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금은화 수용성 추출물의 LPS 유도 염증매개물 억제 효과

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Inhibitory Effect of Aqueous Extract from Lonicera japonica Flower on LPS-induced Inflammatory Mediators in RAW 264.7 Macrophages.

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ABSTRACT

Objective : Lonicera japonica (Caprifoliaceae) has long been used for treatment of infectious diseases in oriental countries. The aim of this study was to investigative the effect by which the aqueous extract from flower of L. japonica (LJFAE) inhibited the lipopolysaccharide (LPS)-induced inflammatory mediators in murine macrophages, RAW 264.7 cells

Methods : The dried flowers of L. japonica were extracted with distilled water at 100°C for 7 h. The extract was filtered through 0.45 μm filter, freeze-dried. The dried extract was dissolved in Hank's balanced salt solution (HBSS) and filtered through 0.22 μm filter before use. Accumulated nitrite, an oxidative product of nitric oxide (NO), was measured in the culture medium by the Griess reaction. The levels of prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 production, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression were measured by enzyme-linked immunosorbent assay and Western blot analysis.

Results : LJFAE (10-400 μg/ml) per se had no cytotoxic effect in unstimulated macrophages, but LJFAE concentration-dependently reduced NO, PGE2, TNF-, IL-1, and IL-6 production and COX-2 activity caused by stimulation of LPS. The levels of iNOS and COX-2 protein expressions were markedly suppressed by the treatment with LJFAE in a concentration dependent manner.

Conclusions : These results suggest that LJFAE suppress the NO and PGE2production in macrophages by inhibiting iNOS and COX-2 expression and these properties may contribute to the anti-inflammatory activity of Lonicera japonica.

Key words: Lonicera japonica, Nitric oxide; Prostaglandin E2 Tumor necrosis factor-α, Interleukin-1β, IL-6, Cyclooxygenase-2

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INTRODUCTION

It is well known that nitric oxide (NO), prostaglandin E2 (PGE2), and cytokines such as interleukin-1 beta (IL-1β), IL-6 and tumor necrosis alpha (TNF-α), are involved in the development of inflammation1-[4]. Macrophages play critical roles in inflammatory diseases, by production of these inflammatory mediators. Production of these mediators from macrophages has been found in many inflammatory tissues, along with increased expression of their mRNAs, following exposure to immune stimulants, including outer bacterial toxins such as lipopolysaccharide (LPS) and lipoteichoic acid-7[5,6]. Although NO and pro-inflammatory cytokines are involved in host defense mechanism, overproduction of these inflammatory mediators contributes to the pathogenesis of several diseases such as sepsis, rheumatoid arthritis, atherosclerosis, pulmonary fibrosis, and chronic hepatitis[7,8]. Thus, inhibition of production of these inflammatory mediators may prevent or suppress a variety of inflammatory diseases.

Lonicera japonica (Caprifoliaceae) flower has long been used for treatment of infectious diseases in oriental countries. It has been known as an anti-inflammatory agent in Korea from ancient times and is used widely for treating upper respiratory tract infections, diabetes mellitus, and rheumatoid arthritis[9,10]. In traditional Chinese medicine, it is supposed that L. japonica dispels noxious heat from blood and neutralizes poisonous effects11). L. japonica significantly increases blood neutrophils activity and promotes the neutrophil phagocytosis at its proper concentrations[12]. Some investigators suggested that the methanol extracts of L. japonica have protective effects on rat hepatic injuries caused by carbon tetrachloride[13] and the aqueous extract of L. japonica flower (LJFAE) may act as an therapeutic agent for inflammatory disease through a selective regulation of nuclear factor kappa B (NF-κB) activation in rat liver[10]. Furthermore, recent studies demonstrated that loniceroside A and C from L. japonica showed in vivo anti-inflammator y activity in a croton-oil induced ear edema model14) and anti-arthritis activity [15]. However, the action of aqueous extract from LJFAE on LPS-stimulated NO, PGE2 and pro-inflammatory cytokines production remained to be defined. Therefore, in this study, we investigated the effect of LJFAE on the production of NO, PGE2 and pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in RAW 264.7 macrophages activated with LPS.

