Aqueous extract of *Paeonia radix* suppresses lipopolysaccharide-induced expressions of cyclooxygenase-2 and inducible nitric oxide synthase in mouse BV2 microglial cells

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SUMMARY

Paeonia radix is the root of *Paeonia aliflora* Pallas, which is a perennial plant classified in the family Paeoniaceae. *Paeonia radix* possesses several pharmacological effects such as analgesic, anti-inflammatory and anti-allergic, anti-oxidative, and anti-coagulant activities. In this study, we investigated the effect of the aqueous extract of *Paeonia radix* on the lipopolysaccharide-induced inflammation in mouse BV2 microglial cells. The aqueous extract of *Paeonia radix* at respective concentration was treated one hour before lipopolysaccharide treatment. In the present results, the aqueous extract of *Paeonia radix* suppressed prostaglandin E₂ synthesis and nitric oxide production by inhibiting the lipopolysaccharide-stimulated mRNA expressions of cyclooxygenase-2 and inducible nitric oxide synthase in mouse BV2 microglial cells. These results demonstrate that *Paeonia radix* exerts anti-inflammatory and analgesic effects probably by suppressing mRNA expressions of cyclooxygenase-2 and inducible nitric oxide synthesis. The present study demonstrates that *Paeonia radix* may offer a valuable mean of therapy for brain inflammatory diseases.

Key words: *Paeonia radix*; Lipopolysaccharide; Cyclooxygenase-2; Prostaglandin E₂; Inducible nitric oxide synthase; Nitric oxide

INTRODUCTION

Paeonia radix is the root of *Paeonia aliflora* Pallas, which is a perennial plant classified in the family Paeoniaceae. Paeony plants, such as *Paeonia radix*, have been used for a lot of therapeutic purpose in traditional medicine. *Paeonia radix* possess several

pharmacological effects such as analgesic (Sugishita *et al.*, 1984), anti-inflammatory and anti-allergic (Yamahara *et al.*, 1982), anti-oxidative (Kim *et al.*, 1998), anti-coagulant activities (Ishida *et al.*, 1987), and inhibitory action on steroid protein binding (Tamaya *et al.*, 1986).

Microglia are considered resident immune cells in central nervous system (CNS) (Liu and Hong, 2003), and they are activated in response to infection or brain injury (Kreutzberg, 1996). The major factors produced by activated microglia are tumor necrosis factor- α (TNF- α), interleukins, nitric

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oxide (NO), prostaglandin E₂ (PGE₂), leukotrienes, growth factors, and fatty acid metabolites such as eicosanoids. Microglia activation in the CNS is associated with neurodegenerative disorders and release of proinflammatory cytokines (Boje and Arora, 1992; Chao *et al.*, 1992; Gonzalez-Scarano and Baltuch, 1999).

Pain is the first response to injury or infection. Injury and infection activate the immune system to produce inflammatory responses (Dickerson *et al.*, 1998). Lipopolysaccharide (LPS) initiates a number of major cellular responses that play vital roles in the pathogenesis of inflammatory response. LPS stimulates the production of inflammatory mediators such as NO, TNF- α , interleukins, PGE₂, and leukotrienes (Lee *et al.*, 1992; Kubes and McCafferty, 2000). Moreover, LPS enhances the pain response to various somatic stimuli (Watkins *et al.*, 1994; Yirmiya *et al.*, 1994).

PGE₂ is a key inflammatory mediator, and an increased level of PGE₂ mediates the cardinal features of inflammation such as pain, edema, and fever (Coleman *et al.*, 1994; Tilley *et al.*, 2001). PGE₂ is converted from arachidonic acid by cyclooxygenase (COX). There are two isoforms of COX: COX-1 and COX-2. COX-1 is a constitutively expressed form in normal physiologic functions, while COX-2 is expressed only in response to inflammatory signals such as cytokines and bacterial endotoxin, LPS. COX-2 produces large amount of PGE₂ that induces inflammation (Mitchell *et al.*, 1995; Crofford *et al.*, 2000).

NO is synthesized from L-arginine by nitric oxide synthase (NOS). NO plays an important role in the regulation of many physiological processes (Dawson *et al.*, 1992). There are three distinct isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Of these, iNOS is an important enzyme involved in the regulation of inflammation (Yui *et al.*, 1991), and LPS upregulates iNOS expression in macrophages (Korhonen *et al.*, 2002) and microglial cells (Vegeto *et al.*, 2001).

