Inhibition of Methanol Extract from the Aerial Parts of Saururus chinensis on Lipopolysaccharide-Induced Nitric Oxide and Prostaglandin E₂ Production from Murine Macrophage RAW 264.7 Cells

Rung-Gyu Kim, Kyung-Min Shin, Young-Kwan Kim, Hyeh-Jeong Jeong, Joohun Ha, Jong-Won Choi, Hee-Juhn Park, and Kyung-Tae Lee*

* College of Pharmacy, Kyung-Hee University; a College of Pharmacy, Daeyeon-Dong, Kyung-Sung University; Pusan 608–736, Korea: and d Division of Applied Plant Sciences, Sang-Ji University; Woosan-Dong, Wonju 220–702, Korea.

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As an attempt to search for bioactive natural products exerting antiinflammatory activity, we have evaluated the effects of the methanol extract from the aerial parts of Saururus chinensis (LOUR.) BAILL (Saururaceae) on lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E₂ (PGE₂) 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 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 the aerial part of S. chinensis (SCM) in a dose-dependent manner. Furthermore, SCM inhibited the LPS-induced DNA binding activity of nuclear factor-κB (NF-κB), which was associated with decreased p65 protein levels in the nucleus. These results suggest that SCM inhibits LPS-induced iNOS and COX-2 expression by blocking NF-κB activation.

Key word Saururus chinensis; nitric oxide; prostaglandin E₂; COX-2; iNOS; NF-κB

The aerial part of Saururus chinensis (LOUR.) BAILL (Saururaceae) has been used for the treatment of edema, jaundice, and gonorrhea as Korean folk medicine. Although several pharmacological effects of S. chinensis have been reported, there are very few reports on its antiinflammatory activity.

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 known to be involved in the pathogenesis of many chronic diseases including multiple sclerosis, Parkinson’s and Alzheimer’s diseases, and colon cancer. The proinflammatory properties of NO are attributed to the excessive production of NO by iNOS and are influenced by factors like interaction of NO with other oxidants (e.g., peroxynitrite formation with superoxide anion), duration of NO production, and substrate availability. Studies have shown that the chronic phase of inflammation in particular correlates with an increase in iNOS activity. The most conclusive evidence for NO as a mediator of tissue injury has been obtained from arthritis, which is based on studies in an animal model, human osteoarthritis, and rheumatoid arthritis. The constitutive epithelial and neuronal forms of NO production 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 isozymes, and COX-2 is an inducible form responsible for the production of large amounts of proinflammatory PGs at the inflammatory site.

One of the most ubiquitous transcription factors that regulate gene expressions involved in cellular proliferation, inflammatory responses, and cell adhesion, is nuclear transcription factor kappa-B (NF-κB). Functionally active NF-κB 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 IκB. Phosphorylation and subsequent ubiquitination of IκB upon exposure of cells to various extracellular stimuli lead to rapid degradation of this inhibitory subunit by proteosomes. The resulting free NF-κB is translocated to the nucleus, where it binds to the κB binding sites in the promoter regions of target genes, thereby controlling their expression.

As a part of our search for the identification of new biologically active substances from medicinal plants, we evaluated the effects of the MeOH extracts of the aerial part of S. chinensis (SCM) on lipopolysaccharide (LPS)-induced NO and PGE₂ release, and then iNOS and COX-2 enzyme and mRNA expression by the macrophage cell line RAW 264.7 in the present study. We also examined whether SCM influences the LPS-induced DNA binding activity of NF-κB and the protein level of its subunits, IκB and p65.

MATERIALS AND METHODS

Plant Materials and Preparation of the Methanol Fraction The aerial parts (stem and leaves) of S. chinensis were collected at an herbal garden (Sambu farm), Geochang City, Korea, in May 1998, and identified by B.-T. Ahn (College of Pharmacy, Chungbuk National University). A voucher specimen has been deposited at the herbarium of the College of Pharmacy of Chungbuk National University (CNUP 3053). The dried plant material (70 g) was cut into small pieces and extracted repeatedly with methanol (3 × 2 l) at 70 °C. The combined methanolic extract was filtered through filter paper, evaporated in vacuo, and freeze-dried to give a powdered extract (7.1 g).

