Inhibition of LPS-Induced iNOS, COX-2 and Cytokines Expression by Poncirin through the NF-κB Inactivation in RAW 264.7 Macrophage Cells

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We previously reported that poncirin, a flavanone glycoside isolated from the EtOAc extract of the dried immature fruits of *Poncirus trifoliata*, is an anti-inflammatory compound that inhibits PGE_2 and IL-6 production. The present work was undertaken to investigate the molecular actions of poncirin in RAW 264.7 macrophage cell line. Poncirin reduced lipopolysaccharide (LPS)-induced protein levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and the mRNA expressions of iNOS, COX-2, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in a concentration-dependent manner, as determined by Western blotting and RT-PCR, respectively. Furthermore, poncirin inhibited the LPS-induced DNA binding activity of nuclear factor-κB (NF-κB). Moreover, this effect was accompanied by a parallel reduction in IκB-α degradation and phosphorylation that in by nuclear translocations of p50 and p65 NF-κB subunits. Taken together, our data indicate that anti-inflammatory properties of poncirin might be the result from the inhibition iNOS, COX-2, TNF-α and IL-6 expression *via* the down-regulation of NF-κB binding activity.

Key words poncirin; *Poncirus trifoliata*; inducible nitric oxide synthase; cyclooxygenase-2; nuclear factor-κB; anti-inflammation

Innate immunity is an ancient form of host defense that is activated rapidly to enable, through a multiplicity of effector mechanisms, defense against bacterial or viral infections and stresses. However, excessive innate immunity response may conditions such as sepsis and chronic inflammation.[1)]

During the inflammatory process, large amounts of the pro-inflammatory mediators, nitric oxide (NO) and prostaglandin E_2 (PGE_2) are generated by the inducible isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2).[2)] In mammalian cells, NO is synthesized by three isoforms of nitric oxide synthase (NOS), *i.e.*, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Moreover, although nNOS and eNOS are constitutively expressed, iNOS is expressed in response to interferon-γ, lipopolysaccharide (LPS), and various pro-inflammatory cytokines.[3,4)] While endothelial-derived NO normally functions to maintain vascular homeostasis by acting as a potent vasodilator, NO can also modulate acute and chronic inflammatory responses.[5)] PGE_2 is generated from arachidonic acid by cyclooxygenase (COX),[6)] and two distinct isoforms of COX were identified in the early 1990s. COX-1 is constitutively expressed in nearly all tissues and provides PGs to maintain physiological functions like cytoprotection of the stomach and the regulation of renal blood flow.[6,7)] In contrast, COX-2 is induced in immune cells, such as macrophages and synoviocytes, in response to infection, injury, or other stresses, and produces large amounts of PGs that act to sensitize nociceptors and induce inflammatory states.[8,9)] In addition, PGs also contribute to tumor growth by inducing angiogenesis and inhibiting apoptosis.[10,11)]

Nuclear transcription factor kappa-B (NF-κB) is one of the most important transcription factors, and is found in cell types that express cytokines, chemokines, growth factors, cell adhesion molecules, and some acute phase proteins in healthy and in disease states.[12,13)] The activation of NF-κB involves the phosphorylation of IκBs at two critical serine residues (Ser32, Ser36) *via* the IκB kinase (IKK) signalosome complex. Once IκBs have been phosphorylated, they are ubiquitinated and degraded by 26S proteosome. The resulting free NF-κB is then translocated to the nucleus, where it binds to κB binding sites in the promoter regions of target genes, and induces the transcription of pro-inflammatory mediators, *e.g.*, iNOS, COX-2, TNF-α and IL-1β, -6 and -8.[14)]

During our on-going screening program designed to identify natural compounds with anti-inflammatory potential, we isolated poncirin (Fig. 1) from the dried immature fruits of *Poncirus trifoliata* and characterized its structural identity using physical and spectroscopic methods,[15)] and compared these data with literature values.[16,17)] The dried immature fruits of *Poncirus trifoliata* (L.) Raf. (Rutaceae) are traditionally used to induce uterine contraction and relaxation, and to treat gastrointestinal and cardiovascular diseases in China.[18)] Moreover, previous phytochemical work on the dried immature fruits of *P. trifoliata* has resulted in the isolation of numerous compounds, including various flavonoids, coumarines, and alkaloids.[19—22)] The extracts and some of isolates of this fruits were found to have anti-allergic,[19,23)] hypocholesterolemic,[20)] anti-helicobacter pylori,[24)] anti-platelet,[21)] and anti- inflammatory effects.[25)] However, no report has been issued on its anti-inflammatory activity or mode of action of its active constituents, although we previously reported that poncirin inhibits PGE_2 and IL-6 production in LPS-activated RAW 264.7 macrophage cells.[15)] Therefore, in an effort to determine the mechanisms underly-

