Anti-inflammatory and Anti-allergic Effects of *Lycii Fructus* Extract

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Lycii Fructus (LF) has been reported to possess various biological activities. In this study, we investigated the anti-inflammatory and anti-allergic effects of LF extract in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells and rat peritoneal mast cells(RPMC). LF extract inhibited NO production and the expressions of inducible NO synthase (iNOS) mRNA and enzyme protein, determined by Griess reactions, RT-PCR and Western blotting, respectively, in LPS-stimulated RAW 264.7 macrophages cells. LF extract significantly inhibited histamine release in compound48/80 stimulated rat peritoneal mast cells compared with the compound48/80 only treated cells. It is suggested that the LF extract can improve the inflammation status that involves the histamine release.

Key words : Lycii Fructus, anti-allergic, anti-inflammatory, NO, Compound 48/80

Introduction

Lycii Fructus(LF) has been reported to exhibit various biological activities, including tonyfying the kidney and liver, nourishing the blood and improve eye brightness in many Asian countries[1,2]. The main active component of LF is known as a carotenoid, polysaccharide and betain. It has been reported to possess immunomodulatory, anti-tumor, hepatoprotective and antifungal activities[3-5]. However, from published studies it is uncertain whether or not LF extract has an anti-inflammatory activity that involved allergic status.

Nitric oxide (NO) is one of the reactive oxygen species (ROS) that plays an important role in diverse physiological processes, including vasodilatation, neurotransmission and immune responses[6]. Pathological roles of NO have been proposed in inflammatory disease[7].

Allergy refers to undesirable reactions produced by the normal immune system that causes tissue inflammation and organ dysfunction[8]. Allergic inflammation is occurred by mast cells, antigen specific CD4+ T cells, basophiles and eosinophils, and caused a characteristic feature of bronchial asthma, allergic rhinitis and atopic dermatitis[9].

In this study, we investigated the effects of anti-inflammatory and anti-allergic of LF extract in LPS-stimulated RAW264.7 macrophage cells and coumpound 48/80-induced rat peritoneal mast cells.

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Materials and Methods

1. Animals

Male Spargue-Dawley rats (250-300g) were provided by Monash University. The rats were housed in standard cages in a light-controlled and air-conditioned with food and water. Experimentation Ethics Committee of RMIT university and conformed to the Australian National Health and Medical Research Council Guidelines.

2. Preparation of Lycii Fructus

Lycii Fructus extract was kindly provided by Professor Zhao Zhong Zhen at School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong.

3. Cell culture

Murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC; TIB71, Maryland, USA). The cells were maintained in complete DMEM supplemented with 10% heat-inactivated FBS, 1%

antibiotic/antimycotic (100 U/ml of penicillin, 25 ㎍/㎖ of amphotericin D and 100 ㎍/㎖ of streptomycin) and 1.5% sodium bicarbonate, at 37℃, in a humidified 5% CO_2 atmosphere. Cells were plated at a density of 1×10^6cells/well in a 60 mm dish and allowed to attach for 2 h. For stimulation, the medium was replaced with DMEM containing 1% FBS, and the cells stimulated with 1 ㎍/㎖ of LPS in the presence or absence of various concentrations of LF extracts for 48 h.

4. Preparation of rat peritoneal mast cells

Rats were asphyxiated in a standard CO_2 atmosphere and decapitated. A 10 ㎖ Tyrode's solution (NaCl, 1.37.0 mM; KCl_2, 1.0 mM; $MgCl_2$, 1.0 mM; $CaCl_2$, 1.6 mM: NaH_2PO_4, 0.41 mM; and 1% glucose, pH 7.4) containing 0.3% BSA and 5.0 units/ml heparin was injected intraperitoneally (i.p.). After gently massaging the abdomen for 2 to 3 min, the collected peritoneal solution containing mast cells was centrifuged at 1200 rpm for 5 min at 4 ℃. The residue containing mast cells was washed 3 times with 10 ml of Tyrode's solution and resuspended in 10 mM HEPES-Tyrode's buffer, pH 7.4 containing 0.1% BSA. For the histamine release experiment, the concentration of mast cells in the suspension was adjusted to 5×10^5cells /㎖.

