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Fructus *Ligustrum lucidi* inhibits inflammatory mediator release through inhibition of nuclear factor-κB in mouse peritoneal macrophages

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Abstract

Fructus *Ligustrum lucidi* (FLL) is a widely used herbal medicine for the treatment of a variety of pathologies. We have investigated the anti-inflammatory mechanism of FLL in mouse peritoneal macrophages. FLL exerted an anti-inflammatory action through inhibition of lipopolysaccharide (LPS)-induced tumour necrosis factor (TNF)-α production in mouse peritoneal macrophages. The maximal inhibition rate of TNF-α production by FLL (0.5 mg mL^{-1}) was $60.88 \pm 0.30\%$. In the inflammatory process, nitric oxide (NO) and prostaglandin E_2 (PGE_2) increased in peritoneal macrophages. FLL decreased the protein level of NO and PGE_2 in LPS-stimulated mouse peritoneal macrophages. In addition, FLL inhibited nuclear factor-κB activation and IκB-α degradation by the decrease in IκB-α phosphorylation. Our study suggested that FLL reduced inflammation via an important molecular mechanism, which might explain its beneficial effect in the regulation of inflammatory reactions.

Introduction

The source of the crude drug, Fructus Ligustri Lucidi (FLL, Oleaceae), is the fruit of *Ligustrum lucidum Ait* (Zhang et al 2006). It has been used in traditional Chinese medicine for over 1000 years, mainly to treat such ailments as menopausal problems, blurred vision, tinnitus, rheumatic pains, palpitations, backache, and insomnia as well as to alleviate age-related symptoms (Liu et al 2003). Earlier pharmacological studies have demonstrated that the crude FLL extract could produce a hypolipidaemic action in a rabbit model of hypercholesterolaemia (Li 1994). In clinical situations, herbal preparations containing FLL were shown to be successful for the treatment of coronary heart disease (Li 1994).

Macrophage activation is known to play an important role in the inflammatory process (Medzhitov & Janeway 1997) and produce potent pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-6, which induce inflammation and recruit other immune cells, e.g. neutrophils and T lymphocytes. Although these pro-inflammatory cytokines are beneficial to the host defense, they can also trigger pathological conditions when expressed in excess. For example, massive stimulation of macrophages after a severe Gram-negative bacterial infection leads to excessive production of pro-inflammatory cytokines and the development of fatal septic shock syndrome and multiple organ failure. In addition, higher levels of pro-inflammatory cytokines are implicated in a variety of chronic inflammatory diseases including rheumatoid arthritis, psoriasis, and Crohn's disease (Parrillo 1993). Nitric oxide (NO) produced by the inducible NO synthase (iNOS) isoform is an essential component of the host innate immune and inflammatory response to a variety of pathogens, such as intracellular bacteria, viruses, fungi, and parasites (Harbrecht et al 1992; Pfeilschifter & Muhl 1999). Nevertheless, as for other components of the host inflammatory and immune response, excessive activation of iNOS results in cardiovascular and organ dysfunction in clinical or experimental situations of inflammatory disease of both septic and nonseptic aetiology (Vallance & Moncada 1993; Petros et al 1994; Weinberg et al 1995).

Activated macrophages produce a variety of pro-inflammatory mediators, including TNF-α, ILs and prostaglandin E_2 (PGE_2). PGE_2 formation results from the release of

arachidonic acid from cell-membrane phospholipids by phospholipase enzymes and is converted to PGE_2 via cyclooxygenase (COX) and PGE_2 synthase enzymes (Harris et al 2006). We examined the effects of FLL on lipopolysaccharide (LPS)-induced cytokine (TNF-α and IL-6) production, PGE_2, and iNOS protein expression from mouse peritoneal macrophages.