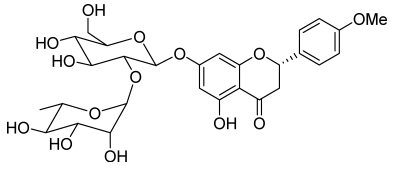

Fig. 1. Chemical Structure of Poncirin

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ing the anti-inflammatory effects of poncirin, we investigated the protein and mRNA expressions of inflammatory mediators in RAW 264.7 macrophage cells. In addition, we also investigated whether poncirin influences the LPS-induced DNA binding activity of NF-κB and the protein levels of its p50 and 65 subunits.

MATERIALS AND METHODS

Materials Poncirin used for this study was isolated from the fruits of *Poncirus trifoliata* (Rutaceae) as previously described.[15] The poncirin isolated was checked by HPLC and was >95% pure. Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.). iNOS, COX-2, COX-1, p65, p50, p-IκB-α, IκB-α, p-ERK, ERK, p-p38, p38, p-JNK, JNK, β-actin, PARP monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The enzyme immunoassay (EIA) kit for TNF-α and IL-6 was obtained from R&D Systems (Minneapolis, MN, U.S.A.). iNOS, COX-2, TNF-α, IL-6 and β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea). Sulfanilamide, aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), L-N^6-(1-iminoethyl)lysine (L-NIL), lipopolysaccharide (LPS) (*Escherichia coli*, serotype 0111:B4), Triton X-100 and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Culture and Sample Treatment The RAW 264.7 macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37 °C in DMEM medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO_2. Cells were incubated with the poncirin at increasing concentrations (25, 50, 100 μM) or positive chemical and then stimulated with LPS (1 μg/ml) for the indicated time.

Nitrite Determination The nitrite accumulated in culture medium was measured as an indicator of NO production based on the Griess reaction. 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], incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured in a microplate reader (Perkin Elmer Cetus, Foster City, CA, U.S.A.). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was measured with the sodium nitrite serial dilution standard curve and nitrite production was measured.

Determination of PGE_2, TNF-α and IL-6 Levels PGE_2, TNF-α and IL-6 levels in macrophage culture medium were quantified by EIA kits according to the manufacturer's instructions.

Western Blot Analysis Cellular proteins were extracted from control and poncirin-treated RAW 264.7 cells. Cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride and 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated with 20 min at 4 °C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions. Forty micrograms of cellular protein from treated and untreated cell extracts was electroblotted onto a nitrocellulose membrane following separation on a 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4 °C, followed by incubation for 4 h with a primary antibody. Blots were washed four times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1 : 1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed three times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science).

RNA Preparation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology, Seoul, Korea) according to the manufacturer's instructions. From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM dNTP, and oligo(dT_{12-18}) 0.5 μg/μl. Then PCR analyses were performed on the aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α and IL-6 and β-actin (as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA, U.S.A.). The reactions were carried out in a volume of 25 μl containing (final concentration) 1 units of *Taq* DNA polymerase, 0.2 mM dNTP, ×10 reaction buffer, and 100 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 95 °C, thirty 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-6 (1 min of 94 °C denaturation, 1 min of 57 °C annealing, and 1 min 72 °C extension). PCR primers used in this study are listed below and were purchased from Bioneer (Seoul, Korea): sense strand iNOS, 5′-AAT GGC AAC ATC AGG TCG GCC ATC ACT-3′, anti-sense strand iNOS, 5′-GCT GTG TGT CAC AGA AGT CTC GAA CTC-3′; sense strand COX-2, 5′-GGA GAG ACT ATC AAG ATA GT-3′ anti-sense strand COX-2, 5′-ATG GTC AGT- AGA CTT TTA CA-3′; sense strand TNF-α, 5′-ATG AGC ACA GAA AGC ATG ATC-3′, anti-sense strand TNF-α, 5′-TAC AGG CTT GTC ACT CGA ATT-3′; sense strand IL-6, 5′-GAG GAT ACC ACT CCC AAC AGA CC-3′, anti-sense strand IL-6, 5′-AAG TGC- ATC ATC GTT GTT CAT ACA-3′; sense strand β-actin, 5′-TCA TGA AGT GTG ACG- TTG ACA TCC GT-3′, anti-sense strand β-actin, 5′-CCT AGA AGC ATT TGC GGT- GCA CGA TG-3′. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA) RAW 264.7 macrophages cells were plated in 100-mm dishes (1×10^6 cells/ml). The cells were treated

