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Licochalcone A Potently Inhibits Tumor Necrosis Factor α-Induced Nuclear Factor-κB Activation through the Direct Inhibition of IκB Kinase Complex Activation

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ABSTRACT

Glycyrrhiza inflata has been used as a traditional medicine with anti-inflammatory activity; however, its mechanism has not been fully understood. Licochalcone A is a major and biogenetically characteristic chalcone isolated from *G. inflata*. Here, we found that licochalcone A strongly inhibited tumor necrosis (TNF)-α-induced nuclear localization, DNA binding activity, and the transcriptional activity of nuclear factor-κB (NF-κB). Whereas licochalcone A had no effect on the recruitment of receptor-interacting protein 1 and IκB kinase β (IKKβ) to TNF receptor I by TNF-α, it significantly inhibited TNF-α-induced IκB kinase complex (IKK) activation and inhibitor of nuclear factor-κB degradation. It is interesting that we found that the cysteine residue at position 179 of IKKβ is essential for licochalcone A-induced IKK inhibition, because licochalcone A failed to affect the kinase activity of the IKKβ (C179A) mutant. In contrast, a structurally related compound, echinatin, failed to inhibit TNF-α-induced IKK activation and NF-κB activation, suggesting that the 1,1-dimethy-2-propenyl group in licochalcone A is important for the inhibition of NF-κB. In addition, TNF-α-induced expression of inflammatory cytokines CCL2/monocyte chemotactic protein-1and CXCL1/KC was clearly inhibited by licochalcone A but not echinatin. Taken together, licochalcone A might contribute to the potent anti-inflammatory effect of *G. inflata* through the inhibition of IKK activation.

Tumor necrosis factor (TNF)-α is mainly produced by macrophages but also by a broad variety of other tissues, including lymphoid cells, mast cells, endothelial cells, fibroblasts, and neuronal tissues (Chen and Goeddel, 2002; Wajant et al., 2003). TNF-α is a pleiotropic proinflammatory cytokine with a wide range of biological effects. TNF-α participates in the inflammatory effect by inducing various inflammatory cytokines, including CCL2/monocyte chemotactic protein (MCP)-1, CXCL1/KC, and interleukin-6, through the activation of a transcription factor, nuclear factor-κB (NF-κB) (Mukaida et al., 1990; Zhang et al., 1990; Ping et al., 1999). Therefore, dysregulated TNF-α function is implicated in the pathological process of many diseases, including rheumatoid arthritis, Crohn's disease, and several neurological diseases (Liu, 2003).

In mammals, the NF-κB family has five members: RelA/p65, RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52 (Hayden and Ghosh, 2008). The NF-κB p50/p65 heterodimer is a typical member of the Rel family of transcription factors that regulate diverse cellular functions, such as immune response, cell growth, and development. In the canonical NF-κB pathway, TNF-α activates the IκB kinase complex (IKK) that is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ. In unstimulated cells, NF-κB remains inactive in the cytoplasm through the association with inhibitor proteins of the IκB family (DiDonato et al., 1997; Mer-

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ABBREVIATIONS: TNF, tumor necrosis factor; MCP, monocyte chemotactic protein; NF-κB, nuclear factor-κB; IKK, IκB kinase complex; IκBα, inhibitor of nuclear factor-κB; TNFR, tumor necrosis factor receptor; TRADD, tumor necrosis factor receptor-associated death domain; RIP, receptor-interacting protein; TRAF, tumor necrosis factor receptor-associated factor; CMV, cytomegalovirus; HEK, human embryonic kidney; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GST, glutathione transferase; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide.

curio et al., 1997; Zandi et al., 1997; Yamaoka et al., 1998). Activated IKKs phosphorylate IκBs, leading to their ubiquitination and proteosomal degradation. These events release NF-κB dimers in the cytosol, allowing them to translocate to the nucleus where they enhance the transcription of target genes (Palombella et al., 1994; DiDonato et al., 1996).

So far, the molecules involved in the TNF-α signaling pathway have been identified, and the signal transduction mechanism leading to NF-κB activation has been fully understood. As with other signaling ligands, TNF-α exerts its cellular effect through two distinct receptors, a 55-kDa receptor 1 (TNFRI) and a 75-kDa receptor (TNFRII) (Heller et al., 1990; Barrett et al., 1991). TNFRI is ubiquitously expressed and seems to be the key mediator of TNF-α signaling in the majority of cells (Ryffel et al., 1991). Binding of TNF-α to TNFRI on the cell surface triggers the trimerization of receptors, and the exposed intracellular domain of TNFRI is recognized by a death domain-containing adaptor protein, the TNF receptor-associated death domain (TRADD) (Hsu et al., 1995). TRADD acts as a scaffold protein that recruits a serine/threonine kinase, receptor-interacting protein (RIP), and an adapter protein TNF receptor-associated factor (TRAF) 2 (Rothe et al., 1994; Hsu et al., 1996; Liu et al., 1996). Consequently, it activates the downstream IKK complex, leading to NF-κB activation (Palombella et al., 1994; DiDonato et al., 1996, 1997; Mercurio et al., 1997; Zandi et al., 1997; Yamaoka et al., 1998).

Liquorice root has been used as a traditional medicine in the East and West for the treatment of gastric ulcer, bronchial asthma, and inflammation (Shibata, 2000). Licochalcone A is a major and biogenetically characteristic chalcone isolated from the root of Xinjiang liquorice (*Glycyrrhiza inflata)* (Hatano et al., 1988). A previous study showed that licochalcone A possessed radical-scavenging effects (Haraguchi et al., 1998), antileishmanial activity, and antimicrobial activity, inhibiting the growth of *Staphylococcus aureus* and the activity of *Helicobacter pylori* (Chen et al., 1993; Fukai et al., 2002). Furthermore, licochalcone A has been reported to inhibit the production of chemical mediators, such as prostaglandin E_2 and interleukin-1-induced cytokines in human skin fibroblasts (Furuhashi et al., 2005). Therefore, drugs consisting only of licochalcone A are expected to have a potent anti-inflammatory effect; however, the detailed anti-inflammatory mechanism has not been clarified.

In this study, we focused on the effect of licochalcone A on the TNF-α signaling pathway. It is interesting that we observed that licochalcone A significantly inhibited TNF-α-induced NF-κB activation by preventing IKK activation. As a result, licochalcone A induced the suppression of NF-κB-regulated gene products and led to the inhibition of TNF-α-induced inflammation.

