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Isoliquiritigenin isolated from the roots of *Glycyrrhiza uralensis* inhibits LPS-induced iNOS and COX-2 expression *via* the attenuation of NF-κB in RAW 264.7 macrophages

Ji-Yeon Kim [a,b], Seung Jae Park [a,b], Kyung-Jin Yun [a,b], Young-Wuk Cho [b], Hee-Juhn Park [c], Kyung-Tae Lee [a,b,*]

[a] *Department of Pharmaceutical Biochemistry, College of Pharmacy, Kyung-Hee University, Dongdaemun-ku, Hoegi-Dong, Seoul 130-701, South Korea*
[b] *Department of Biomedical Science, College of Medical Science, Kyung-Hee University, Seoul 130-701, South Korea*
[c] *Department of Botanical Resources, Sangji University, Wonju 220-702, South Korea*

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Abstract

In this study, the anti-inflammatory effects of flavonoids isolated from the roots of *Glycyrrhiza uralensis* (Leguminosae), namely, isoliquiritin (the glycoside of isoliquirigenin) and isoliquiritigenin (the aglycone of isoliquiritin) were evaluated on lipopolysaccharide (LPS)-treated RAW 264.7 macrophages. Isoliquiritigenin (ILG) more potently inhibited LPS-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production than isoliquiritin (ILT). Consistent with these findings, ILG reduced the LPS-induced expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the protein and mRNA levels in a concentration-dependent manner, as determined by Western blotting and RT-PCR, respectively. In addition, the release of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and the mRNA expression levels of these cytokines were reduced by ILG in a dose-dependent manner. Moreover, ILG attenuated the LPS-induced DNA binding activity and the transcription activity of nuclear factor-kappa B (NF-κB), and this was associated with a decrease in inhibitory kappa B-α (IκB-α) phosphorylation and in the subsequent blocking of p65 and p50 protein translocations to the nucleus. Furthermore, ILG suppressed the phosphorylations of IκB kinase (IKK), ERK1/2, and p38, whereas the phosphorylation of JNK1/2 was unaffected. These results suggest that the anti-inflammatory properties of ILG are caused by iNOS, COX-2, TNF-α, and IL-6 down-regulation due to NF-κB inhibition via the suppression of IKK, ERK1/2 and p38 phosphorylation in RAW 264.7 cells.

Keywords: Isoliquiritigenin; Isoliquiritin; NF-κB; iNOS; COX-2

1. Introduction

Inflammation is the result of host response to pathogenic challenges or tissue injuries, and ultimately leads to the restoration of a normal tissue structure and function. Normal inflammatory responses are self-limited by a process that involves the down-regulations of pro-inflammatory proteins and the up-regulations of anti-inflammatory proteins (Lawrence et al., 2002). Thus, acute inflammation is a limited beneficial process, particularly in response to infectious pathogens, whereas chronic inflammation is an undesirable persistent phenomenon that can lead to the developments of inflammatory diseases (Kaplanski et al., 2003). Prolonged inflammation contributes to the pathogenesis of many inflammatory diseases, such as, bronchitis (Vernooy et al., 2002), gastritis (Sakagami et al., 1997), inflammatory bowel disease (Fichtner-Feigl et al., 2005), multiple sclerosis (Klotz et al., 2005), and rheumatoid arthritis (Ponchel et al., 2002).

Bacterial pathogens, like LPS, activate cytokine networks by inducing many pro-inflammatory genes. Moreover, this induction is mediated via the activations of inducible transcription factors (Liu and Malik, 2006). NF-κB is one of the pivotal regulators of pro-inflammatory gene expression, and aberrant the regulation of NF-κB activity has been implicated in the

* Corresponding author. Department of Pharmaceutical Biochemistry, College of Pharmacy, Kyung-Hee University, Dongdaemun-Ku, Hoegi-Dong, Seoul, 130-701, South Korea. Tel.: +82 2 961 0860; fax: +82 2 966 3885.
E-mail address: ktlee@khu.ac.kr (K.-T. Lee).

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pathogenesis of many diseases, which include immune deficiencies and disorders, neurodegenerative disorders, autoimmune and inflammatory diseases, ischemia reperfusion injury, and cancer (Liu and Malik, 2006). NF-κB proteins in cytoplasm are associated with inhibitory proteins known as IκBs. Moreover, the main activated form of NF-κB is a heterodimer composed of p65 and p50 subunits (Li and Verma, 2002). NF-κB induction in response to pro-inflammatory stimuli involves the phosphorylation of IκBs at two critical serine residues (Ser32, Ser36) by IKK signalosome complex (O'Conell et al., 1996). After IκB has been phosphorylated, it is ubiquitinated and degraded by 26S proteosome (Sanchez-perez et al., 2002), and the resulting free NF-κB is translocated to the nucleus, where it binds to κB binding sites in the promoter regions of target genes, and induces the transcriptions of pro-inflammatory mediators, such as, iNOS, COX-2, TNF-α and IL-1β, -6 and -8 (Surh et al., 2001; Lappas et al., 2002).

iNOS catalyzes the oxidative deamination of L-arginine to produce NO, a potent pro-inflammatory mediator (Ohshima and Bartsch, 1994). Moreover, iNOS expression is significantly induced by LPS or cytokines in a variety of immune cells, including macrophages. COX-2 is another enzyme that plays a pivotal role in the mediation of inflammation, and catalyzes the rate-limiting step in prostaglandin (PG) biosynthesis.

There are two isoforms of COX, designated COX-1 and COX-2 (O'Neill and Hutchinson, 1993). Although both isoforms are involved in the formation of PG endoperoxides (Marnett et al., 1999), they are likely to have fundamentally different biological roles. COX-1 is a housekeeping enzyme and is constitutively expressed in most mammalian tissues. In contrast, COX-2 is barely detectable under normal physiological conditions, but like other early response gene products, it can be rapidly and transiently induced by pro-inflammatory mediators and mitogenic stimuli, which include cytokines, endotoxins, growth factors, oncogenes, and phorbol esters.