In the present study, we investigate the effects of the aqueous extract of *Paeonia radix* from *Paeonia aliflora* Pallas on the LPS-stimulated mRNA expressions of COX-1, COX-2, and iNOS in mouse BV2 microglial cells by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR). The effects of *Paeonia radix* on the PGE₂ synthesis and the NO production were also studied by performing PGE₂ immunoassay and by detecting NO.

MATERIALS AND METHODS

Aqueous extraction of *Paeonia radix* from *Paeonia aliflora pallas*

Paeonia radix used in this experiment was obtained from the Kyungdong market (Seoul, Republic of Korea). To obtain the aqueous extract of *Paeonia radix*, 200 g of *Paeonia radix* was added to distilled water, and extraction was performed by heating at 80°C for 2 h with distilled water, pressure-filtered, concentrated by using a rotary evaporator (Eyela, Tokyo, Japan). The resulting powder weighing 17.3 g (a yield of 8.65%) was obtained through lyophilization by a freezing drier (Ilsin, Kyungkido, Republic of Korea) for 24 h. The aqueous extract of *Paeonia radix* at the respective concentration was treated one hour before the LPS treatment in mouse BV2 microglial cells.

Cell culture

Mouse BV2 microglial 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, 5% CO₂ in a humidified cell incubator. The cells were plated onto culture dishes at a density of 2×10^4 cells/cm² at 24 h prior to drug treatment.

MTT cytotoxicity assay

Mouse BV2 microglial cells were grown in a final

volume of 100 μ l culture medium per well in a 96-well plate. In order to determine the cytotoxicity of *Paeonia radix*, the cells were treated with *Paeonia radix* at concentrations of 0.1 μ g/ml, 1 μ g/ml, 10 μ g/ml, 100 μ g/ml, and 1,000 μ g/ml for 24 h. The cells in the control group were left untreated. After adding 10 μ l of the MTT labeling reagent containing 5 mg/ml of MTT in phosphate-buffered saline (PBS) to each well, the plate was incubated for 4 h. Solubilization solution 100 μ l containing 10% sodium dodecyl sulfate (SDS) 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.) $\times$ 100.

RNA isolation and RT-PCR

To identify the mRNA expressions of COX-1, COX-2, and iNOS, RT-PCR was performed. The total RNA was isolated from BV2 cells using RNeasyTM (TEL-TEST, Friendswood, TX, USA). Two μ g of RNA and 2 μ l of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65°C for 15 min. One μ l of AMV reverse transcriptase (Promega), 5 μ l of 10 mM dNTP (Promega), 1 μ l of RNasin (Promega), and 5 μ l of 10 AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 50 μ l volume 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 μ l containing 1 μ l of the appropriate cDNA, 1 μ l of each set of primers at a concentration of 10 pM, 4 μ l of 10 $\times$ RT buffer, 1 μ l of 2.5 mM dNTP, and 2 units of Taq DNA polymerase (Takara, Shiga, Japan). For mouse COX-1, the primer

sequences were 5'-AGTGCGGTCCAACCTTATCC-3' (a 20-mer sense oligonucleotide) and 5'-CCGCAGGTGATACTGTCGTT-3' (a 20-mer anti-sense oligonucleotide). For mouse COX-2, the primer sequences were 5'-CCAGATGCTATCTTTGGGGAGAC-3' (a 23-mer sense oligonucleotide) and 5'-CTTGCAATTGATGGTGGCTG-3' (a 19-mer anti-sense oligonucleotide). For mouse iNOS, the primer sequences were 5'-ATGAGGTACTCAGCGTGCTCCAC-3' (a 23-mer sense oligonucleotide) and 5'-CCACAATAGTACAATACTACTTGG-3' (a 24-mer anti-sense oligonucleotide). For cyclophilin, the internal control used in the study, the primer sequences were 5'-ACCCACCGTGTCTTCGAC-3' (a 20-mer sense oligonucleotide) and 5'-CATTTGCCATGGACAAGATG-3' (a 20-mer anti-sense oligonucleotide). The expected size of the PCR product was 381 bp for COX-1, 249 bp for COX-2, 395 bp for iNOS, and 299 bp for cyclophilin.

For COX-1, COX-2 and iNOS, the PCR procedures were carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, 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 45 s, annealing at 60°C for 45 s, and extension at 72°C for 60 s, with an additional extension step at the end of the procedure at 72°C for 10 min. For cyclophilin, the PCR procedure was 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 s, annealing at 55°C, and extension at 72°C for 45 s, 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 Molecular AnalystTM version 1.4.1 (Bio-Rad, Hercules, CA, USA).