with various poncirin concentrations (25, 50, 100 μM), stimulated with LPS for 1 h, washed once with PBS, scraped into 1 ml of cold PBS, and pelleted by centrifugation. Nuclear extracts were prepared as described previously[26] with slight modification. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 μg/ml aprotinin) and incubated on ice for 15 min. Then the cells were lysed by the addition of 0.1% Nonidet P-40 and vigorous vortexing for 10 s. The nuclei were pelleted by centrifugation at 12000×g for 1 min at 4 °C and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate). Nuclear extract 10 μg was mixed with the double-stranded NF-κB oligonucleotide. 5′-AGTTGAGGGGACTTTCCCAGGC3′ end-labeled by [γ-^{32}P]dATP (underlying indicates a κB consensus sequence or a binding site for NF-κB/cRel homodimeric and heterodimeric complex). Binding reactions were performed at 37 °C for 30 min in 30 μl of reaction buffer containing 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 μg of poly(dI-dC) and 1 mM DTT. The specificity of binding was examined by competition with the 80-fold unlabeled oligonucleotide. DNA–protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5×TBE buffer. The gels were vacuum dried for 1 h at 80 °C and exposed to X-ray film at −70 °C for 24 h.

Statistical Analysis The results were expressed as the mean±S.D. of triplicate experiments. Statistically significant values were compared using Student–Newman–Keuls and p-values less than 0.05 were considered statistically significant.

RESULTS

Effects of Poncirin on LPS-Induced NO and PGE_2 Production and Cell Viability To analyze the potential anti-inflammatory properties of poncirin, we used RAW 264.7 murine macrophage cells, which can produce NO and PGE_2, upon stimulation with LPS. Cells were pre-incubated with poncirin for 1 h and then stimulated with 1 μg/ml LPS for 24 h. Both LPS and sample were not treated in control (Con) group. After cell culture media were collected, nitrite and PGE_2 levels were determined, and poncirin was found to reduce NO production in a dose-dependent manner (Fig. 2A). L-NIL (10 μM) was used as a positive NO production inhibitor. Poncirin was also found to dose-dependently inhibit PGE_2 production (Fig. 2B). The potential cytoxicity of poncirin was evaluated by MTT assay after incubating cells for 24 h in the absence or presence of LPS, but cell viabilities were not affected at the concentrations used (25, 50, 100 μM) to inhibit NO and PGE_2 (Fig. 2C). Thus, the inhibitory effects were not attributable to cytotoxic effects.

Effects of Poncirin on LPS-Induced iNOS and COX-2 Protein and mRNA Expressions Western blot and RT-PCR analyses were performed to determine whether the inhibitory effects of poncirin on the pro-inflammatory mediators (NO and PGE_2) were related to the modulation of the expressions of iNOS and COX-2. In unstimulated RAW 264.7 cells, iNOS and COX-2 protein and mRNA were not detected, but LPS remarkably upregulated their protein levels, and pre-treatment with poncirin inhibited these upregulations

