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Licochalcone A potently inhibits TNFα-induced NF-κB activation through the direct inhibition of IKK activation

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non-standard abbreviations: DMSO, dimethyl sulfoxide, ELISA, enzyme-linked immunosorbent assay, FBS, fetal bovine serum, IκBα, inhibitor of NF-κB, IKK, IκB kinase, IL-1, interleukin 1, IL-6, interleukin-6, MCP-1, monocyte chemotactic protein-1, NF-κB, nuclear factor κB, PAGE, polyacrylamide gel electrophoresis, PBS, phosphate-buffered saline, PGE2, prostaglandin, PVDF, polyvinylidene difluoride, RIP1 receptor-interacting protein 1, SDS, sodium dodecyl sulfate, TGE, Tris-glycine-EDTA, TNFα, tumor necrosis factor alpha, TNFRI, tumor necrosis factor receptor I, TRADD, tumor necrosis factor receptor-associated death domain, TRAF2, tumor necrosis factor receptor-associated factor 2, TRAF6, tumor necrosis factor receptor-associated factor 6

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 *Glycyrrhiza inflata*. Here, we found that Licochalcone A strongly inhibited tumor necrosis factor α (TNF α)-induced nuclear localization, DNA binding activity and the transcriptional activity of nuclear factor kappB (NF-κB). Whereas Licochalcone A had no effect on the recruitment of receptor-interacting protein 1 (RIP1) and IκB kinase β (IKKβ) to TNF receptor I (TNFRI) by TNFα, it significantly inhibited TNFα-induced IκB kinase (IKK) activation and IκBα degradation. Interestingly, we found that the cysteine residue at position 179 of IKKβ is essential for Licochalcone A-induced IKK inhibition, since Licochalcone A failed to affect the kinase activity of the IKKβ (C179A) mutant. On the other hand, a structurally related compound, Echinatin, failed to inhibit TNFα -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, TNFα-induced expression of inflammatory cytokines, CCL2 /MCP-1 and CXCL1/KC, was clearly inhibited by Licochalcone A but not Echinatin. Taken together, Licochalcone A might contribute to the potent anti-inflammatory effect of *Glycyrrhiza inflata* through the inhibition of IKK activation.

Introduction

Tumor necrosis factor α (TNFα) 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). TNFα is a pleiotropic proinflammatory cytokine with a wide range of biological effects. TNFα participates in the inflammatory effect by inducing various inflammatory cytokines, including CCL2/monocyte chemotactic protein-1 (MCP-1), CXCL1/ KC and interleukin-6 (IL-6), through the activation of a transcription factor, nuclear factor kappa B (NF-κB) (Mukaida et al., 1990; Zhang et al., 1990; Ping et al., 1999). Therefore, dysregulated TNFα 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 5 members: RelA/p65, RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52 (Hayden and Ghosh, 2008). The NF-kappaB p50/p65 heterodimer is a typical member of the Rel family of transcription factors which regulate diverse cellular functions, such as immune response, cell growth, and development. In the canonical NF-κB pathway, TNFα activates the IκB kinase (IKK) complex, which 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; Zandi et al., 1997; Mercurio et al., 1997; Yamaoka et al., 1998). Activated IKKs phosphorylate IκBs, leading to their ubiquitination and proteasomal 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 TNFα signaling pathway have been identified and the signal transduction mechanism leading to NF-κB activation has been fully understood. As with other signaling ligands, TNFα 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 appears to be the key mediator of TNFα signaling in the majority of cells (Ryffel et al., 1991). Binding of TNFα 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 2 (TRAF2) (Rothe et al., 1994; Hsu et al., 1996; Liu et al., 1996). Consequently, it activates the downstream IKK complex, leading to NF-κB activation (DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997; Yamaoka et al., 1998, Palombella et al., 1994; DiDonato et al., 1996).

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), anti-leishmanial activity and anti-microbial activity, inhibiting the growth of *Staphylococcus aureus* and the activity of *Helicobactor 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 (PG) E2 and interleukin 1 (IL-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 TNFα signaling pathway. Interestingly, we observed that Licochalcone A significantly inhibited TNFα-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 TNFα-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 TNFα was purchased from PEPROTECH (Rocky Hill, NJ, USA). Antibodies recognizing p65, lamin, IκBα, TNFRI, IKKα, IKKβ, IKKγ, TRADD, TRAF2, β-actin and c-Myc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against RIP1 was purchased from BD Transduction Laboratories (Lexington, NY, USA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse polyclonal IgG antibodies were purchased from Dako (Dako-Japan, Tokyo).

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, NY, USA). Mutagenesis of amino acid residues in IKKβ C179A was performed using a site-directed mutagenesis kit (Stratagene, CA, USA).

Cell culture

NIH-3T3 stably expressing the NF-κB-dependent luciferase reporter plasmid (KF8) was established as previously described (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 5 repeats of the NFκB transcriptional response element (GGGGACTTTCCC) (Stratagene). NIH-3T3 cells, KF8 cells and HEK293T cells were grown in DMEM medium (Nissui, Tokyo, Japan) supplemented

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

Electrophoretic Mobility Shift Assay (EMSA)

Consensus double-strand oligo-deoxynucleotide probes for NF-κB (TAGTTGAGGGGACTTTCCCAGGC) were radioactively labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase, as described previously (Funakoshi-Tago et al., 2003). Then, 2 μg 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% polyacrylamide gel electrophoresis in TGE (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 hr at 37°C. After treatment with TNFα (10 ng/mL) for 5 hr, 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, Madison, WI, USA), 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 (5X 10^4) were preincubated with various concentrations of Licochalocne A for 1 hr at 37°C. After treatment with TNFα (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 Inc., CA, USA).

Immunofluorescence Assay

NIH-3T3 cells (5×10^5 cells) were seeded on sterile coverslips in a 6-well plate and pretreated with Licochalcone A (20 μM) for 1hr following stimulation with TNFα (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 TritonX-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) diluted with PBS containing 3% FBS at 1:200 dilution for 1hr at room temperature. After washing with PBS three times, the coverslips were incubated with a secondary antibody BODIPY FL® anti-rabbit IgG (Invitrogen, OR, USA) at 1:200 dilution for 1hr at room temperature. After washing with PBS three times, the coverslips were mounted using VECTA SHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Each coverslip was analyzed on an OLYMPUS BX50 microscope (Olympus, Tokyo, Japan) with Olympus 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% TritonX-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 r.p.m, 4°C for 15 min to remove the debris and the protein concentration was determined by Bradford assay. Eluted proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were probed using the designated antibodies and visualized with the ECL detection system (Amersham), 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 pCMV5-Myc-IKKβ or pCMV5-Myc-IKKβ (C179A) using Fugene 6 (Roche Diagnostics, IN, USA). NIH-3T3 cells (1×10^7 cells) were preincubated with Licochalcone A (20 μM) for 1 hr at 37°C following stimulation with TNFα (10 ng/mL) for the indicated times. Cell lysates were immunoprecipitated with anti-Myc antibody or anti-IKKγ antibody with Protein G Sepharose (Zymed) for 2 hr 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 kinase buffer including [γ-^{32}P] ATP was carried out with 1 μg GST-IκBα as a substrate for 20 min at 30°C. Samples were resolved by SDS-PAGE and phosphorylated GST-IκBα was visualized by autoradiography, as described previously (Funakoshi-Tago et al., 2003).