Chemicals RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life

* To whom correspondence should be addressed. e-mail: ktlee@khu.ac.kr © 2003 Pharmaceutical Society of Japan
Technologies Inc. (Grand Island, NY, U.S.A.), COX-2, iNOS, NF-κB, and 1kxBo 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 E2 was obtained from R&D Systems (Minneapolis, MN, U.S.A.). NS398, a COX-2 enzyme inhibitor, was from Calbiochem (CA, U.S.A.). RNA extraction kit was from Invitrogen Biotechnology (Seoul, Korea). iNOS, COX-2, and GAPDH oligonucleotide primers were purchased from Bioneer (Seoul, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), 3-(2-hydroxy-2-nitroso-1-propylhydradrazino)-1-propanamine (PAPA NONOate), sulfanilamide, aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), dithiothreitol, caffeic acid, N6-methyl-L-arginine (NMMA), N-NO-(1-iminoethyl)Lysine (L-NIL), E. 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 Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37 °C in RPMI medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO2. Cells were incubated with SCM at increasing concentrations (1, 5, 10 μg/ml) or positive control and incubated overnight. SCM 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 μl 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 μl. The optical density was measured at 540 nm.

**Nitrite Assay** Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by the Griess reaction. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (equal volumes of 1% (v/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethenediamine–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 calculated from a sodium nitrite standard curve freshly prepared in culture medium.

**PGE2 Assay** PGE2 levels in macrophage culture medium were quantified using EIA kits according to the manufacturer’s instructions.

**Western Blot Assay** Cellular proteins were extracted from control and SCM-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 phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated for 15 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 were electroblotted onto a nitrocellulose membrane following separation on 8—12% sodium dodecyl sulfate (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-κB (p65) antibody, and 1 : 1000 dilution of anti-1kxBo antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). Blots were washed two times with in 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., Santa Cruz, CA, U.S.A.) 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 (Invitrogen 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, and GAPDH (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) 2 units of Taq DNA polymerase, 0.2 mM dNTP, × 10 reaction buffer, and 100 pmol of 5’ and 3’ primers. After initial denaturation for 2 min at 95 °C, a cycle number was used that fell within the exponential range of the iNOS response for iNOS (95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min) and COX-2 (94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min). PCRs were electrophoresed through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation.

**iNOS and COX-2 Activity Assay** RAW 264.7 cells were plated at 2×105 cells/well in a 24-well plate and incubated for 6 h for the COX-2 activity assay or 12 h for the iNOS activity assay with LPS, respectively. Then the cells were washed twice with PBS. For measurement of iNOS activity, test samples were added for an additional 12 h in the absence of LPS. The cell-free culture medium was collected and NO levels determined by the Griess reaction. For COX-2 activity, the cells were washed twice with fresh culture medium and allowed to equilibrate in the absence or presence of sample for 30 min. The cells were further incubated with arachidonic acid 100 μM for 15 min with no LPS in the medium. The supernatants were removed and assayed for PGE2 using an EIA kit.

**Scavenging Effect on PAPA NONOate-released NO Radicals** PAPA NONOate (20 μM) was dissolved serum-containing RPMI-1640 medium (pH 7.6) ready for cell culture, and incubated with various concentrations of test sample at 37 °C for 3 h. After incubation, the concentration of nitrite was measured using the Griess reaction method as described above.
Electrophoretic Mobility Shift Assay (EMSA) RAW macrophages were plated in 100-mm dishes (5 \times 10^6 cells). The cells were treated with various SCM concentrations (1, 5, 10 μg/ml), stimulated with LPS for 1 h, washed once with PBS, scraped into 1 ml of cold PBS, and pelleted by centrifugation. Cytosolic and nuclear fractions were extracted as described previously. Binding reactions were performed at 37 °C for 15 min in 20 μl of reaction buffer containing 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 10% glycerol, 1 μg of poly(dI-dC), 1 mM dithiothreitol, and 30000 cpm 32P-labeled oligonucleotide probes for NF-κB. DNA–protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 80 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.

Data Analysis Data are reported as mean±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.