Nuclear factor-kappa B (NF-κB) plays a critical role in the expression of many genes involved in immune and inflammatory responses (Thanos & Maniatis 1995; Ghosh et al 1998; Tegeder et al 2001). In unstimulated cells, *Rel* protein dimers, mainly p50 and p65 subunits, are sequestered in the cytoplasm in complex with one of the several inhibitors of NF-κB. The activation of NF-κB is the consequence of phosphorylation of two specific serines near the N terminus of IκB-α and its degradation. The phosphorylation of IκB-α leads to ubiquitination, resulting in degradation, which targets the protein for degradation by the 26S proteasome and the translocation of NF-κB to the nucleus (Scherer et al 1995). This study was designed to investigate whether FLL could modulate expression of cytokines (TNF-α and IL-6), PGE_2, and iNOS regulated by a transcription factor, NF-κB, IκB-α, and phosphorylated IκB-α.

Materials and Methods

Reagents

Murine recombinant interferon-γ (rIFN-γ) (10^7 U mL^{-1}) was purchased from R&D Systems (Minneapolis, MI). Dulbecco's Modified Eagle's Medium (DMEM), *N*- (1-naphthyl)-ethylenediamine dihydrochloride, LPS from *Escherichia coli*, and ursolic acid were purchased from Sigma (St Louis, MO). Recombinant (r) TNF-α and IL-6, biotinylated anti-murine TNF-α and IL-6, anti-murine TNF-α and IL-6 were purchased from Pharmingen (San Diego, CA). Rabbit polyclonal antibody to iNOS, IκB-α, and mouse polyclonal antibody to phospho-IκB-α were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, MI). DMEM containing L-arginine (84 mg L^{-1}), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL (Grand Island, NY).

Peritoneal macrophage culture

All studies were performed in accordance with international regulations for the handling and use of laboratory animals. The institutional Animal Care and Use Committee approved the protocols.

Male C57BL/6J mice were purchased from Daehan Biolink (Dae-Jeon, South Korea). Thioglycollate-elicited macrophages were harvested three to four days after intraperitoneal injection of 2.5 mL thioglycollate to the mice and isolated, according to Chung et al (2004). Peritoneal lavage was performed using 8 mL HBSS containing 10 U mL^{-1} heparin. The cells were then distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 24-well tissue culture plates (2.5×10^5 cells/well) incubated for 3 h at 37°C in an atmosphere of 5% CO_2. Cells were washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

Preparation of FLL

The plant sample was obtained from an oriental drug store, Daehak Pharmacy (Iksan, Jeonbuk, Republic of Korea), and classified and identified by local experts. An extract of FLL was prepared by decocting the dried prescription of herbs with boiling distilled water. The duration of decoction was approximately 3 h. The decoction was filtered, lyophilized, and kept at 4°C. Dilutions were made in distilled water and then filtered through a 0.45-μm syringe filter.

MTT assay

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with FLL (0.01–1 mg mL^{-1}). After 6 h, the cells were treated with LPS (10 μg mL^{-1}). Cell samples (2.5×10^5 cells/well) were seeded in microplate wells and incubated with 20 μL of a MTT solution (5 mg mL^{-1}) for 4 h at 37°C under 5% CO_2 and 95% air. Consecutively, 250 μL dimethyl sulfoxide (DMSO) was added to extract the MTT formazan and the absorbance of each well at 540 nm was read by an automatic microplate reader.

Cytokines and PGE_2 assay

The ELISA was devised by coating 96-well plates with mouse monoclonal antibody specific to TNF-α and IL-6. Before subsequent steps in the assay, coated plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20. All reagents used in this assay were incubated for 2 h at 37°C. Recombinant TNF-α and IL-6 were diluted and used as a standard. Serial dilutions starting from 10 ng mL^{-1} were used to establish the standard curve. Assay plates were exposed sequentially to biotinylated mouse TNF-α and IL-6 avidin peroxidase, and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) substrate solution containing 30% H_2O_2. The plates were read at 405 nm. The PGE_2 level was quantified by immunoassay kits according to the manufacture's protocols (Stressgen Biotechnologies, USA).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from mouse peritoneal macrophage cells according to the manufacturers' specification using easy-BLUE RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final elutes was determined by spectrophotometry. Total RNA (2.5 mg) was heated at 65°C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37°C using cDNA synthesis kit (AmershamPharmacia, USA). PCR was performed with the following primers for mouse (m) TNF-α (5′ ATG AGA ACA GAA AGC ATG ATC-3′; 5′ TAC AGG CTT GTC ACT CGA ATT 3′), IL-6 (5′ CGG GAT CCA TGT TCC CTA CTT CAC AA 3′; 5′ CCC AAG CTT GGT TTG CCG AGT AGA-3′), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (5′ GGC ATG GAC TGT GGT

