ARTICLE
Journal of Cellular Biochemistry 112:548–558 (2011)

Journal of Cellular Biochemistry

Stercurensin Inhibits Nuclear Factor-κB-Dependent Inflammatory Signals Through Attenuation of TAK1–TAB1 Complex Formation

Young-Joo Kim,[1] Han-Cheon Kim,[1] Hyeonseok Ko,[1] Evangeline C. Amor,[2] Jong Wha Lee,[2] and Hyun Ok Yang[1*]

[1]*Natural Products Research Center, Korea Institute of Science and Technology, Gangneung, Gangwon-do 210-340, Korea*

[2]*Institute of Chemistry, College of Science, University of the Philippines, Diliman 1101, Quezon City, Philippines*

ABSTRACT

We identified a chalcone, 2′,4′-dihydroxy-6′-methoxy-3′-methylchalcone (stercurensin), as an active compound isolated from the leaves of *Syzygium samarangense*. In the present study, the anti-inflammatory effects and underlying mechanisms of stercurensin were examined using lipopolysaccharide (LPS)-stimulated RAW264.7 cells and mice. To determine the effects of stercurensin in vitro, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression were analyzed by RT-PCR and immunoblotting. Nuclear factor-κB (NF-κB) activation and its upstream signaling cascades were also investigated using a dual-luciferase reporter assay, electrophoretic mobility shift assay, immunoblotting, immunofluorescence, and immunoprecipitation. To verify the effects of stercurensin in vivo, the mRNA expression levels of iNOS and COX-2 were evaluated in isolated mouse peritoneal macrophages by quantitative real-time PCR, and the production of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β were assessed in serum samples from mice using a Luminex system. Pretreatment with stercurensin reduced LPS-induced iNOS and COX-2 expression, thereby inhibiting nitric oxide (NO) and prostaglandin E_2 production, respectively. In addition, an inhibitory effect of stercurensin on NF-κB activation was shown by the recovery of LPS-induced inhibitor of κB (I-κB) degradation after blocking the transforming growth factor-β-activated kinase 1 (TAK1)/I-κB kinase signaling pathway. In mouse models, stercurensin negatively regulated NF-κB-dependent pro-inflammatory mediators and cytokines. These results demonstrate that stercurensin modulates NF-κB-dependent inflammatory pathways through the attenuation of TAK1–TAB1 complex formation. Our findings demonstrating the anti-inflammatory effects of stercurensin in vitro and in vivo will aid in understanding the pharmacology and mode of action of stercurensin. J. Cell. Biochem. 112: 548–558, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: STERCURENSIN; NITRIC OXIDE; PROSTAGLANDIN E2; NUCLEAR FACTOR-κB; TRANSFORMING GROWTH FACTOR-β-ACTIVATED KINASE 1; TAK1-BINDING PROTEIN 1

The flavonoid 2′,4′-dihydroxy-6′-methoxy-3′-methylchalcone (stercurensin) is a naturally occurring chalcone that was previously isolated from *Sterculia urens* [Anjaneyulu and Raju, 1984]. Recently, stercurensin has been identified in the leaves and fruits of *Syzygium samarangense* (Blume) Merr. & L.M. Perry (Myrtaceae), which is locally known in the Philippines as "makopa" [Kuo et al., 2004; Simirgiotis et al., 2008). This compound has been reported to possess anti-diabetic [Resurreccion-Magno et al., 2005], spasmolytic [Amor et al., 2006], and cytotoxic activities [Simirgiotis et al., 2008]. Some chalcones such as hydroxychalcones have been reported to inhibit major pro-inflammatory mediators, including nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), and reactive oxygen species (ROS) production, by suppressing inducible enzyme expression via the inhibition of mitogen-activated protein kinase (MAPK) pathways and the nuclear translocation of critical transcription factors [Ahmad et al., 2006]. The underlying anti-inflammatory mechanisms of stercurensin, however, have not been previously reported.

Lipopolysaccharide (LPS) is recognized by toll-like receptor 4 (TLR4) and activates two downstream signaling pathways, a myeloid differentiation factor 88 (MyD88)-dependent pathway and a MyD88-independent pathway. The MyD88-dependent pathway

Grant sponsor: Korea Institute of Science and Technology, Korea; Grant numbers: 2Z03270, 2Z03401.
*Correspondence to: Dr. Hyun Ok Yang, Natural Products Research Center, Korea Institute of Science and Technology, 290 Daejeon-dong, Gangneung, Gangwon-do 210-340, Korea. E-mail: hoyang@kist.re.kr
Received 9 September 2010; Accepted 27 October 2010 • DOI 10.1002/jcb.22945 • © 2010 Wiley-Liss, Inc.
Published online 15 November 2010 in Wiley Online Library (wileyonlinelibrary.com).

regulates pro-inflammatory cytokine genes, whereas the MyD88-independent pathway regulates type I interferon genes [Lu et al., 2008]. According to recent studies, transforming growth factor-β-activated kinase 1 (TAK1), a member of the mitogen-activated protein kinase MAPKKK family, is involved in an LPS-induced, TLR4-mediated NF-κB signaling pathway that plays a key role in inflammatory processes [Irie et al., 2000; Lee et al., 2000]. The stimulation of TLR4 by LPS promotes MyD88/IRAK/TRAF6 recruitment, resulting in the activation of TAK1 and the formation of a complex with TAK1-associated binding protein -1, -2, and -3 (TAB1, 2, and 3) [Wang et al., 2001; Sakurai et al., 2002]. This TAK1 complex subsequently causes the activation of transcription factors such as NF-κB and AP-1 by phosphorylating the inhibitor of κB (I-κB) kinase complex (IKK) and/or MAPK [Lee et al., 2000; Sakurai et al., 2002; Takaesu et al., 2003]. These activated transcription factors regulate various pro-inflammatory cytokines and mediator genes, including TNF-α, interleukin-6 (IL-6), IL-1β, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) [Pasparakis, 2009; Wang et al., 2009; Wan and Lenardo, 2010].

Nitric oxide synthase (NOS) exists in three main isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS. Mechanistically, NOS catalyzes the conversion of L-arginine to L-citrulline, producing NO in the process. Although nNOS and eNOS are constitutively expressed, iNOS is induced by various inflammatory stimuli such as LPS and inflammatory cytokines present in macrophages, hepatocytes, and endothelial cells [Moncada et al., 1991; Muriel, 2000]. During an inflammatory response, a large amount of NO is produced by iNOS, exceeding the physiological amount normally produced by nNOS or eNOS. The NO production derived from iNOS reflects the degree of inflammation and provides a means by which to assess the effects of chemopreventive agents on inflammatory processes.

Constitutive COX-1 and inducible COX-2 catalyze the conversion of arachidonic acid to prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2), and are rate-limiting enzymes in the synthesis of dienoic eicosanoids such as PGE_2. In most tissues, COX-2 is nearly undetectable under normal conditions; however, it is induced in macrophages and epithelial cells by pro-inflammatory stimuli, including mitogens, cytokines, and LPS [Smith et al., 2000; Hinz and Brune, 2002]. Furthermore, COX-2 is involved in many inflammatory processes and is induced in various carcinomas, which suggests that it may play a key role in inflammation and tumorigenesis [Hendrickse et al., 1994; Koki et al., 2003]. Thus, the identification of a COX-2 inhibitor may be a promising approach to protect against inflammation and tumorigenesis.

In the present study, we show that stercurensin has anti-inflammatory activity and elucidate its underlying molecular mechanism of action for the first time in LPS-stimulated mouse macrophages. Furthermore, our results show that stercurensin can inhibit LPS-induced endotoxin shock in an animal model.

MATERIALS AND METHODS

CHEMICALS

Stercurensin (Fig. 1) was isolated from the leaves of *S. samarangense*, and its identity and purity (>99%) were determined by NMR spectroscopic and HPLC-MS analyses. SB203580 (p38 inhibitor) was purchased from Calbiochem (CA). Each stock solution was prepared in 100% dimethylsulfoxide (DMSO) and stored at −20°C. When required, the stock solutions were diluted with cell culture medium to the appropriate concentration. The final concentration of DMSO was adjusted to 0.5% (v/v) in the culture medium.