RESULTS

Effect of SCM on Cell Viability and LPS-Induced NO Production The cytotoxic effect of SCM was evaluated in the presence or absence of LPS using the MTT assay. SCM alone and in the presence of LPS did not affect the cell viability of RAW 264.7 cells even at 50 μg/ml for 24 h (data not shown). To determine the effects of SCM on NO production in RAW 264.7 cells, the cells were treated with LPS (1 μg/ml) in the presence or absence of SCM for 24 h. The production of NO was measured by using the method of Griess. LPS (1 μg/ml) induced approximately 9-fold greater NO2- production compared with the control, and this induction was inhibited by SCM in a dose-dependent manner (Fig. 1). l-NIL (10 μM) was used as a positive inhibitor.

Inhibition of LPS-Induced PGE2 Production by SCM To examine whether the SCM could inhibit PGE2 production, cells were pre-incubated with the SCM for 1 h, and then activated with 1 μg/ml LPS. As shown in Fig. 2, the production of PGE2 was significantly inhibited by the SCM in a dose-dependent manner.

Effect of SCM on LPS-Induced iNOS and COX-2 Protein and mRNA Expression To determine if the inhibitory effect of SCM on these inflammatory mediators (NO and PGE2) was related to a modulation of iNOS and COX-2 enzymes, we examined their expression levels by Western blot and RT-PCR analysis. In response to LPS, the expression levels of iNOS was markedly augmented, and SCM significantly inhibited the iNOS protein induction in a dose-dependent manner (Fig. 3A). RT-PCR analysis showed that the content of iNOS mRNA was correlated with its protein level (Fig. 3B). A similar pattern was observed when the effect of SCM 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 68.5% in cells treated with SCM 10 μg/ml (Fig. 4A). Under the same conditions, COX-2 mRNA levels were also significantly decreased in a dose-dependent manner (Fig. 4B). In unstimulated RAW 264.7 cells, iNOS and COX-2 protein and mRNA were not detectable. In general, these results are consistent with the profile of the inhibitory effect of the SCM on NO (Fig. 1) and PGE2 release (Fig. 2).

Effect of SCM on iNOS and COX-2 Enzyme Activity and NO Scavenging Effect Next, the effect of SCM on iNOS and PGE2 enzyme activity was examined. SCM 1—10 μg/ml caused no significant inhibition of LPS-induced iNOS and COX-2 enzyme activity in RAW 264.7 cells (data not shown), whereas NMMA (an enzyme substrate analogue) 100 μM and NS-398 (a specific COX-2 activity inhibitor) 10 μM inhibited iNOS and PGE2 enzyme activities by 60.0% and 63.8%, respectively. We also investigate whether SCM has a scavenging effect on NO radicals released from PAPA NONOate. PAPA NONOate, a water-soluble NO/nitrovasodilator complex, is capable of spontaneously dissociating to the free amine and NO radical in a pH-dependent manner following first-order kinetics. One molecule of PAPA NONOate generates two molecules of NO. In the present study, our results
revealed that SCM 1—10 μg/ml did not scavenge the NO radical (data not shown). However, caffeic acid 100 μM, when used as a positive control, clearly inhibited NO radical release from 36.53 ± 0.24 μM to 13.59 ± 0.47 μM. These data indicate that SCM inhibits nitrite production, but SCM is not likely to interact directly with NO, nor did it inhibit iNOS and COX-2 enzyme activity. These results indicate that this extract inhibits NO and PGE2 production due to attenuation of iNOS and COX-2 protein and mRNA expression.

**Inhibition of LPS-Induced NF-κB Activation by the SCM** To investigate further the mechanism of SCM-mediated inhibition of iNOS and COX-2 transcription, we focused on transcription factors known to transactivate iNOS, COX-2, and tumor necrosis factor-α. EMSA analyses demonstrated a reduction in NF-κB DNA binding activity in nuclear extracts obtained from LPS-activated RAW macrophages treated with SCM (1, 5, 10 μg/ml) (Fig. 5). The extent of reduction was in a similar range (around 70%) as those of inducible NO synthase and COX-2 protein and mRNA expression.

