OPEM
www.opem.org

Oriental Pharmacy and Experimental Medicine 2010 **10(3)**, 155-164
DOI 10.3742/OPEM.2010.10.3.155

Aqueous extract of *Lycii fructus* suppresses inflammation through the inhibition of nuclear factor kappa B signal pathway in murine raw 264.7 macrophages

Beum-Seuk Kim[1], Hyung-Ho Lim[1], Yun-Kyung Song[1], Yun-Hee Sung[2], Sung-Eun Kim[2], Hyun-Kyung Chang[2], Mal-Soon Shin[2], Chang-Ju Kim[2], Hyejung Lee[3] and Dong-Hee Kim[4,*]

[1]Department of Rehabilitation Medicine, College of Oriental Medicine, Kyung Won University, Kyunggi-do 461-701; [2]Department of Physiology, College of Medicine, Kyung Hee University, Seoul 130-701; [3]Acupuncture and Meridian Science Research Center, Kyung Hee University, Seoul 130-701; [4]Department of Ophthalmology, College of Medicine, Chungju Hospital, Konkuk University, Chungbuk 380-704, Korea

Received for publication August 24, 2010; accepted September 7, 2010

SUMMARY

Lycii fructus is the fruit of Lycium chinense Miller and is part of the Solanaceae family. *Lycii fructus* produces various effects such as hypotensive, hypoglycemic, anti-pyretic, and anti-stress activities. *Lycii fructus* is known to contain betaine, carotene, nicotinic acid, zeaxanthin, and cerebroside. In the present study, the effects of *Lycii fructus* aqueous extract on lipopolysaccharide (LPS)-induced inflammation in murine raw 264.7 macrophage cells were investigated. In this study we utilized the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, and nitric oxide (NO) detection. *Lycii fructus* aqueous extract suppressed NO production by inhibiting the LPS-induced expressions of inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-α) mRNA and iNOS protein in murine raw 264.7 macrophage cells. Also, *Lycii fructus* aqueous extract suppressed the activation of nuclear factor-kappa B (NF-κB) in the nucleus. These results demonstrated that *Lycii fructus* aqueous extract causes an anti-inflammatory effect that was likely produced by the suppression of iNOS expression through the down-regulation of NF-êB binding activity.

Key words: *Lycii fructus*; Lipopolysaccharide; Inducible nitric oxide synthase; Nitric oxide; Nuclear factor-kappa B

Introduction

Lycium chinense Miller, a plant belonging to the Solanaceae family, is distributed throughout northeast Asia and has been used as a tonic agent in traditional medicine. The plants' fruit, known as *Lycii fructus*, exhibits hypotensive, hypoglycemic, anti-pyretic, and anti-stress activities (Funayama *et al*., 1980; Morota *et al*., 1987).

Lipopolysaccharide (LPS) stimulates the production of inflammatory mediators such as nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), interleukins, prostaglandin E_2 (PGE_2), and leukotrienes in microglia cells (Chao *et al*., 1992; Vegeto *et al*., 2001). Microglia are macrophage-like cells of the central

*Correspondence: Dong-Hee Kim, Department of Ophthalmology, College of Medicine, Chungju Hospital, Konkuk University, Chungbuk 380-704, Korea. Tel.: +82-43-840-8871; Fax: +82-43-848-4046; E-mail: kimdh @kku.ac.kr

nervous system (CNS) and they are generally considered to be immunologically quiescent under normal conditions. Exaggerated pain is induced by the activation of astrocytes and microglia. Astrocytes and microglia are activated by pain-inducing neurotransmitters including substance P, glutamate, and fractalkine. These neurotransmitters are known to excite pain-responsive neurons, resulting in the production of reactive oxygen species (ROS), nitric oxide (NO), prostaglandins (PGs), and growth factors (Watkins and Maier, 2000).

NO is synthesized from L-arginine by nitric oxide synthase (NOS). There are multiple forms of NOS, which are classified according to physical and chemical properties into three groups, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Both nNOS and eNOS are considered constitutive NOS because they exist in the normal state whereas iNOS is expressed in response to special stimulants such as LPS and cytokines and is thus regarded as inducible enzyme (Nathan *et al*., 1992).

TNF-α is one of the inflammatory cytokines and it is produced in various types of cells including macrophages, lymphocytes, neutrophils, and mast cells. TNF-α is known to be a key mediator in the induction of apoptosis and the development of the humoral immune response. However, high levels of TNF-α activity are closely associated with the induction of tissue injury and the potential of septic shock (Marino *et al*., 1997; Ettinger *et al*., 1998). TNF-α may produce homeostatic effects by limiting the extension of the inflammatory response as well as acting as an anti-malarial agent and functioning in intra-membranous bone repair (Ma, 2001; Wise and Yao, 2003).