Materials and Methods

Reagents. Licochalcone A and echinatin were donated by Minophagen Pharmaceutical Co. Ltd. (Akasaka, Tokyo) (Hatano et al., 1988; Shibata, 2000). Murine TNF-α was purchased from PeproTech (Rocky Hill, NJ). Antibodies recognizing p65, lamin, IκBα, TNFRI, IKKα, IKKβ, IKKγ, TRADD, TRAF2, β-actin, and c-Myc were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody against RIP1 was purchased from BD Biosciences Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse polyclonal IgG antibodies were purchased from Dako-Japan (Tokyo, Japan).

Plasmids. Human RIP1 cDNA and IKKβ cDNA were subcloned into pCMV5. The expression vector for His_6-tagged ubiquitin was a gift from Dr. Dirk Bohmann (University of Rochester, Rochester, NY). Mutagenesis of amino acid residues in IKKβ C179A was performed using a site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Cell Culture. NIH-3T3 stably expressing the NF-κB-dependent luciferase reporter plasmid (KF8) was established as described previously (Funakoshi-Tago et al., 2008). The NF-κB-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and five repeats of the NF-κB transcriptional response element (5'-GGGGACTTTCCC-3') (Stratagene). NIH-3T3 cells, KF8 cells, and HEK293T cells were grown in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supple-

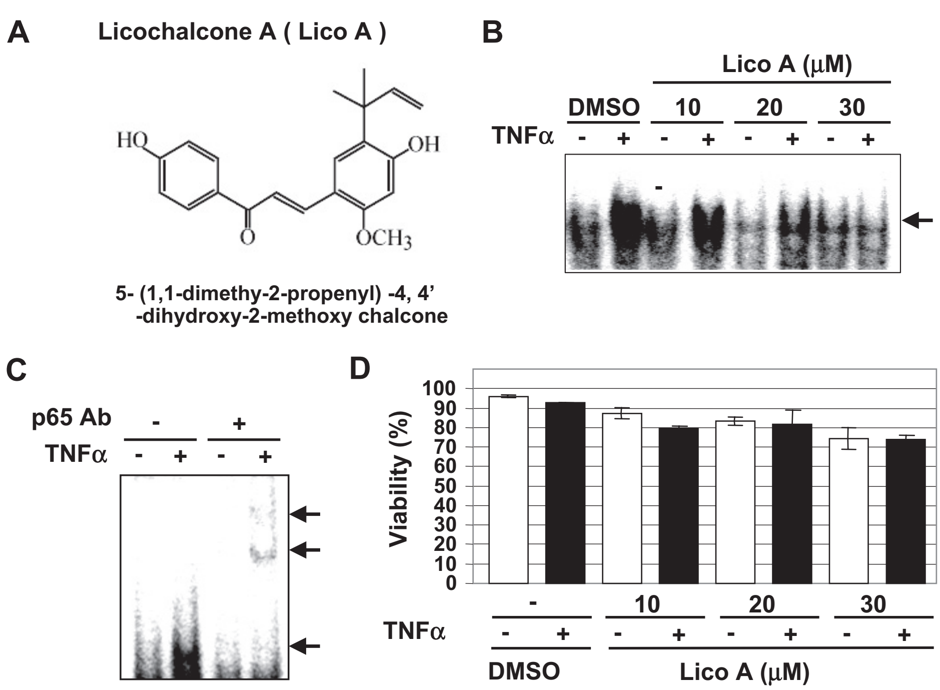

Fig. 1. Licochalcone A (LicoA) potently inhibits TNF-α-induced NF-κB activation. A, structure of licochalcone A. B, NIH-3T3 cells were pretreated with different concentrations of licochalcone A or DMSO (0.1%) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for 30 min, and nuclear extracts were prepared. NF-κB DNA binding activity was measured by electrophoretic mobility shift assay with a radiolabeled probe containing a consensus NF-κB binding site (5′-AGTTGAGGG-GACTTTCCCAGG-3′). C, for supershift assay, nuclear extracts from cells treated with TNF-α (10 ng/ml) for 30 min were incubated in the presence of 1 μg of anti-p65 antibody. D, NIH-3T3 cells (5 × 10^4) were preincubated with various concentrations of licochalocne A for 1 h at 37°C. After treatment with TNF-α (10 ng/ml) for 12 h, cell viability was analyzed by trypan blue exclusion tests using a cell viability analyzer (Beckman Coulter).

mented with 10% heat-inactivated fetal bovine serum (FBS) (Biowest, Nuaillé, France), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 4 mM L-glutamine.

Electrophoretic Mobility Shift Assay. Consensus double-stranded oligodeoxynucleotide probes for NF-κB (5'-TAGTTGAGGGGACTTTCCCAGGC-3') were radioactively labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase, as described previously (Funakoshi-Tago et al., 2003). Then, 2 μg of nuclear extract was incubated with a γ-^{32}P-labeled double-stranded oligonucleotide probe in buffer containing 10 mM HEPES-KOH, pH 7.8, 420 mM KCl, 0.1 mM EDTA, pH 8.0, 5 mM $MgCl_2$, 20% glycerol, 25 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 mM Na_3VO_4. The binding reaction was carried out at 30°C for 20 min in a total volume of 25 μl. Bound complexes were separated by 4%

