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Salicortin suppresses lipopolysaccharide-stimulated inflammatory responses via blockade of NF-κB and JNK activation in RAW 264.7 macrophages

Dong-Joo Kwon[1,2], Young-Soo Bae[3], Sung Mi Ju[1], Gi Soo Youn[1], Soo Young Choi[1] & Jinseu Park[1,]*

[1]Department of Biomedical Science and Research Institute for Bioscience & Biotechnology, Hallym University, Chuncheon 200-702, [2]Hongcheon Institute of Medicinal Herb, Hongcheon 200-930, [3]Department of Forest Biomaterials Engineering, Kangwon National University, Chuncheon 200-701, Korea

We isolated the phenolic glucoside salicortin from a *Populus euramericana* bark extract, and examined its ability to suppress inflammatory responses as well as the molecular mechanisms underlying these abilities, using lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Salicortin inhibited iNOS expression and the subsequent production of NO in a dose-dependent manner in the LPS-stimulated RAW 264.7 cells. Salicortin significantly suppressed LPS-induced signal cascades of NF-κB activation, such as IKK activation, IκBα phosphorylation and p65 phosphorylation in RAW 264.7 cells. In addition, salicortin inhibited the LPS-induced activation of JNK, but not ERK or p38 MAPK. Furthermore, salicortin significantly inhibited production of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 in the LPS-stimulated RAW 264.7 cells. These findings suggest that salicortin may show its anti-inflammatory activity by suppressing the LPS-induced expression of pro-inflammatory mediators through inhibition of NF-κB and JNK MAPK signaling cascades in macrophages. [BMB Reports 2014; 47(6): 318-323]

INTRODUCTION

Macrophages are primary immune cells which play important roles in a variety of disease processes, including autoimmune diseases, infections, and inflammatory disorders (1). Upon inflammatory stimuli, such as lipopolysaccharide (LPS) and interferon (IFN)-γ, activated macrophages produce various pro-inflammatory cytokines/chemokines, and other inflammatory mediators, such as nitric oxide (NO) and prostaglandin E2 (PGE2), which are generated by the induction of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively (2-4). These inflammatory mediators contribute to the pathogenesis of various human diseases (5, 6).

Nuclear factor-κB (NF-κB) is one of the major transcription factors which regulate the expression of numerous genes related to inflammation, including iNOS and COX-2 (7). LPS binding to its receptor, toll-like receptor 4 (TLR4), initiates intracellular signaling cascades via activation of the IκB-kinase (IKK) complex, consisting of 2 kinase subunits (IKKα and IKKβ) and a regulatory subunit IKKγ/NEMO (8). IKK-mediated phosphorylation of IκBα results in its ubiquitination and subsequent proteasomal degradation (9). Dissociated NF-κB then translocates to the nucleus and regulates the transcription of NF-κB target genes.

During inflammation processes, the mitogen-activated protein kinases (MAPKs), including p38, c-Jun NH_2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), are activated and mediate the signaling cascades leading to the expression of pro-inflammatory genes (10). LPS stimulation activates MAPKs, which participates in the signaling cascades leading to activation of various transcription factors, such as NF-κB, in the macrophages (7). Therefore, the IKK-NF-κB and MAPK pathways provide primary targets for the development of therapeutics against various inflammatory diseases.

Salicortin, a phenolic glycoside, is the dominant secondary metabolites in many plants, including Populus and Salix species, and exerts various biological effects, such as anti-amnesic and anti-adipogenic effects (11, 12). Recently, it was reported that salicortin significantly inhibited iNOS expression and NO production in LPS-stimulated macrophages and microglia (12, 13). Salicortin suppressed TNF-α-induced ICAM-1 expression in human endothelial cells (14). These studies suggest that salicortin has immune-modulatory activity, even though the molecular action mechanism for this activity has not been fully determined.