Nuclear factor kappa B (NF-κB) plays a critical role in the expression of many genes involved in the immune and inflammatory responses (Ghosh *et al*., 1998). The expression of iNOS is related to the upregulation of NF-κB through the site that was identified in the promoter region of the iNOS gene (Prabhu *et al*., 2002; Lee *et al*., 2003). NF-κB is constitutively localized in the cytosol as either a homodimer or a heterodimer and is associated with inhibitory-kappa B (IκB) protein. The activation of NF-κB is induced by IκB kinase (IKK), which phosphorylates IκB, leading to its degradation and to the translocation of NF-κB to the nucleus (Griscavage *et al*., 1996).

Many pharmacological actions of *Lycii fructus* have been reported, however, the mechanism of the anti-inflammatory action of *Lycii fructus* has not yet been clarified. In the present study the effects of *Lycii fructus* aqueous extract on the LPS-stimulated expressions of NF-κB, TNF-α and iNOS in murine raw 264.7 macrophages were investigated. The procedures preformed in this study included 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), Western blot and NO detection.

MATERIALS AND METHODS

Cell culture

Murine raw 264.7 macrophages were purchased from Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL) at 37 °C in 5% CO_2 - 95% O_2 in a humidified cell incubator. The cells were plated onto culture dishes at a density of 2×10^4 cells/cm^2 24 h prior to drug treatments.

Reagents

Lipopolysacharride (LPS), pyrrolidine dithiocarbamate (PDTC), 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), hydrochloric acid (HCl), diethylpyrocarbonate (DEPC), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium chloride, potassium chloride, glycerol, Triton X-100, magnesium chloride hexahydrate), ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF),

leupeptin, pepstatin, sodium ortho vanadate, sodium floride, Dithiothreitol (DTT), aprotinin, Nonidet P - 40, tris-hydroxymethylaminomethane (Tris-base), agarose, ethidium bromide (EtBr), acrylamide, bis-acrylamide, and almost drugs used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of *Lycii fructus* aqueous extract

Lycii fructus used in this experiment was purchased from the Dae Young Pharm. Co. Ltd. (Kyunggi, Korea). 50 g of *Lycii fructus* was added to distilled water and extraction was performed by heating at 80 °C for 2 h, concentrating with a rotary evaporator (Eyela, Tokyo, Japan), and lyophilizing using a drying machine (Ilsin, Kyunggi, Korea) for 24 h. The resulting powder, which weighed 19.51 g (yield of 39.02%), was diluted to the desired concentrations with autoclaved distilled water and filtered through a 0.22 μm syringe filter with polyethersulfone (PES) membrane (Millipore Corporation, Billerica, MA, USA) before use.

MTT cytotoxicity assay

Murine raw 264.7 macrophages were grown in 100 μl of culture medium per well in 96-well plates. In order to determine the cytotoxicity of *Lycii fructus,* the cells were treated with *Lycii fructus* at concentrations of 1 μg/ml, 10 μg/ml, 100 μg/ml, 1,000 μg/ml, and 10,000 μg/ml for 24 h. The cells in the control group were left untreated. After adding 10 μl of the MTT labeling reagent containing 5 mg/ml 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide in phosphate-buffered saline to each well, the plates were incubated for 2 h. 100 μl of solubilization solution containing 10% sodium dodecyl sulfate in 0.01 M hydrochloric acid was added to each well and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 nm with a reference wavelength of 690 nm. The optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that observed at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) × 100.