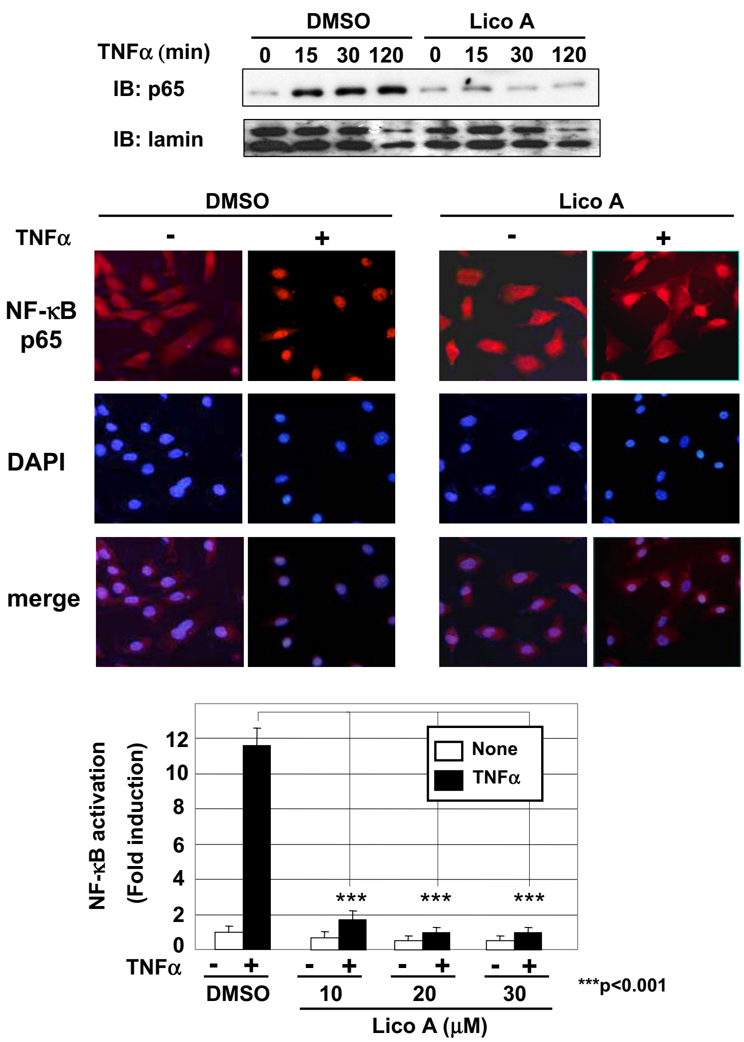

Fig. 2. Licochalcone A potently inhibits TNF-α-induced nuclear translocation of NF-κB p65. A, NIH-3T3 cells were pretreated with licochalcone A (20 μM) or DMSO (0.1%) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for the indicated periods. Nuclear extracts were prepared and immunoblotted with anti-p65 antibody or anti-lamin antibody (as a control). B, NIH-3T3 cells were cultured on sterile coverslips and pretreated with licochalcone A (20 μM) or DMSO (0.1%) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for 30 min. Cells were fixed in 4% paraformaldehyde and the localization of p65 was visualized with an antibody (green). 4,6-Diamidino-2-phenylindole (DAPI; blue) was also applied to visualize nuclei. C, NIH-3T3 cells expressing pNF-κB-Luc (KF8 cells) were pretreated with different concentrations of licochalcone A or DMSO (0.1%) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for 5 h. NF-κB-dependent luciferase activity was normalized to the protein amounts. Data are the mean ± S.D. of the relative luciferase activities of pNF-κB-Luc in four independent experiments. ***, $p < 0.001$.

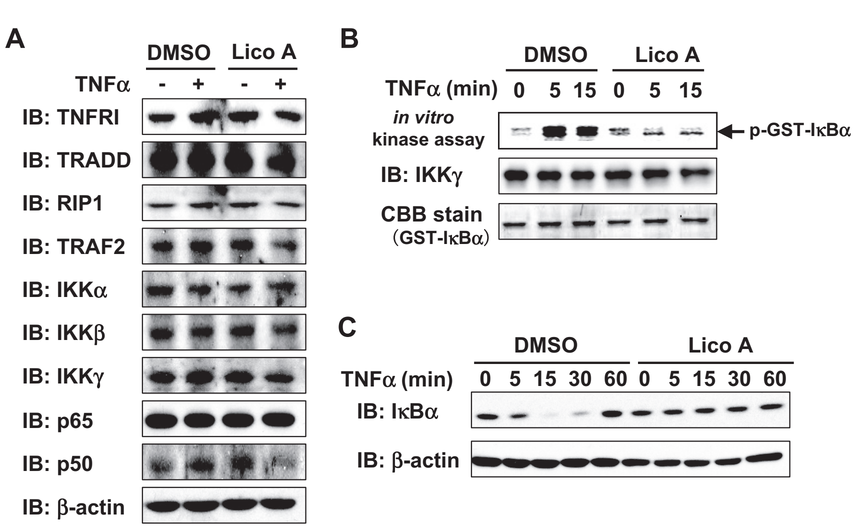

Fig. 3. Licochalcone A significantly inhibits TNF-α-induced IKK activation and IκBα degradation. NIH-3T3 cells were pretreated with licochalcone A (20 μM) or DMSO (0.1%) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for 10 min. A, whole cell lysates were immunoblotted with antibodies recognizing TNFRI, TRADD, RIP1, TRAF2, IKKα, IKKβ, IKKγ, p65, p50, or β-actin. B, cell lysates were immunoprecipitated with anti-IKKγ antibody. IKKγ immunoprecipitates were assayed for kinase activity using purified GST-IκBα as a substrate (top). Immunoprecipitates were immunoblotted with anti-IKKγ antibody (middle). The gel was stained with Coomassie Brilliant Blue (CBB; bottom). C, whole cell lysates were immunoblotted with antibodies recognizing IκBα or β-actin as a control.

polyacrylamide gel electrophoresis in Tris-glycine-EDTA buffer and visualized by autoradiography.

NF-κB Luciferase Assay. KF8 cells (5×10^4 cells) were cultured in a 24-well plate and preincubated with various concentrations of licochalone A or echinatin for 1 h at 37°C. After treatment with TNF-α (10 ng/ml) for 5 h, the cells were harvested and lysed in passive lysis buffer (Promega, Madison, WI). Luciferase activity of the lysates was determined using the luciferase reporter assay system (Promega), according to the manufacturer's instructions. NF-κB-dependent luciferase activity was normalized by the quantity of protein for each sample, as described previously (Funakoshi-Tago et al., 2008).

Cell Viability Analysis. NIH-3T3 cells (5×10^4) were preincubated with various concentrations of licochalocne A for 1 h at 37°C. After treatment with TNF-α (10 ng/ml) for 12 h, the cells were collected by trypsinization and then analyzed by trypan blue exclusion tests using a cell viability analyzer (Beckman Coulter, Fullerton, CA).

Immunofluorescence Assay. NIH-3T3 cells (5×10^5 cells) were seeded on sterile coverslips in a six-well plate and pretreated with licochalcone A (20 μM) for 1 h after stimulation with TNF-α (10 ng/ml) for 30 min. After washing with PBS three times, the cells were fixed in 4% paraformaldehyde for 10 min at room temperature and washed with PBS three times. Cells on coverslips were permeabilized in 0.2% (v/v) Triton X-100 for 5 min at room temperature. After washing with PBS three times, the coverslips were blocked in PBS containing 3% FBS for 5 min and incubated with an antibody recognizing p65 (Santa Cruz Biotechnology, Inc.) diluted with PBS containing 3% FBS at 1:200 dilution for 1 h at room temperature. After washing with PBS three times, the coverslips were incubated with a secondary antibody BODIPY FL anti-rabbit IgG (Invitrogen, Carlsbad, CA) at 1:200 dilution for 1 h at room temperature. After washing with PBS three times, the coverslips were mounted using VECTASHIELD mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Each coverslip was analyzed on a BX50 microscope (Olympus, Tokyo, Japan), with Micro DP70 software (Olympus) as described previously (Funakoshi-Tago et al., 2008).