Thus, there is considerable research interest in the identification of new anti-inflammatory agents from plants used in traditional medicine. Licorice (*Glycyrrhiza uralensis*) has been used for more than 4 millennia as a flavoring agent in foods, beverages, and tobacco, and to treat individuals with gastric or duodenal ulcers (Fintelmann, 1991), sore throats, coughs, bronchitis, arthritis (Kamei et al., 2004), adrenal insufficiency, and allergies (Haggag et al., 2003). Moreover, many studies have revealed that several licorice-derived compounds, i.e., glycyrrhizin, isoliquiritigenin (ILG), licochalcone, and glabridin, have a variety of pharmaceutical effects (Fukai et al., 2003; Yokota et al., 1998; Inoue et al., 1986; Zhou et al., 2004). In addition, ILG (Fig. 1), a simple chalcone-type flavonoid, has been evaluated in terms of its anti-oxidative effects (Haraguchi et al., 1998), anti-platelet aggregation effects (Tawata et al., 1992), and estrogenic properties (Tamir et al., 2001). Moreover, several reports have addressed the anti-carcinogenic activity of ILG. In particular, ILG was found to suppress COX-2 protein in RAW 264.7 cells, and the proliferation in colon cancer cells by inducing apoptosis (Takahashi et al., 2004). However, no report has been issued on the molecular aspects of its anti-inflammatory effect in RAW 264.7 cells. Therefore, as a part of our on-going screening program to evaluate the anti-inflammatory potential of natural compounds, we investigated the anti-inflammatory effects of ILG isolated from the roots of *G. uralensis* in LPS-induced macrophage model.

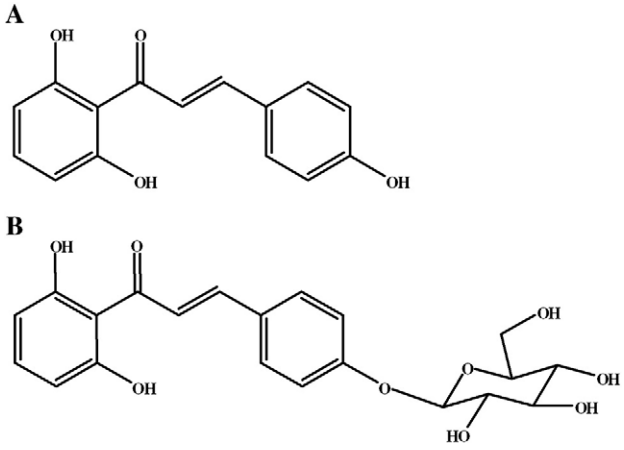

Fig. 1. The chemical structures of isoliquirigenin (A) and isoliquiritin (B).

2. Materials and methods

2.1. Chemicals

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, USA). iNOS, COX-2, p65, p50, p-IκB-α, IκB-α, p-IKK-α/β, IKK-α, IKK-β, p-ERK, ERK, p-p38, p38, p-JNK, JNK and β-actin monoclonal antibodies and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The enzyme immunoassay (EIA) kits for PGE_2, TNF-α and IL-6 were obtained from R&D Systems (Minneapolis, MN, USA). Random oligonucleotide primers and M-MLV reverse transcriptase were purchased from Promega (Madison, WI, USA). dNTP Mix and ex Taq were obtained from TaKaRa (Seoul, Korea). iNOS, COX-2, TNF-α, IL-6, and β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sulfanilamide, aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), L-N^6-(1-iminoethyl)lysine (L-NIL), NS-398, lipopolysaccharide (LPS) (*Escherichia coli*, serotype 0111:B4), Triton X-100, and all other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Plant material and the isolation of ILG and ILT

The roots of cultivated *G. uralensis* were collected on a farm in Pyongchang-kun, Gangwon province, Korea, during July 2004 and identified by Professor Sang-Cheol Lim at the Department of Botanical Resources, Sangji University (voucher specimen (#natchem-29) has been deposited at the same university). Dried roots of *G. uralensis* (2.5 kg) were extracted with methanol three times under reflux. The combined extract was filtered and evaporated under reduced pressure to give a viscous methanol extract (220 g). A portion of this methanol extract (200 g) was

suspended in water (1.0 l) and successively partitioned three times with chloroform (3×800 ml), ethylacetate (3×800 ml) and *n*-butanol (3×800 ml). The ethylacetate- and *n*-butanol-soluble fractions were evaporated under reduced pressure to yield the ethylacetate fraction (49 g) and a *n*-butanol fraction (87 g), respectively. A portion of the butanol extract (20 g) was subjected to silica gel column chromatography (7×60 cm, 650 g) using chloroform–methanol–water (65:35:10) as a mobile phase. Collected fractions were checked on TLC and combined to produce six fractions (GUB-1–GUB-6). The third fraction, GUB-3 (1.5 g), was again purified using Medium Pressure Liquid Chromatography (MPLC) system with the column (SiO_2, 3×21.5 cm, flash column Biotago SF 25+M0712-2, Biotago. Inc., USA) and with the solvent chloroform–methanol–water (7:3:1, lower phase) to yield isoliquiritin (320 mg), the structure of which was identified by comparing its ^{1}H, ^{13}C NMR and FAB-MS spectra with those previously reported in the literature (Lee et al., 2003). The purity of the isoliquiritin so obtained was greater than 98.0% by HPLC.

2.3. Acid hydrolysis of isoliquiritin

Isoliquiritin (500 mg) was dissolved in 100 ml of 5% hydrochloric acid and refluxed for 5 h. After cooling at room temperature, the reaction mixture was extracted with ethylacetate three times. The combined organic phase was then washed with distilled water twice and concentrated *in vacuo*. The residue so obtained was dissolved in methanol and stood overnight to afford isoliquiritigenin as yellowish needles. Its structure was identified by comparing its ^{1}H, ^{13}C NMR and FAB-MS spectra with those previously reported in the literature (Lee et al., 2003). The purity of the isoliquiritigenin obtained was greater than 98.0% by HPLC.

