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Puerariae flos inhibits inflammatory responses in interferon-γ and lipopolysaccharide-stimulated mouse peritoneal macrophages

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SUMMARY

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. In this study, we have examined the inhibition effects of NO production by 85% methanol extract of the flower of *Pueraria thunbergiana* (PF) in mouse macrophages. Extract of PF (1, 10, 100 μg/ml) inhibited NO production, inducible NO synthase and cyclooxygenase-2 expression in interferon-g and lipopolysaccharide-stimulated mouse peritoneal macrophages and it had no cytotoxicity. These data suggest that 85% methanol extract of PF might be useful in controlling macrophages mediated inflammatory disease.

Key words: *Puerariae flos*; Nitric oxide; Inducible NO synthase; Cyclooxygenase-2

INTRODUCTION

The dried flower-heads of *Pueraria thunbergiana* (PF) (Leguminosae) are an oriental drug, which have been used in therapy to counteract the problems associated with alcohol drinking and liver injury (Keung and Vallee, 1998). Inflammation is one mechanism by which alcohol causes liver damage (Szabo, 2007). But it has not been reported that PF has anti-inflammatory effect.

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

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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 (Ambs *et al*., 1998; Goldstein *et al*., 1998; Wilson *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 PF significantly inhibited LPS and interferon (IFN)-γ-induced NO production in a dose-dependent manner. Furthermore, the expression of iNOS and COX-2 protein were decreased.

MATERIALS AND METHODS

Reagents

Murine recombinant (r)IFN-γ was purchased from Pharmingen (Mnchen, Germany). LPS and sodium nitrite were purchased from Sigma (St. Louis, MO). 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 μm 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% CO_2, washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

Preparation of PF

The dried flower-heads of PF were 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 PF (MSS, 24.37 g), 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 MTT to formazan, as described by Mosmann (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).

Measurement of nitrite concentration

Peritoneal macrophages (2.5×10^5 cells/well) were cultured with various concentrations of PF. The

cells were then stimulated with rIFN-γ (20 U/ml). After 6 h, the cells were finally treated with LPS (10 μg/ml). NO synthesis in cell cultures was measured by a microplate assay method, as previously described (Chung *et al.*, 2002.). To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphtyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined by an automatic microplate reader. NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 9 μM of NO_2^-. 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 PF. The cells were then incubated with for 6 h with rIFN-γ (20 U/ml). They were finally stimulated with LPS (10 μg/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 chemiluminesence detection system according to the recommended procedure (Amersham Corp. Newark, NJ).

Statistical analysis

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

RESULTS

Effects of PF on cell viability

To determine the effects of PF on viability of mouse peritoneal macrophages, we carried out MTT assay. When we treated the cells with PF (100 μg/ml), it had no effect on cell viability (Fig. 1).

Effects of PF on NO Production

To determine the effect of PF on the production of NO by mouse peritoneal macrophages, we pretreated the cells with various concentration PF (1, 10, 100 μg/ml). And then we stimulated them with rIFN-γ (20 U/ml) and LPS (10 μg/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. PF had no effect on NO production in resting mouse peritoneal macrophages compared to non-primed conditions. When PF was pretreated in primed cell, PF inhibits NO production dose dependently (Fig. 2).

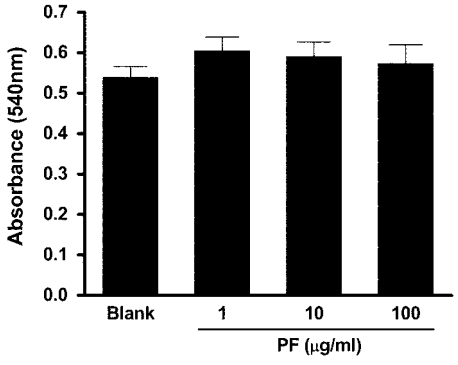

Fig. 1. Effect of PF on the cell viability. Cell viability was evaluated by MTT colorimetric assay 48 h after PF treatment in peritoneal macrophages. Values are the mean ± S.E.M. of three independent experiments duplicate in each run.