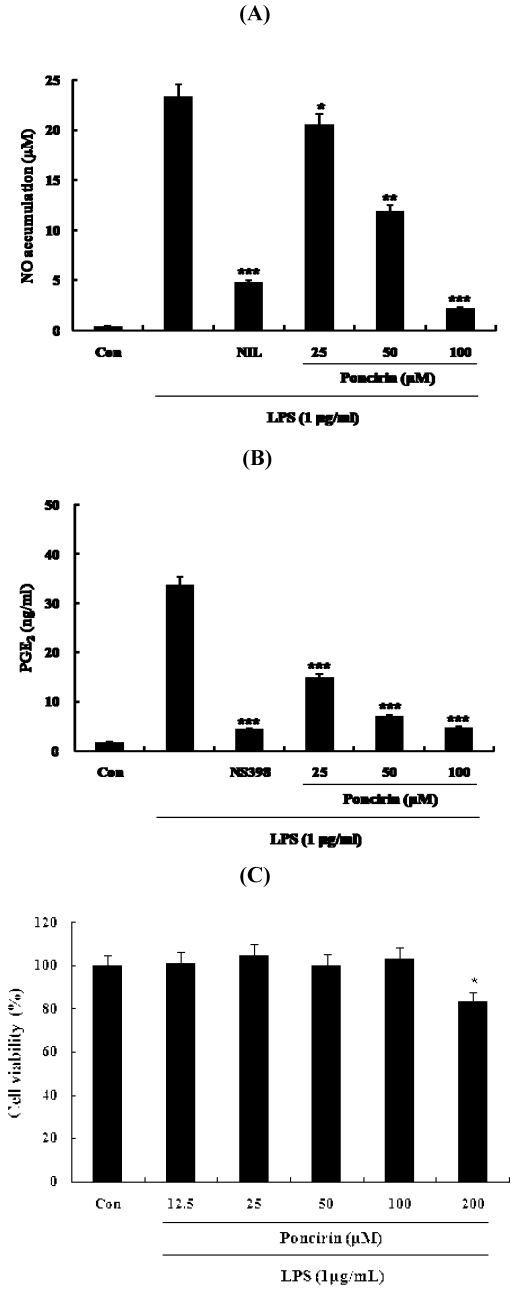

Fig. 2. The Effects of Poncirin on LPS-Induced NO and PGE_2 Production in RAW 264.7 Cells

(A) Cells were pretreated with different concentrations (25, 50, 100μM) of poncirin for 1 h, LPS (1 μg/ml) was then added, and cells were incubated for 24 h. Control (Con) values were obtained in the absence of LPS and of poncirin. L-N^6-(1-Iminoethyl)lysine (L-NIL) was used as a positive control at 10 μM. (B) Poncirin was treated as described above. 10 μM of NS-398 was used as a positive control. Values shown are means±S.D. of three independent experiments. $*p<0.05$, $**p<0.01$, $***p<0.001$ *vs.* the LPS only treated group. (C) Effect of poncirin on the cell viability. RAW 264.7 cells were incubated with or without poncirin (12.5—200 μM) and LPS (1 μg/ml). Values shown are means±S.D. of three independent experiments. $*p<0.05$ *vs.* the none treated control group.

(Fig. 3A). On the other hand, poncirin did not affect on the expression of COX-1, for maintaining normal physiological functions, and β-actin, the housekeeping gene. In general, these results indicate that the inhibitory effects of poncirin on LPS-induced NO and PGE_2 production are caused by iNOS and COX-2 suppression. Furthermore, RT-PCR analysis appears that mRNA expression levels of iNOS and COX-2 are

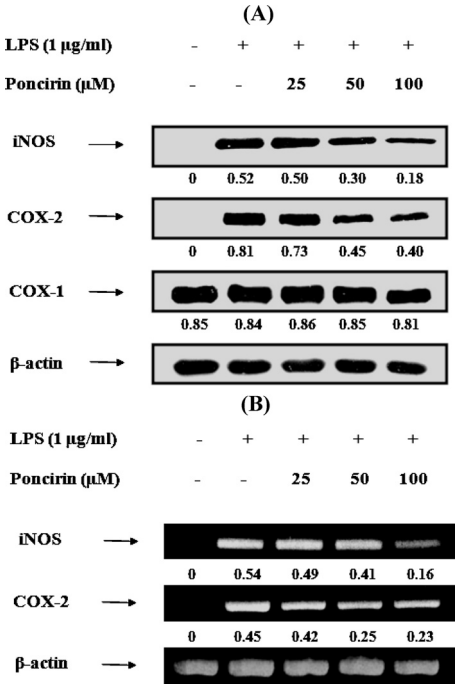

Fig. 3. The Effects of Poncirin on LPS-Induced iNOS and COX-2 Protein (A) and mRNA (B) Expressions in RAW 264.7 Cells