RNA isolation and RT-PCR

RT-PCR was performed in order to identify the expressions of TNF-α and iNOS mRNA as previously described (Chang *et al*., 2005). Total RNA was isolated from murine raw 264.7 macrophages using RNAzol^{TM}B (TEL-TEST, Friendswood, TX, USA). 2 μg of RNA and 2 μl of random hexamers (Promega, Madison, WI, USA) were combined and the mixture was heated at 65 °C for 15 min. One μl of AMV reverse transcriptase (Promega), 5 μl of 2.5 mM dNTP (Promega), 1 μl of RNasin (Promega), and 8 μl of 5 × AMV RT buffer (Promega) were then added to the mixture and the final volume was increased to 40 μl with diethylpyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 °C for 2 h. PCR amplification was performed in a reaction volume of 40 μl containing 1 μl of the appropriate cDNA, 0.5 μl of each set of primers at a concentration of 10 pM, 4 μl of 10 × RT buffer, 1 μl of 2.5 mM dNTP, and 0.2 μl of Taq DNA polymerase (Takara, Shiga, Japan). For mouse TNF-α the primer sequences were 5′-TCATACCAGGGT-TTGAGCTCAG-3′ (a 22-mer sense oligonucleotide) and 5′-TCCCCAAAGGGATGAGAAGTT-3′ (a 21-mer anti-sense oligonucleotide). For mouse iNOS the primer sequences were 5′-CAAGAGTTTGA-CCAGAGGACC-3′ (a 21-mer sense oligonucleotide) and 5′-TGGAACCACTCGTACTTGGGA-3′ (a 21-mer anti-sense oligonucleotide). For cyclophilin, the internal control used in the study, the primer sequences were 5'-ACCCCACCGTGTTCTTCGAC-3' (a 20-mer sense oligonucleotide) and 5'-CATTTGCCATGGACAAGATG-3' (a 20-mer anti-sense oligonucleotide). The expected sizes of the PCR products were 615 bp for TNF-α, 180 bp for iNOS, and 650 bp for cyclophilin. For TNF-α, the PCR procedure was carried out using a PTC-0150 MiniCycler (Bio-Rad, Hercules, CA, USA) under the following conditions: initial denaturation at

94 °C for 5 min, followed by 35 amplification cycles, each consisting of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec, with an additional extension step at the end of the procedure at 72 °C for 10 min. For iNOS, the PCR procedure was carried out under the following conditions: initial denaturation at 94 °C for 5 min followed by 30 amplification cycles, each consisting of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 45 sec, with an additional extension step at the end of the procedure at 72 °C for 10 min. For cyclophilin, the PCR procedure was carried out under the following conditions: initial denaturation at 94 °C for 5 min followed by 25 amplification cycles, each consisting of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, and extension at 72 °C for 45 sec, with an additional extension step at the end of the procedure at 72 °C for 10 min. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Imaging-Pro®Plus (Media Cyberbetics Inc., Silver Spring, MD, USA).

Preparation of whole cell extract

Whole cell extracts were obtained as previously described in detail (Chang *et al*., 2005). The cells were lysed in an ice-cold whole cell lysate buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM magnesium chloride hexahydrate, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM sodium orthovanadate, and 100 mM sodium floride. The mixture was then incubated on ice for 30 min. The cells were centrifuged at 14,000 × g for 15 min at 4 °C. The supernatants were collected and stored at -70 °C by quick freezing.

Preparation of nuclear and cytosolic extracts

Nuclear and cytosolic extracts were obtained as previously described in detail (Jhun *et al*., 2005). The cells were collected and suspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 μg/ml aprotinin) and incubated on ice for 10 min. They were lysed by the addition of 0.1% Nonidet P-40 and vigorous vortexing for 10 sec. The cells were centrifuged at 4,000 × g for 5 min at 4 °C. The supernatants containing cytosolic protein were collected and stored at -70 °C. The pellets acquired from the cytosolic protein extraction were resuspended in a high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium vanadate). The cells were then centrifuged at 14,000 × g for 5 min at 4 °C. The new supernatants containing nuclear protein were collected and stored at -70 °C.

Western blot analysis

Western blot analysis was performed as previously described (Chang *et al*., 2005; Jhun *et al*., 2005). Whole protein extract was used to analyze the expression of iNOS protein. Cytosolic extract was used for the detection of IκB-α protein expression, while nuclear extract was used for the detection of NF-κB (p65) protein expression. The protein concentrations were measured using a colorimetric protein assay kit (Bio-Rad). The protein assay reagent 1 ml is added to a sample 1 ml. After collection of 100 μl of supernatant, the color change is quantitated with a microplate plate reader (Bio-Tek) at a wavelength of 595 nm. Protein standards provided in this kit allow for the generation of a standard curve based on the colors generated by known concentrations of bovine serum albumin. 40 mg of protein were separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Rabbit iNOS antibody (1:500; Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit NF-κB (p65) antibody (1:500; Santa Cruz Biotech), and rabbit IκB-α antibody (1:500; Santa Cruz Biotech) were used as the primary antibodies. Horseradish peroxidase-conjugated anti-goat antibody (1:4000; Santa Cruz Biotech) was

used to probe for iNOS, NF-κB (p65) and IκB-α as the secondary antibody. Band detection was performed using the enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotech).

Determination of nitric oxide production

In order to determine the effect of *Lycii fructus* aqueous extract on NO production, the amount of nitrite in the supernatant was measured using a commercially available NO detection kit (iNtRON Biotechnology, Kyunggi, Korea). After collection of 100 μl of supernatant, 50 μl of N1 buffer containing 1% sulfanilamide was added to each well and the plate was incubated at room temperature for 10 min. N2 buffer containing 0.1% naphtylethylenediamine with 5% phosphoric acid was then added and the plate was incubated at room temperature for 10 min. The absorbance of the contents of each well was measured with a microtiter plate reader (Bio-Tek) at a wavelength of 540 nm. The nitrite concentration was calculated from a nitrite standard curve generated by mixing 0 to 200 μM sodium nitrite solutions with NO detection kit (iNtRON Biotechnology). The standard curve was typically linear between 0 and 200 μM of sodium nitrite.

Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM). The data were analyzed by one-way ANOVA followed by Duncan's *post-hoc* test using SPSS version 12.0. The differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of *Lycii fructus* aqueous extract on the viability of murine raw 264.7 macrophages

In order to assess the cytotoxic effect of *Lycii fructus* aqueous extract on murine raw 264.7 macrophages, the cells were cultured with *Lycii fructus* aqueous extract. The present results showed that the aqueous extract of *Lycii fructus* exerted no cytotoxic effects at concentrations less than 1,000 μg/ml. However, 10,000 μg/ml of *Lycii fructus* aqueous extract reduced cell viability. Thus, we used the concentrations of 100 μg/ml and 1,000 μg/ml of *Lycii fructus* aqueous extract for the next experiments (Fig. 1).

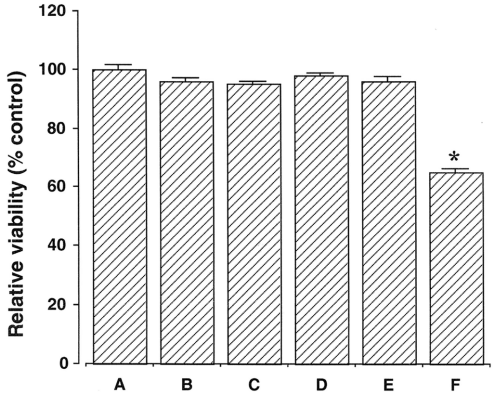

Fig. 1. Effect of *Lycii fructus* on murine raw 264.7 macrophage cell viability. The cells were treated with *Lycii fructus* aqueous extract at concentrations from 1 μg/ml to 10,000 μg/ml for 24 h. The cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and analyzed by enzyme-linked immunosorbent assay (ELISA). The experiments were repeated four times. (A) Control group; (B) 1 μg/ml *Lycii fructus*-treated group; (C) 10 μg/ml *Lycii fructus*-treated group; (D) 100 μg/ml *Lycii fructus*-treated group; (E) 1,000 μg/ml *Lycii fructus*-treated group; (F) 10,000 μg/ml *Lycii fructus*-treated group. The results are presented as the mean ± the standard error of the mean (SEM). $^{*}P < 0.05$ compared to the control.

Effect of *Lycii fructus* aqueous extract on the mRNA expressions of TNF-α and iNOS

RT-PCR analysis to determine the mRNA levels of TNF-α and iNOS was performed in order to provide an estimate of the relative expressions of these genes. Our results showed that LPS enhanced TNF-α and iNOS mRNA expressions in murine raw 264.7 macrophages. The aqueous extract of *Lycii fructus* and PDTC suppressed the LPS-induced expressions of TNF-α and iNOS mRNA (Fig. 2).

Effect of *Lycii fructus* aqueous extract on the expression of iNOS protein

Analysis of the expression of iNOS protein was performed in order to provide an estimate of the