RNA Isolation and Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was prepared using an RNA purification kit (Qiagen, Tokyo, Japan). RT was performed using an oligo (dT) $_{20}$ primer and 1 μg total RNA for first-strand cDNA synthesis, as described previously (Funakoshi-Tago et al., 2003). PCR was performed at an annealing temperature of 57°C with 22 amplification cycles. PCR products were resolved and electrophoresed in a 1.5% agarose gel in TAE (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); CXCL1/KC, 5'-TGGGGACACCTTTTAGCATC-3' (upstream) and 5'-GCCCATCGCCAATGAGCTG-3' (downstream) (Funakoshi-Tago et al., 2003).

Enzyme-linked immunosorbent assay (ELISA)

Cells (5×10^4 cells) were cultured in a 24-well plate and pretreated with chalcones (10 μM) for 1 hr at 37°C. After treatment with TNFα (10 ng/mL) for 24 hr, the supernatants were harvested and the amounts of CCL2/MCP-1 and CXCL1/KC were determined using immunoassay kits (R&D Systems) (Funakoshi-Tago et al., 2008).

Purification of His_6-tagged ubiquitin conjugates

HEK293T cells (1×10^5 cells) were cotransfected with 1 μg pCMV5 or pCMV5-RIP1 and 1 μg His_6-tagged ubiquitin expression vector using FuGENE6 (Roche Diagnostics) according to the manufacturer's protocol. To purify His_6-tagged ubiquitinated proteins, 10% 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, WA, USA) 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 TNFα-induced DNA binding activity of NF-κB.

Licochalcone A is a major component of *Glycyrrhiza inflata* (Fig. 1 A) (Hatano et al., 1988; Shibata, 2000). To investigate the anti-inflammatory effect of Licochalcone A, we evaluated its effect on TNFα-induced NF-κB activation. First, we investigated its effect on NF-κB DNA binding activity by EMSA using nuclear extracts. When NIH-3T3 cells were treated with various concentrations of Licochalcone A for 1 hr following TNFα stimulation for 30 min, Licochalcone A inhibited TNFα-induced DNA binding activity of NF-κB in a dose-dependent manner. On the other hand, 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 super-shifted protein-DNA complexes, indicating that this complex includes p65, which could be involved in two active NF-κB complexes. On the other hand, antibodies against p50 and c-Rel failed to exhibit super-shift activity (data not shown). As shown in Fig. 1D, cell viability was not changed by treatment with Licochalcone A and/or TNFα stimulation, indicating that the inhibition of NF-κB activation by Licochalcone A is not attributable to its cytotoxicity.

Licochalcone A significantly inhibited TNFα-induced nuclear localization and transcriptional activity of NF-κB.

Next, we investigated the effect of Licochalcone A on the TNFα-induced nuclear translocation of NF-κB using nuclear extracts. As shown in Fig. 2A, TNFα induced the

accumulation of the p65 NF-κB subunit in the nucleus at 15 min. Strikingly, Licochalcone A significantly decreased the amount of nuclear NF-κB after TNFα stimulation. In addition, we performed immunofluoresence staining to investigate the effect of Licochalcone A on the translocation of p65 NF-κB subunit. While most NF-κB p65 was translocated into the nucleus after TNFα stimulation in cells treated with DMSO, Licochalcone A clearly inhibited the TNFα-induced translocation of NF-κB p65 (Fig. 2B).

Furthermore, we evaluated the effect of Licochalcone A on NF-κB transcriptional activation using KF 8 cells, which stably express the NF-κB luciferase reporter gene, as previously reported (Funakoshi-Tago et al., 2008). As shown in Fig. 2C, TNFα induced NF-κB transcriptional activation up to about 12 times more than in unstimulated cells. Strikingly, Licochalcone A potently inhibited TNFα-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.

Licochalcone A significantly inhibited TNFα-induced IKK activation and IκBα degradation.

To determine how Licochalcone A inhibited NF-κB activation induced by TNFα, we investigated its effect on the expression levels of major signaling molecules, which are required for the TNFα signaling pathway. Upon TNFα stimulation, a serine/threonine kinase, RIP1, and adaptor molecules, TRADD and TRAF2, were recruited to TNFRI (Rothe et al., 1994; Hsu et al., 1995; Hsu et al., 1996; Liu et al., 1996). Consequently, NF-κB is rapidly activated through activation of the IKK complex (DiDonato et al.,

1997; Zandi et al., 1997; Mercurio 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 TNFα 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 TNFα signaling pathway.

Since IKK activation is a key step in NF-κB activation (DiDonato et al., 1997; Zandi et al., 1997; Mercurio et al., 1997; Yamaoka et al., 1998), we next determined whether Licochalcone A inhibits TNFα-induced IKK activation by *in vitro* kinase assay. Cells were pretreated with Licochalcone A or DMSO as a control following TNFα 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 TNFα-induced IKK activation. Furthermore, when the degradation of IκBα after TNFα stimulation was examined by immunoblotting, Licochalcone A potently inhibited TNFα-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 TNFα 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, as shown in Fig. 3B, we also examined whether Licochalcone A might affect the formation of TNFRI complex, including RIP1 and IKKs, however, a co-immunoprecipitation assay revealed that RIP1 and IKKβ were associated with TNFRI in a TNFα-dependent manner, even in cells treated with Licochalcone A (Fig. 4A).

A previous study showed that RIP1 is poly-ubiquitinated after TNFα stimulation and its poly-ubiquitination is required for not only the recruitment but also the activation of IKK complex (Liao et al., 2008), therefore, we next investigated whether the TNFα-induced ubiquitination of RIP1 is affected by Licochalcone A. In this system, poly-ubiquitination of RIP1 was induced by co-expression of His_6-ubiquitin, however, Licochalcone A had no effect on the ubiquitination of RIP1, as shown in Fig. 4B. Therefore, it is suggested that Licochalcone A inhibits TNFα-induced IKK activation without abrogating its recruitment to TNFRI.

Cysteine 179 in KKβ 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 TNFα-stimulated cells with anti-IKKγ antibody. After being precipitated, immunocomplexes were treated with Licochalcone A or DMSO as a control. Whereas DMSO had no effect on TNFα-induced IKK activation, Licochalcone A significantly inhibited TNFα-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. Interestingly, 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 findings 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 TNFα-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 TNFα. When cells were stimulated with TNFα, marked expressions of CCL2/MCP-1 mRNAs and CXCL1/KC mRNA were induced at 2 hr and detected until 4 hr. On the other hand, treatment with Licochalcone A significantly inhibited the TNFα-induced expression of CCL2/MCP-1 and CXCL1/KC (Fig. 6A). Consistently, Licochalcone A potently reduced the TNFα-induced secretion of CCL2/MCP-1 and CXCL1/KC, as shown in Fig. 6B. These data suggest that Licochalcone A shows anti-inflammatory activity by inhibiting the expression of various TNFα-induced inflammatory cytokines.