(A) Cells were pretreated with different concentrations (25, 50, 100 μM) of poncirin for 1 h, LPS (1 μg/ml) was then added, and the cells were incubated for 24 h. Total cellular proteins (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with specific antibodies, as described in Materials and Methods. A representative immunoblot of three independent experiments is shown. Density ratio over β-actin were measured by densitometer. (B) Samples were treated as described above. Total RNA was prepared for the RT-PCR analysis of the expressions of iNOS and COX-2 in RAW 264.7 macrophages treated with different concentrations (25, 50, 100 μM) of poncirin for 1 h, and then with LPS (1 μg/ml) for 4 h. iNOS-specific sequences (807 bp) and COX-2-specific sequences (721 bp) were detected by agarose gel electrophoresis, as described in Materials and Methods. PCR for β-actin was performed to verify that the initial cDNA contents of the samples were similar. The experiment was repeated three times and similar results were obtained. Density ratio over β-actin were measured by densitometer.

correlated with their protein levels (Fig. 3B).

Effects of Poncirin on LPS-Induced TNF-α and IL-6 Production and mRNA Expression Since poncirin was found to most potently inhibit the pro-inflammatory mediators, we further investigated its effects on LPS-induced TNF-α and IL-6 release by enzyme immunoassay (EIA) and RT-PCR. It was found that poncirin concentration-dependently reduced TNF-α and IL-6 production (Figs. 4A, B) and their mRNA expressions (Fig. 4C).

Effects of Poncirin on LPS-Induced NF-κB Activation and on the Nuclear Translocations of p50 and p65 Because the activation of NF-κB is critically required for iNOS, COX-2, TNF-α and IL-6 activation by LPS, EMSA was performed to determine whether poncirin influences NF-κB activation. Accordingly, EMSA was carried out using nuclear extracts obtained from RAW 264.7 cells stimulated with LPS in the presence or absence of poncirin. Treatment with LPS (1 μg/ml) was found to increase NF-κB–DNA binding, whereas pretreating cells with poncirin prior to LPS decreased LPS-induced NF-κB–DNA binding in a concentration-dependent manner (Fig. 5A).

Since p50 and p65 are major components of NF-κB, which is activated by LPS in macrophages, we examined p50 and p65 translocation to the nucleus by immunoblotting. Thus, RAW 264.7 cells were incubated with LPS in the presence or absence of poncirin for 1 h. Negligible levels of p50 or p65 protein were detected in control cell nuclei, but treatment with LPS for 1 h caused their nuclear translocations. It was found that poncirin pre-treatment concentration-dependently attenuated p50 and p65 levels in nuclear fractions by Western blotting (Fig. 5B). PARP was used as an internal control in these experiments. These observations suggest that poncirin inhibits NF-κB binding by preventing the LPS-induced nuclear translocations of p50 and p65.

