Effect of Forsythiae Fructus Exract on the Release of Inflammatory Mediatorinduced by Lipopolysaccharide in RAW 264.7 Macrophage

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Forsythiae fructus has traditionally been used for the treatment of erysipelas, skin rash and acute or chronic inflammatory disorders. The effect of Forsythiae fructus against lipopolysaccharide-induced inflammation was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), on mouse RAW 264.7 macrophages. Forsythiae fructus extract suppressed the expression of iNOS, COX-2 and NF-κB mRNAs on the lipopolysaccharide-stimulated enhancement in RAW 264.7 macrophages. We examined the expression of iNOS and COX-2 in both mRNA and protein levels to investigate the mechanism by which Forsythiae fructus extract inhibits NO production. Forsythiae fructus extract significantly reduced iNOS, NF-κB and PGE_2, but didn't inhibit COX-2 expression which was induced by LPS treatment in Raw 264.7 cells. These results suggest that Forsythiae fructus exerts anti-inflammatory effects probably by suppression of the iNOS and NF-κB expressions.

Key words : Forsythiae fructus, lipopolysaccharide, iNOS, COX-2, NF-κB, PGE_2

Introduction

Forsythiae fructus has traditionally been used for the treatment of water retention in the body. Administration of the aqueous extract of Forsythiae fructus has the efficiency of removing toxic heat and inducing diaphoresis. It is commonly used for treating acute upper respiratory tract infection, acute bronchitis and light pneumonia[1]. Forsythiae fructus has been known to possess anti-inflammatory activity and modulates the intestinal immune system[2-4].

Lipopolysaccharide (LPS) initiates a number of major cellular responses which play a vital role in the pathogenesis of inflammatory responses including activation of inflammatory cells and production of cytokines and other mediators. Of these, nitric oxide (NO) is endogenously generated from L-arginine by nitric oxide synthase (NOS) and plays an important role in the regulation of many physiological processes[5]. Several isoforms of NOS exist and fall into three major classes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). NO synthesized by iNOS has been implicated as a mediator of rheumatoid arthritis and other autoimmune diseases[6].

In addition, prostaglandins (PGs) are key inflammatory mediator, converted from arachidonic acid by cyclooxygenase. There are two isoforms of cyclooxygenase: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). While COX-1 is a constitutively expressed form in normal physiologic functions, COX-2 is expressed only in response to inflammatory signals such as cytokines and bacterial endotoxin LPS. COX-2 produces large amounts of PGE_2 that induce inflammatory reaction[7,8].

However, the mechanism of anti-inflammatory action of Forsythiae fructus has not been clarified yet. In the present study, the effect of Forsythiae fructus extract on the release of inflammatory mediators induced by LPS in RAW 264.7 macrophages was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), PGE2 immunoassay, and NO detection.

Materials and Methods

1. Cell culture

Cells of the murine macrophage RAW 264.7 were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea).

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· Received : 2007/03/08 · Accepted : 2007/05/25

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) at 37℃ in 5% CO_2-95% O_2 in a humidified cell incubator.

2. Preparation of extract

Forsythiae fructus was obtained from Semyung Oriental medical hospital, and the identification of plant material was done by professor Leem (department of herborogy, college of oriental medicine, Semyung university). The voucher specimen (#SH-145) was deposited at the college of oriental medicine, Semyung university. To obtain the water extract of Forsythiae fructus, 200 g of Forsythiae fructus was added to distilled water, and extraction was performed by heating at 80℃ concentrated with a rotary evaporator and lyophilized. The resulting powder, weighing 30 g, was dissolved in saline.

3. MTT Cytotoxicity Assay

Cell viability was determined using the MTT assay kit (Boehringer Mannheim GmbH, Mannheim, Germany) as per the manufacturer's protocols. In order to determine the cytotoxicity of Forsythiae fructus, cells were treated with Forsythiae fructus at concentrations of 100 ㎍/㎖, 50 ㎍/㎖, 10 ㎍/㎖, 5 ㎍/㎖ for 24 hr. Cultures of the control group were left untreated. Ten ㎕ of the MTT labeling reagent was added to each well, and the plates were incubated for 4 hr. Solubilization solution of 100 ㎕ was then added to each well, and the cells were incubated for another 12 hr. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 ㎚ and a reference wavelength of 690 ㎚. Optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) × 100.

4. RNA isolation and RT-PCR

To identify expressions of mRNAs, RT-PCR was performed. Total RNA was isolated from RAW 264.7 cells using RNAzolTMB (TEL-TEST, Friendswood, TX, USA). Two ㎍ of RNA and 2 ㎕ of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65℃ for 10 min. One ㎕ of AMV reverse transcriptase (Promega), 5 ㎕ of 10 mM dNTP (Promega), 1 ㎕ of RNasin (Promega), and 5 ㎕ of 10 × AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 50 ㎕ with diethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42℃ for 1 hr.

