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Anti-Inflammatory Effects of Sinapic Acid through the Suppression of Inducible Nitric Oxide Synthase, Cyclooxygase-2, and Proinflammatory Cytokines Expressions via Nuclear Factor-κB Inactivation

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To investigate the anti-inflammatory potential of sinapic acid as well as the underlying mechanism involved, we studied the inhibitory effect of sinapic acid on the production of pro-inflammatory mediators in vitro and then evaluated its in vivo anti-inflammatory effect. Sinapic acid inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor (TNF)-α, and interleukin (IL)-1β production in a dose-dependent manner. Consistent with these findings, sinapic acid inhibited LPS-induced expressions of inducible nitric oxide synthase (iNOS) and cyclooxygase (COX)-2 at the protein levels, and iNOS, COX-2, TNF-α, and IL-1β mRNA expression in RAW 264.7 macrophages, as determined by Western blotting and reverse-transcribed polymerase chain reaction, respectively. Sinapic acid suppressed the LPS-induced activation of nuclear factor-κB (NF-κB), a transcription factor pivotal necessary for pro-inflammatory mediators, such as iNOS, COX-2, TNF-α, and IL-1β. This effect was accompanied by a parallel reduction of the nuclear translocation of p65 and p50 NF-κB subunits, as well as IκB-α degradation and phosphorylation. The effects of sinapic acid on acute phase inflammation were investigated on serotonin- and carrageenan-induced paw edema and compared with indomethacin (10 mg/kg, p.o.) or ibuprofen (100 mg/kg, p.o.). Maximum inhibitions of 34.2 and 44.5% were observed at a concentration of 30 mg/kg for serotonin- and carrageenan-induced paw edema, respectively. These results suggest that the suppressions of the expressions of iNOS, COX-2, TNF-α, and IL-1β via NF-κB inactivation are responsible for the anti-inflammatory effects of sinapic acid.

KEYWORDS: Sinapic acid; inducible nitric oxide synthase; cyclooxygenase-2; cytokines; nuclear factor-κB

INTRODUCTION

Inflammation, characterized by redness, swelling, pain, and heat, is one of the most important aspects of host defense mechanisms against invading pathogens. However, inflammation may also aid microbial pathogenesis because the inflammatory response elicited by an invading microorganism can result in considerable host damage, making nutrients available and providing access to host tissues (*1*).

Nuclear transcription factor kappa-B (NF-κB) plays a pivotal role in the pathogenesis of inflammation; therefore, a variety of drugs designed to treat human inflammatory disease are focused on the inhibition of NF-κB activation (*2*). At least five different molecules, NF-κB1 (p105/50), NF-κB2 (p100/p52), Rel A (p65), Rel B, and c-Rel, belong to the NF-κB family. Most commonly, NF-κB dimers are composed of the Rel A (p65) and NF-κB1 (p50) or NF-κB2 (p52) subunits. NF-κB normally resides in the cytoplasm, where it is retained by association with inhibitors of κB (IκBs) protein, an endogenous inhibitor (*3*). However, when activated, it translocates to the nucleus, binds the DNA, activates genes, and induces the transcription of pro-inflammatory mediators, such as inducible nitric oxide synthase (iNOS), cyclooxygase (COX)-2, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β (*4*).

Nitric oxide (NO) and prostaglandins (PGs) are two pleiotropic mediators produced at inflammatory sites by the enzymes such as iNOS and COX-2. In inflammation, overproduction of NO is mainly caused by iNOS, which is up-regulated in macrophages by cytokine and/or bacterial lipopolysaccharide (LPS) stimulation (*5*). Despite its beneficial role in host defense,

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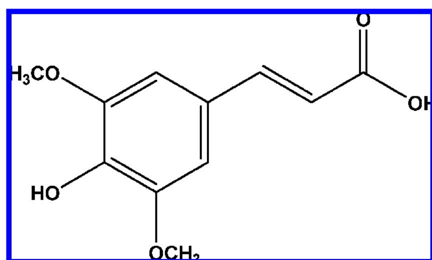

Figure 1. Chemical structure of sinapic acid.

sustained NO production can be deleterious to the host and has been implicated in the pathogenesis of various inflammatory diseases (*6*). Elevated levels of COX-2 expression account for the excessive production of inflammatory PGs. Particularly, PGE_2 is a major COX-2 product at inflammatory sites where it contributes to local blood flow increases, edema formation, and pain sensitization (*7*). TNF-α plays a key role in the induction and perpetuation of inflammation by activating T cells and macrophages and by up-regulating other pro-inflammatory cytokines (*8*). Likewise, IL-1β is one of the most important inflammatory cytokines secreted by LPS in macrophages. During inflammation, the increase of IL-1β leads to cell or tissue damage (*9, 10*), and thus, reduction of IL-1β release from macrophages may retard inflammatory responses to LPS stimulation. Several factors such as TNF-α, IL-1β, and LPS lead to an activation of the transcription factor NF-κB, which in turn regulates the production of pro-inflammatory molecules including NO, PGE_2, TNF-α, and IL-1β (*11*).

Sinapic acid is a cinnamic acid derivative, which possesses 3,5-dimethoxyl and 4-hydroxyl substitutions in the phenyl group of cinnamic acid (**Figure 1**). Sinapic acid is a widely prevalent substance in the plant kingdom and is obtained from various sources such as rye, fruits, and vegetables (*12, 13*). However, with the exception of its antioxidative and anxiolytic-like effects (*14, 15*), almost no information is reported about anti-inflammatory effects. Therefore, as a prelude to reveal the underlying mechanism for the anti-inflammatory effects of sinapic acid, we evaluated variations in inflammatory proteins, mRNAs, and cytokines expression in vitro and then evaluated its in vivo anti-inflammatory effects.

MATERIALS AND METHODS

Materials. 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). iNOS, COX-2, IκB-α, p-IκB-α, p65, p50, and β-actin monoclonal antibodies, GST-IκB-α (1−317) fusion protein, and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enzyme immunoassay (EIA) kits for PGE_2, TNF-α, and IL-1β were obtained from R&D Systems (Minneapolis, MN), and a luciferase assay kit was purchased from Promega (Madison, WI). pNF-κB-Luc reporter plasmid was purchased from BD Biosciences (San Jose, CA). Superfect transfection reagent was from Qiagen (Qiagen GmbH, Hilden, Germany), and RNA extraction kits were from Intron Biotechnology (Seoul, Korea). iNOS, COX-2, TNF-α, IL-1β, and β-actin oligonucleotide primers were from Bioneer (Seoul, Korea). Sinapic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT), aprotinin, leupeptin, phenylmethylsulfonyl-l-fluoride (PMSF), dithiothreitol, *Escherichia coli* LPS, sinapic acid (D7927, 98% titration), carrageenan, serotonin, indomethacin, ibuprofen, and all other chemicals were from Sigma (St. Louis, MO).