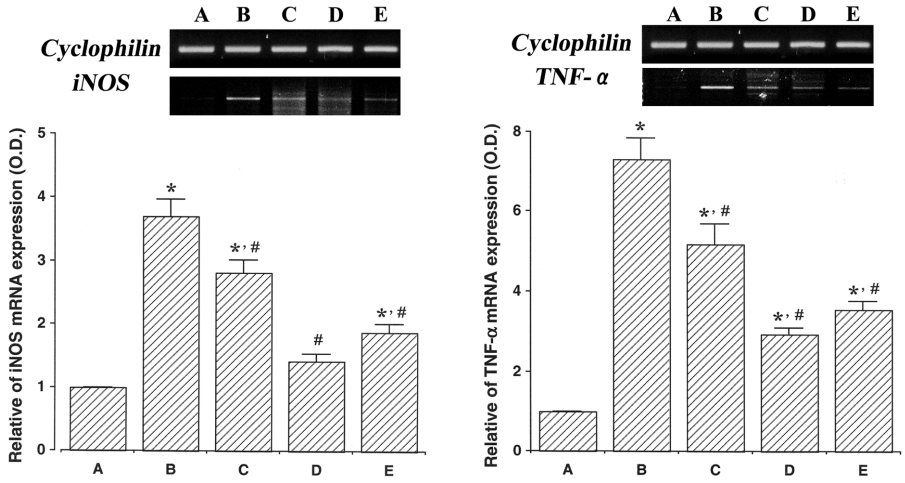

Fig. 2. RT-PCR analysis of the mRNA expression of TNF-α and iNOS. Murine raw 264.7 macrophages were pre-treated with *Lycii fructus* at concentrations of either 100 μg/ml or 1,000 μg/ml for 1 h, followed by treatment with 1 μg/ml lipopolysaccharide (LPS) for 24 h. Cyclophilin mRNA was used as the internal control. (A) Control group; (B) LPS-treated group; (C) LPS- and 100 μg/ml *Lycii fructus*-treated group; (D) LPS- and 1,000 μg/ml *Lycii fructus*-treated group; (E) LPS- and 10 μM pyrrolidine dithiocarbamate (PDTC)-treated group. $^{*}P < 0.05$ compared to the control. $^{\#}P < 0.05$ compared to the LPS-treated group.

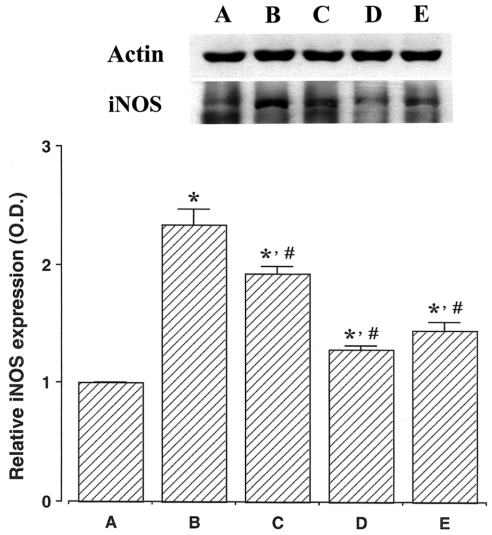

Fig. 3. Western blot analysis of the protein expression of inducible nitric oxide synthase (iNOS). Murine raw 264.7 macrophages were pre-treated for 1 h with *Lycii fructus* at concentrations of either 100 μg/ml or 1,000 μg/ml, and then with 1 μg/ml lipopolysaccharide (LPS) for 24 h. Cyclophilin mRNA was used as the internal control. (A) Control group; (B) LPS-treated group; (C) LPS- and 100 μg/ml *Lycii fructus*-treated group; (D) LPS- and 1,000 μg/ml *Lycii fructus*-treated group; (E) LPS- and 10 μM pyrrolidine dithiocarbamate (PDTC)-treated group. $^{*}P < 0.05$ compared to the control. $^{\#}P < 0.05$ compared to the LPS-treated group.

relative level of expression of this protein. The present results showed that LPS enhanced the expression of iNOS protein in murine raw 264.7 macrophages. The aqueous extract of *Lycii fructus* and PDTC suppressed the LPS-induced expression of iNOS protein (Fig. 3).

Effect of *Lycii fructus* aqueous extract on the expression of NF-êB protein in the nuclear fraction and IêB protein in the cytosolic fraction

The present results showed that LPS enhanced the expression of NF-κB (p65) protein while the aqueous extract of *Lycii fructus* and PDTC decreased the LPS-induced expression of NF-κB (p65) protein in the nuclear fraction. However, LPS suppressed the expression of IκB-α protein, while the aqueous extract of *Lycii fructus* and PDTC increased the expression of IêB-á protein in the cytosolic fraction (Fig. 4).

Effect of *Lycii fructus* aqueous extract on NO production

The present results showed that LPS enhanced NO production in murine raw 264.7 macrophages. The