Immunoblot Analysis. Cells were washed with PBS and lysed in lysis buffer containing 50 mM HEPES, pH 7.5, 0.5% Triton X-100, 100 mM NaF, 10 mM sodium phosphate, 4 mM EDTA, 2 mM Na_3VO_4, 2 mM sodium molybdate, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. Cell lysates were centrifuged at 15,000 rpm at 4°C for 15 min to remove the debris, and the protein concentration was determined by Bradford assay (Bradford, 1976). Eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were probed using the designated antibodies and visualized with the enhanced chemiluminescence detection system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), as described previously (Funakoshi-Tago et al., 2003).

In Vitro IKK Assay. HEK293T cells (1.5×10^6 cells/60-mm dish) were transfected with 2 μg of pCMV5-Myc-IKKβ or pCMV5-Myc-IKKβ (C179A) using FuGENE 6 (Roche Diagnostics, Indianapolis, IN). NIH-3T3 cells (1×10^7 cells) were preincubated with licochalcone A (20 μM) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with anti-Myc antibody or anti-IKKγ antibody with protein G-Sepharose (Zymed Laboratories, South San Francisco, CA) for 2 h at 4°C and then washed three times with lysis buffer and twice with kinase buffer (25 mM HEPES-NaOH, pH 7.5, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM dithiothreitol, and 20 mM p-nitrophenylphosphate). The kinase reaction in 20 μl of kinase buffer including [γ-^{32}P]ATP was carried out with 1 μg of GST-IκBα as a substrate for 20 min at 30°C. Samples were resolved by SDS-polyacrylamide gel electrophoresis, and phosphorylated GST-IκBα was visualized by autoradiography, as described previously (Funakoshi-Tago et al., 2003).

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction. RNA was prepared using an RNA purification kit (QIAGEN, Tokyo, Japan). Reverse transcription was performed using an oligo(dT)$_{20}$ primer and 1 μg of total RNA for first-strand cDNA synthesis, as described previously (Funakoshi-Tago et al., 2003). Polymerase chain reaction was performed at an annealing temperature of 57°C with 22 amplification cycles. Polymerase chain reaction products were resolved and electrophoresed in a 1.5% agarose gel in Tris-acetic acid-EDTA buffer. Primer sequences were as follows: GAPDH, 5′-ACTCCACTCACGGCAAATTC-3′ (upstream) and 5′-CCTTCCACAATGCCAAAGTT-3′ (downstream); CCL2/MCP-1, 5′-TGAGGTGG TTGTGGAAAAGG-3′ (upstream) and 5′-CCTGCTGTTCACAGTTGCC-3′ (downstream); and CXCL1/KC, 5′-TGGGGACACCTTTTAGCATC-3′ (upstream) and 5′-GCCCATCGCCAATGAGCTG-3′ (downstream) (Funakoshi-Tago et al., 2003).

Enzyme-Linked Immunosorbent Assay. Cells (5×10^4) were cultured in a 24-well plate and pretreated with chalcones (10 μM) for 1 h at 37°C. After treatment with TNF-α (10 ng/ml) for 24 h, the supernatants were harvested and the amounts of CCL2/MCP-1 and CXCL1/KC were determined using immunoassay kits (R&D Systems, Minneapolis, MN) (Funakoshi-Tago et al., 2008).

Purification of His$_6$-Tagged Ubiquitin Conjugates. HEK293T cells (1×10^5) were cotransfected with 1 μg of pCMV5 or pCMV5-RIP1 and 1 μg of His$_6$-tagged ubiquitin expression vector using FuGENE6 (Roche Diagnostics) according to the manufacturer's protocol. To purify His$_6$-tagged ubiquitinated proteins, 10%

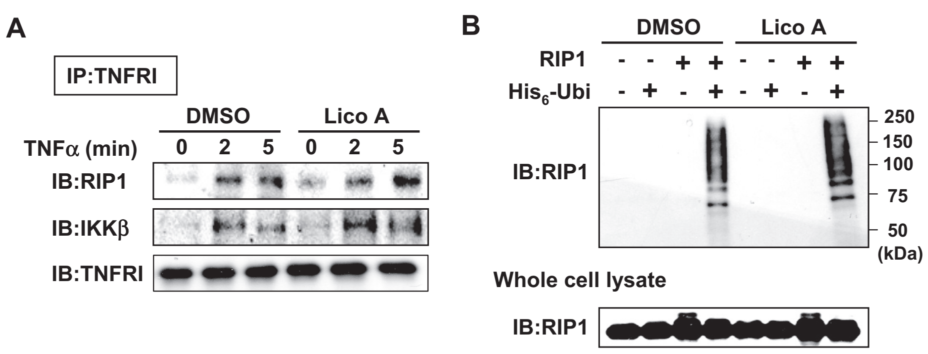

Fig. 4. Licochalcone A had no effect on the recruitment of IKKβ to TNFRI. A, NIH-3T3 cells were pretreated with licochalcone A (20 μM) or DMSO (0.1%) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for the indicated periods. Cell lysates were immunoprecipitated with anti-TNFRI antibody. The immunoprecipitates were blotted with anti-RIP1 antibody, anti-IKKβ antibody, or anti-TNFRI antibody. B, HEK293T cells were cotransfected with the indicated plasmids encoding RIP1 and/or His$_6$-ubiquitin. At 48 h after transfection, cells were pretreated with licochalcone A (20 μM) or DMSO (0.1%) for 1 h at 37°C. The proteins from cell lysates were affinity-purified on nickel resin in buffer containing 8 M urea, electrophoretically separated on denaturing gels containing SDS, and immunoblotted with anti-RIP1 antibody. Whole cell lysates prepared with lysis buffer, as described under *Materials and Methods*, were subjected to immunoblotting with anti-RIP1 antibody (top). Whole cell lysates were immunoblotted with anti-RIP1 antibody (bottom).

transfected cell suspension was taken for direct protein immunoblotting. The remaining cells were resuspended in lysis buffer containing 8 M urea, and His_6-tagged proteins recovered with nickel beads (QIAGEN, Valencia, CA) were eluted with imidazole, diluted with sample buffer, and separated on gels containing SDS. After transferring to membranes, proteins were immunoblotted with anti-RIP1 antibody.