Cell Culture and Sample Treatment. The RAW 264.7 murine macrophage cell line was obtained from Korean Cell Line Bank (Seoul, Korea). Cells were grown at 37 °C in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) in a humidified 5% CO_2 atmosphere. Cells were incubated with the test compounds for 1 h at various concentrations (40, 80, or 160 μM) and stimulated with LPS 1 μg/mL for the indicated time [NO, PGE_2, and cytokines; Western blot, 24 h; reverse-transcribed polymerase chain reaction (RT-PCR), 4 h].

MTT Assay for Cell Viability. RAW 264.7 cells were plated at a density of 10^5 cells/well in 96 well plates. To determine the appropriate concentration not toxic to cells, cytotoxicity studies were performed 24 h after treating cells with various concentrations of sinapic acid. Viabilities were determined using colorimetric MTT assays, as described previously (*16*).

Nitrite Determination. RAW 264.7 cells were plated at 4×10^5 cells /well in 24 well plates and then incubated with or without LPS (1 μg/mL) in the absence or presence of various concentrations (40, 80, or 160 μM) of sinapic acid for 24 h. Nitrite levels in culture media were determined using the Griess reaction and presumed to reflect NO levels (*16*). Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl] and incubated at room temperature for 10 min. The absorbance was then measured at 540 nm using a microplate reader (Perkin-Elmer Cetus, Foster City, CA). Fresh culture media were used as blanks in all experiments. Nitrite levels in samples were read off a standard sodium nitrite curve.

Western Blot Analysis. Cellular proteins were extracted from both the control and the sinapic acid-treated RAW 264.7 macrophage cells. The cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). Washed cell pellets were resuspended in extraction lysis buffer [50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride (NaF), and 0.5 mM Na orthovanadate] containing 5 μg/mL each of leupeptin and aprotinin and then incubated for 30 min at 4 °C. Cell debris was removed by microcentrifugation, and supernatants were quick frozen. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions. Forty micrograms of cellular protein from treated and untreated cell extracts was electroblotted onto a nitrocellulose membrane following separation on a 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4 °C and then incubated for 4 h with a 1:1000 dilution of monoclonal anti-iNOS, anti-COX-2 antibody, and a 1:1000 dilution of anti-p65 and anti-p50 antibody (Santa Cruz Biotechnology Inc.). Blots were washed twice with Tween 20/Tris-buffered saline (TTBS) and then incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Blots were again washed three times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL).

RNA Preparation and RT-PCR. Total cellular RNA was isolated using Easy Blue kits (Intron Biotechnology), according to the manufacturer's instructions. RNA (1 μg) was RT from each sample using MuLV RT, 1 mM dNTP, and 0.5 μg/μL oligo (dT_{12-18}). PCR analyses were performed on aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α, and IL-1β (using β-actin as an internal standard) gene expression using a thermal cycler (Perkin-Elmer Cetus). Reactions were carried out in a volume of 25 μL containing (final concentrations) 1 unit of Taq DNA polymerase, 0.2 mM dNTP, 10× reaction buffer, and 100 pmol of 5′- and 3′-primers. After an 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), TNF-α (1 min of 95 °C denaturation, 1 min of 55 °C annealing, and 1 min 72 °C extension), and IL-1β (1 min of 94 °C denaturation, 1 min of 60 °C annealing, and 1 min 72 °C extension), and β-actin (1 min of 94 °C denaturation, 1 min of 60 °C annealing, and 1 min 72 °C extension). PCR primers used in this study are listed below and were purchased from Bioneer: sense strand iNOS, 5′-AATGGCAACATCAGGTCGGCCATCACT-3′; antisense strand iNOS, 5′-GCTGTGTGTCACAGAAGTCTC-GAACTC-3′; sense strand COX-2, 5′-GGAGAGACTATCAAGATAGT-3′; antisense strand COX-2, 5′-ATGGTCAGTAGACTTTTACA-3′;

sense strand TNF-α, 5′-ATGAGCACAGAAAGCATGATC-3′; antisense strand TNF-α, 5′-TACAGGCTTGTCACTCGAATT-3′; sense strand IL-1β, 5′-TGCAGAGTTCCCCAACTGGTACATC-3′; antisense strand IL-1β, 5′-GTGCTGCCTAATGTCCCCTTGAATC-3′; and sense strand β-actin, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′; antisense strand β-actin, 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′. After amplification, portions of the PCR reaction products were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Determination of PGE_2, TNF-α, and IL-1β Production. RAW 264.7 cells were pretreated with sinapic acid for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. Levels of PGE_2, TNF-α, and IL-1β in the culture media were quantified using EIA kits (R&D Systems).

Transient Transfection and Luciferase Assay (Reporter Gene Assay). RAW 264.7 macrophage cells were transfected using Superfect reagent (Qiagen GmbH) and pNF-κB-Luc reporter plasmid (BD Biosciences), as instructed by the manufacturer's instructions. Cells were incubated for 2 h before the addition of 5 mL of DMEM/10% FBS. Forty-eight hours after the start of transfection, cells were pretreated with sinapic acid for 1 h and stimulated with LPS (1 μg/mL). Following 3 h of stimulation, cells were lysed, and the luciferase activity was determined using the Promega luciferase assay system (Promega) and luminometer (Perkin-Elmer Cetus). The luciferase activity was normalized vs sample protein concentrations.

Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA). RAW 264.7 macrophages in 100 mm dishes (1×10^6 cells/mL) were preincubated with various concentrations of sinapic acid (40, 80, and 160 μM) and then stimulated with LPS (1 μg/mL) for 1 h. The cells were 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*). Cell pellets were 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, and 10 μg/mL aprotinin) and incubated on ice for 15 min. They were then lysed by adding 0.1% Nonidet P-40 and vortexing vigorously for 10 s. Nuclei were pelleted by centrifugation at 12000*g* for 10 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, and 1 mM sodium orthovanadate). Nuclear extract (10 μg) was mixed with double-stranded NF-κB oligonucleotide. 5′- AGTTGAGGGGACTTTCCC-AGGC-3′ was end-labeled by [γ-^{32}P] dATP (underlying indicates a κB consensus sequence or a binding site for NF-κB/cRel homodimeric or 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. DNA−protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5 × TBE buffer. Gels were vaccuum-dried for 1 h at 80 °C and exposed to X-ray film at −70 °C for 24 h.