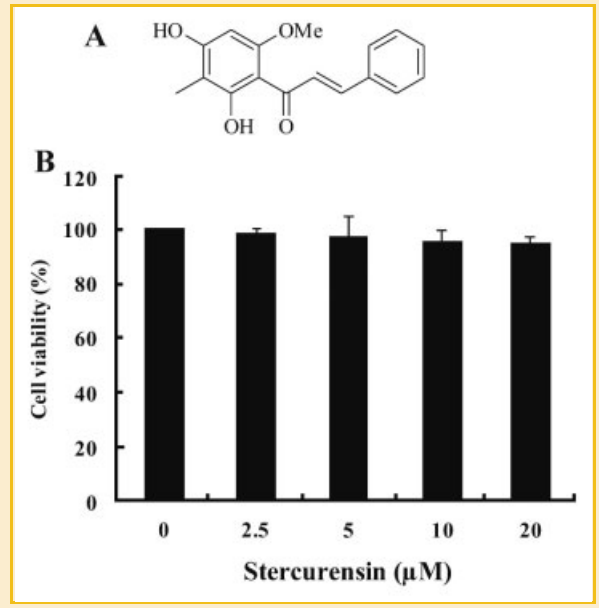

Fig. 1. A: Chemical structure of stercurensin (2′,4′-dihydroxy-6′-methoxy-3′-methylchalcone). B: Effects of stercurensin on RAW264.7 cell viability. Cells were treated with different concentrations of stercurensin for 48 h, and cell viability was measured using a CCK-8 assay. Data from three independent experiments were averaged and are reported as the mean ± SD of the percentage of cell viability. Control cells were treated with 0.5% (v/v) DMSO.

CELL CULTURE

The mouse macrophage RAW264.7 cell line and the human embryonic kidney (HEK) 293T cell line were purchased from the American Type Culture Collection (ATCC, VA). Cells were grown in Dulbecco's modified Eagle's medium (Hyclone, UT) supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C in a humidified atmosphere with 5% CO_2.

ANALYSIS OF CELL VIABILITY

The Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) was used to determine cell viability according to the manufacturer's recommendations.

MEASUREMENT OF NITRIC OXIDE PRODUCTION

We assayed NO production using the Griess reaction, which measures the accumulation of nitrite (NO_2^-) in the culture medium. Briefly, RAW264.7 cells were seeded in 96-well plates (1×10^5 cells/well) and incubated for 6 h to allow attachment to the plates. The attached cells were pretreated with phenol red-free medium containing the indicated concentration of stercurensin for 2 h and

then exposed to 1 μg/ml of *E. coli* LPS (strain 055:B5) for 24 h. The supernatant was collected, mixed with an equal volume (100 μl) of Griess reagent (1% sulfanilamide, 5% phosphoric acid, and 0.1% *N*-(1-naphthyl)-ethylenediamine), and incubated at room temperature for 5–10 min. Absorbance was measured using a microplate reader at a wavelength of 540 nm. Sodium nitrite was used to generate a standard reference curve. Control cells were exposed to phenol red-free medium containing 0.5% (v/v) DMSO, and all experiments were performed in triplicate.

MEASUREMENT OF PGE_2 PRODUCTION

The amount of PGE_2 produced from endogenous arachidonic acid was measured using a PGE_2 Parameter Assay Kit (R&D Systems, MN) according to the manufacturer's protocol.

TOTAL RNA EXTRACTION AND REVERSE TRANSCRIPTASE (RT)-PCR ANALYSIS

After LPS stimulation (1 μg/ml) of stercurensin-exposed cells for 6 h, total RNA was isolated using an RNeasy Mini Kit (Qiagen, MD) and reverse transcribed into cDNA using Superscript™ RNase H^- reverse transcriptase (Invitrogen, CA) according to the manufacturer's recommendations. Subsequent PCR analysis was carried out with aliquots (100 ng) of the cDNA preparation using a GeneAmp PCR System 9700 (Applied Biosystems, CA). The PCR conditions were as follows: predenaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C (COX-2 and GAPDH) or 60°C (iNOS) for 30 s and extension at 72°C for 30 s. The PCR products were visualized in 2% agarose gels. The primer sequences were as follows: mouse iNOS, 5′-TCT Tgg AgC gAg TTg Tgg AT-3′ (sense), 5′-ggg TCg TAA TgT CCA ggA AgT-3′ (anti-sense); mouse COX-2, 5′-Tgg Agg CgA AgT ggg TTT TA-3′ (sense), 5′-gAg Tgg gAg gCA CTT gCA TT-3′ (anti-sense); mouse GAPDH (as an internal control for PCR), 5′-gAT ggC ATg gAC TgT ggT CA-3′ (sense), 5′-gCA ATg CCT CCT gCA CCA CC-3′ (anti-sense).

PREPARATION OF WHOLE-CELL EXTRACTS

RAW264.7 cells were grown in six-well plates and stimulated with 1 μg/ml LPS for various time periods following pretreatment in the presence or absence of stercurensin for 2 h. Whole-cell extracts were then prepared according to the manufacturer's instructions using RIPA buffer (Cell Signaling, MA) supplemented with 1× protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride (PMSF).

PREPARATION OF CYTOSOLIC AND NUCLEAR EXTRACTS

RAW264.7 cells grown in 60 mm dishes were stimulated with 1 μg/ml LPS for 1 h after pretreatment in the presence or absence of stercurensin for 2 h. The treated cells were rinsed twice with cold PBS and collected. The cell pellets were resuspended by repeated pipetting in 120 μl of hypotonic buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1× protease inhibitor cocktail, 1 mM PMSF, and 1 mM Na_3VO_4] for 15 min on ice. Subsequently, 7.5 μl of 10% Nonidet P-40 (USB, OH) was added, and the mixture was vortexed and then centrifuged at 13,200 rpm for 30 s at 4°C. The supernatant containing cytosolic proteins was collected and stored at −80°C until further use. The nuclear pellets were rinsed twice with cold PBS and resuspended in 40 μl of hypertonic buffer [20 mM HEPES (pH 7.9), 0.4 M NaCl, 0.1 mM EDTA, 1 mM DTT, 1× protease inhibitor cocktail, 1 mM PMSF, and 1 mM Na_3VO_4] by rocking at 4°C for 15 min. The resuspended nuclear fraction was then centrifuged at 13,200 rpm for 5 min at 4°C. The supernatant containing nuclear proteins was collected and stored at −80°C until further use.

WESTERN BLOT ANALYSIS

Proteins (whole-cell extracts: 30 μg/lane, nuclear extracts: 10 μg/lane, cytosolic extracts: 30 μg/lane) were separated by electrophoresis in NuPAGE 4–12% Bis-Tris gels (Invitrogen), blotted onto PVDF transfer membranes and analyzed with epitope-specific primary and secondary antibodies. Bound antibodies were visualized using ECL Advance Western Blotting Detection Reagents (GE Healthcare, UK) and a LAS 4000 imaging system (Fujifilm, Japan). Monoclonal antibodies against IKKβ, p-I-κBα (Ser32), p-JNK/SAPK (Thr183/Tyr185), p-NF-κB/p65 (Ser536), and GAPDH and polyclonal antibodies against iNOS, COX-2, p-IKKα/β (Ser176/180), IKKα, I-κBα, HDAC1, p-NF-κB/p65 (Ser276), NF-κB/p65, p-p38 (Thr180/Tyr182), p38, p-ERK 1/2 (Thr202/Tyr204), ERK 1/2, JNK/SAPK, and TAK1 were purchased from Cell Signaling Technology, Inc. (Cell Signaling). The antibody against β-actin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

DUAL-LUCIFERASE REPORTER ASSAY

RAW264.7 cells grown in 12-well plates were cotransfected with a pNF-κB-Luc vector (BD Biosciences, CA) and a pRL-SV40 vector (Promega, WI) using the FuGENE 6 Transfection Reagent (Roche, Germany) according to the manufacturer's protocol. After cotransfection for 24 h, the cells were pretreated with the indicated concentration of stercurensin for 2 h and then stimulated with 1 μg/ml LPS for 6 h. Following LPS treatment, the cells were lysed, and luciferase activity was assessed using a dual-luciferase reporter assay system (Promega) and a luminometer (Turner Biosystems, CA) according to the manufacturers' protocols. The relative Firefly luciferase activity was normalized to Renilla luciferase expression to adjust for variation in the transfection efficiency, and all experiments were performed in triplicate.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

A double-stranded DNA probe containing the NF-κB binding site (5′-AgTTgAggggACTTTCCCAggC-3′) was end-labeled with [γ-^{32}P] dATP using T4 polynucleotide kinase (NEB, MA). Each binding reaction was carried out in 20 μl of binding buffer containing 10 mM HEPES (pH 7.9), 20 mM KCl, 0.8 mM EDTA, 4 mM $MgCl_2$, 5 μM $ZnCl_2$, 1 mM dithiothreitol, 1% BSA, 8% glycerol, and 10 μg of prepared nuclear extract, at room temperature for 30 min. The protein–DNA complexes were resolved from free probe using 4% non-denaturing polyacrylamide gel electrophoresis at room temperature in 0.5× TBE buffer. The gels were dried and then exposed to X-ray film at −70°C with a Kodak intensifying screen (Kodak, NY).