Results

Licochalcone A Significantly Inhibited TNF-α-Induced DNA Binding Activity of NF-κB. Licochalcone A is a major component of *G. inflata* (Fig. 1A) (Hatano et al., 1988; Shibata, 2000). To investigate the anti-inflammatory effect of licochalcone A, we evaluated its effect on TNF-α-induced NF-κB activation. First, we investigated its effect on NF-κB DNA binding activity by electrophoretic mobility shift assay using nuclear extracts. When NIH-3T3 cells were treated with various concentrations of licochalcone A for 1 h after TNF-α stimulation for 30 min, licochalcone A inhibited TNF-α-induced DNA binding activity of NF-κB in a dose-dependent manner. In contrast, licochalcone A alone had no effect on NF-κB activation (Fig. 1B). Next, to decide which type of NF-κB subunit was included in this protein-DNA complex, we added specific antibodies against several NF-κB subunits, including p65, p50, and c-Rel. As shown in Fig. 1C, the addition of anti-p65 antibody resulted in two sizes of supershifted protein-DNA complexes, indicating that this complex includes p65, which could be involved in two active NF-κB complexes. Conversely, antibodies against p50 and c-Rel failed to exhibit supershift activity (data not shown). As shown in Fig. 1D, cell viability was not changed by treatment with licochalcone A and/or TNF-α stimulation, indicating that the inhibition of NF-κB activation by licochalcone A is not attributable to its cytotoxicity.

Licochalcone A Significantly Inhibited TNF-α-Induced Nuclear Localization and Transcriptional Activity of NF-κB. Next, we investigated the effect of licochalcone A on the TNF-α-induced nuclear translocation of NF-κB using nuclear extracts. As shown in Fig. 2A, TNF-α induced the accumulation of the p65 NF-κB subunit in the nucleus at 15 min. It was striking that licochalcone A significantly decreased the amount of nuclear NF-κB after TNF-α stimulation. In addition, we performed immunofluorescence staining to investigate the effect of licochalcone A on the translocation of p65 NF-κB subunit. Although most NF-κB p65 was translocated into the nucleus after TNF-α stimulation in cells treated with DMSO, licochalcone A clearly inhibited the TNF-α-induced translocation of NF-κB p65 (Fig. 2B).

Furthermore, we evaluated the effect of licochalcone A on NF-κB transcriptional activation using KF8 cells, which stably express the NF-κB luciferase reporter gene, as reported previously (Funakoshi-Tago et al., 2008). As shown in Fig. 2C, TNF-α induced NF-κB transcriptional activation up to approximately 12 times more than in unstimulated cells. It is striking that licochalcone A potently inhibited TNF-α-induced NF-κB activation. The concentration of licochalcone A up to 30 μM had no effect on cell viability, as determined by the trypan blue exclusion test (data not shown), suggesting that licochalcone A is a potent inhibitor of NF-κB.

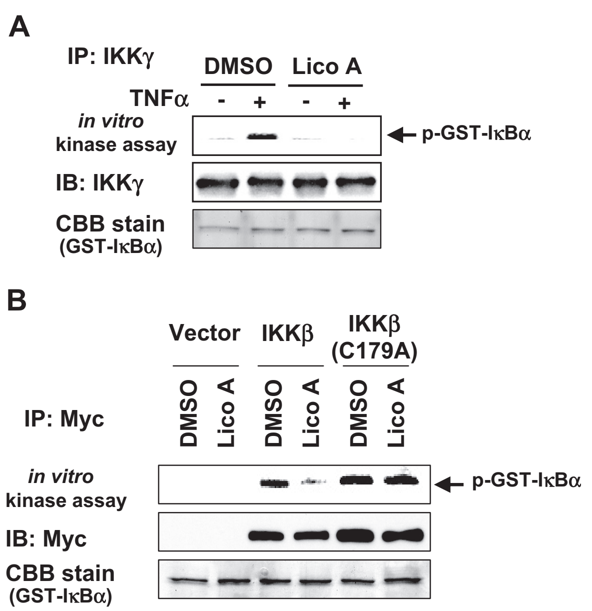

Fig. 5. Cysteine 179 in IKKβ was involved in its inhibition by licochalcone A. A, NIH-3T3 cells were stimulated with TNF-α (10 ng/ml) for 10 min, and cell lysates were prepared. IKKγ immunoprecipitates were incubated with licochalcone A (20 μM) or DMSO (0.1%) for 1 h at 37°C and then assayed for kinase activity using purified GST-IκBα as a substrate (top). Immunoprecipitates were immunoblotted with anti-IKKγ antibody (middle). The gel was stained with Coomassie Brilliant Blue (CBB; bottom). B, HEK293T cells were transfected with the indicated plasmids encoding Myc-IKKβ or Myc-IKKβ mutant (C179A). At 48 h after transfection, cell lysates were immunoprecipitated with anti-Myc antibody. The immunoprecipitates were incubated with licochalcone A (20 μM) or DMSO (0.1%) for 1 h at 37°C and assayed for kinase activity using purified GST-IκBα as a substrate (top). Immunoprecipitates were immunoblotted with anti-IKKγ antibody (middle). The gel was stained with CBB (bottom).

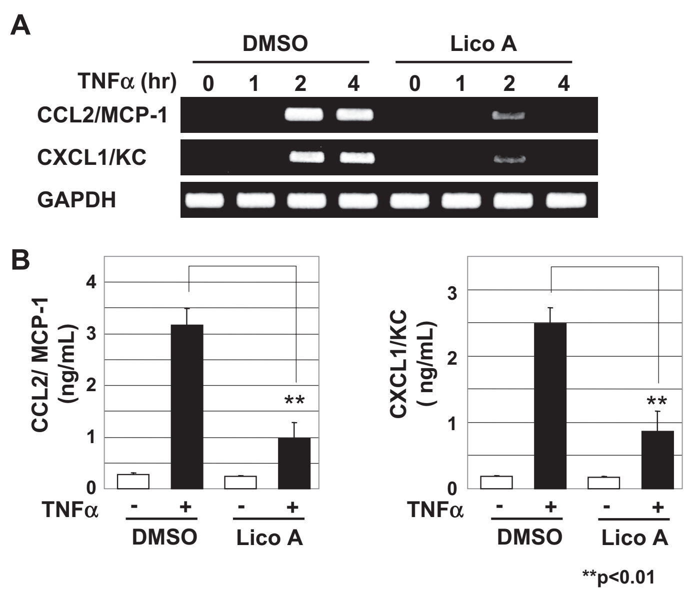

Fig. 6. Licochalcone A significantly inhibits TNF-α-induced expression and secretion of CCL2 /MCP-1 and CXCL1/KC. A, NIH-3T3 cells were pretreated with licochalcone A (10 μM) or DMSO (0.1%) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for the indicated periods. Total RNA was used to perform RT-PCR with gene-specific primers to CCL2/MCP-1, CXCL1/KC, or GAPDH. B, NIH-3T3 cells were pretreated with licochalcone A (10 μM) or DMSO (0.1%) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for 14 h. CCL2/MCP-1 or CXCL1/KC in the cultured supernatants was measured with a commercial ELISA kit (R&D Systems). Data are shown as the mean ± S.D. of three independent experiments. **, $p < 0.01$.