In this study, we determined the inhibitory effect of salicortin on the LPS-induced expression of pro-inflammatory mediators,and its action mechanisms in RAW 264.7 macro-

*Corresponding author. Tel: +82-33-248-2116; Fax: +82-33-256-3420; E-mail: jinpark@hallym.ac.kr

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phages. We observed that salicortin suppressed LPS-induced expression of iNOS, as well as the subsequent production of NO, in RAW 264.7 cells. We found that salicortin significantly suppressed LPS-induced activation of NF-κB and JNK MAPK. In addition, salicortin inhibited production of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, in the LPS-stimulated RAW 264.7 cells. These findings may provide a molecular basis for anti-inflammatory activities of salicortin.

RESULTS

Effects of salicortin on LPS-induced iNOS expression and NO production in RAW 264.7 cells

LPS-stimulated RAW 264.7 macrophages were used as an inflammation model (15). As shown in Fig. 1B, salicortin at concentrations up to 40 μg/ml did not affect cell viability in the presence or absence of LPS. To investigate the suppressive effects of salicortin on LPS-induced iNOS expression, cell lysates were analyzed for protein and mRNA expression using Western blot and RT-PCR analysis, respectively. LPS-induced expression of iNOS protein and mRNA was significantly suppressed by pretreatment with salicortin (Fig. 1C). To examine the effect of salicortin on NO production, we measured the level of nitrite in the medium using the Griess reaction. As shown in Fig. 1D, the nitrite concentration in RAW 264.7 cells was markedly increased through stimulation with LPS. Conversely, pretreatment with salicortin inhibited the LPS-induced production of nitrite in a dose-dependent manner. These results suggest that the salicortin-mediated inhibition of NO production in LPS-stimulated RAW 264.7 cells is associated with the inhibition of iNOS expression at a transcriptional level. However, salicortin did not affect COX-2 expression or PGE_2 production in LPS-stimulated RAW 264.7 cells under the same experimental conditions (data not shown).

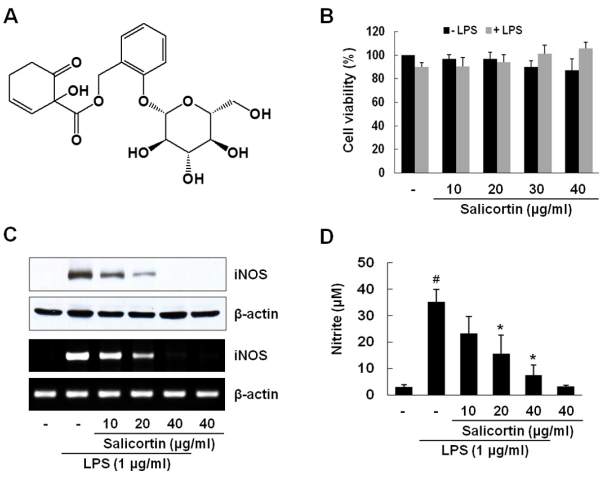

Fig. 1. Effects of salicortin on LPS-induced iNOS expression and NO production in RAW 264.7 macrophages. (A) Chemical structure of salicortin. (B) Cell viability after treatment with salicortin and/or LPS (1 μg/ml) for 24 h was measured by MTT assay. (C) Cells were pretreated with the indicated concentrations of salicortin for 3 h and were then stimulated with LPS (1 μg/ml) for 18 h (for protein) or 4 h (for mRNA). Cell lysates were electrophoresed and the protein expression of iNOS was detected by the specific antibody. Total RNA was prepared and analyzed by RT-PCR. β-actin was used as a control. (D) Cells were pretreated with salicortin for 3 h prior to incubation with LPS (1 μg/ml) for 18 h. The nitrite levels in the cultured media were measured by Griess reaction. Data are presented as the mean ± S.D. of three experiments. $^{\#}P < 0.001$ compared with control group, $^{*}P < 0.001$ compared to the LPS-treated group.