CONFOCAL MICROSCOPY ANALYSIS

RAW264.7 cells were seeded onto sterile coverslips plated in 12-well plates. The next day, the cells were pretreated with 10 μM

stercurensin for 2 h and then stimulated with 1 μg/ml LPS for 1 h. The cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 30 min, blocked with 2% BSA in PBS for 1 h, incubated with an anti-p65 primary antibody at room temperature for 1 h, sequentially incubated with an Alexa Fluor 488-conjugated secondary antibody (Invitrogen) at room temperature for 1 h in the dark, and finally incubated with 1 μg/ml of 4′,6′-diamidino-2-phenylindole (DAPI) at room temperature for 20 min in the dark. The coverslips were sealed to slides that had been precoated with one drop of ProLong Gold antifade reagent (Invitrogen) using nail lacquer. Images were obtained using a Leica TCS SP5 confocal microscope (Leica, Germany).

IMMUNOPRECIPITATION

Unlike the RAW264.7 cells prepared for endogenous protein interactions, HEK 293T cells were transfected with a FLAG-TAB1 expression vector (21C Frontier Human Gene Bank, Korea) using FuGENE 6 Transfection Reagent (Roche) as described by the manufacturer. The cells were pretreated with 10 μM stercurensin for 2 h and then stimulated with 1 μg/ml LPS for 15 min. Following LPS treatment, cell lysates were prepared using 1× RIPA buffer as described by the manufacturer and centrifuged at 13,200 rpm for 20 min at 4°C. Samples of the lysates (500 μg) were immunoprecipitated using an anti-TAB1 antibody (Cell Signaling) and an anti-FLAG antibody (Sigma, MO) for 4 h at 4°C, washed three times with wash buffer and subjected to Western blot analysis.

ISOLATION OF MOUSE PERITONEAL MACROPHAGES

Five-week-old male ICR mice (Orient, Korea) weighing 20 ± 2 g were used to isolate peritoneal macrophages. The mice were kept in a temperature-controlled room (23 ± 1°C) on a 12 h:12 h light:dark schedule with food and water provided ad libitum for 1 week to adapt to the new surroundings. The mice were injected intraperitoneally (i.p.) with 500 μl of 3% thioglycollate medium (Sigma). After 3 days, the animals were sacrificed, and peritoneal macrophages were harvested via an i.p. injection of 10 ml of sterile PBS. The cells were then washed with PBS and resuspended in RPMI 1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin. Prior to subsequent experiments, the cells were equilibrated for 24 h. All animal studies were performed in accordance with the Institutional Animal Care and Use Committee of Korea Institute of Science and Technology guidelines.

QUANTITATIVE REAL-TIME PCR

Isolated mouse peritoneal macrophages were stimulated with 1 μg/ml LPS for 6 h following stercurensin treatment for 2 h. Quantitative real-time PCR analysis was performed using RNA extracts from the peritoneal macrophages with SYBR Green PCR Master Mix reagents (Applied Biosystems). Dissociation Analysis Software was used to verify the singularity and specificity of the amplification products. PCR analysis of iNOS and COX-2 gene expression was performed with a GeneAmp PCR System 9700 (Applied Biosystems). The reaction conditions were as follows: predenaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. All of the products were normalized to GAPDH. The primer sequences were the same as those described above for the RT-PCR experiments.

IN VIVO ENDOTOXIN SHOCK MODEL

Prior to the experiments, the animals were divided four groups containing four mice each that were housed for 1 week to adapt to the new surroundings. Stercurensin was dissolved in saline containing 2% DMSO and administered to the mice by i.p. injection at various concentrations (1 or 10 mg/kg) 2 h before an i.p. injection of LPS (1 mg/kg). Vehicle controls were injected with saline only. The mice were sacrificed 2 h after LPS stimulation, and blood samples were collected via cardiac puncture. To determine the levels of TNF-α, IL-6, and IL-1β release, serum was obtained and assayed for the levels of these cytokines using a Procarta Cytokine Assay Kit (Panomics, CA) according to the manufacturer's recommendations. Quantification was performed using a Luminex 200 system (MiraiBio, CA).

STATISTICAL ANALYSIS

Results are expressed as means ± SD of at least three independent determinations for each experiment. Statistical significance was analyzed using Student's *t*-test ($P < 0.05$ was considered significant).

RESULTS

EFFECTS OF STERCURENSIN ON CELL VIABILITY

The cytotoxic effects of stercurensin on RAW264.7 cells were evaluated using a CCK-8 assay. As shown in Figure 1B, stercurensin did not affect cell viability for 48 h at concentrations up to 20 μM. For the subsequent experiments described in this study, 2.5–10 μM stercurensin was used.

STERCURENSIN INHIBITS NO AND PGE_2 PRODUCTION BY SUPPRESSING THE EXPRESSION OF INOS AND COX-2 IN LPS-STIMULATED RAW264.7 CELLS

Macrophages produce inflammatory mediators such as NO and PGE_2 upon stimulation with LPS. Therefore, we first investigated whether stercurensin might have anti-inflammatory properties in LPS-stimulated RAW264.7 cells. Treatment of the cells with LPS resulted in increased NO and PGE_2 production; however, stercurensin significantly inhibited the production of these factors in a dose-dependent manner (Fig. 2A,D). In particular, NO and PGE_2 secretion decreased to near basal levels in cells exposed to the highest concentration of stercurensin (10 μM). We next assessed whether the inhibitory effects of stercurensin on these inflammatory mediators were related to iNOS and COX-2 protein and mRNA levels using Western blot and RT-PCR analyses, respectively. In unstimulated RAW264.7 cells, iNOS and COX-2 protein and mRNA levels were undetectable; however, the expression levels of both genes were remarkably increased by LPS. Stercurensin significantly suppressed the expression of both genes in a dose-dependent manner (Fig. 2B,C,E,F). Western blot analysis showed that iNOS and COX-2 protein levels were correlated to their mRNA expression. Taken