CAT GA 3′; 5′ TTC ACC ACC ATG GAG AAG GC 3′) to verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 60°C for TNF-α, 50°C for IL-6, and 62°C for GAPDH, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Measurement of nitrite concentration

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with FLL (0.01–0.5 mg mL^{-1}) and ursolic acid (1–10 μM). After 6 h, the cells were treated with LPS (10 μg mL^{-1}). NO synthesis in cell cultures was measured by a microplate assay method after 48 h. To measure nitrite, 100-μL samples were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined by an automatic microplate reader. NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5–9 μM of NO_2^-. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

Preparation of cytoplasmic and nuclear protein extraction

Preparation of nuclear and cytoplasmic extract was performed as described by Yang et al (2006). Briefly, after cell activation for the times indicated cells were washed with ice-cold PBS and resuspended in 60 μL buffer A (in mM: 10 Hepes/KOH, 2 $MgCl_2$, 0.1 EDTA, 10 KCl, 1 DTT, and 0.5 PMSF, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 μL 10% Nonidet P (NP)-40, and centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was collected and used as the cytoplasmic extract. The nuclear pellet was resuspended in 40 μL buffer B (in mM: 50 HEPES/KOH, 50 KCl, 300 NaCl, 0.1 EDTA, 10% glycerol, 1 DTT, and 0.5 PMSF, pH 7.9), left on ice for 20 min, inverted and the nuclear debris was spun down at 15 000 *g* for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen and stored at −70°C until ready for analysis.

Western blot analysis

Peritoneal macrophages (5×10^6 cells/well) were pretreated with FLL (0.01–0.5 mg mL^{-1}). The cells were then stimulated with LPS (10 μg mL^{-1}) for 12 h. Whole cell lysates were made by boiling peritoneal macrophages in a sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5% skim milk in PBS-Tween-20 for 1 h at room temperature and incubated with anti-iNOS, NF-κB, IκB, and phospho-IκB antibodies. After washing in PBS containing 0.05% Tween-20 three times, the blot was incubated with secondary antibody for 30 min and the antibody-specific proteins were visualized by an enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp. Newark, NJ).

Transcription factor enzyme-linked immunoassay

We performed the transcription factor enzyme-linked immunoassay (TF-EIA) for NF-κB. Avidin peroxidase was coated on a 96-well ELISA plate. The coated plate was washed with PBST and then blocked with 3% skim milk solution. It was subsequently incubated with 1 mg mL^{-1} 5′-biotinylated 21 single-strand DNA oligonucleotide sequence for 1 h at room temperature. This sequence contains the previously described NF-kB-binding motif. The sequence used here was: 5′-AGT TGA GGG GAC TTT CCC AGG-3′. DNA-binding reaction was carried out in a total volume of 100 mL containing 10 mg nuclear protein extract in a buffer containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 5% glycerol, 1 mM EDTA, and 1 mM DTT, for 1 h at room temperature and then washed. NF-kB antibodies were then added at a 1:500 concentration in PBS containing 3% BSA for 1 h, followed by the addition of the corresponding alkaline phosphatase-coupled secondary antibody. Between each addition, the wells were extensively washed in PBST. Alkaline phosphatase activity was then detected by the addition of p-nitrophenyl phosphate solution (Sigma). After incubation for 10 min, the reaction was arrested by the addition of 0.5 M H_2SO_4. Colour intensity was detected at 405 nm using an ELISA reader. Alkaline phosphatase activity was normalized to control values (unstimulated cells).