Echinatin failed to inhibit TNFα-induced IKK activation and NF-κB activation.

Glycyrrhiza 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, as shown in Fig. 1A. On the other hand, Echinatin lacks a 5- (1, 1-dimethy-2-propenyl) group, as shown in Fig. 7A. To understand the precise mechanism of Licochalcone A, we examined the correlation of its structure and activity using Licochalcone A and Echinatin. Interestingly, whereas Licochalcone A significantly inhibited TNFα-induced NF-κB activation, Echinatin had no effect on NF-κB activation (Fig. 7B), therefore 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 TNFα-induced IKK activation, Echinatin had no effect on TNFα-induced IKK activation even at high concentrations (Fig. 7C), therefore, it was confirmed that Echinatin failed to inhibit TNFα-induced IKK activation. As shown in Fig. 8, Echinatin had no effect on TNFα-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 TNFα-induced NF-κB activation through the inhibition of IKK activation. Licochalcone A is a major flavonoid isolated from the root of *Glycyrrhiza 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. TNFα 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 MAP kinases and NF-κB in these TNFα-induced cytokine expressions. Although Licochalcone A effectively diminished TNFα-induced inflammatory cytokine expression, it had no effect on TNFα-induced activation of the MAP kinase family, JNK 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). On the other hand, we showed that Licochalcone A effectively inhibits TNFα-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 TNFα signaling pathway.

IKKβ contains an N-terminal protein kinase domain and leucine zipper (LZ) and helix-loop-helix (HLH) 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). Previously, it has been reported that Curcumin and Butein inhibited NF-κB activation through the direct inhibition of IKKβ via cysteine 179 (Jobin et al., 1999; Pandey et al., 2007). Curcumin (diferuloylmethane) is a naturally occurring product isolated from rhizomes of the plant *Curcuma longa*, and Butein (3,4,2,4-Tetrahydroxychalcone) 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). Additionally, Park et al. reported that melittin also exhibited inhibitory effects on NF-κB activation through direct interaction with IKKα and IKKβ (Park et al., 2007).

Interestingly, 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 TNFα-induced signaling pathways leading to NF-κB activation, this observation could be a valuable indication. When compared with signaling cascades stimulating IKK complexes, LPS and TNFα utilize different signaling components. TNFα-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. showed that

poly-ubiquitination of RIP1 is required for the recruitment of IKK to TNFRI and IKK activation (Liao et al., 2008), 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 TNFα-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. Additionally, the different cells used in the two experiment systems could be another explanation. Whereas we utilized murine fibroblasts, NIH-3T3 cells, to examine TNFα 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 TNFα (data not shown). Our current results suggest that signaling molecules leading to NF-κB activation in the TNFα signaling pathway might be deficient in cells and this molecule could be an essential factor for exhibiting sensitivity against Licochalcone A. DiDonato et al. purified a 900 kDa protein kinase complex harboring the ability to phosphorylate IκBα (DiDonato et al., 1997), 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, shown in 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 *Glycyrrhiza inflata*. Interestingly, whereas Licochalcone A significantly inhibited TNFα-induced NF-κB activation, Echinatin had no effect on the TNFα signaling pathway. To consider the different effects of these compounds, log *P* of Licochalcone A and Echinatin was calculated by Spartan '04. 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, as shown in Fig. 7C. These data clearly showed that Echinatin was not able to inhibit IKK activation and the 1, 1-dimethy-2-propenyl group is required for IKK inhibition. Since 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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Footnotes

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Figure legends

Fig. 1. Licochalcone A potently inhibits TNFα-induced NF-κB activation.

(A) Structure of Licochalcone A. LicoA indicates Licochalcone A. (B) NIH-3T3 cells were pretreated with different concentrations of Licochalcone A or DMSO (0.1%) for 1 hr at 37°C following stimulation with TNFα (10 ng/mL) for 30 min and nuclear extracts were prepared. NF-κB DNA binding activity was measured by EMSA with a radiolabeled probe containing a consensus NF-κB binding site (5'-AGTTGAGGGGACTTTCCCAGG-3'). (C) For supershift assay, nuclear extracts from cells treated with TNFα (10 ng/mL) for 30 min were incubated in the presence of 1 μg anti-p65 antibody. (D) NIH-3T3 cells (5X 104) were preincubated with various concentrations of Licochalocne A for 1 hr at 37°C. After treatment with TNFα (10 ng/mL) for 12 hr, cell viability was analyzed by trypan blue exclusion tests using a cell viability analyzer (Beckman Coulter Inc., CA, USA).

Fig. 2. Licochalcone A potently inhibits TNFα-induced nuclear translocation of NF-κB p65.

(A) NIH-3T3 cells were pretreated with Licochalcone A (20 μM) or DMSO (0.1%) for 1 hr at 37°C following stimulation with TNFα (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 hr at 37°C following stimulation with TNFα (10 ng/mL) for 30 min. Cells were fixed in 4% paraformaldehyde and the localization of p65 was visualized with an antibody (green).

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 hr at 37°C following stimulation with TNFα (10 ng/mL) for 5 hr. NF-κB-dependent luciferase activity was normalized to the protein amounts. Data are the mean ± SD of the relative luciferase activities of pNF-κB-Luc in four independent experiments. *** indicates $p<0.001$.

Fig. 3. Licochalcone A significantly inhibits TNFα-induced IKK activation and IκBα degradation.

NIH-3T3 cells were pretreated with Licochalcone A (20 μM) or DMSO (0.1%) for 1 hr at 37°C following stimulation with TNFα (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 (upper). Immunoprecipitates were immunoblotted with anti-IKKγ antibody (middle). The gel was stained with CBB (Coomassie brilliant blue) (lower). (C) Whole cell lysates were immunoblotted with antibodies recognizing IκBα or β-actin as a control.

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 hr at 37°C following stimulation with TNFα (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 hr after transfection, cells were pretreated with Licochalcone A (20 μM) or DMSO (0.1%) for 1 hr at 37°C. The proteins from cell lysates were affinity purified on nickel resin in buffer containing 8 M urea and electrophoretically separated on denaturing gels containing SDS, and were immunoblotted with anti-RIP1 antibody. Whole cell lysates prepared with lysis buffer, as described in Materials and Methods, were subjected to immunoblotting with anti-RIP1 antibody (upper). Whole cell lysates were immunoblotted with anti-RIP1 antibody (bottom).

Fig. 5. Cysteine 179 in KKβ was involved in its inhibition by Licochalcone A .