Licochalcone A Significantly Inhibited TNF-α-Induced IKK Activation and IκBα Degradation. To determine how licochalcone A inhibited NF-κB activation induced by TNF-α, we investigated its effect on the expression levels of major signaling molecules, which are required for the TNF-α signaling pathway. Upon TNF-α stimulation, a serine/threonine kinase, RIP1, and adaptor molecules TRADD and TRAF2 were recruited to TNFRI (Rothe et al., 1994; Hsu et al., 1995, 1996; Liu et al., 1996). Consequently, NF-κB is rapidly activated through activation of the IKK complex (DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997; Yamaoka et al., 1998). However, the expression levels of TNFRI, TRADD, RIP1, TRAF2, IKKα, IKKβ, and IKKγ were not changed in cells treated with 20 μM licochalcone A in the absence and presence of TNF-α stimulation (Fig. 3A). We also analyzed the expression of the mature form of TNFRI on the cell surface by flow cytometry analysis; however, a similar level of cell surface TNFRI was observed in cells untreated and treated with licochalcone A (data not shown). Moreover, the expressions of the NF-κB family, p65, and p50 were also not affected by licochalcone A (Fig. 3A), confirming that the inhibition of NF-κB activation by licochalcone A was not due to the altered expression of signaling molecules in the TNF-α signaling pathway.

Because IKK activation is a key step in NF-κB activation (DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997; Yamaoka et al., 1998), we next determined whether licochalcone A inhibits TNF-α-induced IKK activation by in vitro kinase assay. Cells were pretreated with licochalcone A or DMSO as a control after TNF-α stimulation. IKK complex was immunoprecipitated with anti-IKKγ antibody, and IKK activity was measured using GST-IκBα as a substrate. As shown in Fig. 3B, licochalcone A significantly inhibited TNF-α-induced IKK activation. Furthermore, when the degradation of IκBα after TNF-α stimulation was examined by immunoblotting, licochalcone A potently inhibited TNF-α-induced IκBα degradation (Fig. 3C). Thus, these data indicate that licochalcone A inhibited NF-κB activation by suppressing IKK activity.

Licochalcone A Had No Effect on the Recruitment of IKK to TNFRI. It is known that IKK complex is recruited to TNFRI in response to TNF-α and then activated (Hsu et al., 1996; DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997). Because IKK activity was severely inhibited by licochalcone A (Fig. 3B), we also examined whether licochalcone A might affect the formation of TNFRI complex, including RIP1 and IKKs; however, a coimmunoprecipitation assay revealed that RIP1 and IKKβ were associated with TNFRI in a TNF-α-dependent manner, even in cells treated with licochalcone A (Fig. 4A).

A previous study showed that RIP1 is polyubiquitinated after TNF-α stimulation and that its polyubiquitination is required for not only the recruitment but also the activation of IKK complex (Liao et al., 2008); therefore, we next investigated whether the TNF-α-induced ubiquitination of RIP1 is affected by licochalcone A. In this system, polyubiquitination of RIP1 was induced by coexpression of His$_6$-ubiquitin; however, licochalcone A had no effect on the ubiquitination of RIP1 (Fig. 4B). Therefore, it is suggested that licochalcone A inhibits TNF-α-induced IKK activation without abrogating its recruitment to TNFRI.

Cysteine 179 in IKKβ Was Involved in Its Inhibition by Licochalcone A. To investigate how licochalcone A suppresses IKK activity, we incubated whole cell lysates from untreated cells and TNF-α-stimulated cells with anti-IKKγ antibody. After being precipitated, immunocomplexes were treated with licochalcone A or DMSO as a control. Although DMSO had no effect on TNF-α-induced IKK activation, licochalcone A significantly inhibited TNF-α-induced IKK activation (Fig. 5A).

IKKβ contains various cysteine residues and the cysteine residue at position 179 in the activation loop has been shown to be critical for its biological activity (Byun et al., 2006). Therefore, to determine whether this cysteine is involved in licochalcone A-mediated inhibition of IKK, HEK293T cells were transfected with wild-type Myc-IKKβ or Myc-IKKβ mutant with C179A mutation. After being precipitated using anti-Myc antibody, the immunocomplexes were treated with licochalcone A or DMSO as a control. It is interesting that licochalcone A treatment significantly inhibited wild-type IKKβ. In contrast, licochalcone A had no apparent effect on IKKβ (C179A) activity (Fig. 5B). Taken together, these find-