Statistical analysis

Results were expressed as the mean ± s.e. of independent experiments. The statistical analyses were performed by one-way analysis of variance with Tukey, and Duncan's post-hoc test was used to express the difference among the groups. All statistical analyses were performed using SPSS v12.0 statistical analysis software. A value of $P<0.05$ was considered to indicate statistical significance.

Results

Effects of FLL on cell viability

The MTT assay was used to determine the effects of FLL on the viability of mouse peritoneal macrophages. To begin with the cells were treated with FLL 0.01, 0.1, or 1 mg mL^{-1}, and the viability of the FLL-treated cells was 99.95±2.7%, 103.46±3.5%, and 56.99±11.5%, respectively, compared with the non-treated group as 100%. The highest concentration (1 mg mL^{-1}) of FLL was then lowered to 0.5 mg mL^{-1}. The viability of 0.5 mg mL^{-1} FLL-treated cells was 98.75± 2.4%. Therefore, FLL concentrations of 0.01, 0.1, and 0.5 mg mL^{-1} were used in the study.

Effects of FLL on cytokine production and mRNA expression

The effect of FLL was tested on TNF-α and IL-6 production from LPS-treated mouse peritoneal macrophages. As shown

Table 1 Effect of FLL on the production of inflammatory mediators

Treatment[a]		TNF-α[b] (ng mL^{-1})	IL-6[b] (ng mL^{-1})	PGE_2[c] (pg mL^{-1})
LPS (10 μg mL^{-1})	FLL (mg mL^{-1})			
–	–	0.14 ± 0.01	0.29 ± 0.01	0.71 ± 0.06
+	–	5.13 ± 0.66	11.31 ± 1.03	80.48 ± 1.68
+	0.5	2.01 ± 0.36*	8.77 ± 1.08	47.20 ± 1.77*
+	0.1	2.47 ± 0.33*	10.66 ± 1.45	78.30 ± 3.04
+	0.01	4.12 ± 0.20	8.90 ± 0.29	78.35 ± 1.11

[a] Peritoneal macrophages (2.5×10^5 cells mL^{-1}) were pretreated with FLL (0.01–0.5 mg mL^{-1}) for 30 min, and then stimulated with LPS (10 μg mL^{-1}) for 24 h. [b]Cytokine levels in supernatant were measured using ELISA. [c]The amount of PGE_2 production was measured using immunoassay kits. *$P < 0.05$, significantly different from the LPS-stimulated cells.

in Table 1, TNF-α production in response to LPS was inhibited by pretreatment with 0.01–0.5 mg mL^{-1} FLL in a dose-dependent manner; the maximal inhibition rate of TNF-α production by FLL (0.5 mg mL^{-1}) was 60.88 ± 0.30% (Table 1). IL-6 production was shown to have a tendency to decrease with FLL, but the statistical significance was weak. Cell cytotoxicity by FLL was not observed. To determine whether FLL could modulate LPS-induced TNF-α and IL-6 mRNA expression in peritoneal macrophages, RT-PCR was performed. As shown in Figure 1, the enhanced level of TNF-α mRNA by stimulation of LPS was decreased by FLL. However, IL-6 mRNA expression was not affected by FLL in accordance with protein level.

Effects of FLL on PGE_2 production

To investigate the effect of FLL on LPS-induced PGE_2 production, cells were pretreated with FLL (0.01–0.5 mg mL^{-1}) for 30 min and then treated with LPS for 24 h. FLL 0.5 mg mL^{-1} decreased PGE_2 production (Table 1). The inhibition rate of PGE_2 production by FLL 0.5 mg mL^{-1} was 41.36 ± 1.40%.