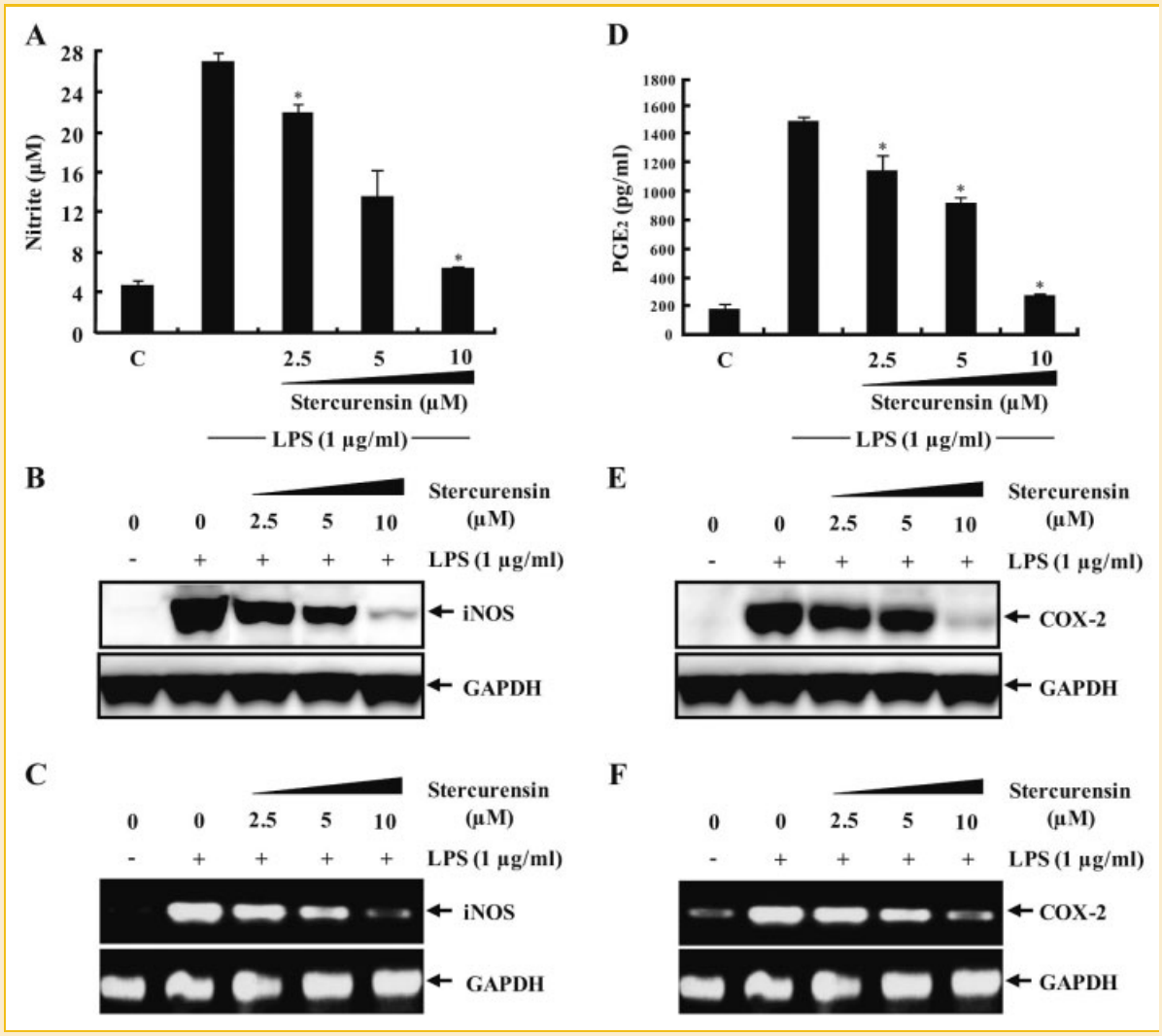

Fig. 2. Stercurensin inhibits NO and PGE2 production by suppressing iNOS and COX-2 expression, respectively, in LPS-induced RAW264.7 cells. A: Stercurensin inhibits NO production. Cells were pretreated with phenol red-free medium containing 2.5–10 μM stercurensin for 2 h and then exposed to 1 μg/ml LPS for 24 h. The culture medium was subsequently collected, and the nitrite concentration was measured by the Griess reaction. Each value represents the mean ± SD of three independent experiments. $^{*}P < 0.05$ compared with the LPS alone. B: Stercurensin inhibits iNOS protein expression. Cells were pretreated with the indicated concentrations of stercurensin for 2 h and then exposed to 1 μg/ml LPS for 24 h. The levels of iNOS and GAPDH proteins were detected by Western blot analysis using their respective antibodies. C: Stercurensin inhibits iNOS mRNA expression. RAW264.7 cells were pretreated with the indicated concentrations of stercurensin for 2 h and then exposed to 1 μg/ml LPS for 6 h. The mRNA levels of iNOS and GAPDH were determined by RT-PCR. The RT-PCR products were separated by electrophoresis in 2% agarose gels and digitally imaged after staining with ethidium bromide (EtBr). D: Stercurensin inhibits PGE_2 release. Cell culture and stercurensin treatment were performed as described for Figure 2A. Each culture supernatant was subsequently collected, and the amount of PGE_2 released was measured using the PGE_2 Parameter Assay Kit. Each value represents the mean ± SD of three independent tests. $^{*}P < 0.05$ compared with the LPS alone. E: Stercurensin inhibits COX-2 protein expression. Cell culture and stercurensin treatment were performed as described for Figure 2B. The protein levels of COX-2 and GAPDH were detected by Western blot analysis using their respective antibodies. F: Stercurensin inhibits COX-2 mRNA expression. Cell culture and stercurensin treatment were performed as described for Figure 2C. The mRNA levels of COX-2 and GAPDH were determined by RT-PCR. The RT-PCR products were separated by electrophoresis in 2% agarose gels and digitally imaged after staining with EtBr.

together, these findings indicate that stercurensin has an inhibitory effect on the production of NO and PGE_2 by repressing pro-inflammatory iNOS and COX-2 gene expression in RAW264.7 cells stimulated with LPS.

STERCURENSIN INHIBITS NF-κB ACTIVATION BY BLOCKING NUCLEAR TRANSLOCATION OF THE NF-κB p65 SUBUNIT

NF-κB is an important transcription factor that regulates cytokines and pro-inflammatory mediators such as TNF-α, IL-6, IL-1β, iNOS, and COX-2 during inflammatory responses. We first examined NF-κB-dependent promoter activity using a reporter gene assay to determine whether LPS-induced NF-κB activation might be regulated by stercurensin. As shown in Figure 3A, LPS increased NF-κB transcriptional activity by about 2.3-fold compared to the control level. Interestingly, stercurensin inhibited the LPS-induced activation of NF-κB in a dose-dependent manner. We next examined the effects of stercurensin on the LPS-induced DNA-binding activity of NF-κB by EMSA. When LPS-treated RAW264.7

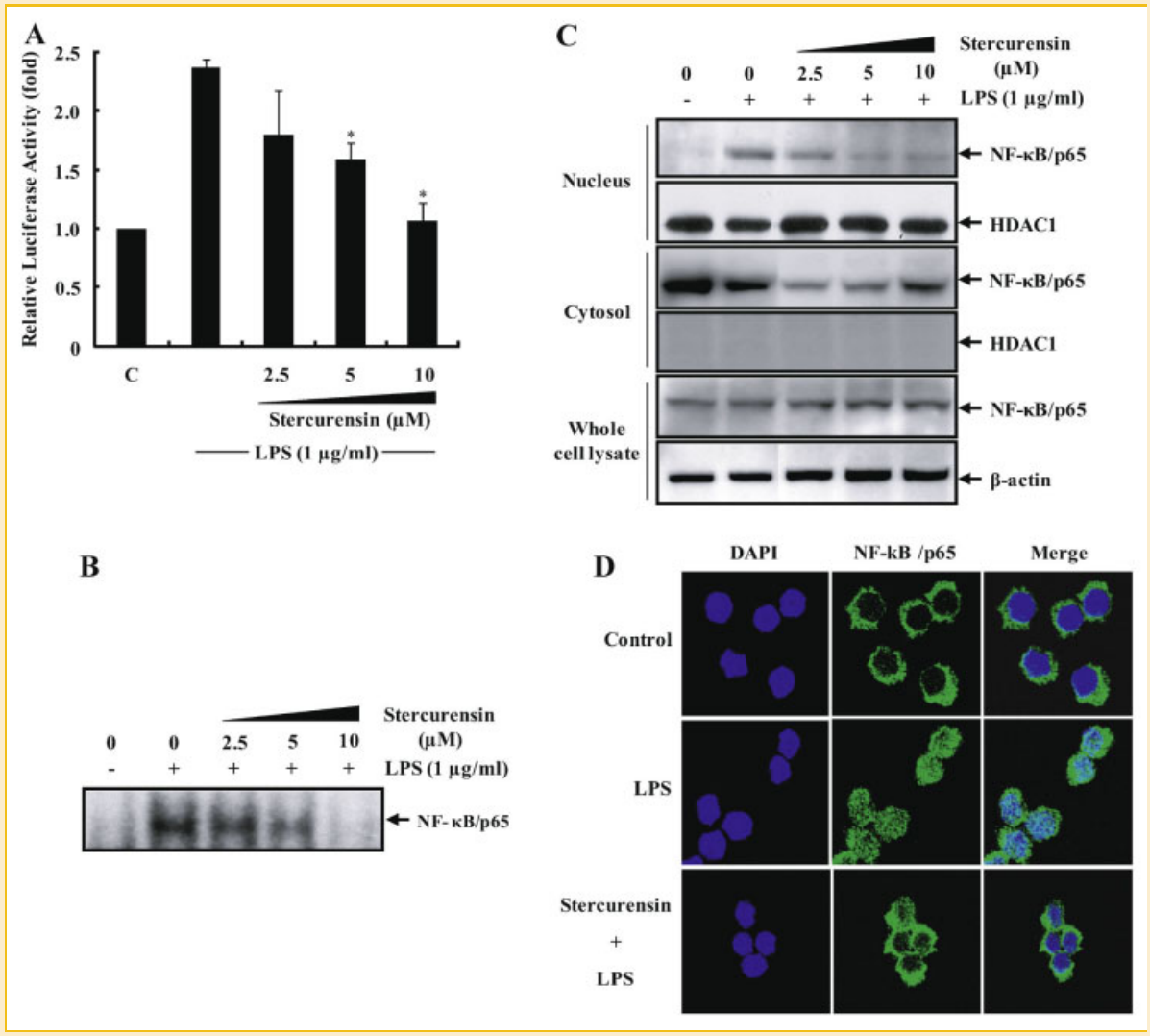

Fig. 3. Stercurensin suppresses NF-κB activation by blocking NF-κB p65 nuclear translocation in LPS-induced RAW264.7 cells. A: Stercurensin suppresses NF-κB promoter activity. Cells were transiently cotransfected with an NF-κB binding site-luciferase reporter plasmid and a Renilla luciferase expression vector. After cotransfection for 24 h, the cells were pretreated with the indicated concentrations of stercurensin for 2 h and then exposed to 1 μg/ml LPS for 6 h. The relative luciferase activity was normalized to Renilla luciferase expression to adjust for variation in the transfection efficiency. Each value represents the mean ± SD of three independent experiments. $^{*}P < 0.05$ compared with the LPS alone. B: Stercurensin suppresses the DNA-binding activity of NF-κB. Cells were pretreated with the indicated concentrations of stercurensin for 2 h and then exposed to 1 μg/ml LPS for 1 h. Nuclear proteins were extracted and subjected to an EMSA to evaluate NF-κB activation as described in the Materials and Methods Section. C: NF-κB p65 localization was assessed by Western blot analysis. Cell culture and stercurensin treatment were performed as described for Figure 3B. The protein levels of p65, HDAC1, and β-actin were detected using their respective antibodies. D: NF-κB p65 localization was assessed by confocal microscopy as described in the Materials and Methods Section. Cells were pretreated with or without stercurensin (10 μM) and then exposed to 1 μg/ml LPS for 1 h. Nuclei were stained with DAPI.

nuclear extracts were incubated with a radiolabeled NF-κB probe, a protein–DNA complex containing the NF-κB probe was observed; however, stercurensin inhibited the formation of this complex in a dose-dependent manner (Fig. 3B). Thus, stercurensin suppresses NF-κB transcriptional activity by preventing the formation of an NF-κB–DNA complex.