2.4. Cell culture and sample treatment

The RAW 264.7 macrophage cell line was obtained from the Korea Cell Line Bank (Seoul). 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 5% CO_2 atmosphere. Cells were incubated with tested samples (ILG or ILT) at concentrations of 0.4, 0.8, or 1.6 μM, or with positive controls (L-NIL or NS-398) and then stimulated with LPS 1 μg/ml for the indicated time.

2.5. 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 ILG and ILT. Viabilities were determined using colorimetric MTT assays, as described previously (Won et al., 2006).

2.6. 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 (0.4, 0.8, or 1.6 μM) of ILG and ILT for 24 h. Nitrite levels in culture media were determined using the Griess reaction and presumed to reflect NO levels (Won et al., 2006). 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. Absorbance was then measured at 540 nm using a microplate reader (Perkin Elmer Cetus, Foster City, CA, USA). Fresh culture media were used as blanks in all experiments. Nitrite levels in samples were read off a standard sodium nitrite curve.

2.7. PGE_2, TNF-α and IL-6 assays

RAW 264.7 cells were pretreated with ILG or ILT for 1 h and then stimulated with LPS (1 μg/ml) for 24 h. Levels of PGE_2, TNF-α and IL-6 in the culture media were quantified using EIA kits (R&D Systems, Minneapolis, MN. USA).

2.8. Western blot analysis

Control and ILG-treated RAW 264.7 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 PMSF, 0.5 mM DTT, 5 mM NaF and 0.5 mM sodium orthovanadate) containing 5 μg/ml of leupeptin and aprotinin, respectively, and incubated for 20 min at 4 °C. Cell debris was removed by microcentrifugation, and supernatants were rapidly frozen. Protein concentrations were determined using Bio-Rad protein assay reagent, according to the manufacture's instruction. Forty micrograms of cellular proteins from treated or untreated cell extracts were separated on 10% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes, which were incubated overnight with blocking solution (5% skim milk) at 4 °C, and then with primary antibody for 4 h. Blots were then washed four times with Tween 20/Tris-buffered saline (TTBS), incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, rewashed three times with TTBS, and then developed by enhanced chemiluminescence (Amersham Life Science).

2.9. RNA preparation and RT-PCR (reverse transcriptase polymerase chain reaction)

Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology, Seoul, Korea). From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM deoxyribonucleotide triphosphate (dNTP), and oligo (dT_{12-18}) 0.5 μg/μl. PCR was performed on aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α, IL-6 and β-actin (the internal standard) gene expressions using a thermal cycler (Perkin Elmer Cetus, Foster City, CA). Reactions were carried out in a volume of 25 μl containing; 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 at 95 °C denaturation, 1 min at 60 °C annealing, and 1.5 min at 72 °C extension), COX-2 (1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C), TNF-α (1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C), and IL-6 (1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C). The 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 under UV after ethidium bromide staining.

2.10. Nuclear extraction and electrophoretic mobility shift assay (EMSA)

RAW 264.7 macrophages cells were plated in 100-mm dishes (1×10^6 cells/ml), and treated with ILG (0.4, 0.8, 1.6 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 (Won et al., 2006). 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, 10 μg/ml aprotinin) and incubated on ice for 15 min. Cells were then lysed by adding 0.1% Nonidet P-40 and vortexed vigorously for 10 s. Nuclei were pelleted by centrifugation at 12,000 ×*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 extracts (10 μg) were mixed with double-stranded NF-κB oligonucleotide. 5′-AGTTGAGGGGACT-TTCCCAGGC3′ end-labeled with [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. 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× Tris Boric acid EDTA (TBE) buffer. Gels were vacuum dried for 1 h at 80 °C and exposed to X-ray film at −70 °C for 24 h.

2.11. Transient transfection and luciferase assay

RAW 264.7 cells were transfected using Superfect reagent (Qiagen GmbH, Germany) and NF-κB-Luc reporter plasmid (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Cells were incubated for 2 h before adding 5 ml of DMEM containing 10% FBS. At 48 h after the start of transfection, cells were pretreated with ILG for 1 h and then stimulated with LPS (1 μg/ml). After 3 h of stimulation, cells were lysed and luciferase activity was determined using the Promega luciferase assay system (Promega, Madison, CA, USA) and a luminometer (Perkin Elmer Cetus, Foster City, CA, USA).

2.12. Statistical analysis

Results are expressed as the means±S.D. of triplicate experiments. Statistically significant values were compared