Effects of FLL on NO production and iNOS expression

To investigate the effect of FLL on LPS-induced NO production, cells were pretreated with FLL (0.01–0.5 mg mL^{-1}) for 30 min and then treated with LPS for 48 h. FLL decreased NO production in a dose-dependent manner (Figure 2A). The maximal inhibition rate of NO production by FLL 0.5 mg mL^{-1} was 88.83 ± 2.15%. In an effort to reveal the major components of FLL that inhibited NO production, we investigated ursolic acid, a component contained in FLL. Instead of FLL, the cells were treated with ursolic acid (1–10 μM). As shown in Figure 2A, ursolic acid significantly decreased NO production in a dose-dependent manner. To determine the effect of FLL on LPS-induced iNOS expression in peritoneal macrophages, Western blotting was performed. As shown in Figure 4B, treatment with LPS caused a significant increase of iNOS expression. Pretreatment of FLL (0.01–0.5 mg mL^{-1}) resulted in inhibition of iNOS expression (Figure 2B).

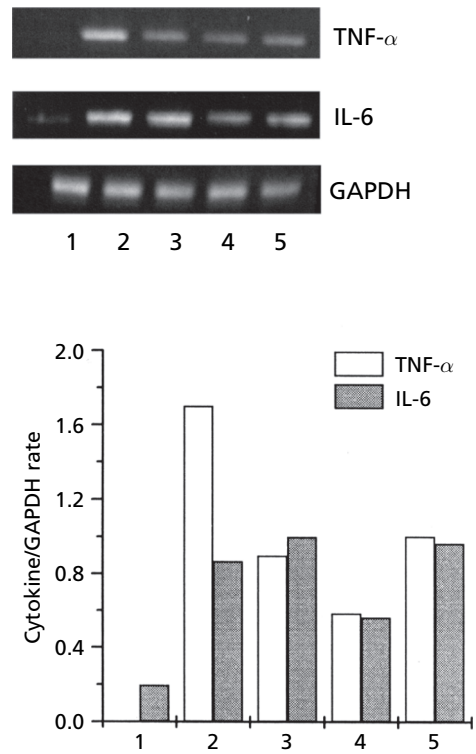

Figure 1 Effect of FLL on cytokine mRNA expression. Cells (3×10^6) were pretreated for 30 min with FLL (0.01–0.5 mg mL^{-1}) followed by activation with LPS (10 μg mL^{-1}) for 6 h. The level of mRNA was measured using RT-PCR. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. 1, Unstimulated cells; 2, LPS (10 μg mL^{-1}); 3, 0.5 mg mL^{-1} FLL plus LPS (10 mg mL^{-1}); 4, 0.1 mg mL^{-1} FLL plus LPS (10 μg mL^{-1}); 5, 0.01 mg mL^{-1} FLL plus LPS (10 μg mL^{-1}). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. All data represent the mean ± s.e. of three independent experiments.

Effects of FLL on NF-κB expression

Since NF-κB activation requires nuclear translocation of the Rel/p65 subunit of NF-κB, we examined the effect of FLL on the nuclear levels of Rel/p65 after LPS-stimulation by Western blot analysis. In LPS-stimulated cells, the expression