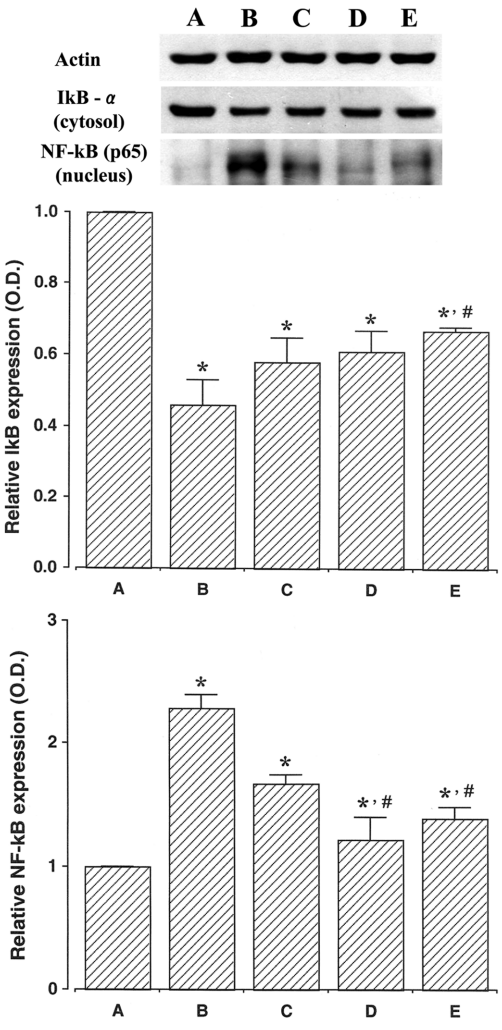

Fig. 4. Western blot analysis of nuclear factor-κB (NF-κB) (p65) and the degradation of inhibitory κB-α (IκB-α) in murine raw 264.7 macrophages. Actin was used as the internal control. The cells were pre-treated for 1 h with *Lycii fructus* at concentrations of either 100 μg/ml or 1,000 μg/ml, followed by treatment with 1 μg/ml lipopolysaccharide (LPS) for 20 min. Murine raw 264.7 macrophages were incubated for 20 min with varying concentrations of *Lycii fructus*. (A) Control; (B) LPS-treated group; (C) LPS- and 100 μg/ml *Lycii fructus*-treated group; (D) LPS- and 1,000 μg/ml *Lycii fructus*-treated group; (E) LPS- and 10 μM pyrrolidine dithiocarbamate (PDTC)-treated. The results are presented as the mean ± standard error of the mean (SEM). $^{*}P < 0.05$ compared to the control. $^{\#}P < 0.05$ compared to the LPS-treated group.

aqueous extract of *Lycii fructus* and PDTC suppressed the LPS-induced NO production (Fig. 5).

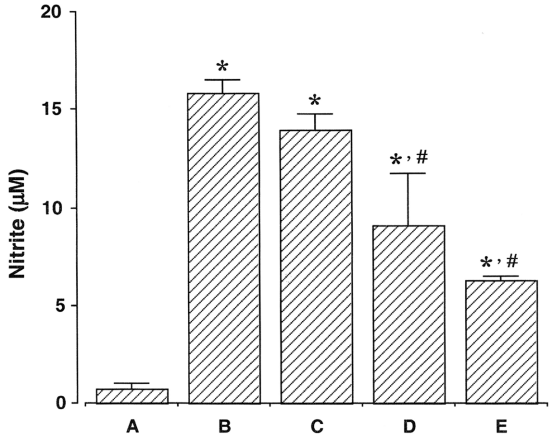

Fig. 5. Nitric oxide (NO) production in murine raw 264.7 macrophages. The cells were pre-treated for 1 h with *Lycii fructus* at concentrations of either 100 μg/ml or 1,000 μg/ml and then treated with 1 μg/ml lipopolysaccharide (LPS) for 24 h. NO was detected and analyzed by enzyme-linked immunosorbent assay (ELISA). The experiments were repeated four times. (A) Control; (B) LPS-treated group; (C) LPS- and 100 μg/ml *Lycii fructus*-treated group; (D) LPS- and 1,000 μg/ml *Lycii fructus*-treated group; (E) LPS- and 10 μM pyrrolidine dithiocarbamate (PDTC). The results are presented as the mean ± standard error of the mean (SEM). $^{*}P < 0.05$ compared to the control. $^{\#}P < 0.05$ compared to the LPS-treated group.

DISCUSSION

Inflammation is a response of the organism to injury related to either physical or chemical noxious stimuli or microbiological substances and is involved in multiple pathologies such as arthritis, asthma, multiple sclerosis, colitis, inflammatory bowel disease, and atherosclerosis (Guzik *et al*., 2003). Activated microglia generate inflammatory cytokines such as TNF-α, IL-1β, NF-κB, and NO that likely cause or exacerbate neuronal degeneration (Combs *et al*., 2000; Tikka *et al*., 2001; Lo *et al*., 2002).

NO is known to regulate inflammatory and immune responses in several ways. For example, iNOS produces large amounts of NO and excessive NO production induces pathologic states (Shin *et al*., 2003). NO causes inflammatory damage, genetic alterations, and autoimmune diseases (McCartney-Francis *et al*., 1993; Kleemann *et al*., 1993). In addition,

some studies have shown a correlation between the release of NO by microglia and the progression of neurodegeneration (Boje and Arora, 1992; Chao *et al*., 1992). In the present results, LPS treatment enhanced iNOS expression and increased the synthesis of NO in murine raw 264.7 macrophages.