MATERIALS AND METHODS

1. Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and antibiotics were purchased from GIBCO BRL (Grand Islang, NY). Rabbit anti-iNOS, rabbit anti-COX-1, and COX-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). LPS (phenol extracted Salmonella enteritidis), Tween 20, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazoliu m bromide (MTT), sodium dodecyl sulphate (SDS), NG-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. (ST Louis, MO). TNF-α, IL-1β, IL-6, and PGE2 immunoassay kits (QuantikineTM) were purchased from R&D System (Minneapolis, MN, USA). Ninety-six well tissue culture plate and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). All reagents were tested for their LPS content with the use of a colorimetric Limulus amoebocyte lysate assay (detection limit, 10 pg/ml; Whitaker Bioproducts, Walkersville, MD).

2. Plant material

The flower of L. japonica were purchased from

the herbal medicine cooperative association of Jeonbuk Province, Republic of Korea. A voucher specimen (no. LGF777) was deposited at the Herbarium of the College of Oriental Medicine, Wonkwang University (Republic of Korea).

3. Preparation of the extract

The dried flowers (1 kg) were extracted with distilled water (5,000 ml) at 100°C for 7 h. The extract was filtered through 0.45 μm filter, freeze-dried (yield, 32.5 g/kg) and kept at -20°C. The dried extract was dissolved in Hank's balanced salt solution (HBSS) and filtered through 0.22 μm filter before use.

4. Cell culture

Murine macrophage RAW 264.7 cell line obtained from the American Type Culture Collection (ATCC, TIB 71, Maryland, USA), was maintained at 1×106 cells/ml culture in DMEM supplemented with 10 % heat inactivated FBS, 1% penicillin G/streptomycin, and L-glutamine (2 mM) and incubated at 37℃ in a humidified atmosphere containing 5 % CO2 and 95% air. On the following day, the medium was replaced with fresh DMEM, and the cells were then stimulated with LPS (1 μg/ml) in the presence or absence of LJFAE for the indicated periods. Extract was dissolved in HBSS, and then diluted with medium to a final concentration.

5. MTT assay for cell viability

Cells were plated at a density of 2.5×105 cells/ml into 96 well plate containing 100 μl of DMEM medium with 10% FBS and incubated overnight. Twenty-four hours after seeding, 100 μl new media or LJFAE (10-400 μg/ml) was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 μl FBS-free medium containing 50 μg/ml MTT. After 4 h of incubation at 37℃, the medium was discarded and the formazan blue, which formed in the cells, was dissolved in 50 μl DMSO. Optical density at 570 nm was determined with a microplate reader. The optical density of formazan formed in control (untreated) cells was taken as 100% of viability.

6. Nitrite Assay

Accumulated nitrite, an oxidative product of NO, was measured in the culture medium by the Griess reaction. Briefly, 100 μl of cell culture medium were mixed with 100 μl of the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenedi amine dihydrochloride/2.5% phosphoric acid) and incubated at room temperature for 10 min, then the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was used as the blank in all experiments. The nitrite levels in the samples were calculated from a nitrite standard curve freshly prepared in culture medium17).

7. PGE2, TNF-α, IL-1β and IL-6 assay

Cells (1×106/ml) were pre-incubated 2 h with LJFAE(10-400 μg/ml)and further cultured 6 h or 18 h with LPS (1 μg/ml) in 24-well plates. Supernatants were removed at the allotted times and PGE2, TNF-α, IL-1β and IL-6 levels were quantified by immunoassay kits according to the manufacture's protocols, respectively.

8. Western Blot

Cellular proteins were extracted from control and LJFAE-treated RAW 264.7 cells. The washed cell pellets were resuspended in cold lysis buffer (10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 1% triton X-100, 5 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin) and incubated for 30 min at 4℃. Nuclei and cell debris were removed by microcentrifugation, followed by quick freezing of the supernatants. Some 50 μg of cellular

proteins from treated and untreated cell extracts were electroblotted onto nitrocellulose membrane following separation on a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4℃, followed by incubation for 4 h with appropriate dilutions of primary antibodies (against rabbit anti-iNOS, rabbit anti-COX-1, and rabbit anti-COX-2). Blots were washed 2 times with PBS and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. Blots were again washed three times in Tween 20/Tris-buffered saline (TTBS) and then developed with 10 ml of a 1:1 mixture of solutions of ECL detection system for 1 min, dried quickly, and exposed to a film for 2-20 min. Protein concentration was determined by Bio-Rad protein assay reagent according to the manufacture's instruction.