Immunoprecipitation (IP) and IκB Kinase (IKK) Assay. Whole cell extracts were lysed with lysis buffer (10% glycerol, 1% Triton X-100, 1 mM EGTA, 5 mM EDTA, 1 mM sodium pyrophosphate, 20 mM Tris-HCl, pH 7.9, 10 mM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 mM NaF, 1 mM sodium orthovanadate, and protease inhibitor cocktail) for 15 min at 4 °C. Equal amounts of total cellular protein were immunoprecipitated with anti-IKKβ monoclonal antibody in TNT buffer (20 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 300 μM sodium orthovanadate, 2 mM PMSF, and protease inhibitor cocktail). The IKKβ−antibody complex was precipitated with protein A/G-Sepharose beads for 4 h at 4 °C. The kinase assay was carried out in kinase buffer containing 5 μCi [γ-^{32}P]ATP and GST-IκB-α (1−317) fusion protein (Santa Cruz, CA) as a substrate and incubated for 30 min at 37 °C. Each sample was mixed with Laemmli's loading buffer, heated for 10 min at 100 °C, and subjected to 10% SDS-PAGE. The gels were dried and visualized by autoradiography.

Animals. ICR male mice weighing 20−25 g and Sprague−Dawley male rats weighing 100−120 g were purchased from the Korean Experimental Animal Co. and maintained under constant conditions (temperature, 20 ± 2 °C; humidity, 40−60%; and light/dark cycle, 12 h) for 2 weeks or more. Twenty-four hours before the experiment, only water was provided. All animal experiments were approved by the University of Kyungsung Animal Care and Use Committee, and all procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health.

Serotonin-Induced Paw Edema in Mice. The initial hind paw volumes of ICR mice were determined volumetrically. A 1% solution of serotonin in saline (0.5 μg per rat) was injected subcutaneously into the right hind paws 1 h after a test solution (10, 20, and 30 mg/kg) had been administered orally. Test samples were first dissolved in 10% Tween 80 and then diluted with saline. The same volume of vehicle was administered to the control group. Paw volumes were measured for up to 30 min after oral administration at 6 min intervals, and volumes of edema were measured using a plethysmometer. Indomethacin, an anti-inflammatory drug, was used as a positive control (*17*).

Carrageenan-Induced Edema in Rats. The initial hind paw volume of the Sprague−Dawley strain rats was determined volumetrically. A 1% solution of carrageenan in saline (0.1 mL per rat) was injected subcutaneously into the right hind paws 1 h after administering test solutions (10, 20, and 30 mg/kg) orally. Test samples were first dissolved in 10% Tween 80 and then diluted with saline. The same volume of vehicle was administered to the control group. Paw volumes were measured for up to 5 h after injections at 1 h intervals, and edema volumes were measured using a plethysmometer. Ibuprofen (100 mg/kg), an anti-inflammatory drug, was used as a positive control (*17*).

Statistical Analysis. In vitro experiments were performed three times independently and analyzed using a nonparametric multiple comparisons test (Kruskal−Wallis test) followed by Dunn's test. All data are expressed as means ± standard deviations (SDs) of 10 animals in vivo experiments. Statistical analysis was performed by analysis of variance for multiple comparisons followed by Dunnett's test. Statistical significance was set at $P < 0.05$.

RESULTS

Inhibition of Sinapic Acid on LPS-Induced NO and PGE_2 Production. We initially investigated the inhibitory effects of sinapic acid on the LPS-induced production of the inflammatory mediators, such as NO and PGE_2 in RAW 264.7 cells. Cells were treated with/without sinapic acid (40, 80, and 160 μM) for 1 h and then treated with LPS (1 μg/mL) for 24 h. Neither LPS nor sinapic acid was added to the control (Con) group. Cell culture media were harvested, and NO concentrations were measured using the Griess reaction, and L-N^6-(1-iminoethyl) lysine (L-NIL) (10 μM) was used as a positive NO production inhibitor. Sinapic acid was found to significantly inhibit LPS-induced NO production in a dose-dependent manner (**Figure 2A**). To examine whether sinapic acid inhibits PGE_2 production, cells were preincubated with sinapic acid for 1 h and then activated with 1 μg/mL LPS for 24 h. As shown in **Figure 2B**, sinapic acid also inhibited the production of PGE_2 in a dose-dependent manner. NS-398 was used as a COX-2 selective inhibitor and showed significant inhibitory effects upon PGE_2 production. In addition, the cytotoxic effect of sinapic acid was evaluated in the presence or absence of LPS using the MTT assay, and this compound did not affect the cell viability of RAW 264.7 cells at least up to 300 μM in either the presence or the absence of LPS after a period of 24 h (**Figure 2C**). Thus, the inhibitory effects observed were not attributable to cytotoxic effects.

Inhibition of Sinapic Acid on LPS-Induced iNOS and COX-2 Expressions. To determine whether the inhibitory effects of sinapic acid on NO and PGE_2 are related to the modulation of iNOS and COX-2 enzymes, we examined their expression levels by Western blotting. In unstimulated RAW 264.7 cells, iNOS and COX-2 protein levels were undetectable. However, in response to LPS, the expression levels of iNOS and COX-2 were markedly upregulated, and sinapic acid significantly inhibited these iNOS and COX-2 expressions in a concentration-

