

Anti-inflammatory activity of methanol extract isolated from stem bark of *Albizia julibrissin*

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SUMMARY

Albizia julibrissin (AJ) has been used widely as a traditional medicine. In macrophages nitric oxide (NO) is released as an inflammatory mediator and has been proposed to be an important modulator of many pathophysiological conditions including inflammation and carcinogenesis. In this study we have examined the NO inhibition effect of 85% methanol extracts of AJ in mouse macrophage. Lipopolysaccharide (LPS) has been reported to induce production of NO. Extracts of AJ (1, 10, 100 µg/ml) suppressed nitric oxide production in LPS-stimulated (100 µg/ml) mouse (C57BL/6) macrophages and analyzed by ELISA. In addition, it also attenuated the expression of inflammatory products like Interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), and inducible NOS (iNOS) as assessed by immunoblotting with specific antibodies. These results suggest that 85% methanol extracts of AJ would be useful in inflammatory diseases.

Key words: *Albizia julibrissin*; Nitric oxide; IL-6; Cyclooxygenase-2

INTRODUCTION

The stem bark of *Albizia julibrissin* DURAZZ. (Leguminosae) is recorded in the Chinese Pharmacopoeia as an anti-inflammatory agent, and specified to treat injuries from falls and remove carbuncles (The Pharmacopoeia Committee of People's Republic of China, 1995). It is well known as sedative drug for treating skin ulcers, wounds or swelling and pain of the lungs (Zhang, 2005).

Macrophage activation is known to play an important role in the inflammatory process

(Medzhitov and Janeway, 1997a; Beutler 2000) and produce potent pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-6 which induce inflammation and recruit other immune cells, e.g., neutrophils and T lymphocytes (Medzhitov and Janeway, 1997b). Although these pro-inflammatory cytokines are beneficial to the host defense, they can also trigger pathological conditions when expressed in excess. 4) 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 (Parrillo, 1993; Beutler, 1995) and activate pro-inflammatory genes. In addition, higher levels of pro-inflammatory cytokines are

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also implicated in a variety of chronic inflammatory diseases including rheumatoid arthritis, psoriasis, and Crohn's disease (Beutler, 1995).

Macrophages are a first line of defence against microbial invaders and malignancies by nature of their phagocytic, cytotoxic and intracellular killing capacities (Adams and Hamilton, 1984). Macrophage activation by lipopolysaccharide (LPS), the major component of gram-negative bacteria cell wall, results in the release of several inflammatory mediators such as nitric oxide (NO). The physiologic or normal production of NO from phagocytes is beneficial for the host defense against microorganism, parasites, and tumor cells (Thiemermann and Vane, 1990). However, overproduction of NO can be harmful and result in septic shock, neurologic disorders, rheumatoid arthritis, and autoimmune diseases (Thiemermann and Vane, 1990; Evans, 1995; O'Shea *et al.*, 2002). Therefore, inhibition of NO production is a very important therapeutic target in the development of anti-inflammatory agents.

The stimulation of macrophages with LPS also induces expression of the inducible isoform of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Cao *et al.*, 2006). High levels of NO have been described in a variety of pathophysiological processes including various forms of circulatory shock (Szabo *et al.*, 1995), inflammation (MaPFicking *et al.*, 1997) and carcinogenesis (Ohshima *et al.*, 1994). iNOS is synthesized in response to several inflammatory mediators and produces NO in micromolar concentrations in a calcium independent manner (Wang *et al.*, 2003). It is well known that NO, synthesized by iNOS, releases from macrophage intimately correlated with the pathophysiology in inflammation and lots of diseases (Thiemermann *et al.*, 1990; Kim *et al.*, 2005) and increased expression of iNOS and its catalytic activity has been observed in several human tissues and in chemically-induced animal tumors and also in inflammatory disorders (Goldstein *et al.*, 1998; Wilson *et al.*, 1998; Ambts *et al.*, 1998). Another enzyme that plays a

important role in mediating inflammation is COX-2. There are two isoform of COX, constitutively expressed COX-1 and the inducible isoform COX-2 (Kanazawa *et al.*, 1995). COX-2 are upregulated in response to inflammatory and pro-inflammatory mediators and their products can influence many aspects of inflammatory cascade.

In the present study, we show that AJ significantly inhibited LPS and interferon (IFN- γ)-induced IL-6 and NO production in a dose-dependent manner. Furthermore, the expression of iNOS and COX-2 protein were decreased.

MATERIALS AND METHODS

Reagents

LPS and sodium nitrite were purchased from Sigma (St. Louis, MO). Murine recombinant (r)IFN- γ was purchased from Pharmingen (Mnchen, Germany). Anti-mouse IL-6, biotinylated anti-mouse IL-6 and recombinant mouse IL-6 were purchased from Pharmingen (San Diego, CA, U.S.A.).