NF-κB is believed to play an important role in the regulation of the inflammatory response. NF-κB activation is associated with the phosphorylation and degradation of IκB-α and the nuclear translocation of p65 (Pahl, 1999). Excessive proinflammatory cytokine and NO production through NF-κB activation play an important role in inflammatory diseases (Southan and Szabo, 1996). Activation of NF-κB is also critical for the induction of iNOS gene expression in LPS-stimulated macrophages (Lo *et al*., 2002; Lee *et al*., 2003). The activation of NF-κB can induce the transcription of cytockines such as TNF-α, interleukin (IL)-1β, and IL-6 genes (Mukaida *et al*., 1996). The NF-κB signaling pathway plays an important role in regulating inflammation through the transcription of COX, iNOS, and cytokine genes (Surh *et al*., 2001). Baeuerle (1998) reported that the expression of iNOS in murine raw 264.7 macrophages is dependent on NF-êB activation. In the present results, LPS treatment enhanced the expressions of NF-κB and TNF-α while decreased the expression of IêB in murine raw 264.7 macrophages.

Lycii fructus is known to contain betaine, carotene, nicotinic acid, zeaxanthin, and cerebroside (Kim *et al*., 1999). Among these, betaine was reported to be the biologically active component. Betaine is known to suppress inflammation via the NF-κB pathway. Go *et al*. (2005) reported that betaine suppresses certain proinflammatory signaling factors via the NF-κB pathway. In addition, Jia *et al.* (2003) reported that *Lycii fructus* shows inhibitory effect on edema in the model of inflammation in rats. In the present results, we have found that *Lycii fructus* aqueous extract inhibited the production of NO, NF-κB and TNF-α, and increased the activation of IκB-α in LPS-induced murine raw 264.7 macrophages. Also, LPS-induced NO production was decreased by PDTC, which is an NF-κB inhibitor. PDTC also suppressed the LPS-induced expressions of NF-κB and TNF-α and increased IκB-α expression in LPS-induced murine raw 264.7 macrophages.

We have shown in this study that *Lycii Fructus* aqueous extract inhibited LPS-induced NO and TNF-α production. Moreover, the effects of *Lycii Fructus* aqueous extract may involve the inhibition of inflammation through the regulation of the NF-κB pathway.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2005 - 0049404).

REFERENCES

Baeuerle PA. (1998) IκB-NF-κB structures: at the interface of inflammation control. *Cell* **95**, 729-731.

Boje KM, Arora PK. (1992) Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. *Brain Res.* **587**, 250-256.

Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK. (1992) Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J. Immunol.* **149**, 2736-2741.

Chang HK, Yang HY, Lee TH, Shin MC, Lee MH, Shin MS, Kim CJ, Kim OJ, Hong SP, Cho S. (2005) *Armeniacae semen* extract suppresses lipopolysaccharide-induced expressions of cyclooxygenase-2 and inducible nitric oxide synthase in mouse BV2 microglial cells. *Biol. Pharm. Bull.* **28**, 449-454.

Combs CK, Johnson DE, Karlo JC, Cannady SB, Landreth GE. (2000) Inflammatory mechanisms in Alzheimer's disease: inhibition of β-amyloid-stimulated proinflammatory responses and neurotoxicity by PPARγ agonists. *J. Neurosci.* **20**, 558-567.

Ettinger R, Mebius R, Browning JL, Michie SA, van Tuijl S, Kraal G, van Ewijk W, McDevitt HO. (1998) Effects of tumor necrosis factor and lymphotoxin on peripheral lymphoid tissue development. *Int.*

Immunol. **10**, 727-741.

Funayama S, Yoshida K, Konno H, Hikkino H. (1980) Structure of kukoamine A, a hypotensive principle of Lycium chinense root bark. *Tetrahedron Lett.* **21**, 1355-1356.

Ghosh S, May MJ, Kopp EB. (1998) NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16**, 225-260.

Go EK, Jung KJ, Kim JY, Yu BP, Chung HY. (2005) Betaine suppresses proinflammatory signaling during aging: the involvement of nuclear factor κB via nuclear factor-inducing kinase/IκB kinase and mitogen-activated protein kinases. *J. Gerontol. A Biol. Sci. Med. Sci.* **60**, 1252-1264.

Griscavage JM, Wilk S, Ignarro LJ. (1996) Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF-κB. *Proc. Natl. Acad. Sci. USA.* **93**, 3308-3312.

Guzik TJ, Korbut R, Adamek-Guzik T. (2003) Nitric oxide and superoxide in inflammation and immune regulation. *J. Physiol. Pharmacol.* **54**, 469-487.

Jia W, Gao WY, Cui NQ, Xiao PG. (2003) Anti-inflammatory effects of an herbal medicine (*Xuan-Ju* agent) on carrageenan and adjuvant-induced paw edema in rats. *J. Ethnopharmacol.* **89**, 139-141.