(A) NIH-3T3 cells were stimulated with TNFα (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 hr at 37°C and then assayed for kinase activity using purified GST-IκBα as a substrate (upper). Immunoprecipitates were immunoblotted with anti-IKKγ antibody (middle). The gel was stained with CBB (Coomassie brilliant blue) (lower). (B) HEK293T cells were transfected with the indicated plasmids encoding Myc-IKKβ or Myc-IKKβ mutant (C179A). At 48 hr 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 hr at 37°C and assayed for kinase activity using purified GST-IκBα as a substrate (upper). Immunoprecipitates were immunoblotted with anti-IKKγ antibody (middle). The gel was stained with CBB (lower).

Fig. 6. Licochalcone A significantly inhibits TNFα-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 hr at 37°C following stimulation with TNFα (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 hr at 37°C following stimulation with TNFα (10 ng/mL) for 14 hr. 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 ± SD of three independent experiments. ** indicates $p<0.01$.

Fig. 7. Echinatin fails to inhibit TNFα-induced IKK activation and NF-κB activation.

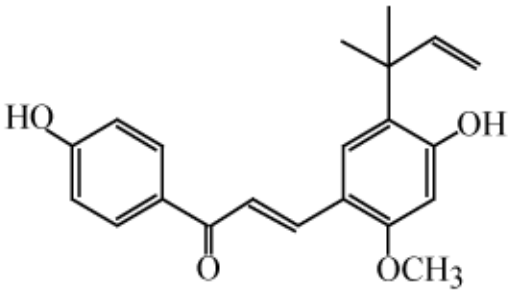
(A) Structure of Echinatin (B) NIH-3T3 cells expressing pNF-κB-Luc (KF8 cells) were pretreated with different concentrations of Echinatin, 10 μM Licochalcone A or DMSO (0.1%) for 1 hr at 37°C following stimulation with TNFα (10 ng/mL) for 5 hr. NF-κB-dependent luciferase activity was normalized to the protein amounts. Data are the mean ± SD of the relative luciferase activities of pNF-κB-Luc in four independent experiments. *** indicates $p<0.001$. (C) NIH-3T3 cells were stimulated with TNFα (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 hr at 37°C and then assayed for kinase activity using purified GST-IκBα as a substrate (upper). Immunoprecipitates were immunoblotted with anti-IKKγ antibody (middle). The gel was stained with CBB (Coomassie brilliant blue) (lower).

Fig. 8. Echinatin has no effect on TNFα-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 hr at 37°C following stimulation with TNFα (10 ng/mL) for 2 hr. 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 hr at 37°C following stimulation with TNFα (10 ng/mL) for 14 hr. 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 ± SD of three independent experiments. ** indicates $p<0.01$.