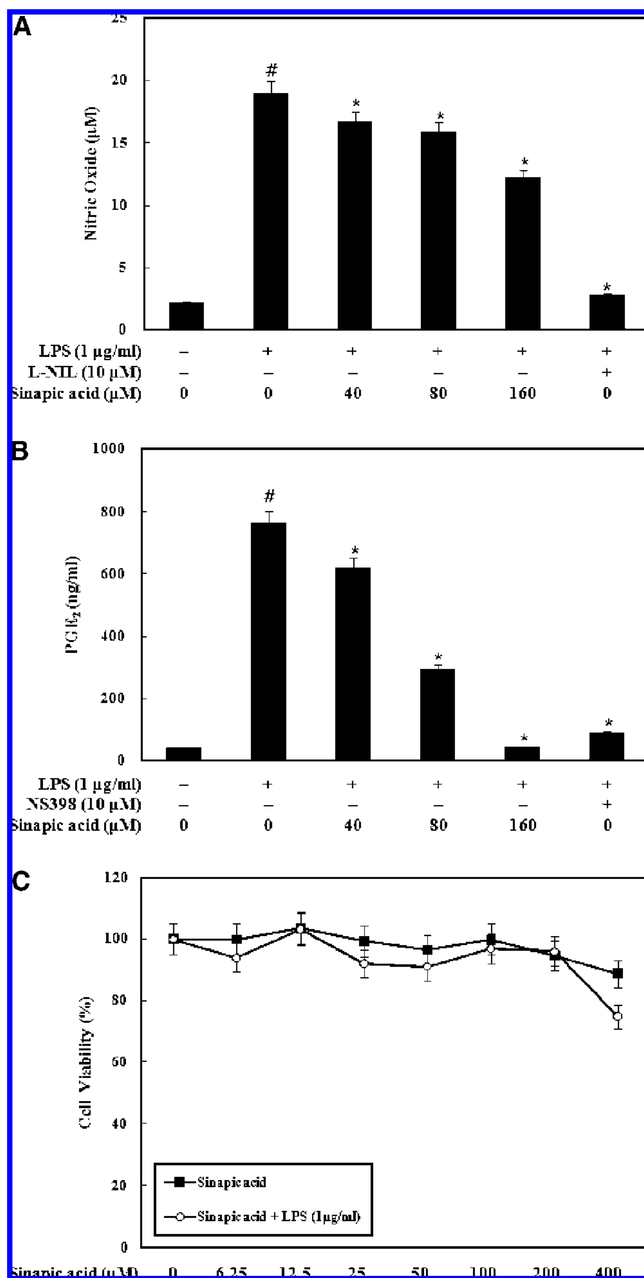

Figure 2. Effects of sinapic acid on LPS-induced NO and PGE_2 in RAW 264.7 cells. (**A**) Cells were treated with different concentrations (40, 80, and 160 μM) of sinapic acid for 1 h, then with LPS (1 μg/mL), and incubated for 24 h. Control (Con) values were obtained in the absence of LPS and sinapic acid. L-NIL was used as a positive control at a concentration of 10 μM. (**B**) Samples were treated as described in the legend of panel **A**. NS-398 (10 μM) was used as a positive control. Values are the means $\pm$ SDs of three independent experiments. (**C**) Cells were treated with various concentrations of sinapic acid in the presence or absence of LPS and then incubated for 24 h. Cell viabilities were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. $^{\#}P < 0.05$ as compared with the control group, and $^{*}P < 0.05$ as compared with the LPS-treated group; significant differences between groups were determined using a nonparametric multiple comparisons test (Kruskal–Wallis test) followed by Dunn's test (**A**, **B**).

dependent manner (**Figure 3A**). Because changes in amounts of iNOS and COX-2 enzyme could reflect altered protein synthesis or degradation, RT-PCR was performed to investigate whether sinapic acid suppressed LPS-mediated induction of iNOS and COX-2 via a pretranslational mechanism. RT-PCR analysis showed that levels of iNOS and COX-2 mRNA expressions were also dose dependently reduced (**Figure 3B**). However, sinapic acid did not affect the expression of β-actin, the housekeeping gene.

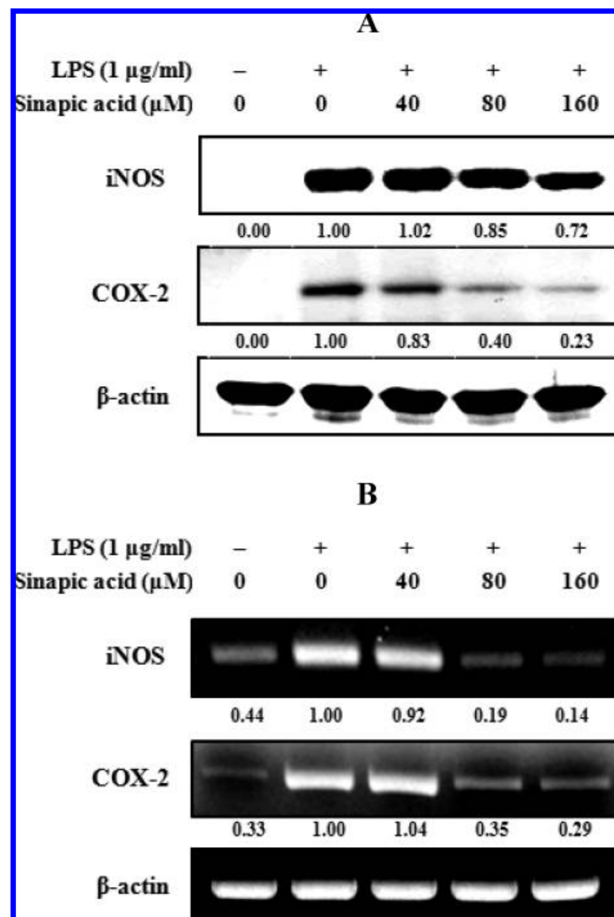

Figure 3. Effects of sinapic acid on LPS-induced iNOS and COX-2 protein and their mRNA expressions in RAW 264.7 cells. (**A**) Lysates were prepared from control or 24 h LPS (1 μg/mL) stimulated cells or from LPS plus sinapic acid (40, 80, and 160 μM). Total cellular proteins (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with specific antibodies, as described in the Materials and Methods. A representative immunoblot of three separate experiments is shown. (**B**) Total RNA was prepared for the RT-PCR of iNOS and COX-2 gene expressions from RAW 264.7 macrophages treated with different concentrations (40, 80, and 160 μM) of sinapic acid for 1 h followed by LPS (1 μg/mL) treatment for 4 h. iNOS-specific sequences (807 bp) and COX-2-specific sequences (721 bp) were detected by agarose gel electrophoresis, as described in the Materials and Methods. PCR of β-actin was performed to verify that the initial cDNA contents of samples were similar. The experiment was repeated three times, and similar results were obtained. The density ratio of the sinapic acid-treated group over the LPS only-treated group or control group was measured by a densitometer.