NF-κB is retained in the cytosol in an inactive state by virtue of its binding to I-κB. Following LPS stimulation, I-κBα/β is phosphorylated, ubiquitinated, and degraded, resulting in the nuclear translocation of NF-κB. We investigated whether stercurensin could inhibit the nuclear translocation of p65 (the major component of NF-κB) after its release from I-κB using Western blot analysis and confocal microscopy. The nuclear accumulation of p65 was increased in LPS-stimulated RAW264.7 cells but reduced in stercurensin-treated cells in a dose-dependent manner (Fig. 3C). These results were confirmed by immunofluorescence staining. Cytoplasmic retention of p65 was observed in cells treated with stercurensin, whereas translocation of p65 into the nucleus was observed in LPS-induced cells (Fig. 3D). Taken together, these results demonstrate that stercurensin inhibits NF-κB activation by preventing LPS-induced p65 nuclear translocation in RAW264.7 cells.

STERCURENSIN SUPPRESSES THE TAK1/IKK/NF-κB SIGNALING CASCADE IN LPS-STIMULATED RAW264.7 CELLS

The phosphorylation and degradation of I-κBα are regulated by the IKKα/β complex, which constitutes part of the NF-κB signaling pathway. Therefore, we assessed whether stercurensin might have effects on IKK or I-κB, both of which are crucial upstream mediators of the inflammation-related NF-κB signaling pathway. LPS strongly induced IKKα/β phosphorylation, whereas stercurensin markedly inhibited this phosphorylation without affecting total IKKα/β levels (Fig. 4A). Moreover, stercurensin significantly suppressed LPS-induced I-κBα phosphorylation and degradation (Fig. 4A). In addition, LPS-induced NF-κB phosphorylation at Ser536 and Ser 276 was reduced by stercurensin (Fig. 4A). Recent studies have shown that LPS activates a TLR4-mediated downstream signaling cascade comprising MyD88-dependent and MyD88-independent pathways. In particular, the MyD88-dependent pathway has been reported to have a profound association with the regulation of inflammation-related genes [Andreakos et al., 2004; Takeda and Akira, 2004; Lu et al., 2008]. We also investigated whether stercurensin might affect TAK1–TAB1 complex formation, which is a pivotal upstream signal for IKK complex activation in the MyD88-dependent pathway. Immunoprecipitation and immunoblotting analyses revealed that stercurensin decreased LPS-induced TAK1–TAB1 complex formation both when TAK1 and TAB1 were present at endogenous levels and when they were overexpressed (Fig. 4B,C). These findings indicate that stercurensin inhibits LPS-induced NF-κB signal transduction by attenuating TAK1–TAB1 complex formation.

EFFECTS OF STERCURENSIN ON LPS-INDUCED MAPK ACTIVATION IN RAW264.7 CELLS

Several MAPKs, including p38, JNK/SAPK, and ERK 1/2, are involved in signal transduction pathways that lead to the regulation of inflammatory mediators. Moreover, they play a critical role in the activation of NF-κB [Surh et al., 2001]. To elucidate the MAPK signaling pathway of stercurensin, we investigated MAPK activation in the presence or absence of stercurensin in LPS-stimulated RAW264.7 cells (Fig. 5A). The phosphorylation of p38, ERK1/2, and

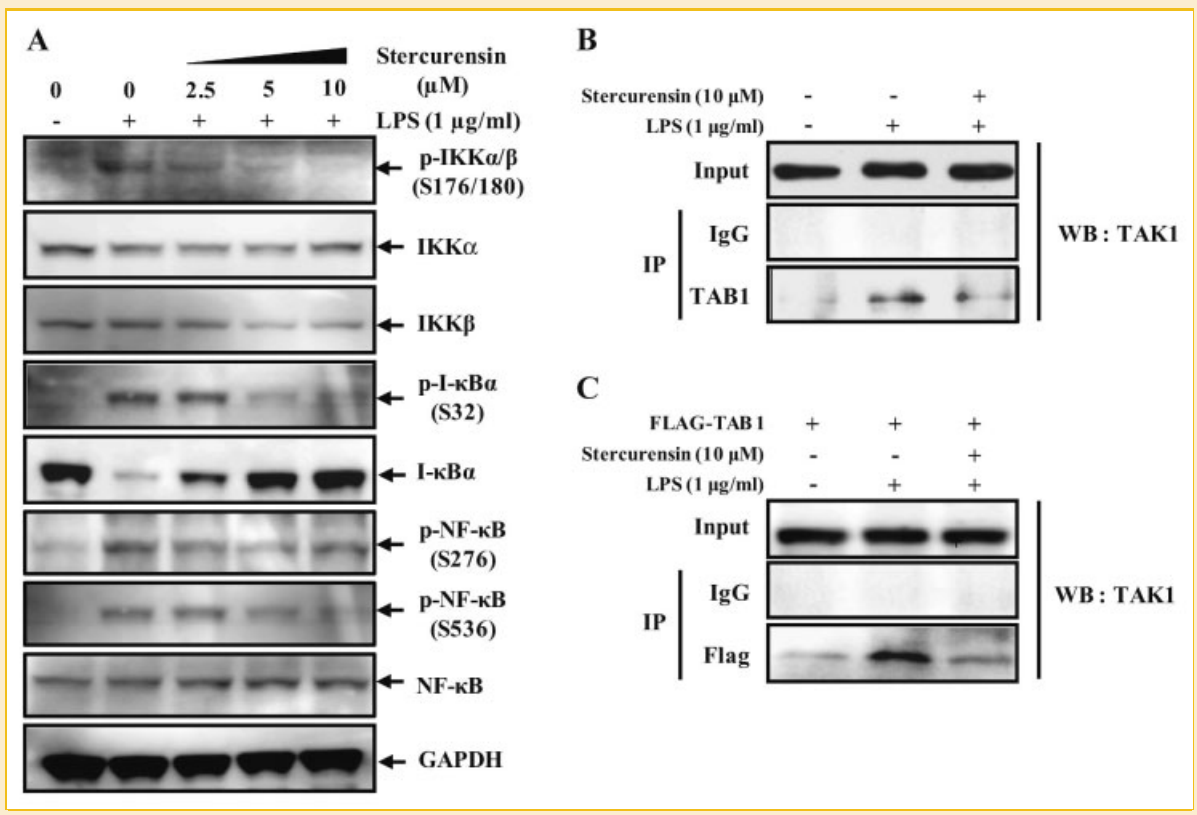

Fig. 4. Stercurensin regulates the LPS-induced TAK1/IKK/NF-κB signaling pathway. A: Stercurensin reduces the phosphorylation of I-κBα (Ser32) and NF-κB (Ser536) by preventing IKKα/β activation in LPS-induced RAW264.7 cells. Cells were pretreated with the indicated concentrations of stercurensin for 2 h and then exposed to 1 μg/ml LPS for 30 min. The protein levels of p-IKKα/β (Ser176/180), IKKα, IKKβ, p-I-κBα (Ser32), I-κBα, p-NF-κB (Ser276), p-NF-κB (Ser536), NF-κB, and GAPDH were detected by Western blot analysis using their respective antibodies. B: Stercurensin reduces the LPS-induced endogenous TAK1–TAB1 interaction in RAW264.7 cells. Cells were pretreated with the indicated concentrations of stercurensin for 2 h and then exposed to 1 μg/ml LPS for 15 min. Whole-cell lysates were subjected to immunoprecipitation with an anti-TAB1 antibody. Rabbit IgG was used as a negative control. Immunoprecipitates and whole-cell lysates were subjected to Western blot analysis using an anti-TAK1 antibody. C: Stercurensin reduces the LPS-induced TAK1–TAB1 interaction in HEK 293T cells. FLAG-TAB1 was transfected into HEK 293T cells. After transfection for 24 h, the cells were pretreated with the indicated concentrations of stercurensin for 2 h and then exposed to 1 μg/ml LPS for 15 min. Whole-cell lysates were subjected to immunoprecipitation using an anti-FLAG antibody. Mouse IgG was used as a negative control. Immunoprecipitates and whole-cell lysates were subjected to Western blot analysis using an anti-TAK1 antibody.