PCR amplification was performed in a reaction volume of 40 ㎕ containing 1 ㎕ of the appropriate cDNA, 1 ㎕ of each set of primers at a concentration of 10 pM, 4 ㎕ of 10 × RT buffer, 1 ㎕ of 2.5 mM dNTP, and 2 units of Taq DNA polymerase (TaKaRa, Shiga, Japan). For mouse iNOS, the primer sequences were 5'-GTGTTCCACCAGGAGATGTTG-3' (a 21-mer sense oligonucleotide) and 5'-CTCCTGCCCACTGAGTTCGTC-3' (a 21-mer anti-sense oligonucleotide). For mouse COX-2, the primer sequences were 5'-TGCATGTGGCTGTGGATGTCATCAA-3' (a 25-mer sense oligonucleotide) and 5'-CACTAAGACAGACCCGTCATCTCCA-3' (a 25-mer anti-sense oligonucleotide). For mouse NF-κB, the primer sequences were 5'-GACATGGCTGCCAACTGGCAGGTA-3' (a 24-mer sense oligonucleotide) and 5'-CTAAATTTTGCCTTCAATAGGTCC-3' (a 24 mer antisense oligonucleotide). For cyclophilin, the internal control used in the study, the primer sequences were 5'-ACCCCACCGTGTTCTTCGAC-3' (a 20-mer sense oligonucleotide starting at position 52) and 5'-CATTTGCCATGGACAAGATG-3' (a 20-mer anti-sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 583 bp (for COX-2), 500 bp (for iNOS), 57 bp (for NF-κB) and 299 bp (for cyclophilin).

For iNOS and COX-2, the PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: initial denaturation at 94℃ for 5 min, followed by 40 amplification cycles, each consisting of denaturation at 94℃ for 30 sec, annealing at 58℃ for 30 sec, and extension at 72℃ for 30 sec, with an additional extension step at the end of the procedure at 72°C for 5 min. For cyclophilin, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular AnalystTM version 1.4.1 (Bio-Rad, Hercules, CA, USA).

5. Determination of Nitric Oxide Synthesis

In order to determine the effect of Forsythiae fructus on NO synthesis, the amount of nitrite in the supernatant was measured using a commercially available NO detection kit (Intron Biotech., Seoul, Korea). After collection of 100 ㎕ of supernatant, 50 ㎕ of N1 buffer was added to each well, and the plate was incubated at room temperature for 10 min. N2 buffer was then added, and the plate was incubated at room temperature for 10 min. The absorbance of the content of each well was measured at 450 ㎚. The nitrite concentration was calculated from a nitrite standard curve.

6. Measurement of Prostaglandin E_2

Synthesis Assessment of PGE_2 synthesis was performed using a commercially available PGE_2 competitive enzyme immunoassay kit (Amersham Pharmacia Biotech. Inc.,

Piscataway, NJ, USA). Cells were lysed and cell lysates and standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE_2 antibody and peroxidase-conjugated PGE_2 were added to each well, and the plate was incubated at room temperature and shook for 1 hr. The wells were drained and washed, and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution was added. The plate was incubated at room temperature with shaking, and the reaction was stopped after 30 min through the addition of H_2SO_4. The absorbance of the content of each well was then measured at 450 ㎚.

7. Statistical Analysis

Results are expressed as mean ± standard error mean (S.E.M.). Data were analyzed by one-way ANOVA followed by student's t-test using SPSS. Differences were considered statistically significant at $p<0.05$.

Results

1. Effect of Forsythiae fructus on RAW 264.7 Cells Viability

The viabilities of cells incubated with Forsythiae fructus at 100 ㎍/㎖, 50 ㎍/㎖, 10 ㎍/㎖ and 5 ㎍/㎖ for 24 hr were 101.27 ± 7.06%, 97.32 ± 3.98%, 92.52 ± 2.16%, and 92.40 ± 3.20% of the control value, respectively. The MTT assay revealed that Forsythiae fructus exerted no significant cytotoxicity in the macrophage RAW 264.7 cells(Fig. 1).

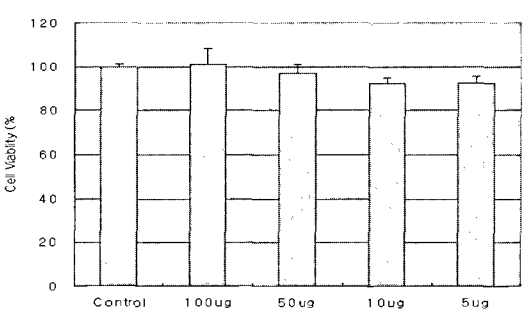

Fig. 1. Effect of Forsythiae fructus on RAW264.7 cell viability. Viability was determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are presented as mean ± standard error mean (S.E.M).