9. Data analysis

All values are expressed as the mean S.D. of three independent determinations. All experiments were done at least three times, each time with three or more independent observations. Statistical analysis was performed with analysis of variance (ANOVA) and Student's t-test. A confidence level ($P<0.05$) was considered significant.

RESULTS

1. Effects of LJFAE on the viability of RAW 264.7 Macrophages

RAW 264.7 macrophages were used to assess the effects of LJFAE on the inflammatory mediators. First, we confirmed that the treated concentrations of LJFAE (10 - 400 μg/ml) used here had no effects on the morphology and the viability of RAW 264.7 macrophages (data not shown). The endotoxin LPS at 1 μg/ml reduced the viability of RAW 264.7 macrophages by 25.4%. Interestingly, LJFAE concentration-dependently reversed LPS-induced toxicity (Fig. 1).

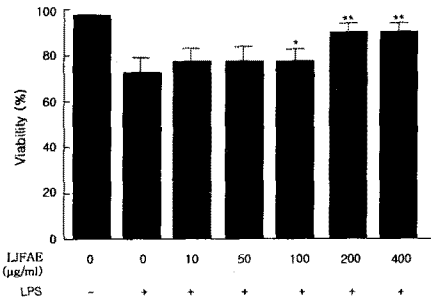

Fig. 1. Effects of LJFAE on the viability of in RAW 264.7 macrophages. Cells (2.5×105/ml) were incubated with or without LPS (1 μg/ml) for 24 h in the presence or absence of LJFAE at the indicated concentrations. The cell viability was determined by the MTT assay as described in Materials and Methods. Each column represents the mean ± S.D. from three independent experiments. *P<0.05 and **P<0.01 indicate significant differences with the LPS treated control group.

2. Effects of LJFAE on NO production and iNOS expression in LPS-stimulated RAW 264.7 Macrophages

The effects of LJFAE on NO production and iNOS protein expression were examined in RAW 264.7 macrophages. First, we confirmed that the treated concentrations of LJFAE (10 - 400 μg/ml) used here had no effects on NO production and iNOS protein expression in non-stimulated RAW 264.7 macrophages with LPS (data not shown). However, LJFAE markedly inhibited NO production in LPS (1 μg/ml) treated RAW 264.7 macrophages. To further evaluate whether the reduction of NO production by LJFAE was correlated with iNOS we examined the expression of iNOS protein by Western blotting analysis. Consistently with the reduction of NO production in Fig. 2A, LJFAE inhibited the expression of iNOS protein in RAW 264.7 macrophages stimulated with LPS in a concentration dependent manner (Fig. 2B). NO inhibitor L-NAME (10 μM),

positive control, also inhibited the production of NO and the expression of iNOS in activated RAW 264.7 (Fig. 2). These results clearly demonstrated that LJFAE produced a concentration-dependent inhibition of NO production and iNOS expression in response to LPS without cytotoxicity.

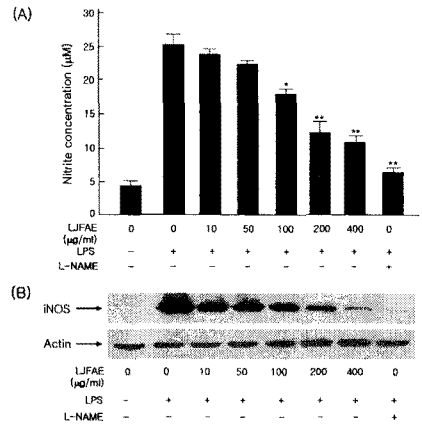

Fig. 2. Effects of LJFAE on NO production (A) and iNOS protein expression (B) in RAW 264.7 macrophages. Cells (2.5×105/ml) were incubated 24 h (for NO assay) or 18 h (for iNOS Western blot) with medium, LPS (1 μg/ml), or LPS plus LJFAE (10-400 μg/ml). NO concentration was determined by the Griess reagent as described in Materials and Methods. Each column represents the mean ± S.D. from three independent experiments. *P<0.05 and **P<0.01 indicate significant differences with the LPS treated control group. For the determination of intracellular iNOS protein, Western immunoblot analysis was carried out as described in Materials and Methods.