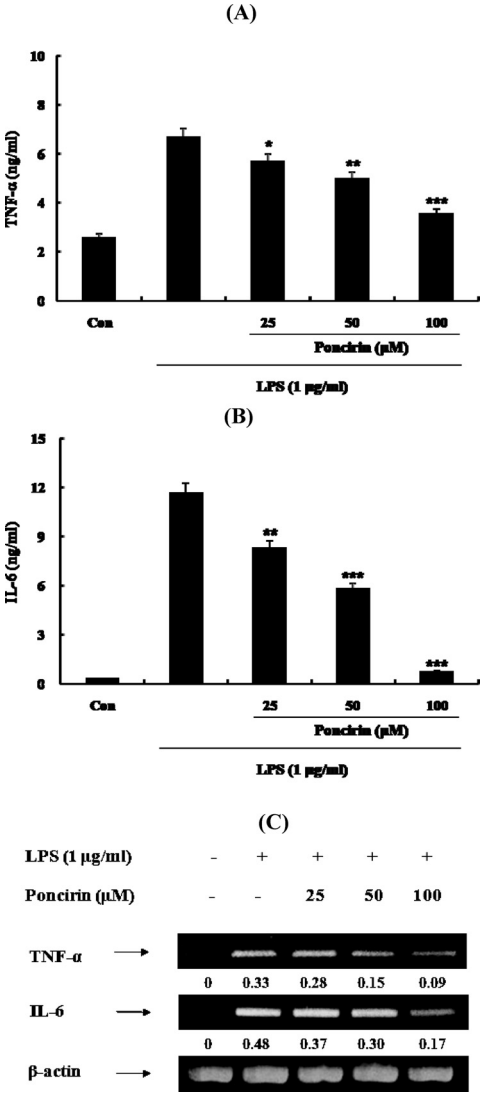

Fig. 4. The Effects of Poncirin on LPS-Induced TNF-α (A) and IL-6 Release (B) and mRNA Expression (C) in RAW 264.7 Cells

(A) Cells were pretreated with different concentrations (25, 50, 100 μM) of poncirin for 1 h, LPS (1 μg/ml) was then added, and cells were incubated for 24 h. Control (Con) values were obtained in the absence of LPS and of poncirin. The experiment was repeated three times and similar results were obtained. Values represent means±S.D. of three independent experiments. $*p<0.05$, $**p<0.01$, $***p<0.001$ *vs.* the LPS only treated group. (B) Samples were treated as described for Fig. 4A. (C) Total RNA was prepared as described in Fig. 3B, and RT-PCR was then performed to compare the mRNA levels of TNF-α and IL-6. TNF-α (351 bp) and IL-6-specific sequences (142 bp) were detected by agarose gel electrophoresis, as described in Materials and Methods. β-Actin was used as a PCR control. The experiment was repeated three times and similar results were obtained. Density ratio over β-actin were measured by densitometer.

Effects of Poncirin on LPS-Induced IκB-α and MAP Kinase Phosphorylation NF-κB is known to be activated when IκB-α phosphorylation and degradation are inhibited.[14] Here, we investigated whether poncirin inhibits the

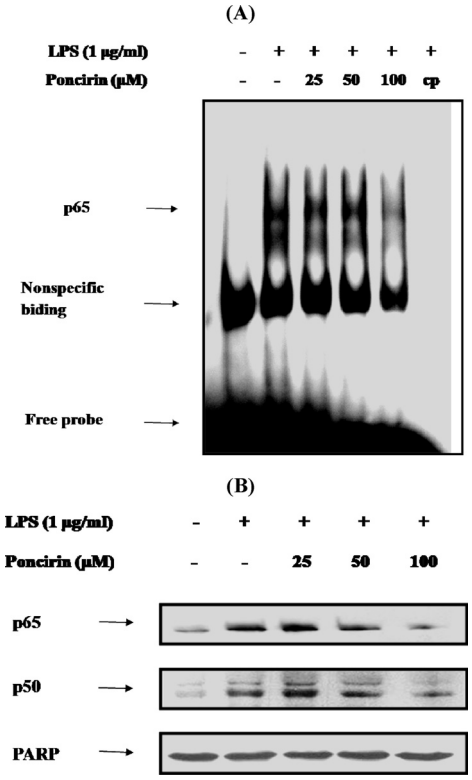

Fig. 5. Inhibition of p65-DNA Binding and NF-κB (p65 and p50) Translocation to the Nucleus by Poncirin

(A) Nuclear extracts were prepared from controls or pretreated with different concentrations (25, 50, 100 μM) of poncirin 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 NF-κB band position. Specificity of binding was examined by competition with the 80-fold unlabeled NF-κB oligonucleotide (cp). Data shown are representative of two independent experiments. (B) Nuclear extracts (40 μg/ml) were prepared for the Western blot analysis of p65 and p50 of NF-κB using specific anti-p65 and anti-p50 NF-κB monoclonal antibodies. Experiments were repeated two times and similar results were obtained.