Effects of salicortin on IKK and NF-κB activation induced by LPS in RAW 264.7 cells

NF-κB is a key transcriptional factor for the regulation of expression of various pro-inflammatory mediators, including iNOS (9). LPS stimulation activates the IKK complex which induces the phosphorylation and degradation of IκBα, leading to the nuclear translocation of NF-κB components, such as p65

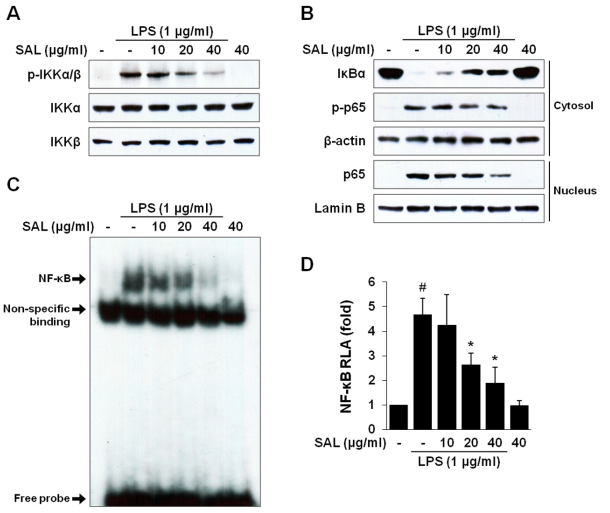

Fig. 2. Effects of salicortin on IKK and NF-κB activation in LPS-stimulated RAW 264.7 macrophages. Cells were pre-incubated for 3 h with or without salicortin at the indicated concentrations and were then stimulated with LPS (1 μg/ml) for 20 min. (A) Cell lysates were analyzed for IKKα/β activation by Western blot analysis using phospho-specific antibodies. (B) The levels of IkBα in the cell lysates, as well as those of p65 in the cytosolic and nuclear fractions, were measured by Western blot analysis. (C) DNA-binding activity of NF-κB in the nuclear extracts of the cells was determined by EMSA, using a NF-κB specific oligonucleotide probe. (D) Cells were transiently transfected with a NF-κB promoter-luciferase construct and a β-galactosidase construct (pCMV-lacZ). Cells were exposed to LPS (1 μg/ml) in the presence or absence of salicortin for 24 h, and the luciferase activity in the cell extracts was then determined. Luciferase activities are normalized to the β-galactosidase activities and are expressed as fold inductions over the control. RLA, relative luciferase activity. Data are presented as the mean ± S.D. of three experiments. $^{\#}P < 0.001$ compared to control group, $^{*}P < 0.001$ compared to the LPS-treated group.

(3,8). We first examined the effect of salicort in on LPS-induced IKKα/β activation. The levels of IKK activation in cell lysates were evaluated using an antibody against phosphorylated IKKα/β. As shown in Fig. 2A, LPS strongly induced IKKα/β phosphorylation, whereas salicortin markedly inhibited phosphorylation. Next, we examined the inhibitory effect of salicortin on LPS-induced downstream signal cascades of NF-κB activation. As shown in Fig. 2B, salicortin significantly suppressed LPS-induced IκBα degradation in the RAW 264.7 cells. Salicortin also inhibited phosphorylation, as well as nuclear localization of p65, in dose-dependent manners (Fig. 2B). In addition, salicortin inhibited LPS-induced NF-κB DNA-binding activity, as measured by an EMSA (Fig. 2C). We further examined the effect of salicortin on NF-κB promoter activity. As shown in Fig. 2D, salicortin reduced LPS-stimulated up-regulation of NF-κB promoter activity in a dose-dependent manner. These results suggest that salicortin exerts its inhibitory effects on LPS-induced iNOS expression via the inhibition of NF-κB signaling cascades.

Effects of salicortin on MAPKs activation induced by LPS in RAW 264.7 cells

The mitogen-activated protein kinase (MAPK) pathways play important roles in mediating the expression of pro-inflammatory mediators in macrophages (7). We examined the effect of salicortin on the LPS-induced activation of ERK, p38 MAPK, and JNK using phosphor-specific antibodies against MAPK proteins. As shown in Fig. 3A, LPS stimulation induced