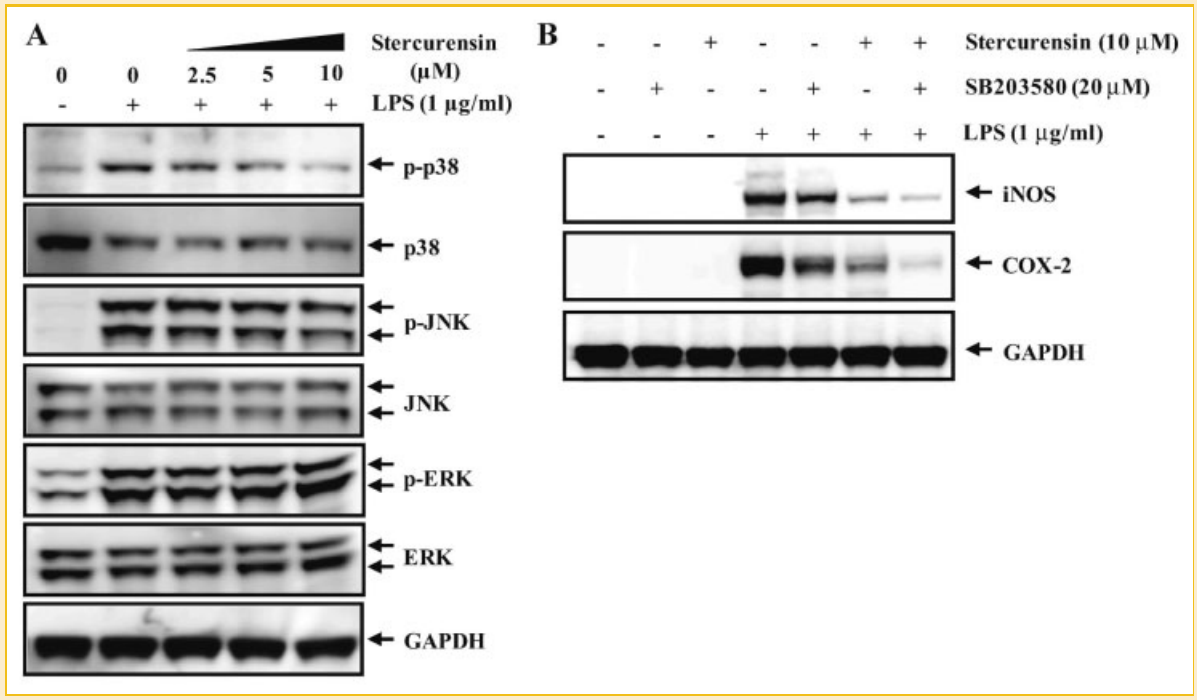

Fig. 5. Effects of stercurensin on MAPK phosphorylation in LPS-induced RAW264.7 cells. A: Stercurensin inhibits LPS-induced p38 phosphorylation. Cell culture and stercurensin treatment were performed as described for Figure 4A. The protein levels of p-p38, p38, p-JNK, JNK, p-ERK, ERK, and GAPDH were detected by Western blot analysis using their respective antibodies. B: Cooperative actions in p38 pathways lead to active iNOS and COX-2 expression in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with stercurensin and SB203580 (p38 inhibitor) alone or combinative for 2 h, and then exposed to 1 μg/ml LPS for 24 h. The protein levels of iNOS, COX-2, and GAPDH were detected by Western blot analysis using their respective antibodies.

JNK was elevated in cells treated with LPS alone. Interestingly, stercurensin exclusively repressed p38 phosphorylation but did not affect JNK and ERK phosphorylation.

To make certain that p38 mechanistically involves in iNOS and COX-2 expression, we further investigated the inter-relation by using p38 specific inhibitor (SB203580). RAW264.7 cells were pretreated with SB203580 and stercurensin alone or combinative for 2 h before exposure to LPS, and iNOS and COX-2 protein levels were then examined by Western blot analysis. As shown in Figure 5B, LPS-induced iNOS and COX-2 expression levels were partially suppressed by SB203580 alone, and were reduced still more by stercurensin alone and its combination with SB203580.

These findings suggest that there are partly cooperative actions in p38 pathways leading to active iNOS and COX-2 expression, and stercurensin might exert its influence on the pro-inflammatory signaling involved in NF-κB activation via p38.

STERCURENSIN DOWNREGULATES LPS-INDUCED INOS AND COX-2 mRNA EXPRESSION IN MOUSE PERITONEAL MACROPHAGES

To assess the effect of stercurensin on iNOS and COX-2 mRNA expression in mouse peritoneal macrophages stimulated with LPS, these cells were isolated from ICR mice and stimulated with 1 μg/ml LPS for 6 h following stercurensin treatment for 2 h. As shown in Figure 6A, stercurensin downregulated LPS-induced iNOS and COX-2 mRNA expression in a dose-dependent manner ex vivo, similar to the results obtained in RAW264.7 cells. No effect on the viability of the peritoneal macrophages exposed to the indicated concentrations of stercurensin was observed.

STERCURENSIN ATTENUATES LPS-INDUCED TNF-α, IL-6, AND IL-1β PRODUCTION IN MOUSE SERUM

To verify in vivo the relevance of our in vitro results demonstrating the anti-inflammatory effects of stercurensin, we used a well-established shock model involving mice sensitized to LPS. The LPS-treated mice demonstrated increased serum levels of TNF-α, IL-6, and IL-1β (452.9 ± 28.06, 7196.6 ± 661.41, and 215.9 ± 17.06 pg/ml, respectively) compared to control mice (2.7 ± 0.19, 34.5 ± 0.95, and 108.0 ± 5.49 pg/ml, respectively); however, the animals pre-treated with stercurensin (1 or 10 mg/kg) showed significantly attenuated LPS-induced cytokine release in a dose-dependent manner (Fig. 6B–D). These data show that stercurensin has anti-inflammatory effects both in vivo and in vitro.

DISCUSSION

Some chalcones such as hydroxychalcones have been reported to inhibit the production of major pro-inflammatory mediators by suppressing inducible enzyme expression via the inhibition of MAPK pathways and the nuclear translocation of critical transcription factors [Ahmad et al., 2006; Kim et al., 2010]. In particular, cardamonin (2′,4′-dihydroxy-6′-methoxychalcone) has been shown to have potent anti-inflammatory effects [Israf et al., 2007].