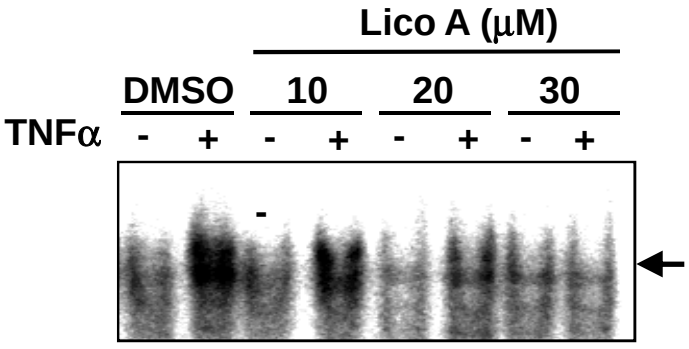
Fig.1

A **Licochalcone A (Lico A)**

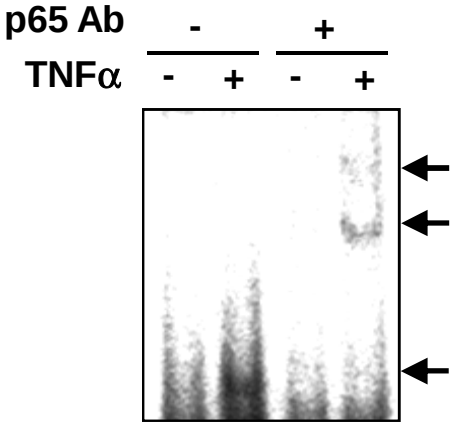


5- (1,1-dimethy-2-propenyl) -4, 4'-dihydroxy-2-methoxy chalcone

B



C



D

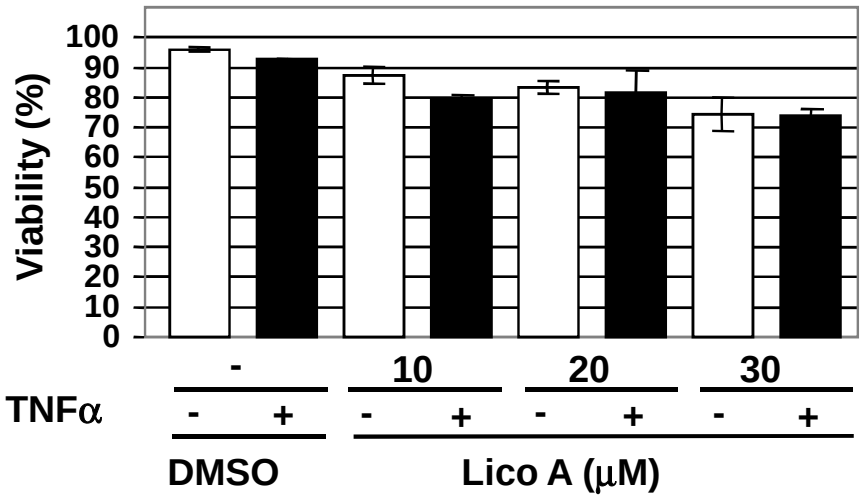
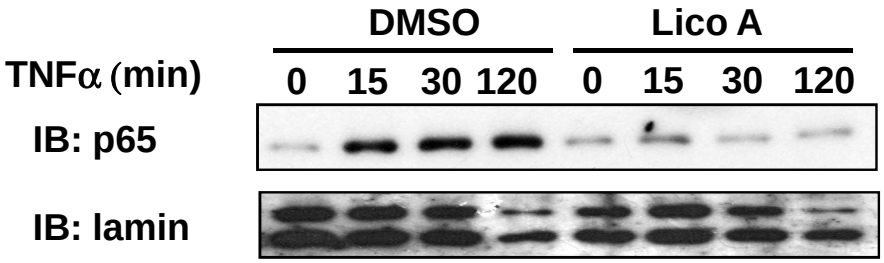
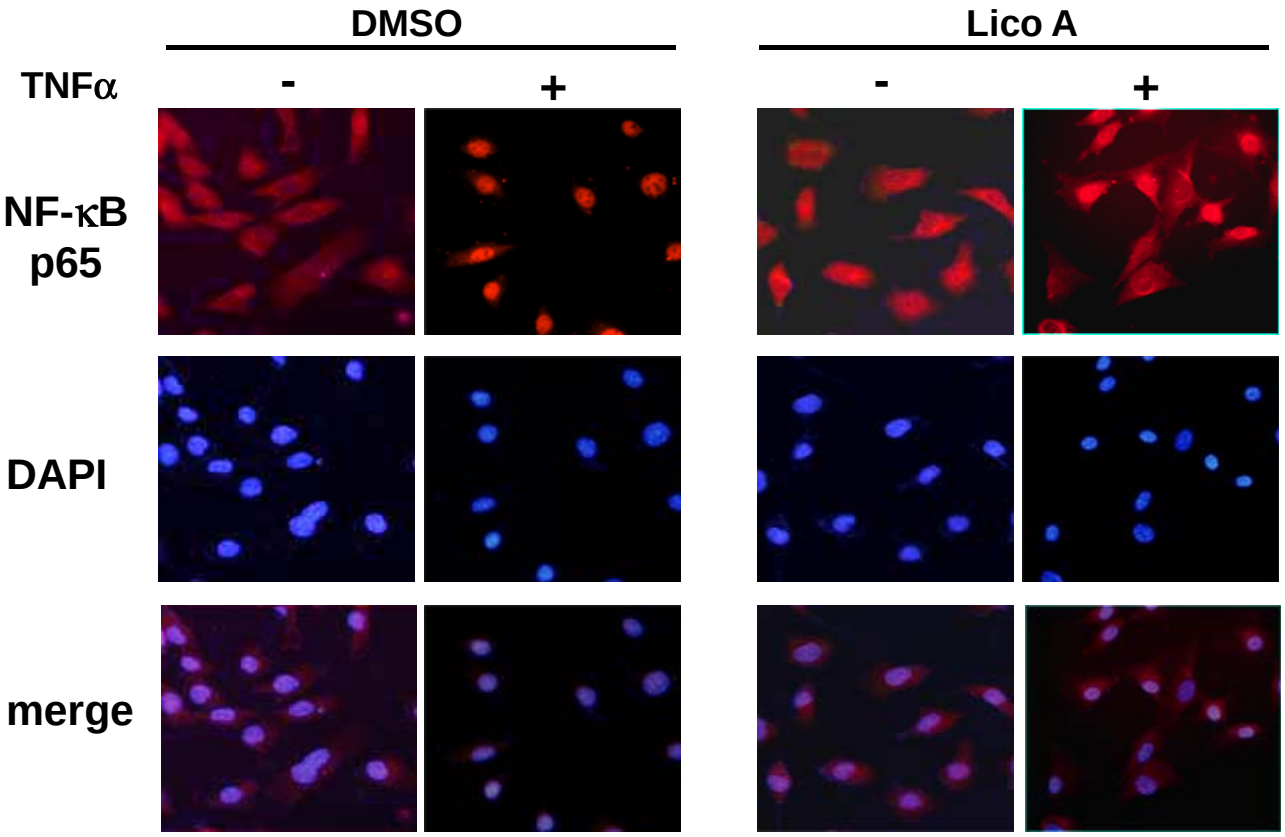


Fig. 2


DMSO
Lico A
TNFα (min)
0 15 30 120 0 15 30 120
IB: p65
IB: lamin


DMSO
Lico A
TNFα
- + - +
NF-κB p65
DAPI
merge

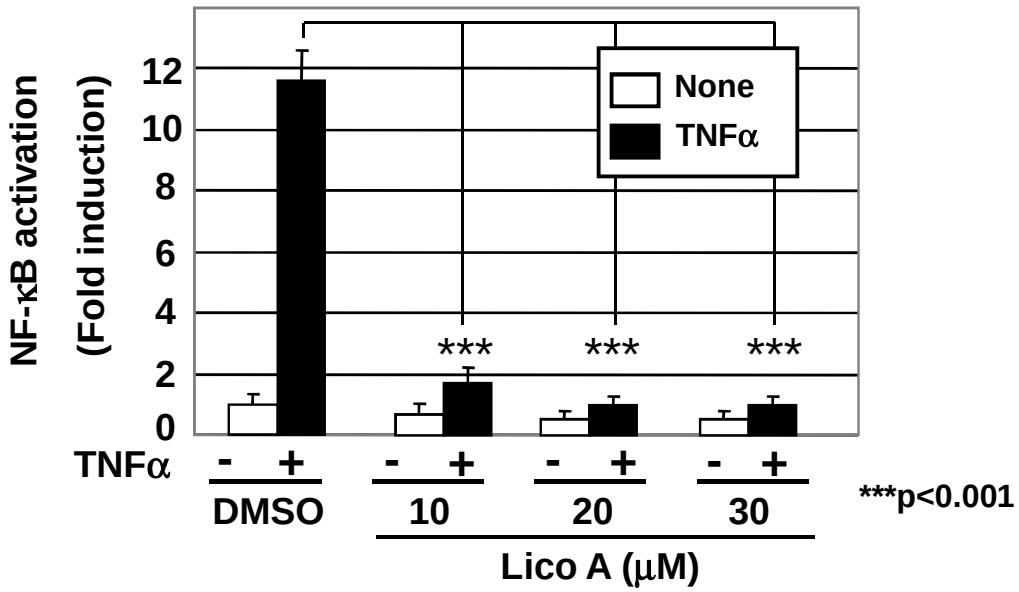
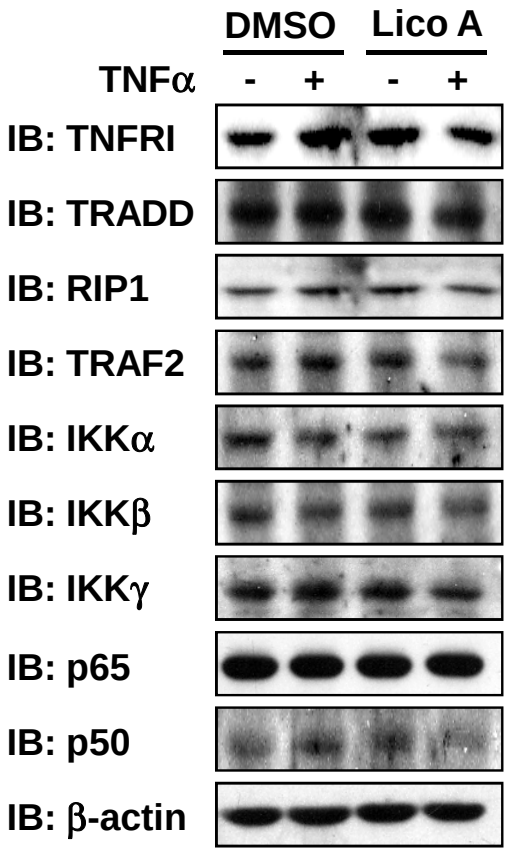
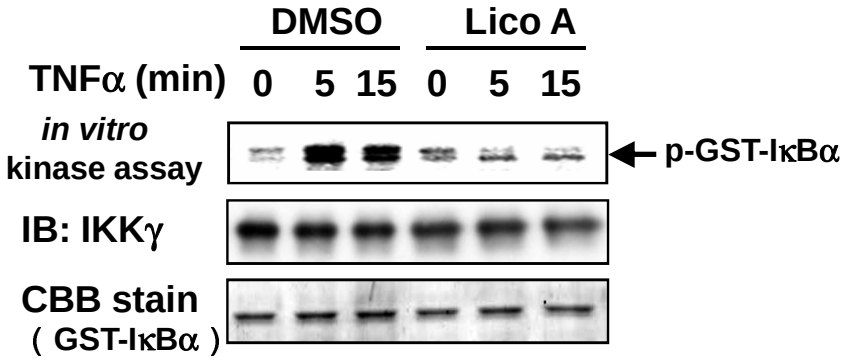