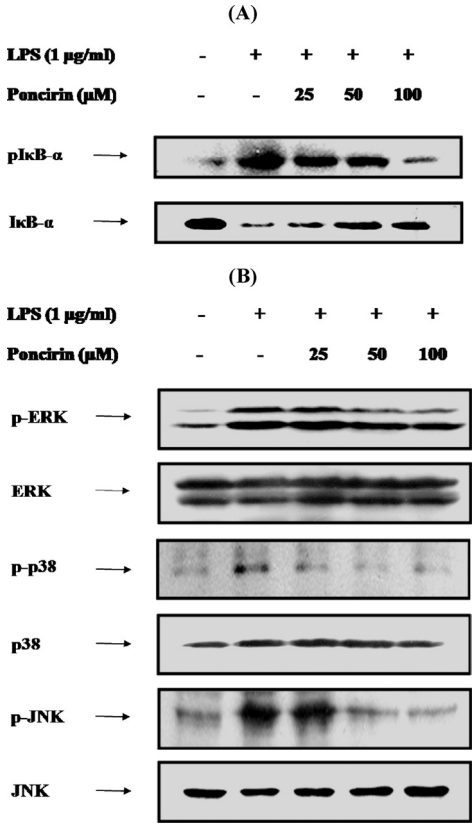

Fig. 6. The Effects of Poncirin on the LPS-Induced Phosphorylations of IκB-α (A) and MAP Kinase (B)

(A) RAW 264.7 macrophage cells were pretreated or not with the indicated concentrations of (25, 50, 100 μM) of poncirin for 1 h and then the cells were incubated with LPS (1 μg/ml) for 15 min. Total cellular proteins were prepared and Western blotted for IκB-α and p-IκB-α using specific IκB-α and p-IκB-α antibodies. β-Actin was used as an internal control. Experiments were repeated three times and similar results were obtained. (B) The conditions of sample treatment were identical to those described for Fig. 6A. Western blot analysis using specific p-ERK, ERK, p-p38, p38, p-JNK and JNK antibodies was repeated two times with similar results.

LPS-induced degradation of IκB-α in RAW 264.7 macrophages by Western blotting with anti-IκB-α antibody. Figure 6A shows that LPS induced IκB-α degradation was significantly blocked by pre-treatment with poncirin in a concentration-dependent manner. To determine whether this degradation of IκB-α is related to IκB-α phosphorylation, we examined the effect of poncirin on LPS-induced p-IκB-α by Western blotting, and found that poncirin reduces LPS-induced IκB-α phosphorylation in a concentration-dependent manner. Corresponding with these results, LPS-induced NF-κB activation and the translocations of the p50 and p65 subunits to the nucleus were dose-dependently blocked by poncirin (Figs. 5A, B).

The mitogen-activated protein (MAP) kinases play critical roles in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses. Moreover, they are also known to be important for the activation of NF-κB.[27] To investigate whether the inhibition of NF-κB activation by poncirin is mediated through the MAP kinase pathway, we examined the effects of poncirin on the LPS-stimulated phosphorylations of ERK1/2, SAPK/JNK and p38 MAP kinases in RAW 264.7 cells by Western blotting. As shown in Fig. 6B, poncirin suppressed the LPS-induced activations of ERK1/2, JNK and p38 MAP kinases in a concentration-dependent manner. However, non-phosphorylated ERK, JNK and P38 kinase expressions were unaffected by LPS or LPS plus poncirin. These results suggest that the phosphorylations of MAP kinase may be involved in the inhibitory effect of poncirin on LPS-stimulated NF-κB binding in RAW 264.7 cells.

DISCUSSION

The fruits of *Poncirus trifoliata* (L.) RAF. (Rutaceae) are widely used in oriental medicine to treat allergic inflammation.[28] Moreover, this material has been shown to have a beneficial effect on type I hypersensitivity, IgE production, and IgE-mediated local anaphylaxis.[29,30] Recently, flavones from *P. trifoliata* were reported to have anti-anaphylactic effects, and in particular, naringenin, hesperetin, and ponciretin were found to potently inhibit the activation and degranulation of mast cells.[19] This paper also reported that ponciretin (the aglycone of poncirin) more potently inhibited IgE-induced β-hexosaminidase release from RBL-2H3 cells than poncirin. However, incubation time of samples in this assay system was only 20 min, which causes no activity because it is not enough to transform to ponciretin, active metabolite of poncirin. To reveal the underlying mechanisms for the biotransformation from poncirin to ponciretin in anti-inflammatory effects on cell based system remains to be clarified.

However, the active components responsible for the anti-inflammatory effects of the crude drug have not been identified.