2. Effect of Forsythiae fructus on mRNA expressions of iNOS, COX-2 and NF-κB

RT-PCR analysis of the mRNA levels of iNOS and COX-2 was performed in order to provide an estimate of the relative levels of expressions of these genes. In the present study, the mRNA levels of iNOS, COX-2 and NF-κB in the control cells were used as a control value 1.00. The levels of mRNAs were markedly increased by treatment with 5 ㎍/㎖ LPS for 24 hr, while significantly decreased in the levels of iNOS and NF-κB mRNAs treated with Forsythiae fructus at 100 ㎍/㎖(Table 1, Fig. 2, 3).

Table 1. Effects of Forsythiae fructus on mRNA expression of iNOS, COX-2 and NF-κB

	Control	LPS	LPS+FF(50㎍/ml)	LPS+FF(100㎍/ml)
iNOS mRNA	1	2.43 ± 0.08[a]	2.30 ± 0.10	1.35 ± 0.44*
COX-2 mRNA	1	2.28 ± 0.63	2.38 ± 0.49	2.15 ± 0.42
NF-κB mRNA	1	1.78 ± 0.08	1.70 ± 0.02	1.48 ± 0.18*

[a] : Mean±standard error. * : Statistical significant value compared with LPS treated group. FF : Forsythiae fructus

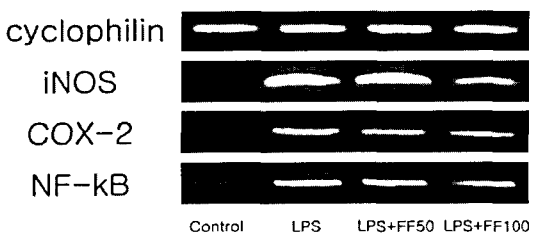

Fig. 2. Results of reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA level of iNOS, COX-2 and NF-κB. As the internal control, cyclophilin mRNA was also reverse - transcribed and amplified.

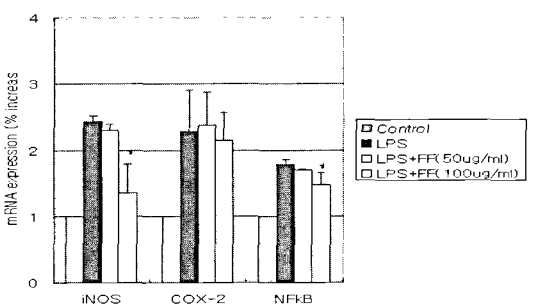

Fig. 3. Results of reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA level of iNOS, COX-2 and NF-κB. As the internal control, cyclophilin mRNA was also reverse - transcribed and amplified. *represents $p<0.05$ compared to the lipopolysaccharide (LPS)-treated group.

3. Effect of Forsythiae fructus on NO Synthesis

From NO detection assay, after 24 hr of exposure to LPS, the amount of nitrite was increased from 2.54 ± 0.08 μM to 14.20 ± 2.58 μM, while decreased to 7.32 ± 0.95 μM and 6.43 ± 0.84 μM by treatment with Forsythiae fructus at 50 ㎍/㎖ and 100 ㎍/㎖(Fig. 4).

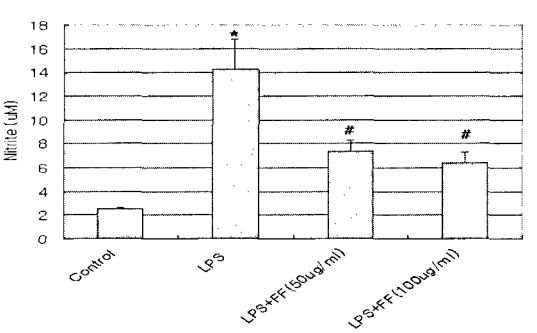

Fig. 4. Measurement of nitric oxide (NO) production in RAW 264.7 cells. *represents $p<0.05$ compared to the control. # represents $p<0.05$ compared to the lipopolysaccharide (LPS)-treated group.

4. Effect of Forsythiae fructus on PGE_2 Synthesis

From PGE_2 immunoassay, after 24 h of exposure to LPS, the amount of PGE_2 was increased from 7.32 ± 2.69 pg/well to 68.27 ± 7.45 pg/well, while decreased to 60.04 ± 4.17 pg/well and 24.95 ± 3.04 pg/well by the treatment with Forsythiae fructus at 50 ㎍/㎖ and 100 ㎍/㎖(Fig. 5).