NF-κB activation (Fold induction)
12
10
8
6
4
2
0
None
TNFα
***
***
***
TNFα
- + - + - + - +
DMSO
10
20
30
Lico A (μM)
***p<0.001

Fig.3

A


DMSO
Lico A
TNFα
- + - +
IB: TNFRI
IB: TRADD
IB: RIP1
IB: TRAF2
IB: IKKα
IB: IKKβ
IB: IKKγ
IB: p65
IB: p50
IB: β-actin

B


DMSO
Lico A
TNFα (min) 0 5 15 0 5 15
in vitro kinase assay
p-GST-IκBα
IB: IKKγ
CBB stain
(GST-IκBα)

C

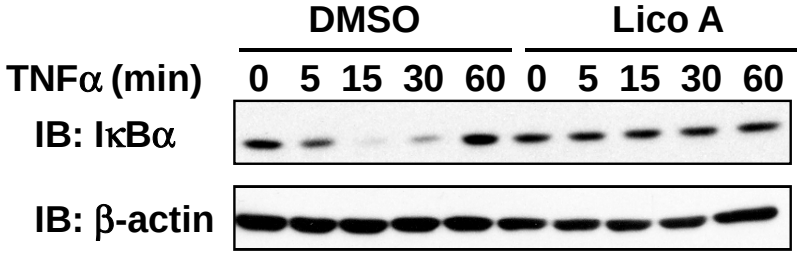

DMSO
Lico A
TNFα (min) 0 5 15 30 60 0 5 15 30 60
IB: IκBα
IB: β-actin

Fig.4

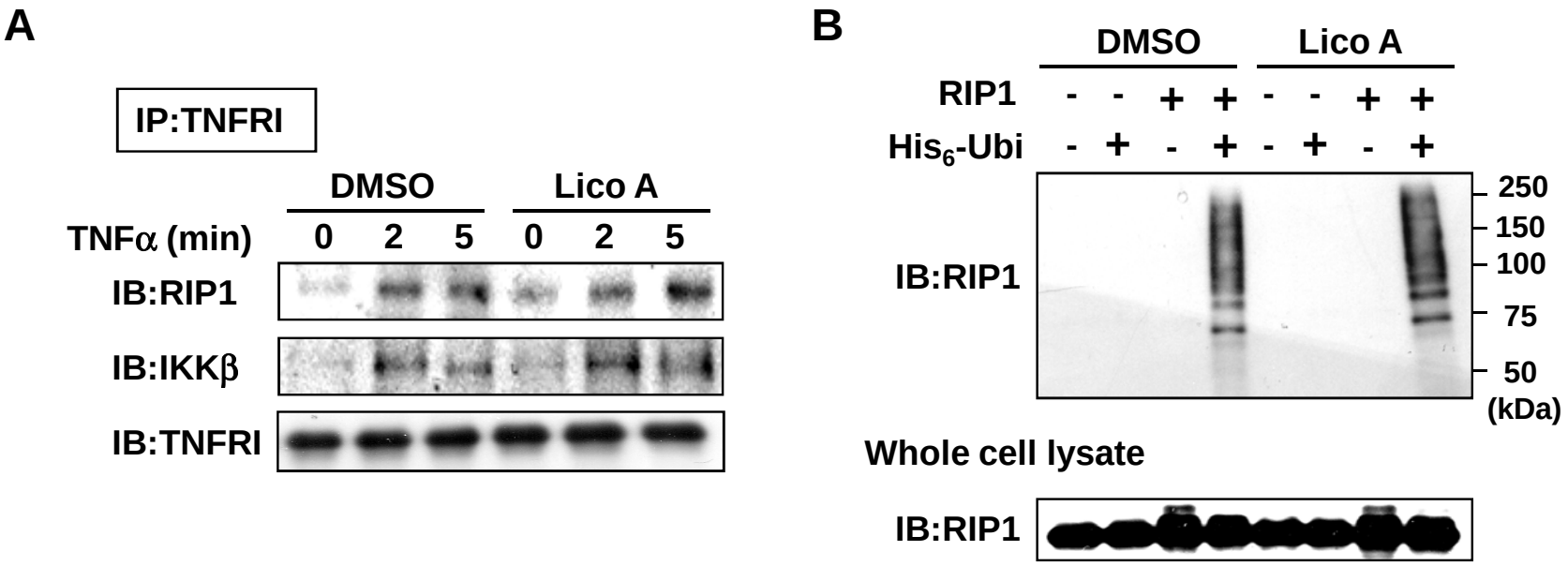

A
IP:TNFRI
DMSO
Lico A
TNFα (min)
0 2 5 0 2 5
IB:RIP1
IB:IKKβ
IB:TNFRI
B
DMSO
Lico A
RIP1
- - + + - - + +
His6-Ubi
- + - + - + - +
IB:RIP1
250
150
100
75
50
(kDa)
Whole cell lysate
IB:RIP1