Inhibition of LPS-Inducible TNF-α and IL-1β Production and mRNA Expression by Sinapic Acid. Because sinapic acid was found to most potently inhibit the pro-inflammatory mediators, for example, NO and PGE_2, we investigated the effects of sinapic acid on LPS-induced TNF-α and IL-1β release using EIAs and RT-PCR. Pretreatment of RAW 264.7 cells with sinapic (40, 80, and 160 μM) significantly reduced TNF-α and IL-1β production (**Figure 4A**, **B**) and mRNA expression (**Figure 4C**) in a dose-dependent manner.

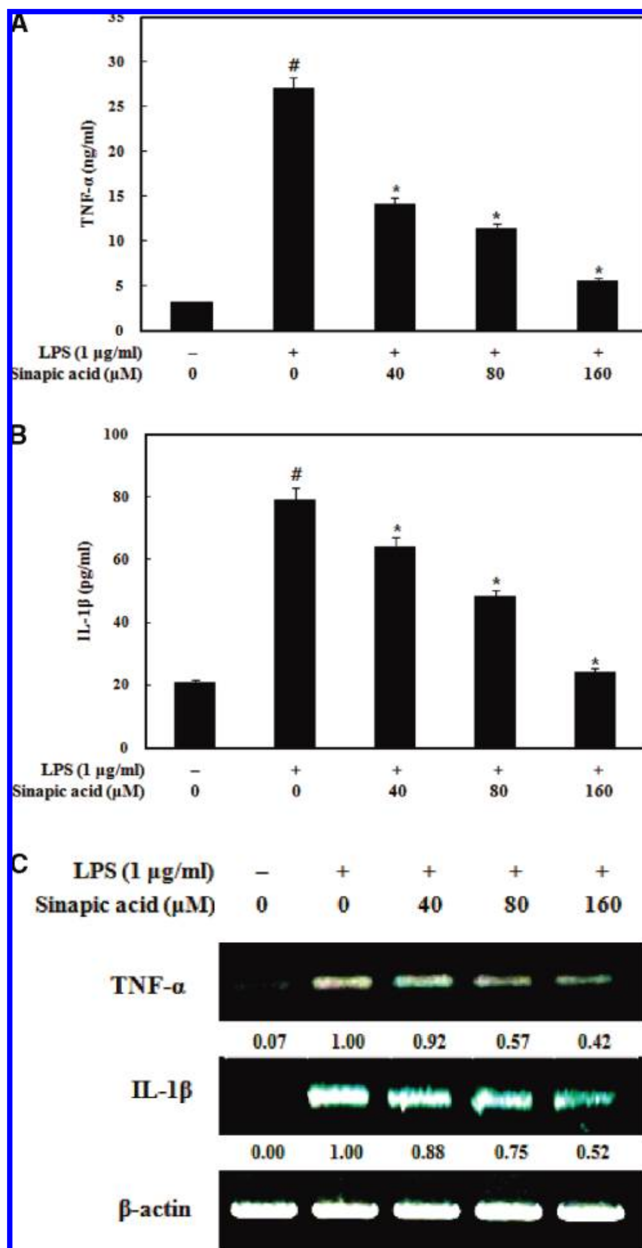

Figure 4. Effects of sinapic acid on LPS-induced TNF-α and IL-1β in RAW 264.7 macrophages. (**A**, **B**) Macrophages were pretreated with different concentrations (40, 80, and 160 μM) of sinapic acid for 1 h, LPS (1 μg/ml) was then added, and cells were incubated for a further 24 h. Control (Con) values were obtained in the absence of LPS or sinapic acid. Values are means ± SDs of three independent experiments. $^{\#}P < 0.05$ as compared with the control group, and $^{*}P < 0.05$ as compared with the LPS-treated group. (**C**) Total RNA was prepared for the RT-PCR of TNF-α and IL-1β gene expression from RAW 264.7 cells pretreated with different concentrations (40, 80, and 160 μM) of sinapic acid for 1 h and then with LPS (1 μg/mL) for 4 h. TNF-α-specific sequences (351 bp) and IL-1β-specific sequences (387 bp) were detected by agarose gel electrophoresis, as described in the Materials and Methods. PCR of β-actin was performed to verify that the initial cDNA contents of the samples were similar. The experiment was repeated three times with similar results. The density ratio of the sinapic acid-treated group over the LPS only-treated group or control group was measured by a densitometer.

Effects of Sinapic Acid on LPS-Induced NF-κB Activation, p65-DNA Binding, and the Nuclear Translocation of NF-κB. To further investigate the mechanism of the sinapic acid-mediated inhibition of iNOS, COX-2, TNF-α, and IL-1β transcription (*4*), luciferase and EMSAs were used to determine whether sinapic acid suppresses NF-κB activation. RAW 264.7 cells were transiently transfected with pNF-κB-luc plasmid and then stimulated with 1 μg/mL LPS either in the presence or in the absence of sinapic acid. Sinapic acid significantly reduced the LPS-induced increase in NF-κB-dependent luciferase enzyme expression (**Figure 5A**). To determine whether sinapic acid affects the transcription of NF-κB, we investigated the DNA binding activity of NF-κB using EMSA. Here, we found that the DNA binding activity of p65 was reduced in nuclear extracts obtained from LPS-activated RAW 264.7 cells pretreated with sinapic acid (40, 60, 80, 120, or 160 μM) (**Figure 5B**). This inhibition of the NF-κB DNA binding activity was also confirmed between 5 and 30 min after the treatment with sinapic acid 160 μM (**Figure 5C**). Moreover, we examined whether sinapic acid prevents the translocation of the p65 and p50 subunits of NF-κB to the nucleus of RAW 264.7 cells. Western blot analysis showed that LPS-induced p65 and p50 levels in nuclear fractions were reduced by sinapic acid with dose-dependent manner (**Figure 5D**). Taken all together, the above finding shows that sinapic acid inhibited NO, PGE_2, iNOS, COX-2, TNF-α, and IL-1β expression at least in part via a NF-κB-dependent mechanism.