Anti-iNOS (SantaCruz, CA, USA) and COX-2 antibody (Cayman, MI, USA) were purchased. Thioglycollate (TG) was purchased from Difco Laboratories (Detroit, MI). 0.4 mm syringe filter and tissue culture plates of 96 wells, 4 wells and 100-mm diameter dishes were purchased from Nunc (Naperville, IL). DMEM containing L-arginine (84 mg/l), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Life Technologies (Grand Island, NY). Male C57BL/6 mice were purchased from Damul Science Co. (Daejon, Republic of Korea).

Peritoneal macrophages culture

TG-elicited macrophages were harvested 3 - 4 days after i.p. injection of 2.5 ml TG to the mice and isolated, as reported previously (Chung *et al.*, 2002). Using 8 ml of HBSS containing 10 U/ml heparin, peritoneal lavage was performed. Then, the cells were distributed in DMEM, which was

supplemented with 10% heat-inactivated FBS, in 4-well tissue culture plates (2.5×10^5 cells/well) incubated for 3 h at 37 °C in an atmosphere of 5% CO₂, washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

Preparation of *Albizia julibrissin* (AJ)

The dried stem bark of AJ was purchased from Korean Oriental Pharmacy Co., Ltd. in 2006. A voucher specimen (dried drug, WME003) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University. An extract was obtained twice from the dried sample (200 g) with 3,000 ml of 85% MeOH under ultrasonification for 2 h. It was evaporated and lyophilized to yield a MeOH extract of AJ, which was then stored at -20 °C until use.

3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell respiration, an indicator of cell viability, was performed by the mitochondrial-dependent reduction of 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, as described by Mosmann (Mosmann *et al.*, 1983). The extent of the reduction of MTT to formazan within cells was quantified by measuring the optical density at 540 nm using an automated microplate reader (GENios, Tecan, Austria).

IL-6 cytokine assay

Cytokine assay was performed by a modified ELISA, as described previously (Kim *et al.*, 2001). The ELISA was devised by coating 96-well plates with mouse monoclonal Ab specific to IL-6. Before subsequent steps in the assay, coated plates were washed with PBS containing 0.05% Tween 20. All reagents used in this assay were incubated for 2 h at 37 °C. Recombinant IL-6 was diluted and used as a standard. Serial dilutions starting from 10 ng/ml were used to establish the standard curve. Assay plates were exposed sequentially to biotinylated

mouse IL-6 avidin peroxidase, and ABTS substrate solution containing 30% H₂O₂. The plates were read at 405 nm.

Measurement of nitrite concentration

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with various concentrations of AJ. The cells were then stimulated with rIFN- γ (20 U/ml). After 6 h, the cells were finally treated with LPS (10 mg/ml). NO synthesis in cell cultures was measured by a microplate assay method, as previously described (Chung *et al.*, 2002). To measure nitrite, 100 μ l aliquots 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% H₃PO₄) at room temperature for 10 min. The absorbance at 540 nm was determined by an automatic microplate reader. NO₂⁻ was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 9 mM of NO₂⁻. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

Western blot analysis

Peritoneal macrophages (5×10^6 cells/well) were pretreated with various concentrations of AJ. The cells were then incubated with for 6 h with rIFN- γ (20 U/ml). They were finally stimulated with LPS (10 mg/ml) for 24 h. Whole cell lysates were made by boiling peritoneal macrophages in 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 then 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 (Sigma) for 1 h at room temperature and then incubated with anti-iNOS or COX-2 antibody. After washing in with phosphate-buffered saline (PBS) containing 0.05% tween-20 three times, the blot was incubated with secondary

antibody for 1 h and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp. Newark, NJ).

Statistical analysis

Results were expressed as the mean \pm S.E.M. of independent experiments, and statistical analysis was performed by one-way analysis of variance (ANOVA) to express the difference among the groups.

RESULTS

Effects of AJ on cell viability

To determine the effects of AJ on viability of mouse peritoneal macrophages, we carried out MTT assay. Cell cytotoxicity by AJ was not observed (Fig. 1).

Effect of AJ on IL-6 production

The effect of AJ was tested on IL-6 production from LPS-treated mouse peritoneal macrophages. As shown in Fig. 2, IL-6 production in response to LPS was inhibited by pre-treatment with 1, 10 and 100 $\mu\text{g/ml}$ AJ in a dose-dependent manner, IL-6 production by AJ (100 $\mu\text{g/ml}$) was significantly inhibited.