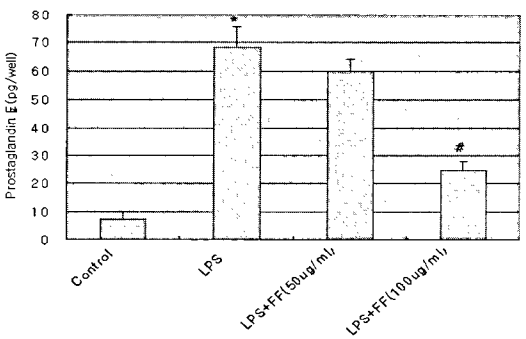

Fig. 5. Measurement of prostaglandin E2 (PGE_2) in RAW264.7 cells. *represents p<0.05 compared to the control. #represents p<0.05 compared to the lipopolysaccharide (LPS)-treated group.

Discussion

During the inflammatory processes, large amounts of pro-inflammatory mediators, nitric oxide (NO) and PGE_2 are generated by the iNOS and COX-2, respectively[9]. In mammalian cells, NO is synthesized by three different isoforms of NOS, namley, nNOS, eNOS and iNOS. Although nNOS and eNOS are constitutively expressed, iNOS is expressed in response to interferon-γ, LPS and to a varity of pro-inflammatory cytokines[10,11]. COX in the enzyme that converts arachidonic acid to PGs. Like NOS, COX existed in two isoforms, COX-1 and COX-2[12]. COX-1 is expressed constitutively in most tissues and appears to be reponsible for maintaining normal physiological functions. In contrast, COX-2 is detectable in only certain types of tissues and is induced transiently by growth factors, proinflammatory cytokines, tumor promoters and bacterial toxins[13,14]. Moreover, elevated levels of COX have been detected in different tumor types and this may account for the excessive production of inflammatory PGs[15].

NF-κB is one of the most ubiquitous transcription factors and regulates gene involved in cellular proliferation, inflammatory responses and cell adhesion. The activation of NF-κB has been reported to induce the transcriptions of many pro-inflammatory mediators, e.g., iNOS, COX-2, TNF-α and interleukin-1β, -6 and -8[16,17].

NF-κB is known to play a critical role in the regulation of cell survival genes and to coordinate the expressions of pro-inflammatory enzymes and cytokines, such as iNOS, and COX-2[18-22]. Since the expressions of these pro-inflammatory mediators are known to be modulated by NF-κB. NF-κB is essentially composed of two proteins, p50 and p65, which are also referred to as RelA and cRel, respectively[23], in the resting state, NF-κB is bound in the cytosol with an inhibitory protein, IκB. However, following its induction by a variety of agents such as LPS, TNF-α and tissue plasminogen activator, IκB is phosphorylated and this process triggers its proteolytic degradation via 26s proteosome, which releases NF-κB from Iκ B and allows its translocated to the nucleus, where it binds to κB binding sites in the promoter regions of its target genes. In the present study, we found that Forsythiae fructus(FF) blocked the LPS-induced activation of NF-κB by inhibiting its translocation to the nucleus[24].

NO produced by the constitutive isoform of NOS is a key regulator of homeostasis, whereas the generation of NO by iNOS plays an important role in inflammation, host-defense responses, and tissue repair[25]. Vane et al.[26] indicated NO as an important mediator of inflammation in animal models. Furthermore, because iNOS is up-regulated by endotoxin, interleukin-1, tumor necrosis factor (TNF)-α, and interferon-γ, the increased synthesis of NO has been implicated in autoimmune disorders, allograft rejection, and systemic response to sepsis.

PGE_2, a major metabolite of the COX-2 pathway, has emerged as an important lipid mediator of inflammatory and immunoregulatory processes. PGE_2 is implicated in the pathogenesis of acute and chronic inflammatory states[27], and specific COX-2 inhibitors attenuate the symptoms of inflammation[28]. COX activity and subsequent production of PGE_2 are closely related to the generation of NO radicals[29]. Salvemini et al.[30] reported that NO modulates the activity of COX-2 as cGMP-independently and plays a critical role in the release of PGE_2 by direct activation of COX-2. Inhibition of the iNOS expression in murine macrophages can be suggested as another possible mechanism of non-steroidal anti-inflammatory drugs[31].

In the present study, we examed the effects of FF on LPS-induced pro-inflammatory molecules, including NO and PGE2. FF was found to be the effective inhibitor of these mediators. To further explore the possible mechanism of these inhibitions by FF, the expression levels of iNOS, COX-2 and NF-kB mRNA were examined. The inhibition by FF of the LPS-stimulated expressions of these molecules in RAW 264.7 cells was no attributable to FF cytotoxicity, as assessed by MTT assay. The inhibition of iNOS and NF-κB gene expression was evidenced by reductions in their mRNA levels. Thus, the inhibition of NOS and PGE_2 release may be attributed to the suppression of iNOS and NF-κB mRNA transcription.

The results of the present study indicate that FF is a

potent inhibitor of the LPS-induced NO and PGE_2 production by blocking iNOS and NF-kB activation in RAW 264.7 macrophages. These findings suggest that FF is a potential therapeutic agent for the tratment of inflammation.

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