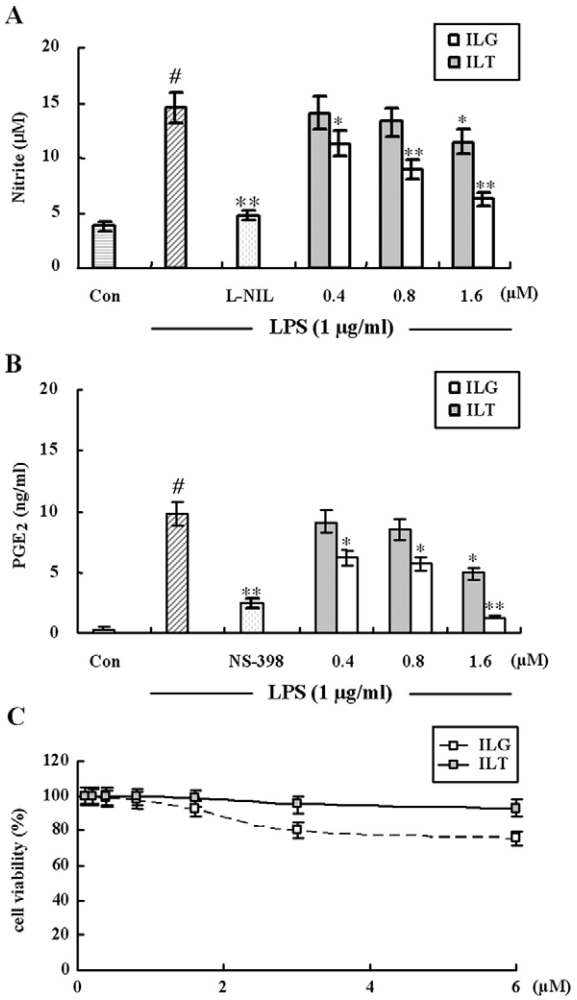

Fig. 2. The effects of ILG and ILT on LPS-induced NO (A) and PGE_2 (B) production and cell viability (C) in RAW 264.7 cells. Cells were treated with different concentrations of ILG or ILT (0.4, 0.8, 1.6 μM) for 1 h, then with LPS (1 μg/ml), and incubated for 24 h. Control (Con) values were obtained in the absence of LPS, ILG and ILT. L-N^6-(1-iminoethyl) lysine (L-NIL) was used as a positive control at a concentration of 10 μM (A). Samples were treated as described in the legend of Fig. 2A. NS-398 (10 μM) was used as a positive control. Values are means±S.D. of three independent experiments. $^{\#}p<0.05$ vs. the control group; $^{*}p<0.05$, $^{**}p<0.01$ vs. the LPS-treated group; significances between treated groups were determined using ANOVA and Dunnett's post-hoc test (B). Cells were exposed to ILG or ILT (from 0.1 μM to 6 μM), and cell viabilities were assessed using assay (C).

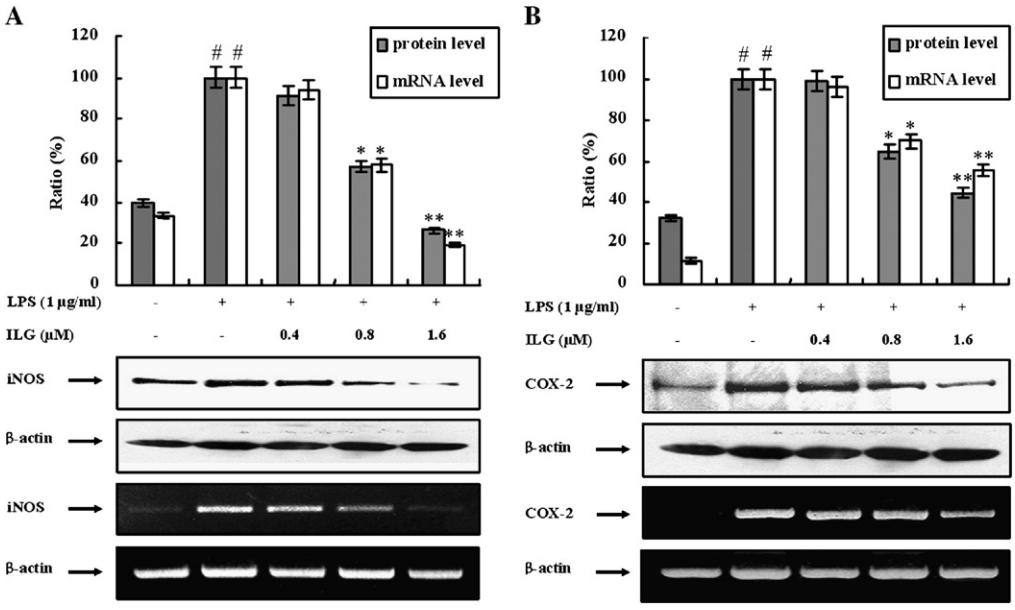

Fig. 3. The effects of ILG on LPS-induced iNOS (A) and COX-2 (B) protein and their mRNA expressions in RAW 264.7 cells. Lysates were prepared from control or 24 h LPS (1 μg/ml) stimulated cells or from LPS plus ILG (0.4, 0.8, 1.6 μM). Total cellular proteins (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected using specific antibodies, as described in Materials and methods. A representative immunoblot of three separate experiments is shown. Total RNA was prepared for RT-PCR of iNOS and COX-2 gene expressions from RAW 264.7 macrophages stimulated with LPS (1 μg/ml) with/without ILG at 0.4, 0.8, or 1.6 μM 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 of β-actin was performed to confirm that the initial cDNA contents of samples were similar. The experiment was repeated three times and similar results were obtained. Values shown are means±S.D. of three independent experiments. $^{\#}p<0.05$ vs. the control group; $^{*}p<0.05$, $^{**}p<0.01$ vs. the LPS-treated group; significant differences between groups were determined using ANOVA and Dunnett's post-hoc test (A), (B).