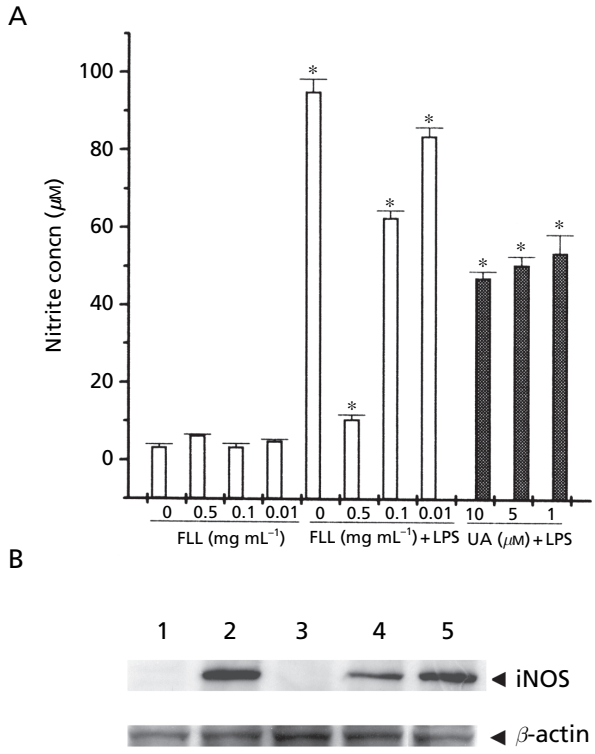

Figure 2 Effects of FLL on NO production and iNOS expression. Cells (2.5×10^5 cells) were pretreated with FLL (0.01–0.5 mg mL^{-1}) and ursolic acid (UA; 1–10 μM) for 30 min, and then treated with LPS (10 μg mL^{-1}) for 48 h. NO production in the medium was measured by the Griess reaction. The amount of NO production was quantitatively assessed using $NaNO_2$ as a standard (A). Cells (5×10^6 cells) were pretreated with FLL (0.01–0.5 mg mL^{-1}) for 30 min, and then stimulated with LPS (10 μg mL^{-1}) for 12 h. The protein extracts were assayed by Western blot analysis for iNOS (B). 1, Unstimulated cells; 2, LPS (10 μg mL^{-1}); 3, 0.5 mg mL^{-1} FLL plus LPS (10 mg mL^{-1}); 4, 0.1 mg mL^{-1} FLL plus LPS (10 μg mL^{-1}); 5, 0.01 mg mL^{-1} FLL plus LPS (10 μg mL^{-1}). All data represent the mean ± s.e. of three independent experiments. *$P < 0.05$, significantly different from the LPS-stimulated cells.

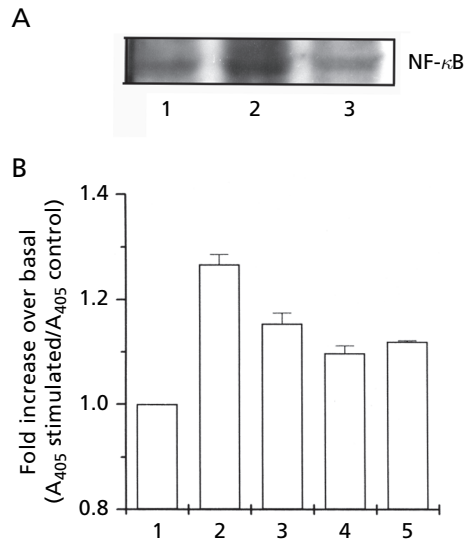

Figure 3 Effect of FLL on nuclear translocation of Rel/p65. Cells (5×10^6) were pretreated for 30 min with FLL (0.5 mg mL^{-1}) and then treated with LPS (10 μg mL^{-1}) for 1 h. Nuclear extracts were prepared as described in Materials and Methods and determined for RelA/p65 by Western blot analysis (A). Nuclear protein was incubated in a 96-well plate coated with an oligonucleotide containing the NF-κB-binding site. The presence of an NF-κB transcription complex was evaluated with an NF-κB antibody. The result is expressed as the fold increase of the absorbance at 405 nm over control conditions (B). 1, Unstimulated cells; 2, LPS (10 μg mL^{-1}); 3, 0.5 mg mL^{-1} FLL plus LPS (10 μg mL^{-1}); 4, 0.1 mg mL^{-1} FLL plus LPS (10 μg mL^{-1}); 5, 0.01 mg mL^{-1} FLL plus LPS (10 μg mL^{-1}).

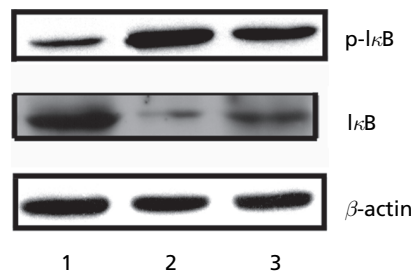

Figure 4 Effect of FLL on IκB-α degradation and phosphorylation. Cells (5×10^6) were pre-incubated for 30 min with FLL (0.5 mg mL^{-1}) and then treated with LPS (10 μg mL^{-1}) for 1 h. The cytosolic extracts were prepared as described in Materials and Methods and determined for IκB-α and phospho-IκB-α by Western blot analysis. 1, Unstimulated cells; 2, LPS (10 μg mL^{-1}); 3, FLL (0.5 mg mL^{-1}) plus LPS (10 μg mL^{-1}).