Measurement of PGE₂ synthesis

In order to determine the effect of *Paeonia radix* on PGE₂ synthesis, the amount of PGE₂ in the supernatant was assessed by using a commercially

available PGE₂ competitive enzyme immunoassay kit. (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA) Supernatant 100 µl from the culture medium and the standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE₂ antibody and peroxidase-conjugated PGE₂ were added to each well, and the plate was incubated at room temperature with shaking for 1 h. 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 H₂SO₄. The absorbance of the content of each well was then measured at a wavelength of 450 nm.

Determination of NO production

In order to determine the effect of *Paeonia radix* on NO production, the amount of nitrite in the supernatant was measured by using the method based on the Griess reaction as an indicator of NO production. After collection of 100 µl of cell culture medium, 50 µl of 1% sulfanilamide was added to each well, and the plate was incubated at room temperature for 10 min. Then 0.1% naphthylethylenediamine containing 5% phosphoric acid was added, and the plate was incubated at room temperature for 10 min. The absorbance of the content in each well was measured at a wavelength of 450 nm. The nitrite concentration was calculated from a nitrite standard curve generated by mixing 0 to 200 µM sodium nitrite solutions with the Griess reagent. The standard curve was typically linear between 0 and 200 µM of sodium nitrite.

Statistical analysis

The results are presented as the mean S.E.M. The data were analyzed by one-way ANOVA followed by Duncan's *post-hoc* test using SPSS. The differences were considered statistically significant at $P < 0.05$.

RESULTS

MTT cytotoxicity assay of *Paeonia radix*

As shown in Fig. 1, the viability of cells incubated with *Paeonia radix* at concentrations of 0.1 µg/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml, and 1,000 µg/ml for 24 h was $100.54 \pm 3.55\%$, $118.88 \pm 5.16\%$, $116.04 \pm 6.31\%$, $128.77 \pm 9.49\%$, and $68.26 \pm 6.34\%$ of the control value, respectively (Fig. 1).

The present results showed that the *Paeonia radix* exerted no significant cytotoxicity until it reached at a concentration of 1,000 µg/ml. *Paeonia radix* at concentrations of 1 µg/ml, 10 µg/ml, and 100 µg/ml showed a slight enhancing effect on cell proliferation in BV2 cells.

Effect of *Paeonia radix* on mRNA expressions of COX-1, COX-2, and iNOS

RT-PCR analysis of the mRNA levels of COX-1, COX-2, and iNOS was performed in order to provide an estimate of the relative level of expressions of these genes. In the present study, the mRNA levels of COX-1, COX-2, and iNOS in the control cells were set as 1.00.

The level of COX-1 mRNA following treatment with 2 µg/ml LPS for 24 h was 0.90 ± 0.08 . The

Fig. 1. Cytotoxicity of *Paeonia radix*. (A) Control cells, (B) 0.1 µg/ml *Paeonia radix*-treated cells, (C) 1 µg/ml *Paeonia radix*-treated cells, (D) 10 µg/ml *Paeonia radix*-treated cells, (E) 100 µg/ml *Paeonia radix*-treated cells, (F) 1,000 µg/ml *Paeonia radix*-treated cells. The data are presented as the mean ± S.E.M.

Fig. 2. Results of the mRNA level of COX-1. The cells at a density of 2×10^6 were treated with *Paeonia radix* at concentrations of 10 μ g/ml and 100 μ g/ml one hour before treatment of 2 μ g/ml LPS for 24 h. Cyclophilin mRNA was used as the internal control. * $P < 0.05$ compared to the control. # $P < 0.05$ compared to the LPS-treated group. (A) Control cells, (B) 2 μ g/ml LPS-treated cells, (C) LPS and 10 μ g/ml *Paeonia radix*-treated cells, (D) LPS and 100 μ g/ml *Paeonia radix*-treated cells, (E) LPS and 500 μ M ASA-treated cells.

level of COX-1 mRNA in the cells pre-treated with *Paeonia radix* at concentrations of 10 μ g/ml, 100 μ g/ml, and 500 μ M acetylsalicylic acid (ASA) one hour before LPS exposure was decreased to 0.56 ± 0.09 , 0.13 ± 0.02 , and 0.09 ± 0.14 , respectively (Fig. 2).