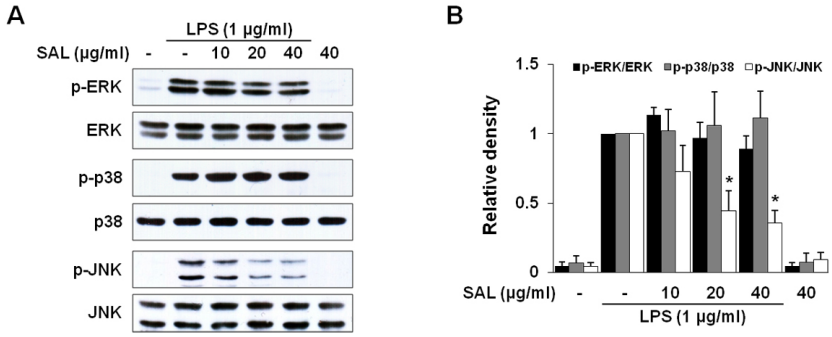

Fig. 3. Effect of salicortin on LPS-induced MAPKs activation in RAW 264.7 macrophages. (A) Cells were pretreated with salicortin for 3 h, and were then stimulated with LPS (1 μg/ml) for 20 min. The phosphorylation levels of ERK, p38, and JNK in the cell lysates were measured by Western blot analysis using phospho-specific antibodies. (B) Relative protein levels were quantified by scanning densitometry and were normalized to control protein levels.

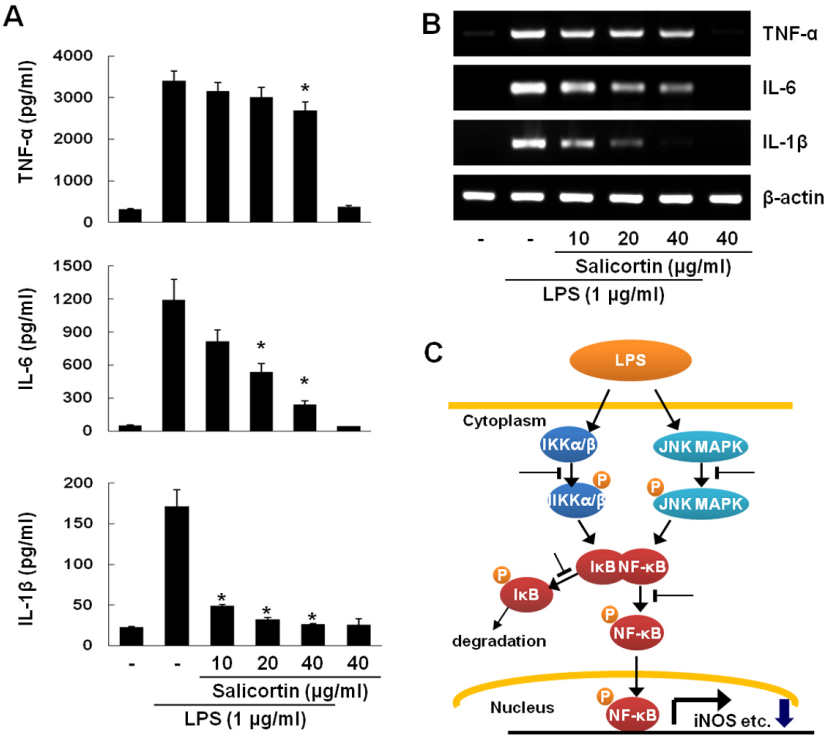

Fig. 4. Effects of salicortin on the expression of proinflammatory cytokines in the LPS-stimulated RAW 264.7 macrophages. Cells were pretreated with salicortin for 3 h, and were then stimulated with LPS (1 μg/ml) for 24 h (for protein) or 4 h (for mRNA). (A) The levels of TNF-α, IL-6, and IL-1β in the culture medium were measured by ELISA kit. Data are presented as the mean ± S.D. of three experiments. *P < 0.001 compared to the LPS-treated group. (B) The levels of TNF-α, IL-6, and IL-1β mRNA were determined by RT-PCR using specific primers. (C) The schematic representation of the action mechanisms by which salicortin exerts its inhibitory effects on LPS-induced inflammatory responses in RAW 264.7 macrophages.

the activation of all three MAPKs. However, LPS-induced phosphorylation of JNK was significantly suppressed by salicortin in a dose-dependent manner, whereas phosphorylation of ERK and p38 MAPK was not affected.