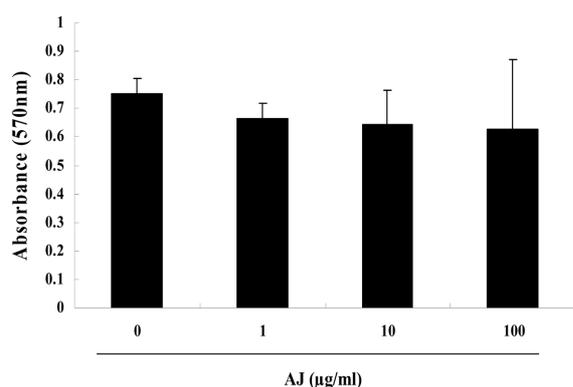


Fig. 1. Effect of AJ on the cell viability. Cell viability was evaluated by MTT colorimetric assay 48 h after AJ treatment in peritoneal macrophages. Values are the mean \pm S.E.M. of three independent experiments duplicate in each run.

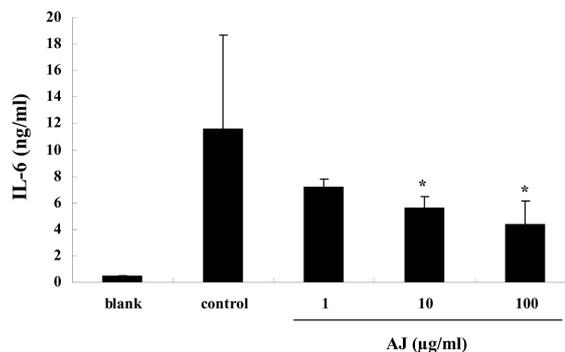


Fig. 2. Effect of AJ on Cytokine Production. Cells (3×10^6 cells/ml) were pretreated with AJ (0.01-1 mg/ml) for 30 min, and then stimulated with IFN- γ + LPS (10 mg/ml) for 24 h. Cytokine levels in supernatant were measured using ELISA. All data represent the mean \pm S.E.M. of three independent experiments. $P < 0.05$, significantly different from the IFN- γ + LPS-stimulated cells.

Effects of AJ on NO production

To determine the effect of AJ on the production of NO by mouse peritoneal macrophages, we pretreated the cells with various concentration AJ (1, 10, 100 $\mu\text{g/ml}$) and stimulated them with rIFN- γ (20 U/ml) and LPS (10 mg/ml). The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants after 48 h treatment. When mouse peritoneal macrophages were primed for 6 h with murine rIFN- γ and then treated with LPS, NO production was increased about 10 folds. AJ had no effect on NO production in resting mouse peritoneal macrophages compared to non-primed conditions. When AJ was pretreated in primed cell, AJ inhibits NO production dose dependently (Fig. 3).

Effects of AJ on expression of iNOS and COX-2 protein

In order to investigate the mechanism of action of AJ on the inhibition of NO production, this experiment was performed. We investigate the effect of the AJ at translational level by western blotting. As shown in Fig. 4, the expression of iNOS was increased after rIFN- δ (20 U/ml) plus LPS (10 $\mu\text{g/ml}$) challenge for 24 h. This enhanced expression of iNOS was significantly reduced by AJ (100 $\mu\text{g/ml}$). We

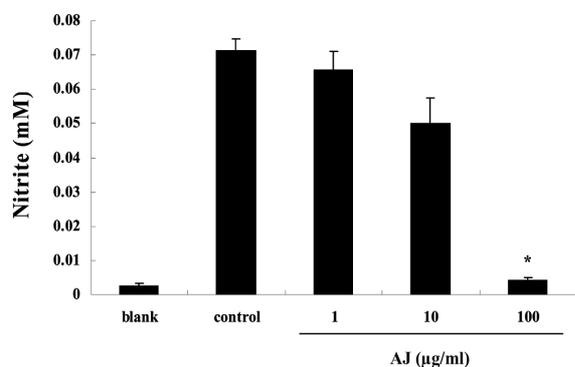


Fig. 3. Dose-dependent effects of AJ on NO inhibition in rIFN- γ and LPS-treated peritoneal macrophages. Peritoneal macrophages (2.5×10^5 cells/well) were cultured with various concentration AJ. The peritoneal macrophages were then stimulated with rIFN- γ (20 U/ml) and LPS (10 mg/ml). After 48 h of culture, NO release was measured by the Griess method (nitrite). NO (nitrite) released into the medium is presented as the mean \pm S.E.M. of three independent experiments duplicate in each run. * $P < 0.001$ compared to rIFN- γ + LPS.