Jhun BS, Oh YT, Lee JY, Kong Y, Yoon KS, Kim SS, Baik HH, Ha J, Kang I. (2005) AICAR suppresses IL-2 expression through inhibition of GSK-3 phosphorylation and NF-AT activation in Jurkat T cells. *Biochem. Biophys. Res. Commun.* **332**, 339-346.

Kim SY, Lee EJ, Kim HP, Kim YC, Moon A, Kim YC. (1999) A novel cerebroside from *lycii fructus* preserves the hepatic glutathione redox system in primary cultures of rat hepatocytes. *Biol. Pharm. Bull.* **22**, 873-875.

Kleemann R, Rothe H, Kolb-Bachofen V, Xie QW, Nathan C, Martin S, Kolb H. (1993) Transcription and translation of inducible nitric oxide synthase in the pancreas of prediabetic BB rats. *FEBS. Lett.* **328**, 9-12.

Lee AK, Sung SH, Kim YC, Kim SG. (2003) Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF-α and COX-2 expression by sauchinone effects on I-κBα phosphorylation, C/EBP and AP-1 activation. *Br. J. Pharmacol.* **139**, 11-20.

Lo AH, Liang YC, Lin-Shiau SY, Ho CT, Lin JK. (2002) Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor-κB in mouse macrophages. *Carcinogenesis.* **23**, 983-991.

Ma X. (2001) TNF-α and IL-12: a balancing act in macrophage functioning. *Microbes. Infect.* **3**, 121-129.

Marino MW, Dunn A, Grail D, Inglese M, Noguchi Y, Richards E, Jungbluth A, Wada H, Moore M, Williamson B, Basu S, Old LJ. (1997) Characterization of tumor necrosis factor-deficient mice. *Proc. Natl. Acad. Sci. USA.* **94**, 8093-8098.

McCartney-Francis N, Allen JB, Mizel DE, Albina JE, Xie QW, Nathan CF, Wahl SM. (1993) Suppression of arthritis by an inhibitor of nitric oxide synthase. *J. Exp. Med.* **178**, 749-754.

Morota T, Sasaki H, Chin M, Sato T, Katayma N, Fukuyama K, Mitsuhashi H. (1987) Studies on the crude drug containing the angiotensin-I converting enzyme inhibitors on the active principles of *Lycium chinense* Muller. *Shoyakugaku. Zasshi*. **41**, 169-773.

Mukaida N, Ishikawa Y, Ikeda N, Fujioka N, Watanabe S, Kuno K, Matsushima K. (1996) Novel insight into molecular mechanism of endotoxin shock: biochemical analysis of LPS receptor signaling in a cell-free system targeting NF-κB and regulation of cytokine production/action through β_2 integrin in vivo. *J. Leukoc. Biol.* **59**, 145-151.

Nathan C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB. J.* **6**, 3051-3064.

Pahl HL. (1999) Activators and target genes of Rel/NF-κB transcription factors. *Oncogene.* **18**, 6853-6866.

Prabhu KS, Zamamiri-Davis F, Stewart JB, Thompson JT, Sordillo LM, Reddy CC. (2002) Selenium deficiency increases the expression of inducible nitric oxide synthase in Raw 264.7 macrophages: role of nuclear factor-κB in up-regulation. *Biochem. J.* **366**, 203-209.

Shin KM, Park YM, Kim IT, Hong SP, Hong JP, Lee KT. (2003) *In vitro* antiinflammatory activity of amygdalin in murine marcrohpage raw 264.7 cells. *Kor. J. Pharmacogn.* **34**, 223-227.

Southan GJ, Szabo C. (1996) Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem. Pharmacol.* **51**, 383-394.

Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, Lee SS. (2001) Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and

iNOS through suppression of NF-κB activation. *Mutat. Res.* **480-481**, 243-268.

Tikka T, Fiebich BL, Goldsteins G, Keinanen R, Koistinaho J. (2001) Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. *J. Neurosci.* **21**, 2580-2588.

Vegeto E, Bonincontro C, Pollio G, Sala A, Viappiani S, Nardi F, Brusadelli A, Viviani B, Ciana P, Maggi A. (2001) Estrogen prevents the lipopolysaccharide-induced inflammatory response in microglia. *J. Neurosci.* **21**, 1809-1818.

Watkins LR, Maier SF. (2000) The pain of being sick: implications of immune-to-brain communication for understanding pain. *Annu. Rev. Psychol.* **51**, 29-57.

Wise GE, Yao S. Expression of tumor necrosis factor-alpha in the rat dental follicle. (2003) *Arch. Oral Biol.* **48**, 47-54.