The pro-inflammatory cytokines, prostaglandins, and NO produced by activated macrophages play critical roles in inflammatory diseases such as sepsis and arthritis.[31,32] Hence, the inhibition of pro-inflammatory cytokines or iNOS and COX-2 expressions in inflammatory cells, offers us a new therapeutic strategy for the treatment of inflammation. In the present study, it was found that poncirin inhibits COX-2 and iNOS expressions in macrophage cells, and that it probably act at the transcriptional level, as evidenced by dose-dependent reductions in their mRNA levels. The inhibition of the LPS-stimulated expressions of these molecules in RAW 264.7 cells by poncirin was not due to poncirin cytotoxicity, as assessed by MTT assay and the expression of the housekeeping gene β-actin. Furthermore, it didn't change the expression level of COX-1, constitutively expressed in most tissues and seems to be responsible for housekeeping roles for normal physiological functions including maintenance of the integrity of the gastric mucosa and regulation of renal blood flow.[6,7]

It has been reported that cytokines, such as TNF-α, IL-6 and IL-1β are pro-inflammatory *in vitro* and *in vivo*.[33] Moreover, the production of TNF-α is crucially required for the synergistic induction of NO synthesis in IFN-γ and/or LPS-stimulated macrophages.[34] TNF-α elicits a number of physiological effects, such as septic shock, inflammation, cachexia, and cytotoxicity,[35] and IL-6 is believed to be an endogenous mediator of LPS-induced fever. In the present study, it was found that poncirin also significantly inhibits TNF-α and IL-6 release and their mRNA expressions.

NF-κB is known to play a critical role in the regulation of cell survival genes, and to coordinate the expressions of pro-inflammatory enzymes and cytokines, such as iNOS, COX-2, TNF-α, and IL-6.[14] Hence, we examined the DNA-binding activity of p65 to confirm the inhibitions of the expressions of iNOS, COX-2, TNF-α, and IL-6 because we considered that the inhibitory effects of poncirin on NO, PGE_2, TNF-α, and IL-6 production were probably regulated by the NF-κB signaling pathway. The results obtained suggested that the DNA binding activity of p65 is in fact inhibited in a concentration-dependent manner by poncirin. NF-κB (a heterodimer of p65 and p50) is located in the cytoplasm as an inactive complex bound to IκB-α, which is phosphorylated and subsequently degraded, and then dissociates to produce activated NF-κB.[14] In the present study, it was found that the translocation of NF-κB was inhibited by poncirin in a concentration-dependent manner, and the phosphorylation and degradation of IκB-α, which is required for p65 activation, were abolished in cells treated with poncirin. Moreover, the phosphorylation of IκB-α bound NF-κB is considered to be mediated by IKK at two conserved serines in the N-terminal domain of IκB-α,[14] and it has been reported that some natural flavonoids suppress the activity of IκB kinase.[36,37] For example the green tea polyphenol, epigallocatechin-3-gallate, was found to directly block IκB kinase activity in an intestinal epithelial cell line.[37]

The MAP kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses.[27] Moreover, MAP kinases are involved in the LPS-induced iNOS expression signaling pathway.[38] Recently, it was found that JNK may associate with the cRel subunit of NF-κB and directly enhance NF-κB activation in the yeast two-hybrid system.[39] In the present study, we also investigated the effects of poncirin on the LPS-induced phosphorylation of MAP kinases in Raw 264.7 cells. Treatment with poncirin was found to significantly inhibit LPS-induced JNK, ERK and p38 phosphorylation, suggesting that JNK, ERK and p38 are involved the inhibition of LPS-stimulated NF-κB binding by poncirin in Raw 264.7 cells.

In summary, the findings of the present study suggest that poncirin isolated from the fruits of *Poncirus trifoliata* is a potent inhibitor of LPS-induced NO, PGE_2, TNF-α and IL-6 production in macrophage cells, and that it acts at the transcription level. Moreover, these inhibitory effects of poncirin were found to be associated with NF-κB inactivation *via* the blockade of IκB-α phosphorylation. Since NF-κB is a transcription factor that regulates the transcriptions of many genes associated with inflammation, its inhibition by poncirin offers a possible approach to the treatment of severe inflammatory diseases.

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