Effect of salicortin on production of proinflammatory cytokines induced by LPS in RAW 264.7 cells

LPS stimulation induces the production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, which play important roles in pathogen-derived inflammatory responses (7). To investigate whether salicortin has any effects on the production of pro-inflammatory cytokines, cells were stimulated with LPS in the presence or absence of salicortin. We measured the protein levels of the cytokines by ELISA. Pretreatment with salicortin resulted in a significant decrease in LPS-induced TNF-α, IL-6, and IL-1β protein levels, in a dose-dependent manner (Fig. 4A). As shown in Fig. 4B, salicortin markedly decreased the mRNA expression levels of TNF-α, IL-6, and IL-1β in LPS-stimulated RAW 264.7 cells.

DISCUSSION

Since NF-κB and MAPK signaling pathways play important roles in the inflammation processes, we evaluated the regulatory effects of salicortin on these signaling pathways and the expression of pro-inflammatory genes, such as iNOS, and cytokines in LPS-stimulated RAW 264.7 macrophages. Salicortin significantly inhibited LPS-induced iNOS expression and subsequent NO production in RAW 264.7 cells. In addition, salicortin suppressed the expression of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, in the LPS-stimulated RAW 264.7 cells. We found that salicortin exerts its inhibitory effects on these inflammatory responses via the suppression of NF-κB and JNK MAPK activation in the LPS-stimulated RAW 264.7 cells (Fig. 4C).

LPS stimulation of macrophages up-regulates various pro-inflammatory mediators such as cytokines/chemokines, NO, and PGE_2 (Reviewed in [7]). Dysregulated production of pro-inflammatory mediators leads to many pathologic states, including cancer (5,6). Therefore, modulation of pro-inflammatory mediators, such as iNOS and NO, provide possible targets for the development of therapeutics against various inflammatory diseases. We observed that salicortin from a *Populus euramericana* bark extract significantly suppressed LPS-induced iNOS expression and NO production in RAW 264.7 cells (Fig. 1C and D). Consistent with our results, it was recently reported that salicortin from Populus and Salix species significantly inhibited LPS-induced nitric oxide production in RAW264.7 cells and in BV2 microglial cells, although the molecular mechanisms of action has not yet been demonstrated (12, 13). Besides the inhibitory effects on iNOS expression and NO production, salicortin also suppressed the expression of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β in LPS-stimulated RAW 264.7 cells (Fig. 4A and B). These results suggest that salicortin has a modulatory activity towards the inflammation process.

Since activation of NF-κB is central to the expression of inflammatory mediators induced by LPS (16), we analyzed the effects of salicortin on the signaling cascades leading to NF-κB activation. LPS interaction with its receptor, toll-like receptor 4 (TLR4), on the cells activates the IκB-kinase (IKK) complex, consisted of two kinase subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ/NEMO), which in turn phosphorylates IκBα, leading to its ubiquitination and subsequent proteasomal degradation (9). As shown in Fig. 2A, salicortin markedly inhibited LPS-induced IKKα/β phosphorylation, Salicortin also exerted its inhibitory effect on LPS-induced downstream signal cascades of NF-κB activation. Pretreatment of salicortin inhibited LPS-induced IκBα degradation, as well as NF-κB activation, in the RAW 264.7 cells (Fig. 2). Taken together, these data suggest that salicortin exerts its inhibitory effects on pro-inflammatory gene expression by suppressing the NF-κB dependent pathway.

The expression of many pro-inflammatory genes by LPS is mediated by the MAPK pathway [10]. As demonstrated in Fig. 3A, ERK, JNK and p38 MAPK are major MAPK subfamily members which are activated in LPS-stimulated RAW 264.7 cells (Fig. 3A). Salicortin significantly suppressed LPS-induced phosphorylation of JNK, but not ERK or p38 MAPK. Previous studies have reported that the JNK activity is involved in iNOS expression in LPS-stimulated RAW264.7 macrophages (17). Taken together, these results suggest that the suppression of LPS-induced iNOS expression by salicortin is mediated by the inhibition of JNK activation.

