) Barnes P. J., Karin M., *N. Engl. J. Med.*, **336**, 1066—1071 (1997).
- 13) Matsuda H., Dai Y., Ido Y., Ko S., Yoshikawa M., Kubo M., *Biol. Pharm. Bull.*, **20**, 1086—1091 (1997).
- 14) Matsuda H., Dai Y., Ido Y., Yoshikawa M., Kubo M., *Biol. Pharm. Bull.*, **20**, 1165—1170 (1997).
- 15) Choi J. W., Lee K. T., Jung H. J., Park H. J., *Arch. Pharm. Res.*, **25**, 336—342 (2002).
- 16) Kim R. G., Shin K. M., Kim Y. G., Jeong H. J., Ha J. H., Choi J. W., Park H. J., Lee K. T., *Biol. Pharm. Bull.*, **26**, 481—486 (2003).
- 17) Forstermann U., Kleinert H., *Naunyn Schmiedeberg Arch. Pharmacol.*, **352**, 351—364 (1995).
- 18) Clancy R. M., Abramson S. B., *Proc. Soc. Exp. Biol. Med.*, **210**, 93—101 (1995).
- 19) Vane J. R., *Nature* (London), **231**, 232—235 (1971).
- 20) Seibert K., Zhang Y., Leahy K., Hauser S., Masferrer J., Perkins W., Lee L., Isakson P., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 12013—12017 (1994).
- 21) Masferrer J. L., Zweifel B. S., Manning P. T., Hauser S. D., Leahy K. M., Smith W. G., Isakson P. C., Seibert K., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 3228—3232 (1994).
- 22) Xie W., Merrill J. R., Bradshaw W. S., Simmons D. L., *Arch. Biochem. Biophys.*, **300**, 247—252 (1993).
- 23) Xie Q. W., Kashiwabara Y., Nathan C., *J. Biol. Chem.*, **269**, 4705—4708 (1994).
- 24) Chen F., Kuhn D. C., Sun S. C., Gaydos L. J., Demers L. M., *Biochem. Biophys. Res. Commun.*, **214**, 839—846 (1995).
- 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) Roshak A. K., Jackson J. R., McGough K., Chabot-Fletcher M., Mochan E., Marshall L. A., *J. Biol. Chem.*, **271**, 31496—31501 (1996).