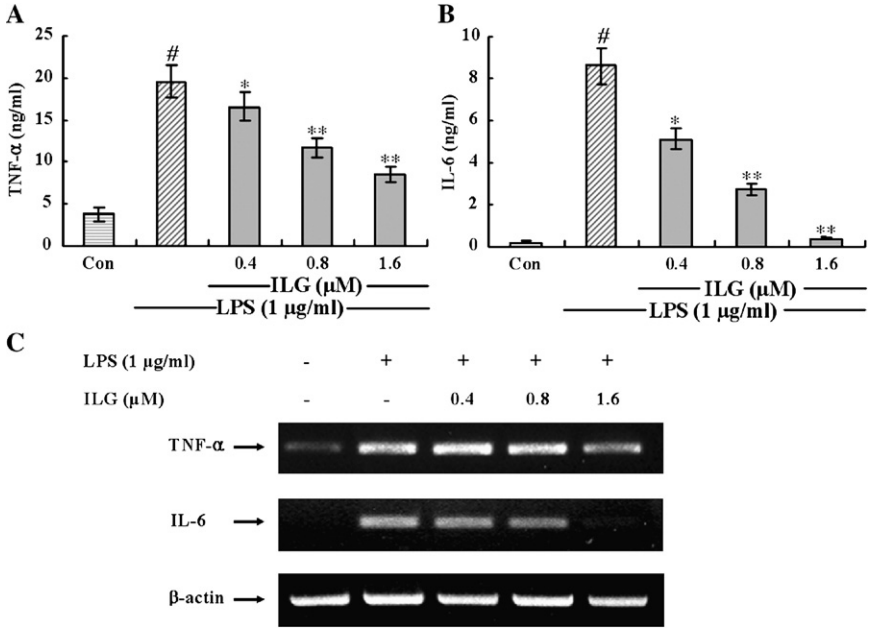

Fig. 4. The effects of ILG on LPS-induced TNF-α (A) and IL-6 (B) release and their mRNA expressions (C) in RAW 264.7 cells. Cells were treated with different concentrations (0.4, 0.8, 1.6 μM) of ILG for 1 h, LPS (1 μg/ml) was then added, and cells were incubated for a further 24 h. Control (Con) values were obtained in the absence of LPS or ILG. Samples were treated as described in the legend of Fig. 2A. Values represent means±S.D. of three independent experiments. $^{\#}p<0.05$ vs. the control group; $^{*}p<0.05$, $^{**}p<0.01$ vs. the LPS-treated group; significant differences between groups were determined using ANOVA and Dunnett's post-hoc test (A), (B). Total RNA was prepared for RT-PCR of TNF-α and IL-6 gene expressions from RAW 264.7 cells pretreated with different concentrations (0.4, 0.8, 1.6 μM) of ILG for 1 h, and then with LPS (1 μg/ml) for 4 h. TNF-α-specific sequences (351 bp) and IL-6-specific sequences (142 bp) were detected by agarose gel electrophoresis, as described in Materials and methods. PCR for β-actin was performed to confirm that initial cDNA contents were similar. The experiment was repeated three times with similar results (C).

using ANOVA and Dunnett's post-hoc test, and p-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of ILG or ILT on LPS-induced NO and PGE_2 production and cell viability

To compare the effects of ILG and ILT on LPS-induced NO and PGE_2 production in RAW 264.7 cells, culture media were harvested and nitrite and PGE_2 levels were measured. ILG more significantly inhibited LPS-induced NO and PGE_2 production than ILT, and did so in a dose-dependent manner (Fig. 2A and B). Moreover, ILG potently reduced NO production with an IC_{50} value of 1.3 μM, whereas ILT had less effect at the same concentration. In addition, treatments with ILG or ILT at 1.6 μM reduced LPS-induced PGE_2 production by 86% and 49%, respectively. L-NIL (10 μM) and NS-398 (10 μM) were used as positive NO and PGE_2 production controls, respectively. Furthermore, the cytotoxic effects of ILG and ILT were evaluated in RAW 264.7 cells using MTT assays, but neither affected cell viabilities at the concentrations used (0.4, 0.8, 1.6 μM) to inhibit NO and PGE_2 production (Fig. 2C).

3.2. Effects of ILG on LPS-induced iNOS and COX-2 protein and mRNA expressions

Since ILG was found to inhibit NO and PGE_2 production, we used Western blotting and RT-PCR to determine whether these inhibitory effects were related to iNOS and COX-2 modulation. iNOS and COX-2 protein levels were markedly up-regulated in response to LPS, and ILG strongly inhibited these protein expression in a concentration-dependent manner. Moreover, RT-PCR analysis showed that the expressions of iNOS and COX-2 mRNA correlated with their protein levels (Fig. 3A and B). However, ILG had no effect on the expression of the house-keeping gene β-actin.

3.3. Effects of ILG on LPS-induced TNF-α and IL-6 release and their mRNA expressions

Since our data showed that ILG inhibited the pro-inflammatory mediators, such as NO and PGE_2, we further examined its effects on the LPS-induced TNF-α and IL-6 release by enzyme immunoassay and RT-PCR. It was found that pretreatment of cells with ILG considerably reduced the production of TNF-α and IL-6 (Fig. 4A and B) and their mRNA expressions (Fig. 4C) in a dose-dependent manner. Moreover, changes in TNF-α and IL-6 production were similar to the observed reduction in their mRNA expressions.

3.4. Effects of ILG on LPS-induced NF-κB-DNA binding and NF-κB transcriptional activity

Since NF-κB activation is critically required for the expressions of iNOS, COX-2, TNF-α and IL-6 by LPS (Surh et al., 2001; Lappas et al., 2002), electrophoretic mobility shift assays (EMSA) and luciferase assays were used to determine whether ILG suppresses NF-κB activation. Accordingly, a DNA binding assay of NF-κB was carried out using nuclear extracts obtained from RAW 264.7 cells stimulated with LPS in the presence or absence of ILG. Treatment with LPS (1 μg/ml) was found to increase NF-κB-DNA binding, and pretreating cells with ILG prior to LPS decreased NF-κB-DNA binding in a concentration-dependent manner (Fig. 5A). In an additional study, we examined the effects of ILG on NF-κB-dependent reporter gene expression induced by LPS. In this experiment, we used a pNF-κB-Luc plasmid, which was generated by inserting four spaced NF-κB binding sites into pLuc-promoter vector. Accordingly, 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 absence of ILG. ILG treatment at 0.4, 0.8, or 1.6 μM significantly reduced LPS-