A better understanding of the action mechanisms by which salicortin modulates the expression of various pro-inflammatory mediators may be a prerequisite to develop its application against inflammatory diseases. In conclusion, we provide a molecular basis that salicortin has an immune-modulatory activity via the suppression of NF-κB and JNK MAPK activation in LPS-stimulated RAW 264.7 macrophages.

MATERIALS AND METHODS

Plant materials and isolation of salicortin

The bark of *Populus euramericana* was collected from the experimental forest of Kangwon National University, Korea in April 2009. We have deposited a voucher specimen (No. 0904-PEB) at the herbarium in the Department of Forest Biomaterials Engineering, Kangwon National University. The air-dried and powdered barks of *P. euramericana* (1.5 kg) were extracted with 70% aqueous acetone (three times, each 5 L) at room temperature. The acetone extract was concentrated *in vacuo* to yield a dark brown residue (262 g, 17.46%). Distilled water (1 L) was added to the residue, and the resultant solution was successively extracted with *n*-hexane, $CHCl_3$ and ethyl acetate (EtOAc). Evaporation of the respective solvent gave the *n*-hexnae (2 g, 0.13%), $CHCl_3$ (16 g, 1.06%) and EtOAc (43 g,

2.87%) soluble fractions. The EtOAc soluble fraction (20 g) was applied to a Sephadex LH-20 column, eluting with MeOH-H_2O (4 : 1, v/v) to obtain six fractions. Fraction 2 (6 g) was further purified on a Sephadex LH-20 column with MeOH-H_2O (1 : 2, 1 : 3, v/v) and EtOH-hexane (2 : 1, v/v) to obtain salicortin (1.42 g). The structure of salicortin (Fig. 1A) was identified by spectroscopic data analysis (NMR and MS) including 2D-NMR (COSY, HSQC and HMBC) and comparison with data reported in literature (18).

Cell culture and Reagents

RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin) at 37°C in a humidified incubator containing 5% CO_2 and 95% air. DMEM and PBS were purchased from Cellgro Mediatech Inc. (Herndon, VA, USA). Antibodies for p65, Lamin B, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for iNOS, IκBα, p-p65, p-IKKα/β, IKKα, IKKβ, p-ERK, ERK, p-p38, p38, p-JNK, and JNK were purchased from Cell Signaling Technology (Beverly, MA, USA). Lipopolysaccharide (LPS, *Escherichia coli* 0127:B8) and horseradish peroxidase (HRP)-conjugated anti-rabbit or goat antibodies were supplied by Sigma (St. Louis, MO, USA). Greiss reagent and luciferase assay kits were obtained from Promega (Madison, CA, USA). ELISA kits for TNF-α, IL-6, and IL-1β were purchased from R&D systems (Minneapolis, MN, USA). iNOS, TNF-α, IL-6, IL-1β, and β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea).

MTT assay

Cell viability was estimated using a colorimetric assay with MTT [3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide] (Duchefa, Haarlem, Netherlands). Cells were treated with various concentrations of salicortin for 24 h in serum-free media, and then MTT was added to the wells at a final concentration of 1 mg/mL for 2 h at 37°C. The MTT medium was removed, and 0.5 mL isopropanol was added to solubilize the formazan crystals. The absorbance at 570 nm was then measured using a microplate reader.

Nitrite determination

The concentration of nitrite, the oxidized product of NO, was determined by the Griess reaction, as previously described (19). Briefly, cells (1×10^6 cells/mL) were cultured in 6-well plates and were pretreated with salicortin for 3 h, followed by stimulation with LPS for 18 h. Then 100 μL of Greiss reagent was mixed with 100 μL of the cell culture media. Total nitrite was calculated from the absorbance of the mixture at 550 nm, using a standard curve of $NaNO_2$.