Effects of Sinapic Acid on LPS-Induced IκB-α Phosphorylation and IKK Activity. We further investigated whether sinapic acid could inhibit the LPS-stimulated phosphorylation of IκB-α in RAW 264.7 cells by Western blotting. In unstimulated cells, NF-κB is sequestered in the cytosol by its inhibitory protein, IκB. When stimulated by LPS, IκB is phosphorylated by its IKKs, ubiquitinated, and rapidly degraded via 26S proteosome, to release NF-κB (*18*). **Figure 6A** shows that LPS-induced IκB-α degradation was significantly blocked by sinapic acid pretreatment. Moreover, to determine whether this IκB-α degradation is related to IκB-α phosphorylation, we examined the effect of sinapic acid on LPS-induced pIκB-α by Western blotting and found that sinapic acid also significantly reduced LPS-induced IκB-α phosphorylation in a concentration-dependent manner. In an attempt to explore the effects of sinapic acid on the inhibition of IKK activity in RAW 264.7 cells, we immunoprecipitated IKK from LPS-stimulated RAW 264.7 cells and then determined its kinase activity in the presence of sinapic acid (40, 80, and 160 μM). Sinapic acid directly inhibited LPS-induced IKK activity in a dose-dependent manner (**Figure 6B**).

Effect of Sinapic Acid on Serotonin- and Carrageenan-Induced Edema in Mice or Rats. Because sinapic acid effectively inhibited iNOS, COX-2, and proinflammatory cytokines inductions in macrophages, studies were extended to determine whether sinapic acid affected acute phase inflammation in animal models. In this study, we used serotonin- and carrageenan-induced edema because these models are widely employed for screening the effects of anti-inflammatory drugs. Maximal edema inhibition was observed at 24 min after serotonin-induced edema in mice. Particularly, sinapic acid treatment (30 mg/kg, p.o.) reduced edema of mice by 34.2% at 24 min, whereas the positive control indomethacin (10 mg/kg, p.o.) decreased the edema rate by 36.9% at 24 min (**Figure 7A**). The significances of these reductions were maintained for more than 30 min after edema induction. In parallel, the anti-inflammatory activities of sinapic acid (10, 20, and 30 mg/kg, p.o.) were observed 1 h after carrageenan injection in rats. Maximal edema inhibition was observed at 3 h after edema induction. It is notable that treatment with sinapic acid (30 mg/kg, p.o.) reduced edema of rats by 40.4% at 3 h, whereas the