level of Rel/p65 was increased. However, pretreatment of FLL decreased the expression level of Rel/p65 (Figure 3A). We also investigated the effect of FLL on LPS-induced NF-κB transcription complex. To perform these studies, we used the NF-κB TF-EIA method. This assay has the advantage of being 10-times more sensitive than electrophoretic mobility shift assay and allows greater flexibility in the experimental step. As shown in Figure 3B, LPS increased DNA-binding activity for NF-κB. However, the increased binding activity was decreased by treatment with FLL.

Effect of FLL on IκB-α degradation and phosphorylation

Most of the inhibitors of NF-κB activation mediate their effect through suppressing IκB-α degradation (Ghosh et al 1998). To determine whether the inhibitory action of FLL was due to its effects on IκB-α degradation and phosphorylation, the cytosol levels of IκB-α and phospho-IκB-α were examined after LPS-stimulation by Western blot analysis. As shown in Figure 4, LPS treatment effectively caused an induction of IκB-α degradation and that pretreatment of FLL 0.5 mg mL^{-1} significantly inhibited LPS-induced IκB-α degradation by the decrease in IκB-α phosphorylation.

Discussion

Dai et al (1989) showed that in-vivo FLL inhibited rat hind-paw oedema induced by injection of 1% carrageenan 0.1 mL,

fresh egg 0.1 mL or 2.5% formaldehyde 0.1 mL, and suppressed the increased vascular permeability induced by 0.7% acetic acid in mice and the proliferation of granuloma induced by cotton pellet in rats. The xylene-induced ear swelling in mice and the content of PGE in inflammatory tissue of rats was also decreased by FLL. However, the anti-inflammatory effects of FLL in-vitro have not been reported. This study is the first to investigate the anti-inflammatory effects via NF-κB suppression of FLL in macrophages.

Pro-inflammatory cytokines such as TNF-α and IL-6 mediate the development of various inflammatory reactions (Dinarello 2000). In this study, we showed that FLL effectively inhibited the production and mRNA expression of TNF-α cytokines on LPS-stimulated peritoneal macrophages. These results suggested that FLL might have an anti-inflammatory activity. On the contrary to our expectation, LPS-induced IL-6 production was not affected by pretreatment of FLL. The results demonstrated that FLL inhibited TNF-α and NO production, but not IL-6 production in activated peritoneal macrophages. This implied that the production of TNF-α and NO occurred through different signal pathways from IL-6 production. In other reports, Punzon et al (2003) reported that this difference could indicate some interference with IFN-γ signalling, which had a co-stimulatory effect on IL-6 production.

The elevation of PGE_2 is brought about by the actions of pro-inflammatory cytokines, such as IL-1β and TNF, which are produced by activated immune cells in the periphery and in some cases in the brain, or by the actions of the exogenous pyrogen LPS (Murakami & Kudo 2004). This study has shown that FLL 0.5 mg mL^{-1} inhibited the production of PGE_2. This suggested that FLL may have provided its beneficial effect on anti-inflammation.

Although NO played an important role in the host defense against various pathogens, the overproduction of NO can be harmful and can result in septic shock, rheumatoid arthritis, and autoimmune diseases (Leiro et al 2004). Therefore, therapeutic agents that inhibit iNOS may be useful for relieving these inflammatory conditions. In this study, FLL inhibited iNOS expression in a dose-dependent manner. This suggested that the anti-inflammatory action of FLL may have been associated with the reduction of iNOS protein expression.