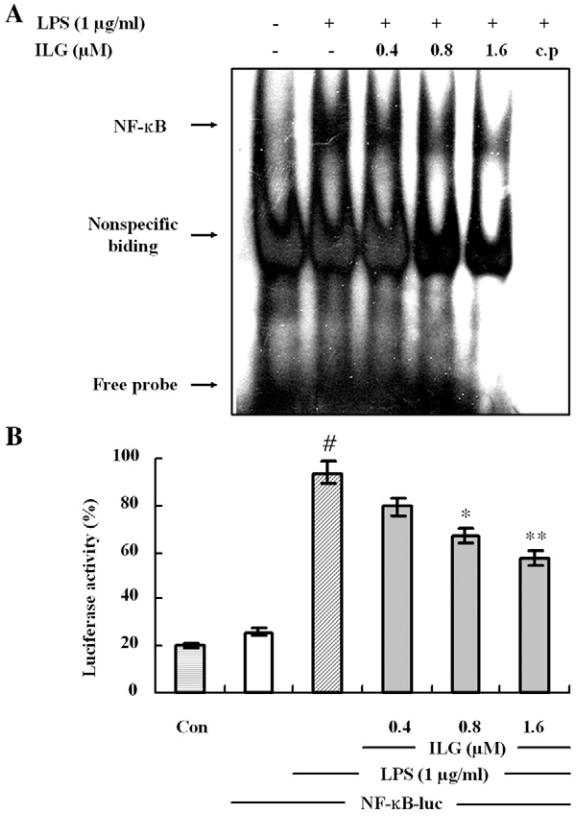

Fig. 5. The inhibitory effects of ILG on LPS-induced NF-κB-DNA binding (A) and luciferase activity (B). Nuclear extracts were prepared from control or pretreated with different concentrations (0.4, 0.8, 1.6 μM) of ILG for 1 h, then with LPS (1 μg/ml) for 1 h, and analyzed for NF-κB binding by EMSA. The arrow indicates the position of the NF-κB band. The data shown are representative of three independent experiments (A). Cells were transiently cotransfected with pNF-κB-luc reporter and then either left untreated (Con) or were pretreated with different concentrations (0.4, 0.8, 1.6 μM) of ILG 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 ± S.D. of three independent experiments. $^{\#}p<0.05$ vs. the control group; $^{*}p<0.05$, $^{**}p<0.01$ vs. the LPS-treated group. Significant differences between treated groups were determined using ANOVA and Dunnett's post-hoc test (B).

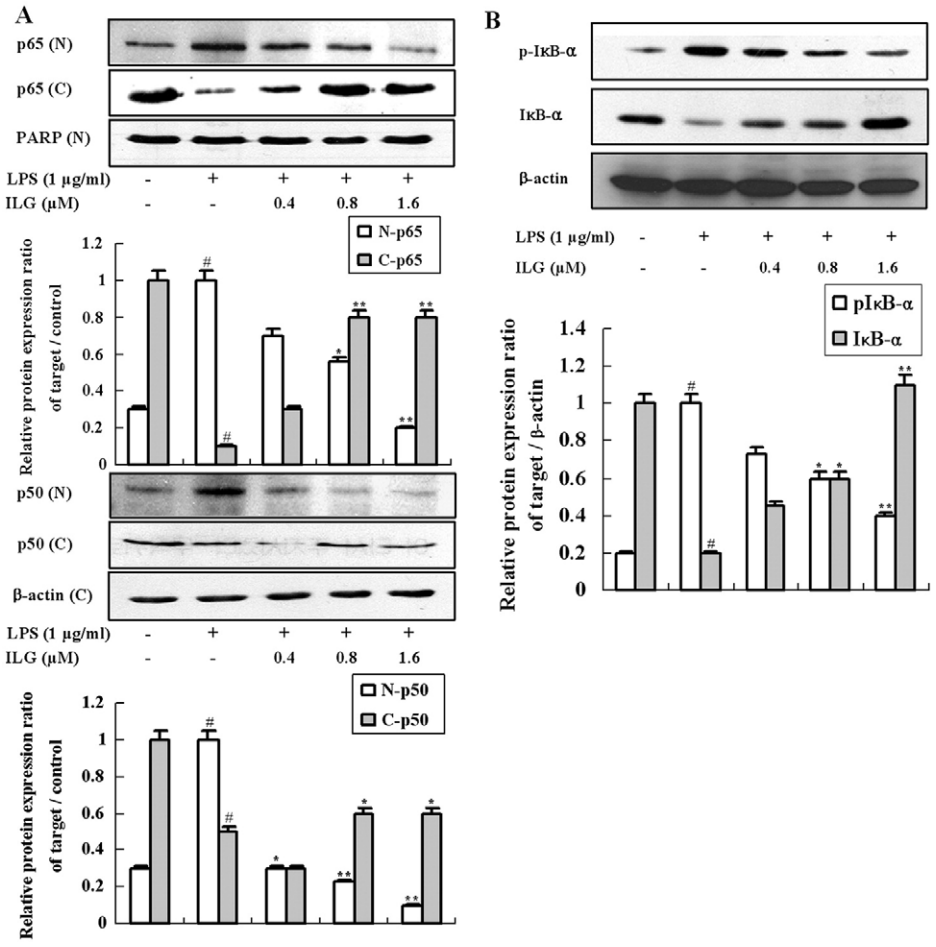

Fig. 6. Inhibition of the nuclear translocation of NF-κB (A) and of the phosphorylation of IκB-α (B) by ILG. RAW 264.7 macrophages were treated with LPS (1 μg/ml) alone or with LPS (1 μg/ml) plus different concentrations (0.4, 0.8, 1.6 μM) of ILG for 1 h. Nuclear extracts were prepared for the Western blotting of p65 and p50 of NF-κB using specific anti-p65 and p50 NF-κB monoclonal antibodies. PARP and β-actin were used as internal controls. The data shown are representative of three independent experiments (A). Cells were pretreated with the indicated concentrations of ILG for 1 h and then with LPS (1 μg/ml) for 15 min. Total cellular proteins were then prepared and Western blotted for IκB-α and p-IκB-α using specific IκB-α and p-IκB-α antibodies (B).

induced increases in NF-κB-dependent luciferase activity (Fig. 5B).