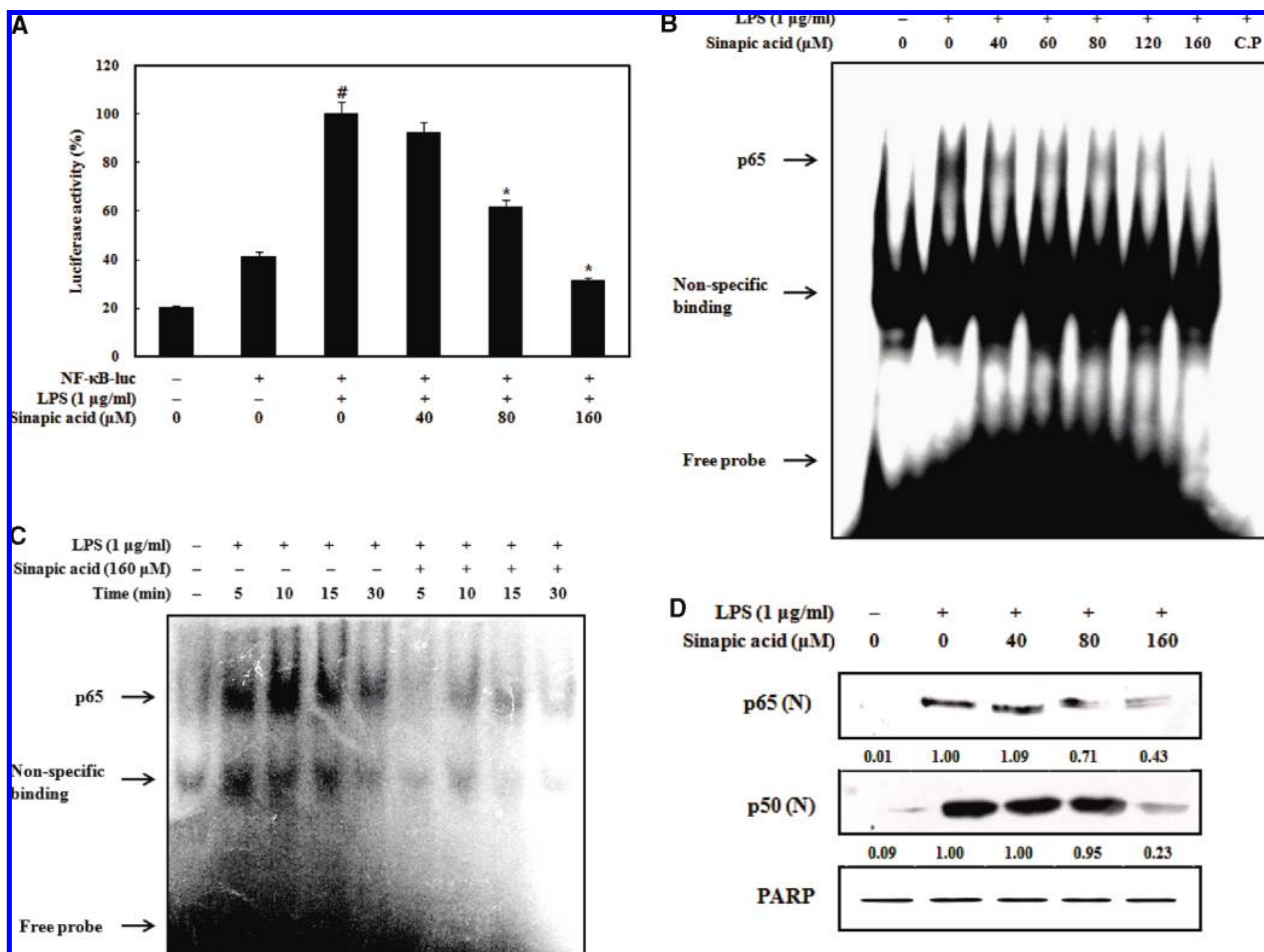

Figure 5. Inhibitory effects of sinapic acid on LPS-induced luciferase and NF-κB-DNA binding activity. (**A**) Cells were transiently cotransfected with pNF-κB-luc reporter and then either left untreated (Con) or were pretreated with different concentrations (40, 80, and 160 μM) of sinapic acid for 1 h. LPS (1 μg/mL) was then added, and cells were further incubated for 3 h. Cells were then harvested, and luciferase activities were determined using a Promega luciferase assay system and a luminometer. The values shown are means ± SDs of three independent experiments. $^{\#}p < 0.05$ vs the control group, and $^{*}p < 0.05$ vs the LPS-treated group. Significant differences between treated groups were determined using a nonparametric multiple comparisons test (Kruskal−Wallis test) followed by Dunn's test. (**B**) Nuclear extracts were prepared from control or pretreated with different concentrations (40, 60, 80, 120, and 160 μM) of sinapic acid for 1 h and then with LPS (1 μg/mL) for 1 h and analyzed for NF-κB binding by EMSA. The arrow indicates the position of the NF-κB band. The specificity of binding was examined by competition with the 80-fold unlabeled NF-κB oligonucleotide (cp). The data shown are representative of three independent experiments. (**C**) The nuclear protein from the LPS-stimulated cells was incubated for the indicated time with 160 μM sinapic acid and then with LPS (1 μg/mL) for 1 h and analyzed for NF-κB binding by EMSA. The arrow indicates the position of the NF-κB band. (**D**) RAW 264.7 cells were pretreated with different concentrations (40, 80, and 160 μM) of sinapic acid for 1 h and then with LPS (1 μg/mL) for 1 h. Nuclear extracts (40 μg/mL) were prepared for Western blot analysis of p65 and p50 of NF-κB protein using a specific anti-p65 and anti-p50 NF-κB monoclonal antibody, respectively. PARP was used as an internal control. The density ratio of sinapic acid-treated group over LPS only-treated group or control group was measured by a densitometer.

positive control, ibuprofen (100 mg/kg, p.o.) decreased the edema rate by 58.9% at 3 h (**Figure 7B**). These results suggest that repression of iNOS and COX-2 induction and of the productions of pro-inflammatory cytokines by sinapic acid may represent important mechanisms involved in the inhibition of paw edema formation by serotonin or carrageenan.

DISCUSSION

The pathology of inflammation is initiated by complex processes triggered by microbial pathogens such as LPS, which is a prototypical endotoxin (*10*). LPS can directly activate macrophages, which trigger the production of inflammatory mediators, such as NO, COX-2, TNF-α, ILs, and leukotrienes (*7, 9*). The pharmacological reduction of LPS-inducible inflammatory mediators (for example NO, TNF-α, and ILs) is regarded as one of the essential conditions to alleviate a variety of disorders caused by activation of macrophages. Thus, RAW 264.7 macrophages provide us with an excellent model for anti-inflammatory drug screening and for subsequently evaluating the inhibitors of the pathways that lead to the induction of pro-inflammatory enzymes and to the production of pro-inflammatory cytokines. Sinapic acid is a phenylpropanoid derivative with antioxidant activities (*14, 15*); however, no report has been issued on the anti-inflammatory effect of sinapic acid and the mode of action involved. Thus, this study was aimed to evaluate anti-inflammatory effect of sinapic acid by screening the effects of sinapic acid on LPS-induced pro-inflammatory molecules in vitro and on acute phase inflammation in vivo. To further explore the possible underlying mechanism of these inhibitions, the effect of sinapic acid on NF-κB activity was examined.

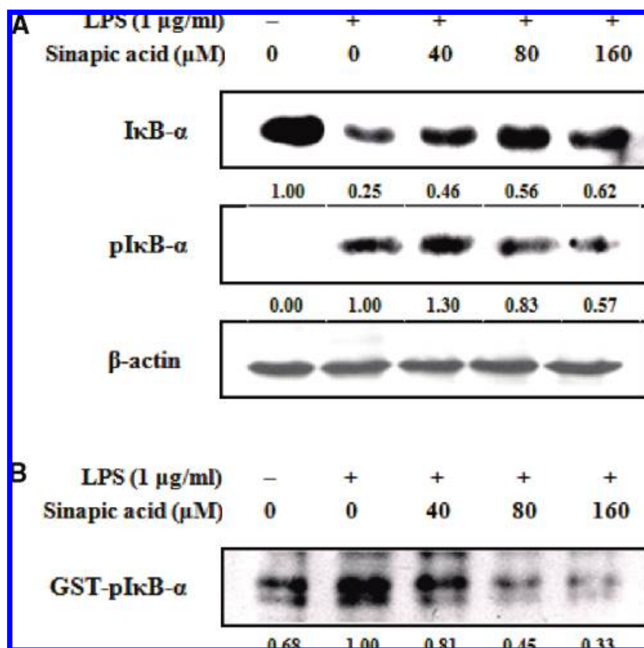

Figure 6. Inhibition of IκB-α phosphorylation and IKK activity by sinapic acid. (**A**) Cells were pretreated with sinapic acid for 1 h and then with LPS (1 μg/mL) for 15 min. Total cellular proteins were prepared and Western blotted for p-IκB-α and IκB-α using specific p-IκB-α and IκB-α antibodies. β-Actin was used as an internal control. (**B**) Samples were treated as described in the legend of panel **A**. IKK-immuno complex was prepared from total cell lysates. The kinase activity was assayed with GST-IκBα substrate performed as described in the Materials and Methods. The density ratio of sinapic acid-treated group over LPS only-treated group or control group was measured by a densitometer.