3. Effects of LJFAE on PGE2 secretion in LPS-stimulated RAW 264.7 Macrophages

The effects of LJFAE on PGE2 secretion were examined in RAW 264.7 macrophages. As shown in Fig. 3, LPS alone secreted large amounts of PGE2 synthesis in RAW 264.7 macrophages. Treatment with the different concentrations of LJFAE inhibited LPS-induced PGE2 secretion in a dose dependent manner (Fig. 3).

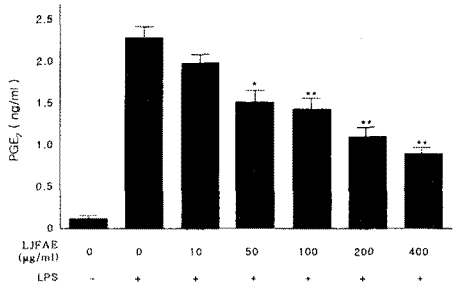

Fig. 3. Efects of LJFAE on PGE2 production in RAW 264.7 macrophages. Cells (1×106/ml) were incubated with or without LPS (1 μg/ml) for 18 h in the presence or absence of LJFAE at the indicated concentrations. PGE2 production in the culture medium was determined as described in Materials and Methods. Each column represents the mean ± S.D. from three independent experiments. *P<0.05 and **P<0.01 indicate significant differences LPS treated control group.

4. Effects of LJFAE on COX activity and secretion in LPS-stimulated RAW 264.7 Ma crophages

To investigate the mechanism of the inhibition of PGE2 secretion by LJFAE, we examined the activity of COX and the expression of COX protein in stimulated RAW 264.7 macrophages with LPS. As shown in Fig. 4B, LPS alone induced large amounts of COX-1 and COX-2 protein expression in stimulated RAW 264.7 with LPS. Treatment with the different concentrations of LJFAE inhibited LPS-induced COX-1 and COX-2 activity in a dose-dependant manner (Fig. 4A). In the range of doses used, LJFAE showed a dose-dependent decrease in LPS-induced COX-1 and COX-2 protein expression (Fig. 4B). The activity and the protein expression of COX-2 inhibited more than COX-1 in the same dose of LJFAE. These results clearly demonstrated that LJFAE produced a concentration-dependent inhibition of PGE2 production by inhibiting COX activity and protein expression in stimulated RAW 264.7 macrophages with LPS.

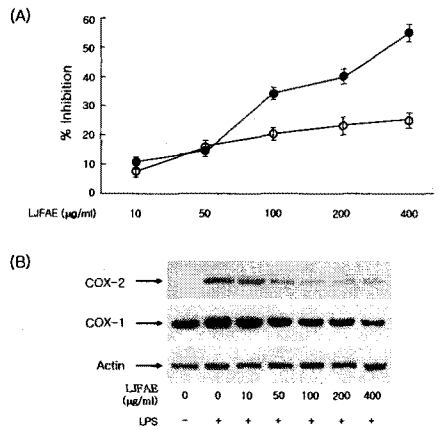

Fig. 4. Effects of LJFAE on COX activity (A) and COX protein expression (B) in RAW 264.7 macrophages. Cells (1×106/ml) were incubated with or without LPS (1 μg/ml) for 18 h in the presence or absence of LJFAE at the indicated concentrations. COX activity in the culture medium was determined as described in Materials and Methods. Each column represents the mean ± S.D. from three independent experiments. *P<0.05 and **P<0.01 indicate significant differences LPS treated control group. Western immunoblot analysis was carried out as described in Materials and Methods.

5. Effects of LJFAE on TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW 264.7 Macrophages

To determine whether LJFAE modulates the production of pro-inflammatory cytokines, we examined the productions of TNF-α, IL-1β, and IL-6 using ELISA methods in stimulated RAW 264.7 macrophage supernatants. There was no significant effect of LJFAE alone on TNF-α, IL-1β, and IL-6 secretions in unstimulated RAW 264.7 macrophages (data not shown). Treatment with the different concentrations of LJFAE resulted in a dose-dependent inhibition of LPS-induced TNF-α, IL-1β, and IL-6 secretions (Fig. 5).