3.5. Inhibitory effects of ILG on the LPS-induced nuclear translocation of NF-κB and the phosphorylation of IκB-α

Here, we investigated whether ILG prevents the translocation of the subunits of NF-κB, i.e., p65 and p50, from the cytosol to the nucleus after its release from IκBs. It was found that pretreatment with ILG prior to LPS attenuated p65 and p50 levels in nuclear fractions and recovered p65 and p50 levels in cytosolic fractions (Fig. 6A). β-actin and PARP were used as internal controls. We also explored whether ILG inhibits the LPS-stimulated degradation of IκB-α in RAW 264.7 cells by Western blotting with anti-IκB-α antibody. Fig. 6B shows that LPS-induced IκB-α degradation was significantly blocked by ILG pretreatment. Furthermore, to determine whether this IκB-α degradation was related to IκB-α phosphorylation, we examined the effect of ILG on the LPS-induced p-IκB-α by Western blotting, and found that ILG also significantly reduced LPS-induced IκB-α phosphorylation in a concentration-dependent manner.

3.6. Effects of ILG on LPS-induced phosphorylation of IKK and the activations of mitogen-activated protein (MAP) kinases

Since IKK-α and -β are upstream kinases of IκB in the NF-κB signal pathway, and in particular, phosphorylate IκB-α and -β (Surh et al., 2001), we examined the effects of ILG on LPS-induced IKK-α/β activation by immunoblotting using IKK-α, -β, and phosphorylated IKK-α/β antibodies. RAW 264.7 cells were pretreated with ILG (0.4, 0.8, 1.6 μM) for 1 h and then stimulated with LPS (1 μg/ml). It was found that LPS strongly induce IKK-α/β phosphorylation, whereas ILG markedly reduced this LPS-induced phosphorylation (Fig. 7A). ILG did not affect the total amounts of IKK-α and IKK-β. β-actin was used as internal control.

MAP kinases play critical roles in the regulation of cell growth and differentiation, and control cellular responses to cytokines and stresses (Vandenberghe et al., 1998). In addition, they are also

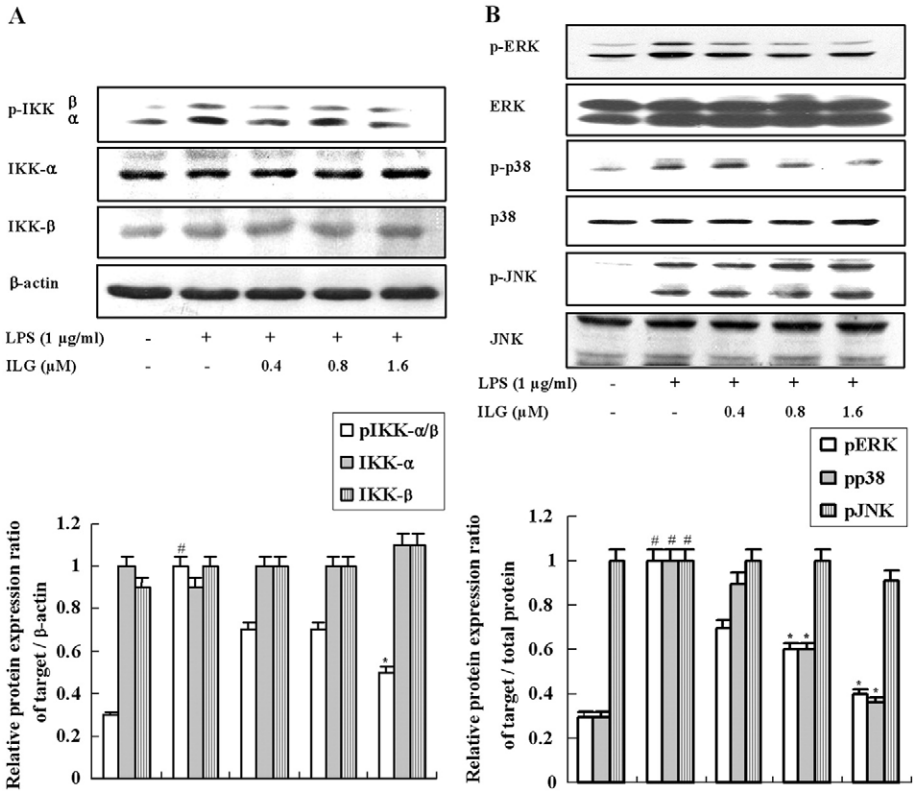

Fig. 7. The effects of ILG on the LPS-induced phosphorylations of IKK-α/β (A) and MAP kinases (B) in RAW 264.7 cells. RAW 264.7 macrophages were treated with LPS (1 μg/ml) in the absence or presence of the indicated concentrations of ILG for 5 min. Lysates were analyzed by Western blotting using specific p-IKK-α/β, IKK-α, and IKK-β antibodies. β-Actin was used as an internal control (A). RAW 264.7 macrophages were treated with LPS (1 μg/ml) alone or with LPS (1 μg/ml) plus the indicated concentrations of ILG for 15 min. Whole cell lysates were analyzed by Western blotting using antibodies against the activated MAP kinases. The data shown are representative of three independent experiments (B).

known to be important for the activation of NF-κB (Vandenberghe et al., 1998). To investigate whether the inhibition of NF-κB activation by ILG is mediated through the MAP kinases pathway, we examined the effect of ILG on the LPS-stimulated phosphorylations of ERK1/2, JNK1/2, and p38 MAP kinases in RAW 264.7 cells by Western blotting. As shown in Fig. 7B, ILG suppressed the LPS-induced activations of ERK1/2 and p38 MAP kinases, but did not affect the phosphorylation of JNK1/2. Non-phosphorylated ERK, JNK, and p38 kinase expressions were unaffected by LPS or by LPS plus ILG.