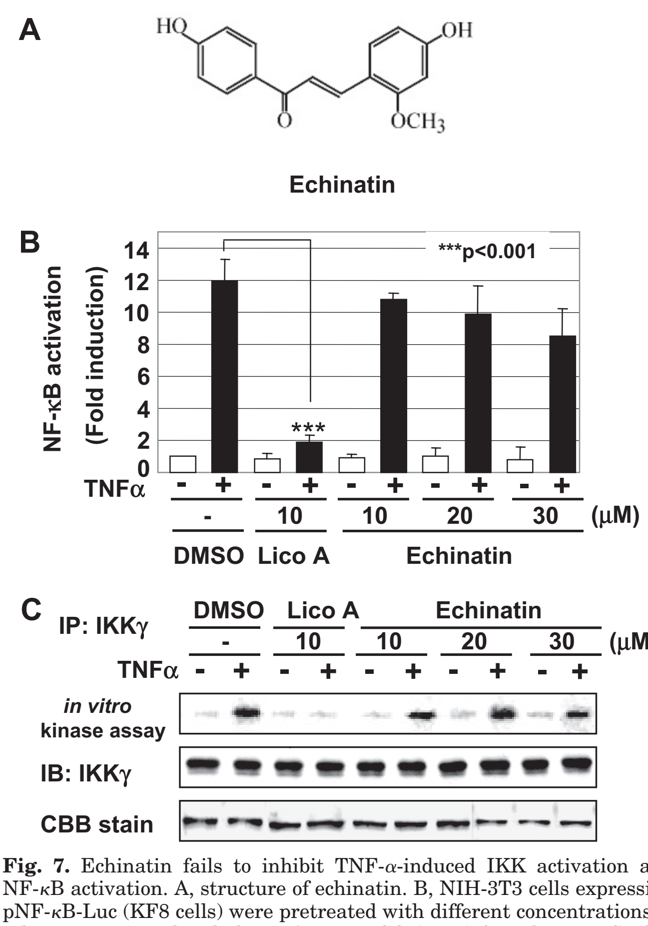

Fig. 7. Echinatin fails to inhibit TNF-α-induced IKK activation and NF-κB activation. A, structure of echinatin. B, NIH-3T3 cells expressing pNF-κB-Luc (KF8 cells) were pretreated with different concentrations of echinatin, 10 μM licochalcone A, or DMSO (0.1%) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for 5 h. NF-κB-dependent luciferase activity was normalized to the protein amounts. Data are the mean ± S.D. of the relative luciferase activities of pNF-κB-Luc in four independent experiments. ***, $p < 0.001$. C, NIH-3T3 cells were stimulated with TNF-α (10 ng/ml) for 10 min, and cell lysates were prepared. IKKγ immunoprecipitates were incubated with different concentrations of echinatin, 10 μM licochalcone A, or DMSO (0.1%) for 1 h at 37°C and then assayed for kinase activity using purified GST-IκBα as a substrate (top). Immunoprecipitates were immunoblotted with anti-IKKγ antibody (middle). The gel was stained with Coomassie Brilliant Blue (CBB; bottom).

ings suggest that cysteine 179 in IKKβ is a structure or part of the structure that is necessary for inhibition by licochalcone A.

Licochalcone A Significantly Inhibited TNF-α-Induced Expression of Inflammatory Cytokines. RT-PCR was performed to examine whether the inhibition of NF-κB by licochalcone A could be translated to its inability to activate target genes such as CCL2/MCP-1 and CXCL1/KC in response to TNF-α, When cells were stimulated with TNF-α, marked expressions of CCL2/MCP-1 mRNAs and CXCL1/KC mRNA were induced at 2 h and detected until 4 h. In contrast, treatment with licochalcone A significantly inhibited the TNF-α-induced expression of CCL2/MCP-1 and CXCL1/KC (Fig. 6A). Licochalcone A potently and consistently reduced the TNF-α-induced secretion of CCL2/MCP-1 and CXCL1/KC (Fig. 6B). These data suggest that licochalcone A shows anti-inflammatory activity by inhibiting the expression of various TNF-α-induced inflammatory cytokines.

Echinatin Failed to Inhibit TNF-α-Induced IKK Activation and NF-κB Activation. *G. inflata* contains not only licochalcone A but also echinatin, which has a related structure (Hatano et al., 1988). Licochalcone A is 5-(1,1-dimethy-2-propenyl)-4,4′-dihydroxy-2-methoxy chalcone (Fig. 1A). In contrast, echinatin lacks a 5-(1,1-dimethy-2-propenyl) group (Fig. 7A). To understand the precise mechanism of licochalcone A, we examined the correlation of its structure and activity using licochalcone A and echinatin. It is interesting that licochalcone A significantly inhibited TNF-α-induced NF-κB activation, but echinatin had no effect on NF-κB activation (Fig. 7B), indicating that the 5-(1,1-dimethy-2-propenyl) group in licochalcone A is important for the inhibition of NF-κB. However, it is speculated that the importance of 1,1-dimethy-2-propenyl group in NF-κB inhibition might be due to enhanced hydrophobility.

To examine these functions without considering the cell permeability of each compound, IKK immunocomplexes were treated with licochalcone A, echinatin, or DMSO as a control in vitro. Whereas licochalcone A significantly inhibited TNF-α-induced IKK activation, echinatin had no effect on TNF-α-induced IKK activation even at high concentrations (Fig. 7C); therefore, it was confirmed that echinatin failed to inhibit TNF-α-induced IKK activation. As shown in Fig. 8, echinatin had no effect on TNF-α-induced expression and the production of inflammatory cytokines, such as CCL2/MCP-1 and CXCL1/KC. Taken together, the 1,1-dimethy-2-propenyl group in licochalcone A is required for NF-κB inhibition and anti-inflammatory effects.

Discussion

In the current study, we showed that licochalcone A significantly inhibited TNF-α-induced NF-κB activation through the inhibition of IKK activation. Licochalcone A is a major flavonoid isolated from the root of *G. inflata*. The several reports indicate that licochalcone A harbors potent anti-inflammatory effects; however, the detailed molecular mechanism of its anti-inflammatory activity has not been explored. TNF-α plays a pivotal role in immune and inflammatory responses by inducing many inflammatory cytokines. In addition, many previous studies have reported the essential role of the activation of mitogen-activated protein kinases and NF-κB in these TNF-α-induced cytokine expressions. Although licochalcone A effectively diminished TNF-α-induced inflammatory cytokine expression, it had no effect on TNF-α-induced activation of the mitogen-activated protein kinase family, c-Jun NH_2-terminal kinase, and p38 (data not shown). Therefore, the inhibition of cytokine expression by licochalcone A seems to have occurred from its specific inhibitory effects on NF-κB activation.

In the previous report, we also discovered an inhibitory effect of licochalcone A on LPS-induced NO production through the inhibition of NF-κB activation (Furusawa et al., 2009). In the LPS signaling pathway, licochalcone A specifically inhibits NF-κB activation by suppressing the phosphorylation of p65; however, it failed to inhibit LPS-induced activation of IKK complex. Our findings indicate that, in the LPS signaling pathway, licochalcone A markedly inhibited the phosphorylation of p65 at serine 276 and then reduced NF-κB transactivation by preventing the interaction of NF-κB p65 and p300 (Furusawa et al., 2009). In contrast, we