5. Total RNA isolation and RT-PCR

Murine macrophage RAW 264.7 cells in the TRIzol reagent were well homogenized and vortexed with a 1/10 volume of chloroform. After incubating the mixture on ice for 15 min, the samples were centrifuged at 12,000 rpm, for 15 min at 4℃. The aqueous phase was transferred to a 1.5 ml micro centrifuge tube. RNA from the aqueous phase was precipitated by mixing it with an equal volume of isopropyl alcohol, and centrifuging at 12,000 rpm for 15 min at 4℃. Precipitated RNA pellets were washed once with 70% ethyl alcohol, and then redissolved in DEPC-treated water (Quality Biological Inc., Gaithersburg, MD, USA). RT reaction of 4 ㎍ of RNA was performed in a 20 ㎕ RT reaction mixture containing 0.5 ㎕ of MMLV reverse transcriptase (200 U/ ml Promega), 4.0 ㎕ of 5 MMLV RT buffer (Promega), 2.0 ㎕ of dNTP mixture (10 mM, BM), 0.5 ㎕ of RNasin (RNase inhibitor, 40 U/ ml, Promega), and 2.0 ㎕ of oligo dT (50 mM) in DEPC-treated water. The reaction was performed under the conditions of 42℃ for 1 hr and 95℃ for 5 min. The cDNAs were amplified by PCR with following primer sets: 5′-GAC AAG CTG CAT GTG ACA TC-3′, 5′-GCT GGT AGG TTC CTG TTG TT -3′ for iNOS and 5′-CCA CCC AGA AGA CTG TGG ATG GC-3′, 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3 for G3PDH. PCR was carried out with the use of 1.0 ㎕ of RT products as templates at 94℃ for 3 min followed by 35 cycles of denaturation at 94℃ for 45 s, annealing at 57℃ for 45 s, and elongation at 72℃ for 45 s. The last cycle was followed by a 10 min extension step at 72℃. The amplified products were analyzed by ethidium bromide-stained agarose gel electrophoresis.

6. Western blotting

Cellular lysates were prepared by suspending 1×10^6cells in 100 ㎕ of lysis buffer (50 mM Tris-Cl, 25 mM EDTA, 650mM NaCl, 5% Triton X-100, 100X PMSF, a 100X protease inhibitor cocktail, 5X lysis buffer). Proteins in the cell lysates were then separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to a Protran nitrocellulose membrane (S&S, GmbH, Germany). The membrane was then blocked with 5% skim milk in PBS-Tween-20 and then incubated with an anti-iNOS antibody (Transduction lab, Lexington, KY). After washing in PBS-Tween-20 three times, the blot was incubated with a secondary antibody. Detection of specific proteins was carried out with an ECL Western blotting kit (Amersham Pharmacia Biotech, USA) according to the manufacturers instructions.

7. Histamine release

A 50㎕ of LB extract was added to 425 ㎕ of the cell suspension (5×10^5cells/㎖). After incubation at 37℃ for 10 min, 50 ㎕ of Compound 48/80 (Sigma, St. Louis, MO, USA, final concentration: 0.5㎍/㎖) were added to it and additional incubation was carried out at 37℃ for 10 min. The reaction stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at 12,000 rpm for 2 min. Residual histamine in the cells was released by disrupting the cells with 30% percholic acid and centrifugation at 12,000 rpm fro 2 min. The histamine content was measured by HPLC procedure of Ikarashi et al [10].

8. HPLC system

An STR ODS-II reverse-phase column (4.6mm I.D.x 150 mm length, Shimadzu was used to separate histamine and spermidine(Sigma, St. Louis, MO, USA). The mobile phase consisted of a mixture (A: B=2:1) of A solution (100mM sodium teraric acid buffer, pH4.4, containing 10mM sodium-octanesulfonate) and solution B (99.7% methanol for HPLC). The flow rate was maintained at 1.0 ㎖/min. The effluent from the column was mixed with a post-column solution (400mM sodium borate buffer, pH 9.2: 10mM OPA in methanol = 2:1) at a flow rate of 0.5 ㎖/min. The mixture of the effluent and the post-collumn solution flowed to a reaction

coil (0.5mm I.D.X5.0 length of stainless steel tubing at a final flow rate of 1.5 ㎖/min. and pass through a flourescent detactor where the OPA-derivatives of histamine and spermidine were detected at excitation of 360 nm and emission of 440nm. Temperature of the separation column and reaction coil was maintained at 50℃. The retention times were 4.22 min for histamine and 8.35 for spermidine. The detection limit of histamine in the injected sample was 10ng.

9. Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the statistical significance of changes in all indices as a function of dose and treatment, followed by Duncan's new multiple range test using $p<0.05$ as the level of significance.

Results

1. Effect of LF extract on NO production by LPS-stimulated RAW 264.7 cells

NO modulation was examined to investigate anti-infammmatory effect of LF extract. NO production was measured in supernatants using the method of Griess[11]. LPS markedly induced NO production in RAW 264.7 cells, compared with un-stimulated cells. LF extract significantly inhibited NO production the in LPS-stimulated RAW 264.7 cells(Fig. 1). The observed effect was not due to cytotoxicity of LF extract, since LF extract did not alter of cell viability (data not shown).

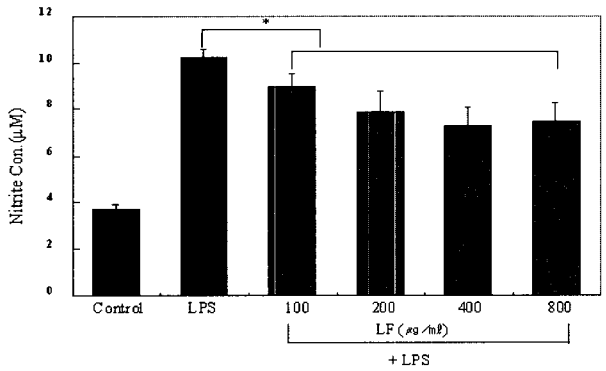

Fig. 1. Effect of LF extract on NO production by LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were incubated with or without lipopolysaccharide (LPS; 1 ㎍/㎖) for 24 hr in the presence or absence of LF extract at indicated doses. The amount of NO released by cells was measured by the method of Griess. Data are the means±SD of three independent experiments. * significantly different from samples treated with LPS alone, (ANOVA, P(0.05).

2. Inhibitory effct of LF extract on iNOS mRNA expression in LPS-stimulated RAW 264.7 cells

We investigated iNOS mRNA expression in order to elucidate the mechanism of NO inhibition of LF extract. LF extract (100-800 ㎍/㎖) markedly decreased iNOS mRNA expression in LPS-stimulated cells in a dose-dependent manner, but the expression of control gene, G3PDH, was not affected with the same LF extract concentration (Fig. 2).

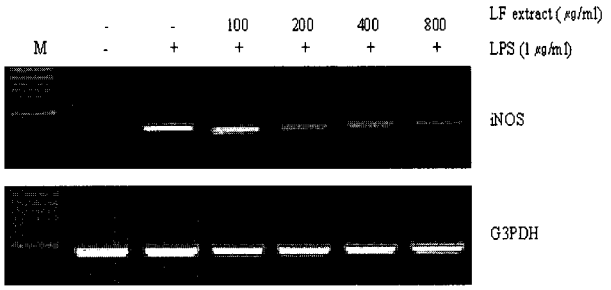

Fig. 2. Inhibitory effect of LF extract on iNOS mRNA expression in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated with 1㎍ /ml LPS in the presence or absence of various concentration of LF extract for 24h. Total RNA was prepared, and iNOS mRNA was analyzed by RT-PCR. G3PDH was used as control genes.

3. Effect of LF extract on the iNOS protein expression in LPS-stimulated RAW 264.7 cells

Whether LF extract could affect iNOS protein expression was also examined. LF extract caused a dose-dependent inhibition of the expression of iNOS protein as determined by Western blot analysis (Fig. 3). On the other hand, the expression of control protein, HSP70, was not affected with same LF extract concentrations. These amounts of iNOS protein correlated with the reduced iNOS mRNA expression.

Fig. 3. Effects of LF extract on the expression of iNOS protein in LPS stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated with 1㎍ /ml LPS in the presence or absence of various concentration of LF extract for 24h. The protein extracts were prepared, and then the samples analyzed for iNOS expression by Western blotting as described in the method. HSP70 was used as control protein.

4. Inhibition effect of LB extract on compound48/80-induced histamine release in rat peritoneal mast cells

We examined whether LF extract has effect of anti-histamine release on compound48/80-induced RPMC. LF extract significantly inhibited the compound48/80-induced histamine release from RPMC. Percentage inhibition of histamine release in was 38.1+2.9, respectively. RPMC were incubated with four different concentrations (0.001-1mg/ml) of LF extract to investigate the dose effect of LF extract on

compound48/80-induced histamine release. LF extract inhibited the compound48/80-induced histamine release in a dose dependently.