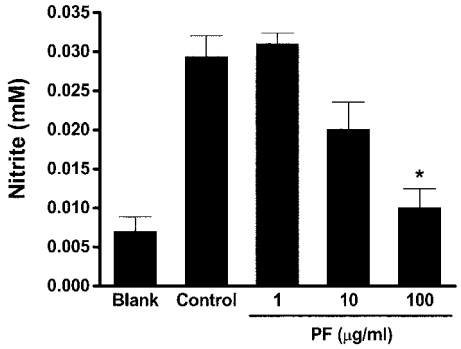

Fig. 2. Dose-dependent effects of PF on NO inhibition in rIFN-γ and LPS-treated peritoneal macrophages. Peritoenal macrophages (2.5×10^5 cells/well) were cultured with various concentration PF. The peritoneal macrophages were then stimulated with rIFN-γ (20 U/ml) and LPS (10 μg/ml). After 48 h of culture, NO release was measured by the Griess method (nitrite). NO released into the medium is presented as the mean ± S.E.M. of three independent experiments duplicate in each run. $^*P < 0.001$ compared to rIFN-γ + LPS.

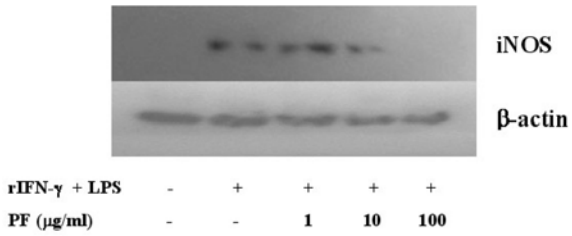

Fig. 3. Effects of PF on the expression of iNOS by rIFN-γ plus LPS-induced peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were pretreated with PF and then stimulated for 6 h with rIFN-γ (20 U/ml). The peritoneal macrophages were then stimulated with LPS (10 μg/ml) for 24 h. The protein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

Effects of PF on expression of iNOS

In order to investigate the mechanism of action of PF (100 μg/ml) on the inhibition of NO production, this experiment was performed. We investigate the effect of the PF at translational level by western blotting. As shown in Fig. 3, the expression of iNOS was increased after rIFN-γ (20 U/ml) plus LPS (10 μg/ml) challenge for 24 h. This enhanced expression of iNOS was significantly reduced by PF (Fig. 3).

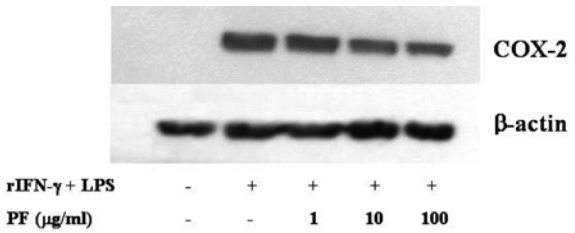

Fig. 4. Effects of PF on the expression of COX-2 by rIFN-γ plus LPS-induced peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were pretreated with PF and then stimulated for 6 h with rIFN-γ (20 U/ml). The peritoneal macrophages were then stimulated with LPS (10 μg/ml) for 24 h. The protein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

Effects of PF on expression of COX-2 protein

We investigated the effect of the PF (100 μg/ml) at translational level. As shown in Fig. 4, the expression of COX-2 protein was markedly increased after IFN-γ (20 U/ml) plus LPS (10 μg/ml) challenge for 24 h. This increased expression of COX-2 protein was significantly reduced by PF (Fig. 4).

DISCUSSION

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

Murine macrophage exhibits a particularly vigorous response to endotoxin, which induces production of variety of inflammatory modulators such as NO, PGE_2 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 (Blackman *et al.*, 2000; Koyanagi *et al.*, 2000; Sharma *et al.*, 2000). At this point of view, we evaluated the effect of methanol extract of PF 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 PF without notable cytotoxicity (Figs. 1 and 2).

NO produced by one of three kinds of NO synthases (NOS) that neuronal NOS (nNOS), endothelial NOS (eNOS), 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 PF might inhibit iNOS expression was examined and PF suppressed the expression of iNOS significantly in IFN-γ and LPS-stimulated mouse peritoneal macrophages (Fig. 3).

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 PF may inhibits PGE_2 production. However, further studies are required to determine whether PM is selective inhibitor of COX-2.

Here in our study, we have shown that PF 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 PF 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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