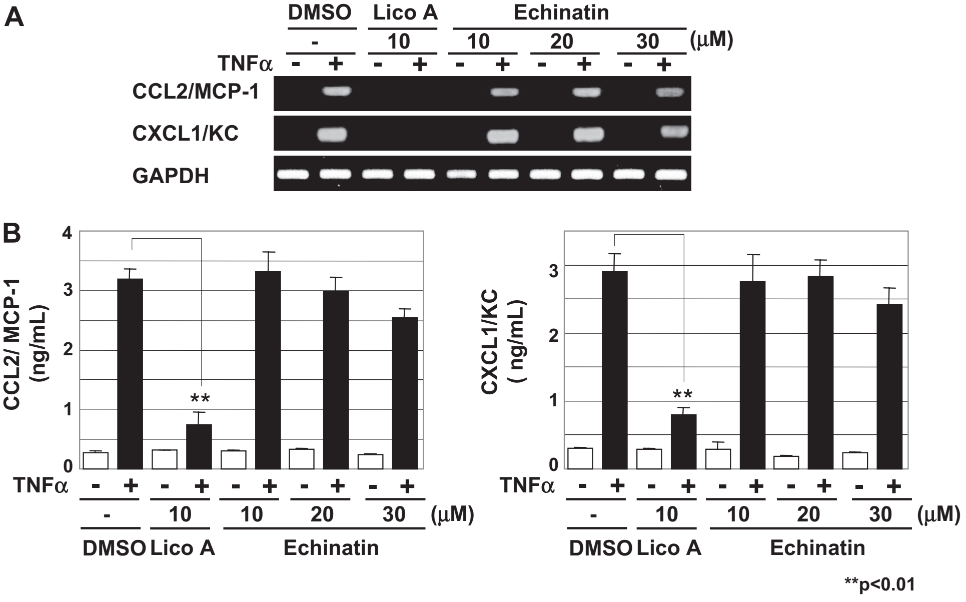

Fig. 8. Echinatin has no effect on TNF-α-induced expression and secretion of CCL2/MCP-1and CXCL1/KC. A, NIH-3T3 cells were pretreated with different concentrations of echinatin, licochalcone A (10 μM), or DMSO (0.1%) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for 2 h. Total RNA was used to perform RT-PCR with gene-specific primers to CCL2/MCP-1, CXCL1/KC, or GAPDH. B, NIH-3T3 cells were pretreated with different concentrations of echinatin, licochalcone A (10 μM), or DMSO (0.1%) for 1 h at 37°C after stimulation with TNF-α (10 ng/ml) for 14 h. CCL2/MCP-1 or CXCL1/KC in the cultured supernatants was measured with a commercial ELISA kit (R&D Systems). Data are shown as the mean ± S.D. of three independent experiments. **, $p < 0.01$.

showed that licochalcone A effectively inhibits TNF-α-induced activation of IKK complex, which is completely different from the LPS data. There is currently insufficient explanation of the different inhibitory mechanisms in the LPS signaling pathway and TNF-α signaling pathway.

IKKβ contains an N-terminal protein kinase domain and leucine zipper and helix-loop-helix motifs in its C-terminal half (Zandi et al., 1997). The cysteine 179 residue in the activation loop of IKKβ is known to be the target site for IKK inhibitors (Rossi et al., 2000). It has been reported that curcumin (diferuloylmethane) and butein (3,4,2,4-tetrahydroxychalcone) inhibited NF-κB activation through the direct inhibition of IKKβ via cysteine 179 (Jobin et al., 1999; Pandey et al., 2007). Curcumin is a naturally occurring product isolated from rhizomes of the plant *Curcuma longa*, and butein has been identified from numerous plants, including the stem bark of cashews (*Semecarpus anacardium*), the heartwood of *Dalbergia odorifera*, and traditional Chinese and Tibetan medicinal herbs *Caragana jubata* and *Rhus verniciflua* Stokes (Jobin et al., 1999; Pandey et al., 2007). In addition, Park et al. (2007) reported that melittin also exhibited inhibitory effects on NF-κB activation through direct interaction with IKKα and IKKβ.

It is interesting that we also observed that licochalcone A inhibited the activity of wild-type IKKβ but not an IKKβ mutant (C179A) (Fig. 5B), suggesting that cysteine 179 in IKKβ is also necessary for IKK inhibition by licochalcone A; however, we have no evidence supporting the direct interaction between IKKs and licochalcone A. In the current study, we observed that licochalcone A is also effective on immunoprecipitated IKK complex (Fig. 5A), suggesting that the target molecule of licochalcone A should be included in immunoprecipitated protein complex. Considering the different inhibitory mechanisms of licochalcone A in the LPS- and TNF-α-induced signaling pathways leading to NF-κB activation, this observation could be a valuable indication. Compared with signaling cascades stimulating IKK complexes, LPS and TNF-α use different signaling components. TNF-α-induced IKK activation requires the recruitment of several cytosolic proteins, RIP1 and TRAF2 to TNFRI, which are required for the recruitment and activation of IKK (Hsu et al., 1996; Mercurio et al., 1997). Furthermore, Liao et al. (2008) showed that polyubiquitination of RIP1 is required for the recruitment of IKK to TNFRI and IKK activation, and this is not observed in the LPS signaling pathway (Chow et al., 1999). However, licochalcone A had no effect on the ubiquitination of RIP1 (Fig. 4B) or on the TNF-α-induced recruitment of IKKβ to TNFRI (Fig. 4A), suggesting that the targeting molecule of licochalcone A should exist downstream of RIP1/TRAF2, maybe in the IKK complex. In addition, the different cells used in the two experiment systems could be another explanation. Whereas we used murine fibroblasts, NIH-3T3 cells, to examine TNF-α signaling in this study, a murine macrophage cell line, RAW264.7, was used to analyze the LPS signaling pathway, as shown previously. Although it has been reported that TNFRI is expressed ubiquitously (Heller et al., 1990; Ryffel et al., 1991), NF-κB activation was not induced when RAW264.7 cells were stimulated with TNF-α (data not shown). Our current results suggest that signaling molecules leading to NF-κB activation in the TNF-α signaling pathway might be deficient in cells and that this molecule could be an essential factor for exhibiting sensitivity against licochalcone A. DiDonato et al. (1997) purified a 900-kDa protein kinase complex harboring the ability to phosphorylate IκBα; therefore, it is expected that the IKK complex could consist of a number of unknown molecules in addition to IKKα, -β, -γ, and so on.

Licochalcone A is a 5-(1,1-dimethy-2-propenyl)-4,4′-dihydroxy-2-methoxy chalcone (Fig. 1A). To understand the precise mechanism of licochalcone A by examining the correlation of the structure and its activity, we compared the effect of echinatin, which is also contained in *G. inflata*. It is interesting that although licochalcone A significantly inhibited TNF-α-induced NF-κB activation, echinatin had no effect on the TNF-α signaling pathway. To consider the different effects of these compounds, log P of licochalcone A and echinatin was calculated by Spartan'04 (Wavefunction, Inc., Irvine, CA). Log P is an index showing the hydrophobility of chemical compounds and the calculated log P were 4.71 and 2.92, respectively. Thus, it is easily speculated that echinatin had difficulty penetrating the cell to exhibit its activity because of its low hydrophobicity. However, when the effect of echinatin on IKK activity was examined in vitro, IKK activity was not inhibited by the addition of echinatin (Fig. 7C). These data clearly showed that echinatin was not able to inhibit IKK activation and that the 1,1-dimethy-2-propenyl group is required for IKK inhibition. Because NF-κB plays a central role in inflammation, a study of the compounds related to licochalcone A would provide clues to develop more specific therapeutic drugs against inflammatory diseases.

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