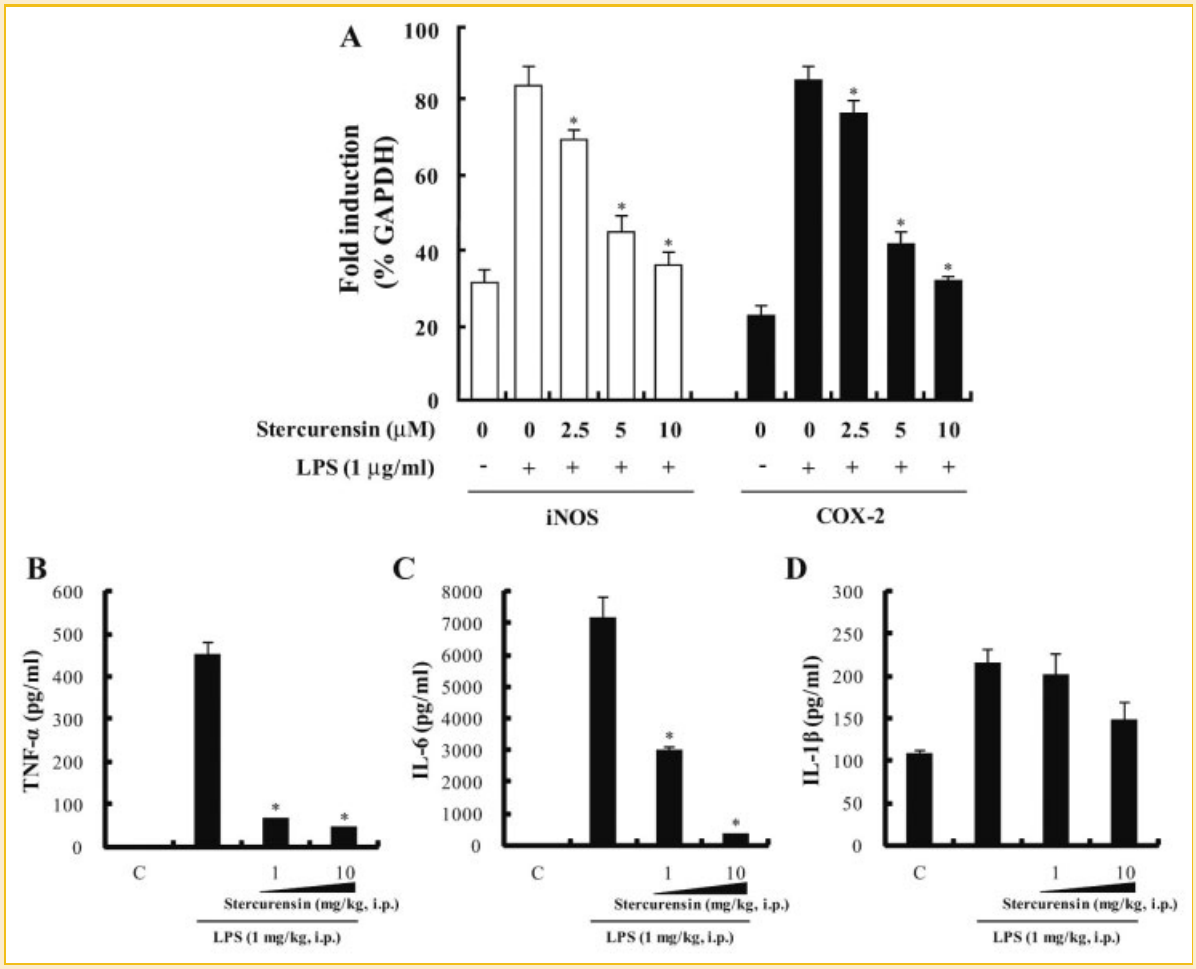

Fig. 6. The effects of stercurensin ex vivo and in vivo. A: Stercurensin decreases iNOS and COX-2 mRNA expression in LPS-induced mouse peritoneal macrophages. Isolated macrophages were pretreated with the indicated concentrations of stercurensin for 2 h and then exposed to 1 μg/ml LPS for 6 h. Quantification of mRNA levels was performed using real-time PCR. The histogram showing the fold difference in iNOS and COX-2 mRNA levels was normalized to GAPDH. Each value represents the mean ± SD of three separate experiments. $^{*}P < 0.05$ compared with the LPS alone. The effects of stercurensin on LPS-induced TNF-α (B), IL-6 (C), and IL-1β (D) production in the serum. Mice were injected with stercurensin (1 or 10 mg/kg, i.p.) and, 2 h later, stimulated with LPS (1 mg/kg, i.p.). The animals were sacrificed 2 h after LPS injection, and serum samples were obtained by cardiac puncture. The concentrations of TNF-α, IL-6, and IL-1β were measured using a Luminex system. Each value represents the mean ± SD of four animals. $^{*}P < 0.05$ compared with the LPS alone.

Stercurensin (2′,4′-dihydroxy-6′-methoxy-3′-methylchalcone) is a naturally occurring chalcone and is one of the main constituents of the leaves and fruits of *S. samarangense* [Anjaneyulu and Raju, 1984]. In the present study, we focused on the similarity of the chemical structure of stercurensin to that of cardamonin, which suggests that stercurensin might also possess anti-inflammatory activity. The underlying mechanisms of the anti-inflammatory effects of stercurensin, however, have not been previously reported.

The inflammatory products NO and PGE_2 are induced by iNOS and COX-2 expression, respectively, as part of the innate immune system. Inhibition of NO and PGE_2 can be used as a therapeutic tool to treat inflammatory diseases. Consequently, the development of inflammatory inhibitors has been focused on negatively regulating the transcription of pro-inflammatory mediators. The expression of iNOS and COX-2 following LPS stimulation is mediated by the transcription factor NF-κB, which is also linked to the transcription of pro-inflammatory cytokines. Therefore, we investigated whether stercurensin might regulate an inflammatory signaling cascade by modulating NF-κB activation in LPS-stimulated RAW264.7 cells. In the present study, we found that stercurensin was an effective inhibitor of LPS-induced NO and PGE_2 production via the suppression of both the transcription and translation of iNOS and COX-2, respectively. Stercurensin also suppressed NF-κB transcriptional activity and complex formation between NF-κB and the NF-κB DNA-binding element present in the iNOS and COX-2 gene promoter regions. Taken together, our data indicate that stercurensin inhibits NF-κB-dependent inflammatory responses such as NO and PGE_2 production by targeting NF-κB promoter binding.

LPS stimulation is associated with a TLR4-mediated NF-κB signaling pathway, which is a key signal transduction mechanism in inflammatory processes. In the present study, we examined whether stercurensin might influence the upstream signaling transduction of NF-κB activation in LPS-stimulated RAW264.7 cells. LPS induced the phosphorylation of IKKα/β, resulting in disassociation of the I-

κB/NF-κB complex, and the activated NF-κB p65 translocated into the nucleus with phosphorylation at Ser536. Stercurensin inhibited I-κB phosphorylation and degradation, however, by suppressing IKK phosphorylation, which prevented NF-κB p65 nuclear translocation. These results are similar to those found for some chemopreventive compounds such as capsaicin [Kim et al., 2003], apigenin [Liang et al., 1999], and diarylheptanoid [Yadav et al., 2003], all of which inhibit the LPS-mediated IKK/NF-κB signaling cascade in mouse macrophages.

A recent study using TAK1 knockout mice revealed that TAK1 mediates IKK phosphorylation; thus, TAK1 is an essential upstream kinase in inflammatory signaling [Shim et al., 2005]. In particular, activation of the endogenous TAK1–TAB1 complex triggers TAK1-induced signaling pathways such as those involving IKK, p38, and JNK [Sakurai et al., 2002]. Therefore, we investigated whether stercurensin might influence TAK1 in LPS-stimulated RAW264.7 and TAB1-transfected HEK293T cells. Western blot analysis and immunoprecipitation studies revealed that stercurensin inhibited TAK1–TAB1 complex formation, which is one of the key components of LPS-mediated IKK/NF-κB upstream signaling. Taken together, these findings indicate that stercurensin inhibits LPS-induced inflammatory responses by blocking the TLR4-mediated TAK1/IKK/NF-κB signaling pathway.

Three families of MAPKs (ERK 1/2, JNK 1/2, and p38 MAPK) play critical roles in cell growth regulation and differentiation and in the control of cellular responses to cytokines and stressors [Van den Berg et al., 1998; Johnson and Lapadat, 2002]. MAPK phosphorylation activates the transcription of NF-κB-mediated pro-inflammatory cytokines [Ajizian et al., 1999; Rajapakse et al., 2008]; therefore, MAPKs are targets for the development of novel anti-inflammatory drugs. Recent in vitro studies using RAW264.7 macrophages have shown that p38 is involved in NF-κB activation [Chen et al., 2006; Cho et al., 2008], whereas JNK controls AP-1 activation [Cho et al., 2003; Shan et al., 2009]. In the present study, stercurensin exclusively regulated p38 phosphorylation in LPS-stimulated RAW264.7 cells. This result supports the idea that stercurensin affects pro-inflammatory signaling not through AP-1 activation via JNK but through NF-κB activation via p38, similar to chemopreventive phytochemicals such as diarylheptanoid [Yadav et al., 2003], capsaicin [Chen et al., 2003], and sesquiterpene lactones [Hwang et al., 1996] that have been reported to inhibit COX-2 by targeting MAPK signaling pathways, including the p38 kinase pathway.

Finally, to verify the in vivo relevance of our in vitro results demonstrating the anti-inflammatory effects of stercurensin, we evaluated the levels of iNOS and COX-2 mRNA expression in isolated mouse peritoneal macrophages. Stercurensin significantly repressed the LPS-induced expression of these genes ex vivo as well as in vitro. In addition, we examined the serum levels of TNF-α, IL-6, and IL-1β, all of which are NF-κB-dependent cytokines, in mice. The peak serum concentrations of TNF-α and IL-6 were reached at 1–2 h following i.p. injection of 1 mg/kg LPS, whereas IL-1β release occurred 2–4 h after injection (data not shown). Therefore, we measured the serum levels of TNF-α, IL-6, and IL-1β at 2 h after LPS injection. Interestingly, stercurensin significantly inhibited LPS-induced cytokines production. A little effect on IL-1β, compared to the others, is considered to be due to the insufficient time to reach the peak by LPS. These date provide evidence that stercurensin exerts its anti-inflammatory actions in vivo via a mechanism similar to that shown in vitro.

In conclusion, our results provide evidence indicating that stercurensin is a potential anti-inflammatory agent that acts via attenuation of the TAK1/IKK/NF-κB signaling pathway, thereby inhibiting pro-inflammatory cytokines and mediators. Thus, stercurensin could be a useful pharmacologic tool to enhance our understanding of basic cellular functions and may constitute a new class of therapeutic or chemopreventive drugs for the reduction of inflammation.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Wonil Choi for technical assistance with the EMSAs.