Western blot analysis

Whole cell, cytoplasmic and nuclear extracts were prepared as previously described (20), and protein concentrations were measured using the Bradford assay (Bio-Rad, Hercules, CA, USA). Protein samples (10-30 μg) were subjected to a 10% SDS-polyacrylamide gel and were transferred to a nitrocellulose membrane by electroblotting. The membranes were blocked for 1 h in TBST (137 mM NaCl, 20 mM Tris-HCl, pH 7.6, 0.1% Tween 20) containing 10% nonfat dry milk, and were probed with primary antibodies overnight at 4°C. After washing three times with TBST, membranes were exposed to horseradish peroxidase-conjugated secondary antibodies. Antigen-antibody complexes were detected using an enhanced chemiluminescence system (Amersham Life Sciences).

RT-PCR analysis

Total RNA was prepared from RAW 264.7 cells using a Trizol reagent (Invitrogen, Gaithersburg, MD, USA). The RNA (2 μg) was reverse-transcribed into cDNA with 10,000 U of reverse transcriptase and 0.5 μg/μL oligo-(dT)15 primer (Promega, Madison, WI, USA) (21). cDNA aliquots were amplified by PCR, using the following sense and antisense primers (5'→'): iNOS sense, CCC TTC CGA AGT TTC TGG CAG CAG C; iNOS antisense, GGC TGT CAG AGC CTC GTG GCT TTG G; COX-2 sense, ACT CAC TCA GTT TGT TGA GTC ATT C; COX-2 antisense, TTTGAT TAG TAC TGT AGG GTT AAT G; TNF-α sense, ACA AGC CTG TAG CCC ACG; TNF-α antisense, TCC AAA GTA GAC CTG CCC; IL-6 sense, CAA GAA AGA CAA AGC CAG AGT CCT T; IL-6 antisense, TGG ATG GTC TTG GTC CTT AGC C; IL-1β sense, TGC AGA GTT CCC CAA CTG GTA CAT C; IL-1β antisense, GTG CTG CCT AAT GTC CCC TTG AAT C; β-actin sense, AGT GTG ACG TTG ACA TCC GTA AAG A; and β-actin antisense, GGA CAG TGA GGC CAG GAT GG. The conditions of PCR amplification were as follows: iNOS, COX-2, TNF-α, IL-6, and β-actin, 94°C, 1 min, 55°C, 1 min, 72°C, 1 min for a total of 32 cycles; IL-1β, 94°C, 30 sec, 64°C, 30 sec, 72°C, 1 min for a total of 30 cycles. PCR products were electrophoresed in 1.5% agarose gels containing ethidium bromide, and the gels were observed under UV light.

Electrophoretic mobility shift assay (EMSA)

RAW 264.7 cells were pretreated with salicortin for 3 h and were then stimulated with LPS for 20 min. The nuclear extracts of cells were prepared and analyzed for NF-κB binding activity by EMSA, as described previously (22).

Transfection and luciferase assay

RAW 264.7 cells were seeded at a density of 3×10^5 cells/ml in a 6-well plate. After 24 h of incubation, a transfection experiment was performed using TransIT-2020 (Mirus, Madison, WI, USA). Cells were transfected transiently with 2.5 μg of pNF-κB-luc vector (Stratagene, La Jolla, CA, USA) and 0.5 μg of pCMV-β-galactosidase vector (Clontech, Mountain View, CA, USA). At 24 h after transfection, the cells were pretreated

with salicortin for 3 h, and were then stimulated with LPS for 24 h. Cell lysates were prepared for the determination of luciferase and β-galactosidase activities. The luciferase activity of each sample was normalized to that of β-galactosidase activity, and the results were expressed as fold transactivation.

Enzyme-linked immunosorbant assay (ELISA)

RAW 264.7 cells were pretreated with salicortin for 3 h and were then exposed to LPS for 24 h. The concentrations of TNF-α, IL-6, and IL-1β in the culture medium were measured using an ELISA kit, according to the manufacturer's instructions.

Statistical analysis

The results were expressed as the means ± S.D. from at least three independent experiments. The results were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple ranges tests, usingGraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA). Data were considered as statistically significant at $P < 0.001$.

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