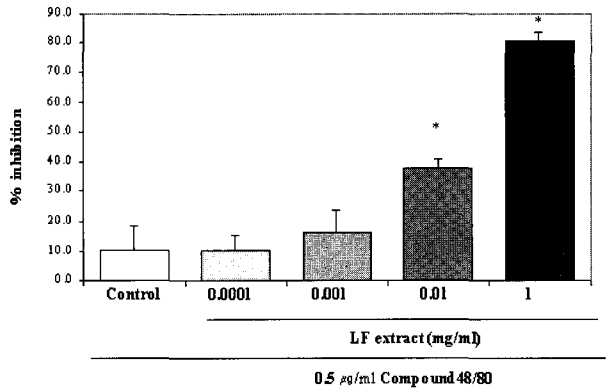

Fig. 4. Inhibitory effect of LF extract on histamine release from peritoneal mast cells in rat by compound48/80. Data calculated as % inhibition of histamine release of total histamine level of mast cells are represented as the mean ± S.D(n=5). *p<0.001 vs compound 48/80-treated control group.

Discussions and Conclusion

LF contains several kinds of compounds including carotenoids, vitamine, fatty acid, polysaccharide, and betaine. It has been also reported that carotenoid, polysaccharide and betaine possess a biologically activities[1-5]. However, there are no reports about the mechanism of the anti-inflammatory effect that involves an allergic status. In this study, it was demonstrated that LF extract inhibited NO production in LPS-stimulated RAW cells and also inhibited compound 48/80-induced histamine release from RPMCs.

NO is a short-lived bioactive molecule that participate in physiology and pathophysiology of many systems. NO is synthesized in vivo from L-arginine by NOS with NADPH and oxygen as co-substrates. The excess production of NO is associated with various disease such as arthritis, diabetes, stroke, septic shock, autoimmune disease, chronic inflammatory disease, and arteriosclerosis[7]. In inflammatory disease, excessive NO production by activated macrophages has been observed[6,7,12,13]. A number of plant materials have been reported to inhibit NO production[1-5].

The study demonstrated that LF extract significantly inhibits NO production, as well as iNOS mRNA and protein expressions in LPS-stimulated RAW 264.7 cells. Furthermore, there seems a dose-related correlation between the inhibition of NO production and the inhibition of iNOS mRNA and of iNOS protein expressions by LF extracts in LPS-stimulated RAW 264.7 cells. These effects of LF extract are unlikely to the due to any cytotoxicity of LF extract, since it did not affect cell viability or the normal expression of HSP70 under the experimental conditions employed. The results suggest that LF extracts reduce LPS-induced NO production through inhibition of iNOS mRNA expression, which leads to a decreased iNOS protein expression. It is likely that this effect of LF extracts may relate to its anti-inflammatory activities, since it is known that anti-inflammatory molecules can decrease the gene expression of iNOS[12-16].

Mast cells were thought to be associated with allergic response resulting from the release of chemical mediators such as histamine from secretary granules. The mast cell has been thought to play a major role in the development of many physiologic changes during allergic response. Mast cell degraulation can be elicited by a number of positively charged substances[17-21]. Compound48/80 is a mixture of polymers synthesized by condensing N-methyl-p-methoxy-phenyl ethylamine with formaldehyde and one of the most potent secretagogues of mast cells[17-19]. Compound48/80 with the natural process, a high concentration of compound48/80 induces almost a 90% release of histamine from mast cells[17]. Compound 48/80 has been used wide as a selective histamine release agent or degranulator from mast cells in rat and mice[10]. Among the preformed and newly synthesized inflammatory substances released on degranulation of mast cells, histamine remains the best characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity[18]. Tasaka et al reported that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane[19]. This result indicates that the membrane permeability increase may be an essential trigger for the release of the mediator from the mast cells. LF extract might act on the lipid bilayer membrane affection the prevention of perturbation being induced by compound 48/80. These results suggest that LF extract has anti-inflammatory activities and these activities may be improving the inflammation caused by histamine release. Studies on the purified components of LF extract are in progress.

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