REFERENCES

Ahmad S, Israf DA, Lajis NH, Shaari K, Mohamed H, Wahab AA, Ariffin KT, Hoo WY, Aziz NA, Kadir AA, Sulaiman MR, Somchit MN. 2006. Cardamonin, inhibits pro-inflammatory mediators in activated RAW 264.7 cells and whole blood. Eur J Pharmacol 538:188–194.

Ajizian SJ, English BK, Meals EA. 1999. Specific inhibitors of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways block inducible nitric oxide synthase and tumor necrosis factor accumulation in murine macrophages stimulated with lipopolysaccharide and interferon-gamma. J Infect Dis 179:939–944.

Amor EC, Villaseñor IM, Ghayur MN, Gilani AH, Choudhary MI. 2006. Presence of calcium antagonist activity explains the use of *Syzygium samarangense* in diarrhea. Phytother Res 20:49–52.

Andreakos E, Sacre SM, Smith C, Lundberg A, Kiriakidis S, Stonehouse T, Monaco C, Feldmann M, Foxwell BM. 2004. Distinct pathways of LPS-induced NF-kappa B activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. Immunobiology 103:2229–2237.

Anjaneyulu ASR, Raju SN. 1984. Stercurensin, a new C-methylchalcone from leaves of *Sterculia urens*. Indian J Chem 23B:1010–1011.

Chen CW, Lee ST, Wu WT, Fu WM, Ho FM, Lin WW. 2003. Signal transduction for inhibition of inducible nitric oxide synthase and cyclooxygenase-2 induction by capsaicin and related analogs in macrophages. Br J Pharmacol 140:1077–1087.

Chen CH, Sheu MT, Chen TF, Wang YC, Hou WC, Liu DZ, Chung TC, Liang YC. 2006. Suppression of endotoxin-induced proinflammatory responses by citrus pectin through blocking LPS signaling pathways. Biochem Pharmacol 72:1001–1009.

Cho YH, Lee CH, Kim SG. 2003. Potentiation of lipopolysaccharide-inducible cyclooxygenase 2 expression by C2-ceramide via c-Jun N-terminal kinase-mediated activation of CCAAT/enhancer binding protein beta in macrophages. Mol Pharmacol 63:512–523.

Cho W, Park SJ, Shin JS, Noh YS, Cho EJ, Nam JH, Lee KT. 2008. Anti-inflammatory effects of the methanol extract of *Polytrichum commune* via NF-kappaB inactivation in RAW264.7 macrophage cells. Biomol Ther 16:385–393.

Hendrickse CW, Kelly RW, Radley S, Donovan IA, Keighley B, Neoptolemos JP. 1994. Lipid peroxidation and prostaglandins in colorectal cancer. Br J Surg 81:1219–1223.

Hinz B, Brune K. 2002. Cyclooxygenase-2–10 years later. J Pharmacol Exp Ther 300:367–375.

Hwang D, Fischer NH, Jang BC, Tak H, Kim JK, Lee W. 1996. Inhibition of the expression of inducible cyclooxygenase and proinflammatory cytokines by sesquiterpene lactones in macrophages correlates with the inhibition of MAP kinases. Biochem Biophys Res Commun 226:810–818.

Irie T, Muta T, Takeshige K. 2000. TAK1 mediates an activation signal from toll-like receptor(s) to nuclear factor-kappaB in lipopolysaccharide-stimulated macrophages. FEBS Lett 467:160–164.

Israf DA, Khaizurin TA, Syahida A, Lajis NH, Khozirah S. 2007. Cardamonin inhibits COX and iNOS expression via inhibition of p65NF-kappaB nuclear translocation and Ikappa-B phosphorylation in RAW 264.7 macrophage cells. Mol Immunol 44:673–679.

Johnson GL, Lapadat R. 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298:1911–1912.

Kim CS, Kawada T, Kim BS, Han IS, Choe SY, Kurata T, Yu R. 2003. Capsaicin exhibits anti-inflammatory property by inhibiting IkB-a degradation in LPS-stimulated peritoneal macrophages. Cell Signal 15:299–306.

Kim YJ, Ko H, Park JS, Han IH, Amor EC, Lee JW, Yang HO. 2010. Dimethyl cardamonin inhibits lipopolysaccharide-induced inflammatory factors through blocking NF-kappaB p65 activation. Int Immunopharmacol 10:1127–1134.

Koki AT, Leahy KM, Harmon JM, Masferrer JL. 2003. Cyclooxygenase-2 and cancer. In: Harris RE, editor. COX-2 blockade in cancer prevention and therapy. Totowa: Humana Press. pp 185–203.

Kuo YC, Yang LM, Lin LC. 2004. Isolation and immunomodulatory effect of flavonoids from *Syzygium samarangense*. Planta Med 70:1237–1239.

Lee JD, Mira-Arbibe L, Ulevitch RJ. 2000. TAK1 regulates multiple protein kinase cascades activated by bacterial lipopolysaccharide. J Leukoc Biol 68:909–915.

Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF, Lin JK. 1999. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 20:1945–1952.

Lu YC, Yeh WC, Ohashi PS. 2008. LPS/TLR4 signal transduction pathway. Cytokine 42:145–151.

Moncada S, Palmer RM, Higgs EA. 1991. Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109–142.

Muriel P. 2000. Regulation of nitric oxide synthesis in the liver. J Appl Toxicol 20:189–195.

Pasparakis M. 2009. Regulation of tissue homeostasis by NF-kappaB signaling: Implications for inflammatory diseases. Nat Rev Immunol 9:778–788.

Rajapakse N, Kim MM, Mendis E, Kim SK. 2008. Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-stimulated RAW264.7 cells by carboxybutyrylated glucosamine takes place via down-regulation of mitogen-activated protein kinase-mediated nuclear factor-kappaB signaling. Immunology 123:348–357.

Resurreccion-Magno MHC, Villasenor IM, Harada N, Monde K. 2005. Anti-hyperglycaemic flavonoids from *Syzygium samarangense* (Blume) merr. and perry. Phytother Res 19:246–253.

Sakurai H, Nishi A, Sato N, Mizukami J, Miyoshi H, Sugita T. 2002. TAK1–TAB1 fusion protein: A novel constitutively active mitogen-activated protein kinase kinase kinase that stimulates AP-1 and NF-kappaB signaling pathways. Biochem Biophys Res Commun 297:1277–1281.

Shan J, Fu J, Zhao Z, Kong X, Huang H, Luo L, Yin Z. 2009. Chlorogenic acid inhibits lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 cells through suppressing NF-kappaB and JNK/AP-1 activation. Int Immunopharmacol 9:1042–1048.

Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee KY, Bussey C, Steckel M, Tanaka N, Yamada G, Akira S, Matsumoto K, Ghosh S. 2005. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathway in vivo. Genes Dev 19:2668–2681.

Simirgiotis MJ, Adachi S, To S, Yang H, Reynertson KA, Basile MJ, Gil RR, Weinstein IB, Kennelly EJ. 2008. Cytotoxic chalcones and antioxidants from the fruits of *Syzygium samarangense* (Wax Jambu). Food Chem 107:813–819.

Smith WL, DeWitt DL, Garavito RM. 2000. Cyclooxygenase: Structural, cellular, and molecular biology. Annu Rev Biochem 69:145–182.

Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, Lee SS. 2001. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: Down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. Mutat Res 480–481:243–268.

Takaesu G, Surabhi RM, Park KJ, Ninomiya-Tsuji J, Matsumoto K, Gaynor RB. 2003. TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway. J Mol Biol 326:105–115.

Takeda K, Akira S. 2004. TLR signaling pathways. Semin Immunol 16:3–9.

Van den Berg AP, Twilhaar WN, van Son WJ, van der Bij W, Klompmaker IJ, Slooff MJ, The TH, de Leij LH. 1998. Quantification of immunosuppression by flow cytometric measurement of intracellular cytokine synthesis. Transpl Int 11(Suppl 1): S318–S321.

Wan F, Lenardo MJ. 2010. The nuclear signaling of NF-kappaB: Current knowledge, new insights, and future perspectives. Cell Res 20:24–33.

Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 412:346–351.

Wang S, Liu Z, Wang L, Zhang X. 2009. NF-kappaB signaling pathway, inflammation and colorectal cancer. Cell Mol Immunol 6:327–334.

Yadav PN, Liu Z, Rafı MM. 2003. A diarylheptanoid from lesser galangal (*Alpinia officinarum*) inhibits proinflammatory mediators via inhibition of mitogen-activated protein kinase, p44/42, and transcription factor nuclear factor-kappa B. J Pharmacol Exp Ther 305:925–931.