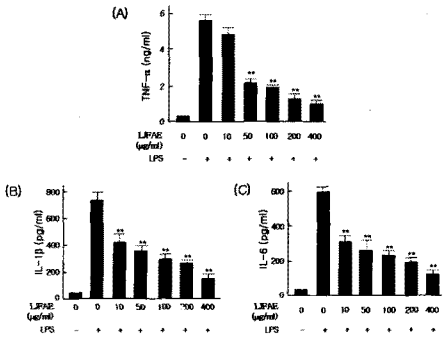

Fig. 5. Effects of LJFAE on TNF-α (A), IL-1β (B), and IL-6 (C) in RAW 264.7 macrophages. Cells (1×106/ml) were incubated with or without LPS (1 μg/ml) for 6h (TNF-α and IL-6) or 12 h (IL-1β) in the presence or absence of LJFAE at indicated concentrations. The productions of TNF-α, IL-1β, and IL-6 in the culture medium were determined as described in Materials and Methods. Each column represents the mean ± S.D. from three independent experiments. **P<0.01 indicates significant differences from LPS treated control group.

DISCUSSION

In order to validate the use of L. japonica flower as an anti-inflammatory drug in traditional Korean medicine, we have investigated the effects of LJFAE on the productions of NO, PGE2, TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW 264.7 macrophages. We showed that LJFAE inhibits the production of the major macrophage-derived inflammatory mediators in a dose-dependent manner.

NO and PGE2, which are produced by iNOS and COX-2, respectively, have been implicated as important mediators in endotoxemia and inflammatory condition[16]. It has been demonstrated that NO plays a important role as immune regulator, neurotransmitter, vasodilator in a variety of tissues at physiological concentration[17]. High levels of NO produced by iNOS, however, have been defined as a cytotoxic molecule in inflammation and endotoxemia18). PGE2, like NO, is a pleiotropic mediator produced at inflammatory sites by COX-2 and gives rise to pain, swelling, and stiffness[19]. Thus, potential inhibitors of iNOS

and COX-2 have been considered to be anti-inflammatory drugs. We here demonstrated that LJFAE inhibits significantly the protein expression of iNOS and COX-2 and results in the suppression of NO and PGE2 production in LPS-stimulated RAW 264.7 macrophages (Fig. 1-4).

A body of evidence indicates that pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 have been shown to control inflammation in vitro as well as in vivo[20-22] and these cytokines appear to be interlinked in a cascade, being produced serially by macrophages during an inflammatory response. Cumulative evidence indicates that an abnormality in the production or function of TNF-α, IL-1β, and IL-6 plays an essential role in many inflammatory lesions[23]. Exposure to LPS causes inflammatory liver damage and septic shock due to production of the high levels of these cytokines[24]. Inhibition of cytokine production or function serves as a key mechanism in the control of inflammation[25]. The present studyshowed that LJFAE suppresses the production of the major macrophage-derived pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in LPS-stimulated RAW 264.7 macrophages.

It would be valuable to develop potent inhibitors of NO, PGE2, and pro-inflammatory cytokines for potentialtherapeutic use. Some natural products are candidates of the potential source for inflammatory mediator inhibitors. According to the literature, the therapeutic effects ofmany Chinese herbs are attributable to the treterpenoids, flavonoids, and ilidoids[26]. Indeed, these compound-producing plants have often been used as an anti-inflammatory medicine[12,26]. L. japonica is one of these compound-producing plants[15,27], and the flower of this plants has been empirically used as an anti-inflammatory drug in traditional Korean medicine[10]. Triterpenoid saponins, iridoid glycosides, and LJFAE from L. japonica exhibit strong in vivo anti-inflammatory activities against mouse ear edema provoked by croton oil or proteinase-activated receptor[19,14,28,29]. We found that LJFAE suppress inflammatory mediators and pro-inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages.

On the other hand, nuclear factor-B (NF-κB) response elements have been demonstrated to be essential for the expressions of iNOS, COX-2, and pro-inflammatory cytokine gene, which are involved in inflammatory process by providing NO, PGE2, and pro-inflammatory cytokines, respectively[30,31]. Therefore, further investigation will be required to confirm whether LJFAE suppresses NF-κB activation and components leading to NF-κB activation

In conclusion, LJFAE can suppress NO, PGE2, TNF-α, IL-1β, and IL-6 production and COX-2 expression in LPS-stimulated RAW 264.7 macrophages. This anti-inflammatory effect occurs by down-regulation of iNOS and COX-2. Our results may provide important information towards understanding the mechanisms by which the traditional Korean medicine L. japonica mediates its therapeutic effects.

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