The level of COX-2 mRNA following treatment with 2 μ g/ml LPS for 24 h was markedly increased to 14.74 ± 1.24 . The level of COX-2 mRNA in the cells pre-treated with *Paeonia radix* at concentrations of 10 μ g/ml, 100 μ g/ml, and 500 μ M ASA one hour before LPS exposure was decreased to 11.39 ± 2.18 , 9.42 ± 1.26 , and 9.61 ± 1.46 respectively (Fig. 3).

The level of iNOS mRNA following treatment with 2 μ g/ml LPS for 24 h was markedly increased to 28.58 ± 3.18 . The level of iNOS mRNA in the cells pre-treated with *Paeonia radix* at concentrations of 10 μ g/ml, 100 μ g/ml, and 500 μ M ASA one

Fig. 3. Results of the mRNA level of COX-2. The cells at a density of 2×10^6 were treated with *Paeonia radix* at concentrations of 10 μ g/ml and 100 μ g/ml one hour before treatment of 2 μ g/ml LPS for 24 h. Cyclophilin mRNA was used as the internal control. * $P < 0.05$ compared to the control. # $P < 0.05$ compared to the LPS-treated group. (A) Control cells, (B) 2 μ g/ml LPS-treated cells, (C) LPS and 10 μ g/ml *Paeonia radix*-treated cells, (D) LPS and 100 μ g/ml *Paeonia radix*-treated cells, (E) LPS and 500 μ M ASA-treated cells.

hour before LPS exposure was decreased to 27.91 ± 3.04 , 13.59 ± 2.33 , and 4.13 ± 0.99 , respectively (Fig. 4).

The present results showed that LPS exerted no significant effect on COX-1 expression, while LPS enhanced COX-2 and iNOS mRNA expressions in BV2 cells. *Paeonia radix* suppressed COX-1 mRNA expression and LPS-induced COX-2 and iNOS mRNA expressions in BV2 cells.

Effect of *Paeonia radix* on PGE₂ synthesis

From PGE₂ immunoassay, the amount of PGE₂ from the culture medium was increased from 81.85 ± 0.34 pg/ml to 121.60 ± 1.69 pg/ml after 24 h of exposure to LPS. PGE₂ synthesis was decreased to 115.11 ± 2.89 pg/ml, 102.01 ± 3.65 pg/ml, and 81.71 ± 3.82 pg/ml by pre-treatment with *Paeonia radix* at concentrations of 10 μ g/ml, 100 μ g/ml,

Fig. 4. Results of the mRNA level of iNOS. The cells at a density of 2×10^6 were treated with *Paeonia radix* at concentrations of 10 µg/ml and 100 µg/ml one hour before treatment of 2 µg/ml LPS for 24 h. Cyclophilin mRNA was used as the internal control. * $P < 0.05$ compared to the control. # $P < 0.05$ compared to the LPS-treated group. (A) Control cells, (B) 2 µg/ml LPS-treated cells, (C) LPS and 10 µg/ml *Paeonia radix*-treated cells, (D) LPS and 100 µg/ml *Paeonia radix*-treated cells, (E) LPS and 500 µM ASA-treated cells.

Fig. 5. Measurement of PGE₂ in BV2 microglial cells. The cells at a density of 2×10^5 were treated with *Paeonia radix* at concentrations of 10 µg/ml and 100 µg/ml one hour before treatment of 2 µg/ml LPS for 24 h. * $P < 0.05$ compared to the control. # $P < 0.05$ compared to the LPS-treated group. (A) Control cells, (B) 2 µg/ml LPS-treated cells, (C) LPS and 10 µg/ml *Paeonia radix*-treated cells, (D) LPS and 100 µg/ml *Paeonia radix*-treated cells, (E) LPS and 500 µM ASA-treated cells.

Fig. 6. Measurement of NO produced in BV2 microglial cells. The cells at a density of 2×10^5 were treated with *Paeonia radix* at concentration of 10 µg/ml and 100 µg/ml one hour before treatment of 2 µg/ml LPS for 24 h. * $P < 0.05$ compared to the control. # $P < 0.05$ compared to the LPS-treated group. (A) Control cells, (B) 2 µg/ml LPS-treated cells, (C) LPS and 10 µg/ml *Paeonia radix*-treated cells, (D) LPS and 100 µg/ml *Paeonia radix*-treated cells, (E) LPS and 500 µM ASA-treated cells.

and 500 µM ASA one hour before LPS exposure, respectively (Fig. 5).