NF-κB is a transcription factor that is important for the activation of many inflammatory mediators, cytokines (e.g. TNF-α and IL-6), PGE_2, and iNOS enzyme (Lappas et al 2002). In the inactivated state, NF-κB is sequestered in the cytoplasm bound to its inhibitory protein, IκB-α, which, with stimulation, is degraded thus allowing NF-κB to translocate into the nucleus and activate pro-inflammatory genes (Ghosh et al 1998). Degradation of IκB-α is a key step for NF-κB-induced transcription of certain pro-inflammatory genes including inducible COX-2 and iNOS. Other workers have reported that anti-inflammatory agents suppressed NF-κB activation through stabilization of IκB-α (Yamamoto et al 1999; Lappas et al 2002). In this study, FLL inhibited transcription factor, activation of NF-κB by blocking the Rel/p65 translocation to the nucleus. FLL inhibited NF-κB activation through suppression of IκB-α degradation and phosphorylation, and Rel/p65 translocation in mouse peritoneal macrophages.

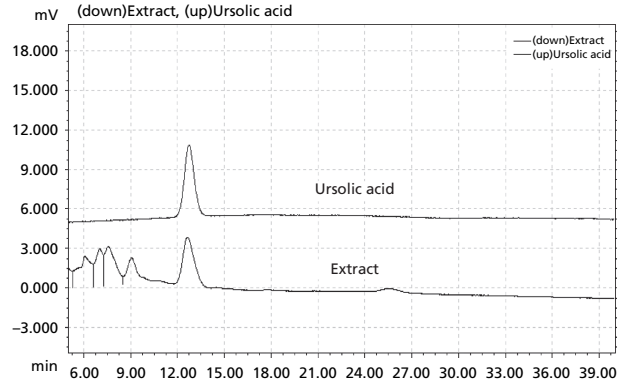

Figure 5 Reverse-phase HPLC for the quantification of ursolic acid in FLL. Contrast solution was prepared by dissolving 10 mg ursolic acid in 5 mL methanol. A 10-mg sample of methanol extract of FLL was accurately weighed and dissolved in 1 mL methanol. A 10-μL portion of the solution was subjected to HPLC (JAI LC, Japan), and analysed with an UV 220 nm. The separation conditions were as follows: column, Phenomenex C_{18} column (250 × 4.60 mm); column temperature 20°C; mobile phase, methanol:water:acetic acid (87:13:0.1, v:v:v); and flow rate 1.5 mL min^{-1}.

Liu et al (2003) determined the amount of ursolic acid in *Ligustrum lucidum Ait* to be 6.52 mg g^{-1} by HPLC and 20.7 mg g^{-1} by MECC. In this study, we confirmed the presence of ursolic acid in FLL by reverse-phase HPLC (Figure 5). Ursolic acid has been proven to be effective on restraining inflammation, protecting the liver, relieving pain, strengthening the heart and calming. Ursolic acid possesses hepatoprotective, anti-inflammatory, anti-tumour, antihyperlipidaemic and antimicrobial activity (Lui 1995). Suh et al (1998) demonstrated that ursolic acid inhibited the release of inflammatory mediators in LPS-activated RAW264.7 cells. In addition, in transient transfection experiments, LPS-stimulated luciferase expression through NF-κB response elements was abolished by treatment with ursolic acid. These observations may provide the mechanistic basis for the anti-inflammatory properties of ursolic acid. We have confirmed an anti-inflammatory effect for ursolic acid by examining the inhibition of NO. We observed that ursolic acid (1–10 μM) inhibited LPS-induced NO in peritoneal macrophages. Therefore, we propose that the anti-inflammatory effect of FLL through NF-κB inhibition was due to ursolic acid. Our results provide a novel mechanism that regulates NF-κB activation in mouse peritoneal macrophages by FLL.

Conclusions

The anti-inflammatory activity of FLL in peritoneal macrophages could be attributed at least in part to inhibition of pro-inflammatory cytokine production, PGE_2, and iNOS protein expression. These effects of FLL caused the inhibition of LPS-induced NF-κB activation and IκB-α degradation and phosphorylation. We suggest that these effects were caused by ursolic acid, a major component of FLL. These results may provide evidence for a novel mechanism of the anti-inflammatory effect of FLL.

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