4. Discussion

Macrophages play a central role in inflammatory response and serve as an essential interface between innate and adaptive immunity (Iontcheva et al., 2004). In particular, they are responsible for antigen processing and for presenting antigens to antigen-specific T cells. Following activation, macrophages modulate the expression of toll-like receptor (TLR) 4 (Iontcheva et al., 2004), and the stimulation of TLR4 by LPS triggers its recruitment of cytoplasmic adaptor protein MyD88, which results in the activation of two distinct downstream signaling pathways; the NF-κB pathway and the MAP kinase pathway (Akira and Takeda, 2004). These two pathways induce the expressions of various inflammatory mediators, which include NO, PGs, and the pro-inflammatory cytokines (Gomez et al., 2005). 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 leads to the induction of pro-inflammatory enzymes and to the production of pro-inflammatory cytokines.

During our continued search for novel anti-inflammatory agents from natural products, we found that ILG (isoliquiritigenin) and ILT (isoliquiritin) isolated from the roots of *G. uralensis* potently inhibit LPS-induced NO and PGE_2 production in RAW 264.7 macrophages. In addition, we also found that ILG has a greater inhibitory effect on LPS-induced NO and PGE_2 production than ILT, which is possibly due to their different affinities for cellular membranes, because affinity for cellular membranes appears to play an important role in the cellular uptakes of lipophilic compounds by passive diffusion, and because ILT possesses a sugar moiety, which would reduce its lipophilicity and compatibility with cellular membranes.

Experimental evidence suggests that the ethanol extract of roasted licorice root has potent anti-inflammatory effects because it reduces NO and PGE_2 levels, and pro-inflammatory cytokine production in LPS-stimulated RAW 264.7 cells. However, constituents responsible have not been identified (Kim et al., 2006). ILG is a chalcone, and is one of the major active components in this licorice extract. Earlier comparative studies on various molecules with a chalcone structure found that ILG contains a unique structure (4,2′,4′-trihydroxychalcone) and that

this structure could confer considerable anti-inflammatory activity (Madan et al., 2000; Takano-Ishikawa et al., 2003). Although (Kumar et al., 2007) recently reported that ILG inhibits the TNF-α induced adhesion of neutrophils to endothelial monolayers by blocking the expressions of ICAM-1, VCAM-1 and E-selectin, and that it blocks NF-κB activity by inhibiting IκB kinase activity and TNF-α induced ROS production in endothelial cells, the mechanisms underlying the anti-inflammatory action of ILG in macrophages have not been addressed. Thus, we investigated the effect of ILG on pro-inflammatory molecules in LPS-treated RAW 264.7 cells. To investigate the inhibitory effects of ILG on pro-inflammatory mediators, we examined the expressions of iNOS and COX-2 protein and of iNOS, COX-2, TNF-α and IL-6 mRNA. We found that the inhibition of iNOS and COX-2 proteins by ILG paralleled those of their mRNAs in a concentration-dependent manner. Thus, we considered that the observed reductions in NO production and PGE_2 release by ILG might have been due to the transcriptional suppressions iNOS and COX-2. Moreover, the inhibitory effects of ILG on the LPS-induced expressions of these molecules in RAW 264.7 cells were not due to the cytotoxicity of ILG, as assessed by MTT assay and by the expression of β-actin.

Cytokines like TNF-α and IL-6 have been reported to be pro-inflammatory *in vitro* and *in vivo* (Molly et al., 1993), and the production of TNF-α is known to be crucial for the induction of NO synthesis in IFN-γ and/or LPS-stimulated macrophages (Jun et al., 1995). TNF-α elicits a number of physiological effects that include septic shock, inflammation, cachexia, and cell death (Aggarwal and Natarajan, 1996), and similarly, IL-6 is also considered to be a pivotal pro-inflammatory cytokine, for example, it is regarded an endogenous mediator of LPS-induced fever. Hence, we scrutinized the inhibitory effect of ILG on TNF-α and IL-6 production and transcription. Our findings indicate that ILG significantly inhibits the release of TNF-α and IL-6 to media and their mRNA expressions.

NF-κB is known to play a key role in the regulation of cell survival genes, and to coordinate the expressions of pro-inflammatory enzymes and cytokines, like iNOS, COX-2, TNF-α and IL-6 (Karin and Ben-Neriah, 2000). Since the expressions of these pro-inflammatory mediators are known to be modulated by NF-κB, our findings suggest that the transcriptional inhibitions of these pro-inflammatory mediators by ILG are due to its blocking the NF-κB signal pathway. In the cytoplasm, NF-κB is bound to 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 ILG, and that the degradation and phosphorylation of IκB-α were also inhibited by ILG in a concentration-dependent manner. These findings indicate that ILG may inhibit NF-κB activation by suppressing the phosphorylation of IκB-α and the translocations of the p50 and p65 subunits of NF-κB from the cytosol to the nucleus in LPS-induced RAW 264.7 cells.

IKK-α and IKK-β (known as the IκB kinases) are responsible for phosphorylating IκBs (May and Ghosh, 1999). These kinases form a large multiprotein complex with a scaffold protein called NF-κB essential modulator (Yamaoka et al., 1998). 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 (Karin and Ben-Neriah, 2000). Other studies have shown that specific inhibitors of the ERK and p38 MAP kinase pathways block nuclear NF-κB activity and the transactivation activity of p65 (Beyaert et al., 1996; Vandenberghe et al., 1998). In the present study, we observed that ILG inhibited the phosphorylations of IKKα/β and MAP kinases. Furthermore, our findings indicate that the inhibition of the phosphorylations of IKKα/β, ERK1/2 and p38 kinase is involved in NF-κB inactivation. Thus, we suggest that the inhibition of IKK, ERK1/2 and p38 kinase by ILG underlies its inhibition of NF-κB activation.

In summary, the present study demonstrates that ILG effectively inhibits LPS-induced iNOS and COX-2 protein expressions and iNOS, COX-2, TNF-α and IL-6 transcriptions by inhibiting the degradation and phosphorylation of IκB-α, and by blocking the activation of NF-κB in RAW 264.7 macrophages. The anti-inflammatory effects of ILG may also be accomplished by reducing NF-κB activation *via* the IKK and MAP kinases signal pathways. Thus, our results suggest that ILG should be considered a potential treatment for inflammatory diseases.

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