The present results showed that LPS enhanced PGE₂ synthesis in BV2 cells and that *Paeonia radix* suppressed LPS-induced PGE₂ synthesis.

Effect of *Paeonia radix* on NO production

From the NO detection assay, the amount of nitrite from the culture medium was increased from 6.06 ± 0.99 µM to 50.60 ± 2.41 µM after 24 h of exposure to LPS. NO production was decreased to 40.44 ± 4.57 µM, 37.28 ± 2.60 µM, and 25.04 ± 2.67 µM by pre-treatment with *Paeonia radix* at concentrations of 10 µg/ml, 100 µg/ml, and 500 µM ASA one hour before LPS exposure, respectively (Fig. 6).

The present results showed that LPS enhanced NO production in BV2 cells and that *Paeonia radix* suppressed LPS-induced NO production.

DISCUSSION

In the present results, LPS treatment enhanced the mRNA expressions of COX-2 and iNOS, and resulted

in the increase of PGE₂ synthesis and NO production in mouse BV2 microglial cells.

Inflammation is a complex process that involves numerous mediators that are of cellular and plasma origin, and these mediators have elaborate and interrelated biological effects. PGE₂ and NO are implicated in a variety of pathophysiological processes including inflammation and carcinogenesis. COX-2 and iNOS are mainly responsible for the production of large amounts of PGE₂ and NO (Schmidt and Walter, 1994; Simon, 1999).

The present results demonstrated that the aqueous extract of *Paeonia radix* inhibited LPS-stimulated enhancement of COX-2 mRNA expression and PGE₂ synthesis in the mouse BV2 microglial cells. Elevation of COX-2 activity has also been suggested to be closely associated with the occurrence of cancers, arthritis, and several types of neurodegenerative disorders. PGE₂ is a major metabolite of the COX-2 pathway. PGE₂ has emerged as an important lipid mediator of inflammation and immune regulation processes, and it is also been implicated in the pathogenesis of acute and chronic inflammatory disease states (Hinz *et al.*, 2000). Specific COX-2 inhibitors are known to attenuate the symptoms of inflammation (Crofford *et al.*, 2000; Shao *et al.*, 2000).

The present results demonstrated that the aqueous extract of *Paeonia radix* inhibited LPS-stimulated enhancement of iNOS mRNA expression and NO production in the mouse BV2 microglial cells. NO has been reported to modulate the activity of COX-2 in a cGMP-independent manner, and NO plays a critical role in the release of PGE₂ by direct activation of COX-2 (Salvemini *et al.*, 1993). NO production has been shown as a key regulator of homeostasis and NO is known as an important mediator of inflammation in several animal models (Vane *et al.*, 1994). Especially, the generation of NO by iNOS plays an important role in inflammation, host-defense responses, and tissue repair (Nathan and Xie, 1994). After cells are exposed to endogenous and exogenous stimulators such as LPS and viral

infections, iNOS is quantitatively induced in a variety of cells. Excessive iNOS expression triggers several deleterious cellular responses and induces inflammation, sepsis, and stroke (Yui *et al.*, 1991; Nakashima *et al.*, 2003). Inhibition of iNOS expression in murine macrophages has been suggested as another possible mechanism of non-steroidal anti-inflammatory drugs (Amin *et al.*, 1995).

Paeonia radix is known to nourish and restore the blood in Oriental medicine, and it has been widely used as a component of blood-building decoctions and for reducing fatigue. Paeoniflorin is a bioactive monoterpene glucoside in *Paeonia radix*. Paeoniflorin is known to possess an anti-coagulant (Ishida *et al.*, 1987), analgesic (Sugishita *et al.*, 1984), anti-inflammatory and anti-allergy (Yamahara *et al.*, 1982), and anti-oxidative activities (Kim *et al.*, 2002).

Here in this study, we have shown that *Paeonia radix* extracted from *Paeonia aliflora* Pallas suppressed PGE₂ synthesis and NO production by inhibiting LPS-stimulated enhancement of COX-2 and iNOS mRNA expressions in the BV2 microglial cells. Our present results demonstrated that *Paeonia radix* exerts anti-inflammatory and analgesic effects probably by suppressing COX-2 and iNOS mRNA expressions, and results in inhibition of PGE₂ synthesis and NO production. Based on the present study, *Paeonia radix* has a potency to be developed as a new natural herbal analgesic.

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