Fig.5

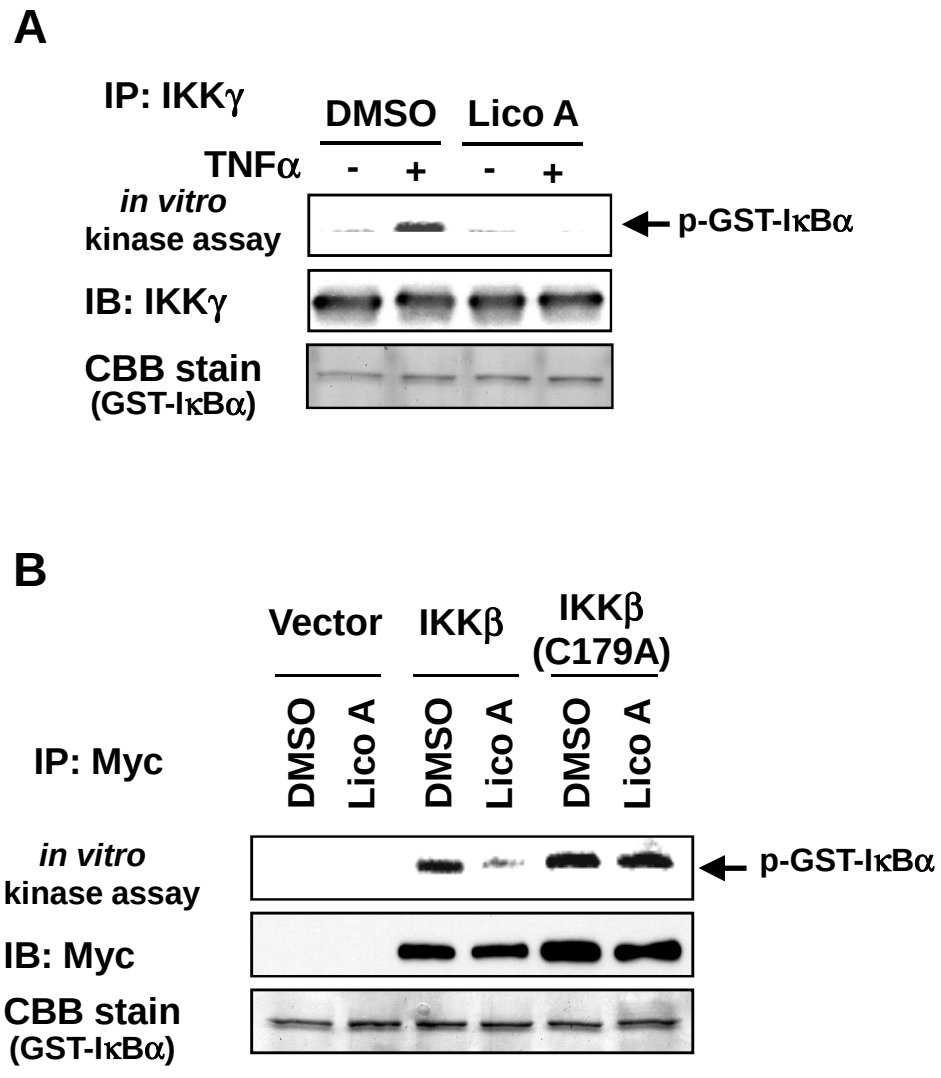
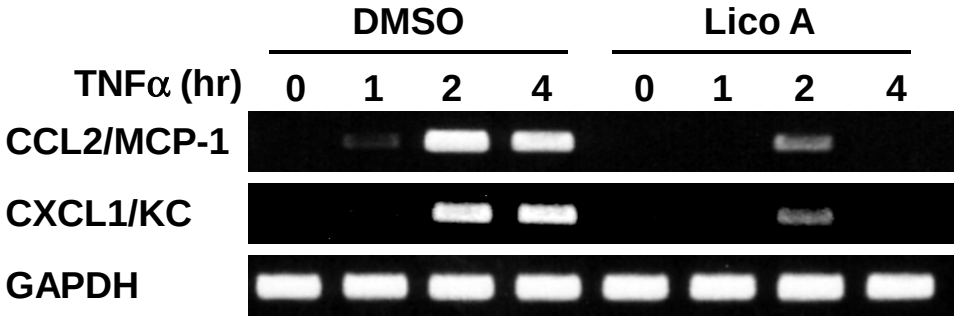
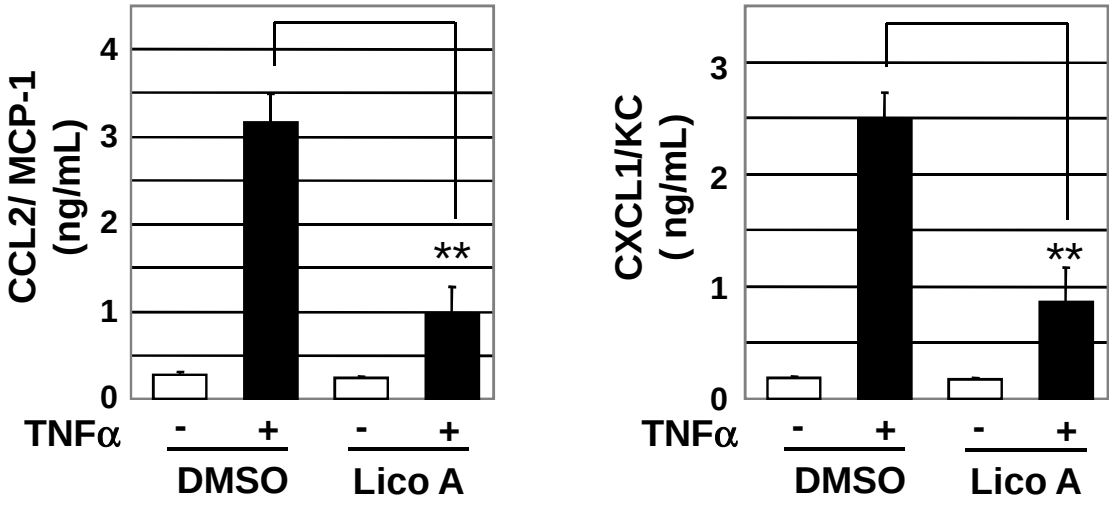

A
IP: IKKγ
DMSO
Lico A
TNFα
- + - +
in vitro
kinase assay
p-GST-IκBα
IB: IKKγ
CBB stain
(GST-IκBα)
B
Vector
IKKβ
IKKβ
(C179A)
DMSO
Lico A
DMSO
Lico A
DMSO
Lico A
IP: Myc
in vitro
kinase assay
p-GST-IκBα
IB: Myc
CBB stain
(GST-IκBα)

Fig.6

A


DMSO
Lico A
TNFα (hr)
0 1 2 4 0 1 2 4
CCL2/MCP-1
CXCL1/KC
GAPDH

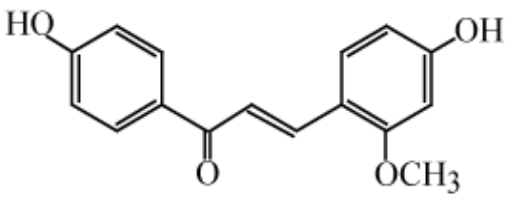
B


CCL2/ MCP-1 (ng/mL)
4
3
2
1
0
**
TNFα - + - +
DMSO
Lico A
CXCL1/KC (ng/mL)
3
2
1
0
**
TNFα - + - +
DMSO
Lico A