**Degradation of IκB and Nuclear Translocation of p65** In unstimulated cells, NF-κB is sequestered in the cytosol by its inhibitor IκB, which upon LPS stimulation is phosphorylated by its inhibitor IκB kinase, ubiquitinated, and rapidly degraded via the 26 S proteosome, thus releasing NF-κB. SCM (10 μg/ml) had no influence on LPS-induced degradation of IκBα, constituting the IκB complex in cytosol until 180 min (Fig. 6A). It was investigated whether SCM prevented the translocation of the subunit of NF-κB, p65, from the cytosol to nucleus after release from IκB. Western blot analyses showed that the LPS-induced p65 level in the nuclear fraction was decreased by SCM in a concentration-dependent manner (Fig. 6B).
function. In contrast, COX-2 has been found to be highly in-
logic level of PGs for normal platelet, stomach, and kidney
cell types. COX-1 has been suggested to provide a physio-
and COX-2, each with a distinct expression pattern in various
indicated periods of time before cytosol fractions were subjected to Western blot analy-

Translocation of p65 (B)

A, RAW macrophages were treated with LPS (1 μg/ml) and 10 μg/ml SCM for the
periods of time before cytosol fractions were subjected to Western blot analy-

DISCUSSION

Because the mechanism of the antiinflammatory effects of
SCM has not been reported, we examined the effects of SCM
on the release of two inflammatory mediators (NO and
PGE₂), the expression levels of iNOS and COX-2, and NF-
xB activity on macrophages activated by LPS. The results of
the present study indicate that SCM is able to inhibit the
induction of iNOS in LPS-activated murine macrophages at the
transcriptional level. This notion is based on the following
lines of evidence. (1) LPS-induced NO production was re-
duced by SCM in a concentration-dependent manner. (2)
Western blot analysis demonstrated markedly reduced levels
of iNOS protein in LPS-activated cells treated with SCM as
compared with untreated cells. (3) RT-PCR showed a lower
expression of iNOS mRNA in RAW 264.7 macrophages exposed to SCM. (4) SCM does not show a di-
rect effect on the enzyme activity of iNOS.

In contrast to continuously expressed NO synthesis, iNOS
is regulated primarily at the transcriptional level. 14) Thus sub-
stances that interfere with the induction of iNOS are not
likely to affect the constitutively expressed NO synthase.
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 NO
synthase system. 15) In this respect, NO synthase inhibitors for
the treatment of NO-mediated inflammatory processes re-
quire high specificity for iNOS, and thus inhibitors of iNOS
induction may function as a rather safe modulator of NO for
various pathological conditions.

The mechanism of various antiinflammatory drug action is
at least shared by the inhibition of PG synthesis, which is
mediated by COX. 16) 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 physio-
logic level of PGs for normal platelet, stomach, and kidney
function. In contrast, COX-2 has been found to be highly in-
duced at inflammatory sites in animals as well as patients
with inflammatory diseases, 17,18) and thus it is considered to
be responsible for proinflammatory PG production. We demon-
strated that SCM also significantly inhibits PGE₂ pro-
duction and COX-2 protein and mRNA expression without
affecting COX-2 activity in LPS-treated RAW 264.7
macrophages.

The expression of iNOS in murine macrophages has been
shown to be dependent on NF-κB activation. 19) The possibil-
ity that SCM may inhibit the activity of NF-κB was exam-
ined. The results indicate that SCM inhibition of expression
of both iNOS and COX-2 proteins and mRNA was most
likely due to SCM suppression of NF-κB. This is consistent
with the reports that NF-κB response elements are present
on the promoters for both the iNOS and COX-2 genes. 19—23)

NF-κB is composed mainly of two proteins, p50 and p65. 50)
Under unstimulated conditions, NF-κB is present in the
cytosol and is bound to the inhibitory protein IκB. After induc-
tion by a variety of agents such as LPS, IκB is phosphory-
lated to trigger proteolytic degradation of IκB. NF-κB is
then released from IκB and is translocated into the nucleus.
In the present study, we found an inhibition of LPS-induced
IκB degradation in the cytosol with an inhibition of p65 pro-
tein increase in the nucleus by SCM, indicating that SCM
blocked LPS-induced activation of NF-κB. These results
suggest that SCM inhibition of LPS-induced expression of
the iNOS and COX-2 genes occurs through blocking NF-κB
activation, although inhibition of other factors such as AP-1,
the interferon response element, and r-activated site (GAS)
may be involved.

In summary, the results of the present study indicate that
SCM is an effective inhibitor of LPS-induced iNOS and
COX-2 protein and gene expression by blocking NF-κB acti-
vation in RAW 264.7 macrophages. SCM appears to be a po-
tential therapeutic agent for treating LPS-induced sepsis syn-
drome.

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