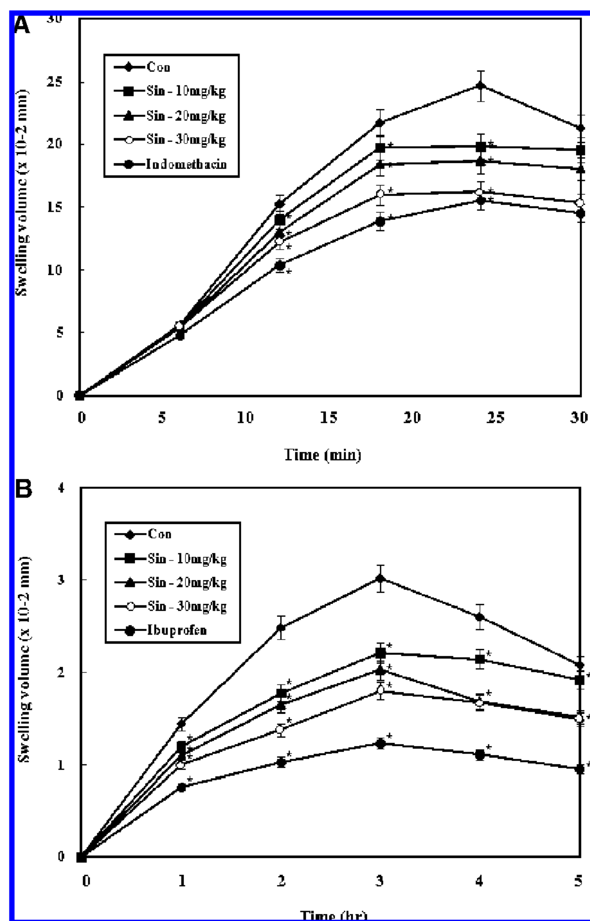

Figure 7. Inhibitory effect of sinapic acid pretreatment on (**A**) serotonin-induced paw edema in mouse and (**B**) carrageenan-induced paw edema in rats. Sinapic acid was administered orally at 10, 20, and 30 mg/kg. Indomethacin (10 mg/kg, p.o) and ibuprofen (100 mg/kg, p.o) were used as a positive control, respectively. Vehicle was administered to control groups, and edema volumes were measured using a plethysmometer. Values represent means ± SDs ($n = 10$). *$P < 0.05$ as compared with the control group.

Although iNOS plays a pivotal role in immunity against infectious agents by producing an excess amount of NO, this enzyme has come into the spotlight for its detrimental roles in inflammation-related diseases (*6*). In fact, macrophages derived from iNOS knockout mice are protected from LPS/interferon-γ-induced inflammation (*19*). Recent evidence suggests that PGs are involved in inflammatory processes, and an inducible isoform of cyclooxygenase (COX-2) is mainly responsible for the production of large amounts of these mediators (*20*). Therefore, we investigated in this study whether sinapic acid decreased the level of LPS-induced NO and PGE_2 productions and iNOS and COX-2 expressions. In the present study, immunoblot and RT-PCR analyses revealed that sinapic acid effectively blocked the induction both of iNOS and COX-2 proteins and of their mRNA levels. In parallel, sinapic acid inhibited LPS-induced NO and PGE_2 productions in a dose-dependent manner. Thus, inhibitions of the releases of NO and PGE_2 may be attributed to the expressional inhibitions of iNOS and COX-2 proteins followed by their suppression at the transcriptional level. Moreover, the inhibitory effects of sinapic acid on the LPS-induced expressions of these molecules in RAW 264.7 cells were not due to the cytotoxicity of sinapic acid, as assessed by MTT assay and by the expression of β-actin.

The proinflammatory cytokines such as TNF-α and IL-1 are small secreted proteins, which mediate and regulate immunity and inflammation (*21*). The production of TNF-α is crucial for the synergistic induction of NO synthesis in IFN-γ and/or LPS-stimulated macrophages. TNF-α induces a number of physiological effects including septic shock, inflammation, and cytotoxicity (*22*). Moreover, IL-1β is one of the most important inflammatory cytokines secreted by macrophages, leading to cell or tissue damage during inflammatory process (*23*). Here, we evaluate the inhibitory effects of sinapic acid on LPS-stimulated macrophages. Our results indicated that sinapic acid showed significantly inhibited LPS-induced TNF-α and IL-1β production as well as their mRNA expression.

It is well-known that NF-κB is involved in the modulation of cell survival genes and the expressions of pro-inflammatory enzymes and cytokines, such as iNOS, COX-2, TNF-α, and IL-1β (*24*). Because the expressions of these pro-inflammatory molecules are regulated by NF-κB, the possibility that sinapic acid inhibits NF-κB activity was investigated in vitro. The present study demonstrates that sinapic acid effectively prevented LPS-inducible NF-κB activation and the DNA binding activity of p65. In the cytoplasm, NF-κB is bound and tightly controlled by its inhibitory subunit, IκB. In the present study, we also found that the translocation of activated NF-κB to the nucleus was inhibited in a concentration-dependent manner by sinapic acid and that the degradation and phosphorylation of IκB-α were also inhibited by sinapic acid in a concentration-dependent manner.

IKK-α and IKK-β (known as the IKKs) are responsible for phosphorylating IκBs (*25*). Transient IKK activation or deactivation is physiologically important because persistent NF-κB activity can result in deleterious or even fatal conditions, such as septic shock or acute inflammation (*26*). In the present study,

we observed that sinapic acid significantly inhibited the kinase activity of the IKK complex precipitated from cell lysate induced by LPS. It is well-recognized that activation of IKK complex phosphorylates IκB-α, and the IKK complex may be activated by various upstream kinases, which include protein kinase C and tyrosine kinase family members (*27, 28*). Therefore, it remains to be established whether sinapic acid acts on these upstream kinases and, if so, what is the true pharmacological target of sinapic acid.

To investigate the anti-inflammatory effects of sinapic acid in vivo, we turned our attention to serotonin- or carrageenan-induced edema tests in animal models. Increased vascular permeability and swelling are common during the early stages of inflammations, and the paw swelling induced by serotonin is principally dependent on increased vascular permeability. Recent studies have shown that carrageenan induces peripheral release of NO as well as that of PGE_2 (*29*). NO plays a major role in edema formation and development of hyperalgesia in inflammatory responses and tissue injury. In addition, it has been reported that carrageenan induces the release of TNF-α, which subsequently promotes IL-1 production in the tissue (*30*). Our serotonin- or carrageenan-induced rat paw edema models enabled us to demonstrate the ability of sinapic acid to inhibit edema induced by acute inflammation. These results in conjunction with the marked inhibition of LPS-induced NO, PGE_2, TNF-α, and IL-1β productions by sinapic acid in macrophages imply that the antiedema effects of sinapic acid might result from its inhibition of NO, PGE_2, TNF-α, and IL-1β syntheses in the peripheral tissues.

In summary, our results demonstrate that sinapic acid exerts anti-inflammatory effects, which results from the inhibition of NF-kB activation in macrophages, thereby inhibiting the production of iNOS, COX-2, and proinflammatory cytokines. More importantly, sinapic acid was found to have an antiedema effect in the carrageenan- or serotonin-induced paw edema models in mouse or rats, one of the well-established acute inflammatory models in vivo. Our findings showing inhibition by sinapic acid of paw edema as well as inflammatory gene induction may help to understand the pharmacology and mechanism of action of sinapic acid.

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