**p<0.01

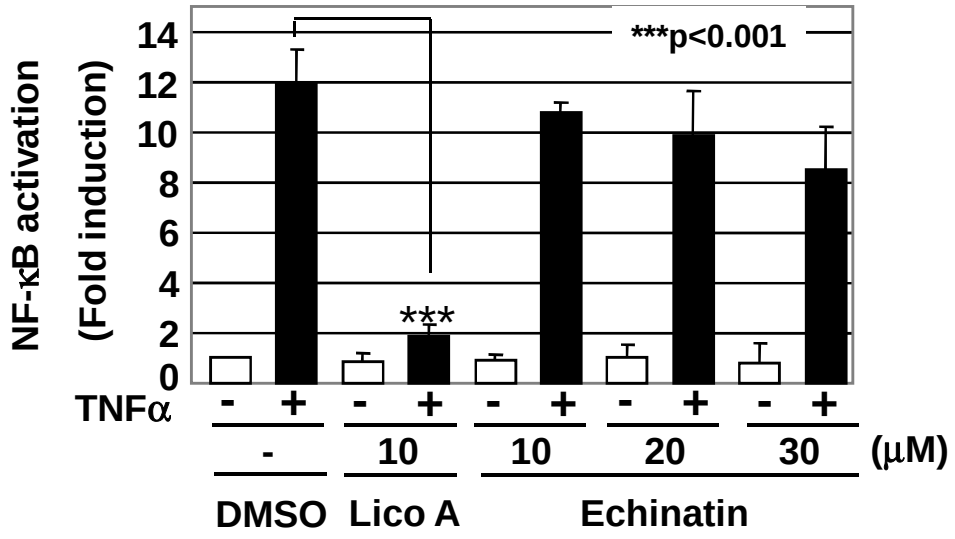
Fig.7

A



Echinatin

B



C

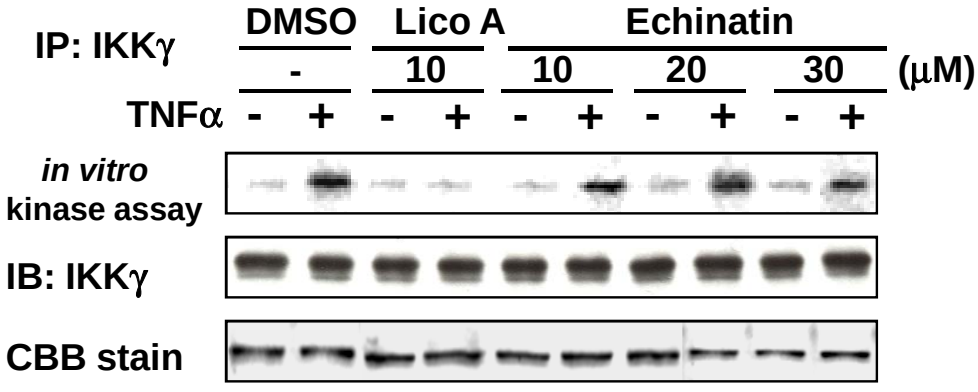
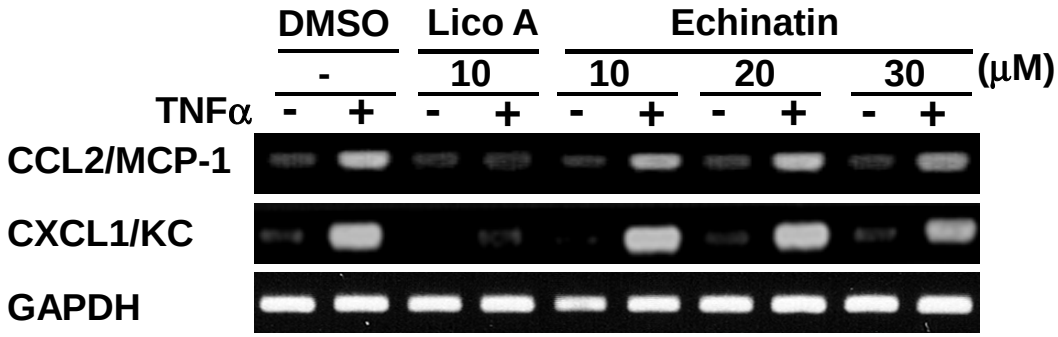
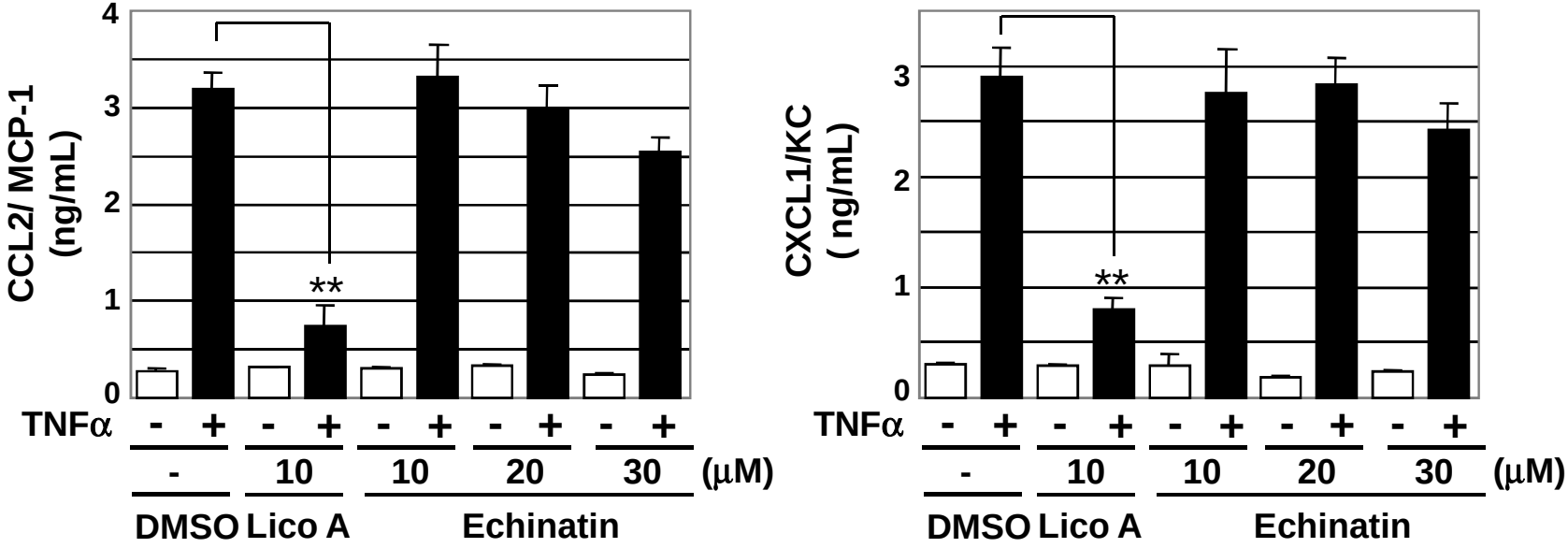


Fig.8

A


DMSO
Lico A
Echinatin
-
10
10
20
30
(μM)
TNFα - + - + - + - + - +
CCL2/MCP-1
CXCL1/KC
GAPDH

B


CCL2/ MCP-1 (ng/mL)
4
3
2
1
0
**
TNFα - + - + - + - + - +
-
10
10
20
30
(μM)
DMSO
Lico A
Echinatin
CXCL1/KC (ng/mL)
3
2
1
0
**
TNFα - + - + - + - + - +
-
10
10
20
30
(μM)
DMSO
Lico A
Echinatin

**p<0.01