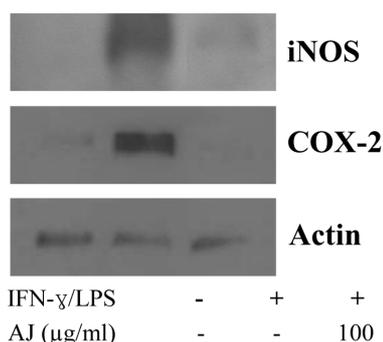


Fig. 4. Effects of AJ on the expression of iNOS and COX-2 by rIFN- γ plus LPS-induced peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were pretreated with AJ and then stimulated for 6 h with rIFN- γ (20 U/ml). The peritoneal macrophages were then stimulated with LPS (10 mg/ml) for 24 h. The protein extracts were prepared, and then samples were analyzed for iNOS and COX-2 expression by Western blotting as described in the method. 1, blank; 2, rIFN- γ + LPS; 3, AJ (100 mg/ml) + rIFN- γ + LPS.

investigated the effect of the AJ at translational level. The expression of COX-2 protein was markedly increased after IFN- γ (20 U/ml) plus LPS (10 mg/ml) challenge for 24 h. This increased expression of COX-2 protein was significantly reduced by AJ (Fig. 4).

DISCUSSION

We show that AJ significantly inhibited LPS and IFN- γ -induced IL-6 and NO production in a dose-dependent manner. Furthermore, the expression of iNOS and COX-2 protein was decreased.

Pro-inflammatory cytokine such as IL-6 mediates the development of various inflammatory reactions (Dinarello, 2000). In this study, we showed that AJ effectively inhibited the production and mRNA expression of IL-6 cytokine on LPS-stimulated peritoneal macrophages. These results suggest that AJ might have an anti-inflammatory activity.

Murine macrophage exhibits a particularly vigorous response to endotoxin, which induces production of variety of inflammatory modulators such as NO, PGE₂ by iNOS and COX-2 respectively. NO has been recognized to be an important mediator of cellular communication in several preparations such as macrophages, neutrophils, smooth muscle, autonomic nervous system, and central nervous system (Koyanagi *et al.*, 2000; Blackman *et al.*, 2000; Sharma *et al.*, 2000). At this point of view, we evaluated the effect of methanol extract of AJ on NO production in IFN- γ and LPS stimulated mouse peritoneal macrophages. In this study, exposure of macrophages to IFN- γ and LPS for 48 h was associated with an accumulation of nitrite in the medium, suggesting an enhanced NO production. This IFN- γ and LPS-induced NO production was inhibited dose-dependently by AJ without notable cytotoxicity (Fig. 1 and 3).

NO produced by one of three kinds of NO synthases (NOS) that neuronal NOS (nNOS), endothelial NOS (eNOS), inducible NOS (iNOS). nNOS and eNOS were critical to normal physiology and thus, inhibition of these enzymes caused damage. In the contrary, the level of iNOS playing a crucial role of excess production of NO in activated macrophages. Therefore, suppression of NO production via inhibition of iNOS expression levels might be an attractive therapeutic target for

the treatment of numerous pathological conditions, including inflammation. Thus the possibility that AJ might inhibit iNOS expression was examined and AJ suppressed the expression of iNOS significantly in IFN- γ and LPS-stimulated mouse peritoneal macrophages (Fig. 4).

COX, another key enzyme in inflammation, is the rate-limiting enzyme that catalyzes the formation of prostaglandins (PGs) from arachidonic acid. Levels of PGs increase early in the course of the inflammation (Wallace *et al.*, 1999). Like NOS, COX also exists in both constitutive (COX-1) and inducible (COX-2) forms. It is well known that the COX-1 is a housekeeping protein in most tissues and it catalyzes the synthesis of PGs for normal physiological functions. In constant, inducible isoform, COX-2, is rapidly stimulated by tumor promoters, growth factors, cytokines and pro-inflammatory molecules (Minghetti *et al.*, 1998) and responsible for the production of the high levels of PGs in several pathological conditions such as inflammation. Since, COX-2 is induced by stimulation in inflammatory cells, inhibitors of COX-2 induction might candidates for the new type of nonsteroidal anti-inflammatory drugs. We documented the increased production of COX-2 protein by macrophages exposed to IFN- γ and LPS. IFN- γ and LPS in combination with PF led to a significant reduction in COX-2 protein expression (Fig. 4). Thus, it seems quite reasonable to speculate that AJ may inhibits PGE₂ production. However, further studies are required to determine whether AJ is selective inhibitor of COX-2.

Here in our study, we have shown that AJ exerts its anti-inflammatory effects probably by the suppression of iNOS and COX-2 expression, and the final result is the inhibition of NO synthesis. Based on our present results, it is possible that AJ can offer a valuable means of therapy for the treatment of inflammatory diseases by attenuating IFN- γ and LPS-induced NO synthesis and controlling of iNOS and COX-2 expression.

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