

Antiallergic Effect of the Root of *Paeonia lactiflora* and Its Constituents Paeoniflorin and Paeonol

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(Received September 7, 2007)

The root of *Paeonia lactiflora* PALL (PL, Family Paeoniaceae) has been used frequently as an antipyretic and anti-inflammatory agent in traditional medicines of Korea, China and Japan. To evaluate antiallergic effect of PL, we isolated its main constituents, paeoniflorin and paeonol, and evaluated *in vivo* their inhibitory effects against passive cutaneous anaphylaxis (PCA) reaction induced by IgE-antigen complex and scratching behaviors induced by compound 48/80. PL, paeoniflorin and paeonol potently inhibited PCA reaction and scratching behaviors in mice. Paeoniflorin exhibited the most potent inhibition against scratching behaviors and the acetic acid-induced writhing syndrome in mice. Paeonol most potently inhibited PCA reaction and mast cells degranulation. These findings suggest that PL can improve IgE-induced anaphylaxis and scratching behaviors, and may be due to the effect of its constituents, paeoniflorin and paeonol.

Keywords: Allergy, *Paeonia lactiflora*, Paeoniflorin, Paeonol

INTRODUCTION

Allergic diseases, such as allergic rhinitis, atopic dermatitis, asthma and food allergy, are now rapidly increasing chronic health problem in most countries (Wuthrich, 1989). Mast cells and basophils are well-known as critical participants in various biologic processes of allergic diseases (Stevens and Austin, 1989; Plaut *et al.*, 1989; Bruhns *et al.*, 2005). These cells express surface membrane receptors with high affinity and specificity for IgE. The interaction of antigen-bound IgE in surface membrane receptors releases histamine, prostaglandins, leukotrienes and cytokines (Nicoloff, 1998; Mitre and Nutman, 2006). These cytokines activate chemotaxis and phagocytosis of neutrophils and macrophages. Finally cytokine-induced reactions cause tissue inflammation. Therefore, antiallergic agents, such as anti-histamines, steroids and immunosuppressants, have been using against allergic diseases

(Schafer-Korting *et al.*, 1996; Sakuma *et al.*, 2001; Simons, 1992). However, improving these diseases is too difficult. Therefore, herbal medicines have been advanced for allergic diseases, and their effectiveness have received increasing attention (Bielory, 2004; Yang *et al.*, 2001).

The root of *Paeonia lactiflora* PALL (PL, family Paeoniaceae) has been used frequently as a antipyretic and anti-inflammatory agent in traditional medicines of Korea, Japan and China. PL, which contains paeoniflorin and paeonol as main constituents, is reported to have many biological properties including antipyretic, cerebral infraction-inhibitory, antioxidant, antiinflammatory, convulsant, anxiolytic, and lipid peroxidation-inhibitory activities (Khan *et al.*, 2005; Chou, 2003; Kim *et al.*, 2004; Mi *et al.*, 2005; Xiao *et al.*, 2005; Yang, 2004; Hsieh *et al.*, 2006). Paeonol was reported to inhibit the anaphylactic reaction induced by compound 48/80. Nevertheless, its pharmacological studies mainly have been focused on anti-inflammatory activity.

Therefore, to evaluate antiallergic activity of PL, we isolated main constituents paeoniflorin and paeonol from PL and *in vivo* evaluated their inhibitory activities against scratching behaviors and passive cutaneous anaphylaxis reaction.

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MATERIALS AND METHODS

Materials

Bovine serum, ovalbumin (OVA), dinitrophenyl-human serum albumin (DNP-HSA), cremophor EL, evans blue and compound 48/80 were purchased from Sigma Co. (St. Louis, MO, U.S.A.). Aluminum hydroxide gel (alum, Rehydralgel) was purchased from Reheis (Berkeley Heights, NJ, U.S.A.).

Extract of paeoniae radix and isolation of paeoniflorin and paeonol

PL was purchased from Kyung Dong Market (Seoul, Korea) and identified by Prof. Sam-Sik Kang, College of Pharmacy, Seoul National University. The PL was extracted with 80% ethanol. Paeoniflorin (purity, >95%) and paeonol (purity, > 95%) were isolated from its extract according to the previous report (Goto *et al.*, 1996) and their purities were analyzed by Dr. Kang S.S., Institute of Natural Products, Seoul National University.

Animals

The male BALB/c and ICR mice (20–25 g) and male Sprague-Dawley rats (180–220 g) were supplied from the Orient Experimental Animal Breeding Center (Seoul, Korea). All the animals were housed in wire cages at 20–22°C and 50 ± 10% humidity, fed standard laboratory chow (Orient Experimental Animal Breeding Center, Seoul, Korea) and given water *ad libitum*. All the procedures relating to the animals and their care conformed to the international guidelines 'Principles of Laboratory Animals Care' (NIH publication no. 85-23, revised 1985).

Assay of passive cutaneous anaphylaxis reaction in mice

An IgE-dependent cutaneous reaction was examined using the method reported by Choo *et al.* (2003). The male ICR mice were injected intradermally with 10 µg of anti-dinitrophenol IgE into two dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with an indelible red marker. Forty-eight hours later, each mouse was injected with 200 µL of 3% Evans blue PBS containing 200 µg of DNP-HSA through the tail vein. The test agents were administered orally 1 h before the DNP-HSA injection. Thirty minutes after the DNP-HSA injection, the mice were sacrificed and their dorsal skin was removed in order to measure the pigment area. After extraction with 1 mL of KOH 1.0 N and 4 mL of a mixture of acetone and phosphoric acid 0.6 N (13:5), the amount of dye was determined colorimetrically (absorbance at 620 nm).

Scratching behavioral experiment

Scratching behavioral experiment was performed ac-

cording to the method reported by Sugimoto *et al.* (1998). Male BALB/c mice were placed into acrylic cages (22×22×24 cm) for approximately 10 min to allow for acclimation. Test agents were administered orally 1 h before administering the scratching agent, compound 48/80. Compound 48/80 was dissolved in saline. The rostral part of the skin on the back of the mice was clipped, and 50 µg/50 µL of compound 48/80 was injected intradermally into each mouse using a 29 gauge needle. The control mice received a saline injection in place of compound 48/80. Immediately after the intradermal injection, the mice (one animal/cage) were placed back into their cage and their scratching behaviors were recorded automatically using an 8-mm video camera (SV-K80, Samsung, Seoul, Korea). The frequency of scratching of the injection site by the hind paws was determined and compared with that of other sites, such as the ears. Each mouse was used for only one experiment. The mice generally scratched for 1 s, and a series of these behaviors was counted as a single scratching incident for 1 h.

Assay of serum IgE in mouse asthmatic mice induced by OVA

The male BALB/c mice were sensitized by an intraperitoneal injection of the mixture of 1 mL of 0.005% OVA and 1 mL of aluminum hydroxide gel. The non-sensitized mice received an intraperitoneal injection of alum alone. On the 10th day, the mice were given an intraperitoneal booster injection of the same antigen or alum. On the 17th day after sensitization, the mice were challenged with aerosolized 5% OVA, which was generated by an ultrasonic nebulizer (Ultra-Neb 99; DeVilbiss, Somerset, PA, U.S.A.). The aerosol was circulated through a large acrylic cylindrical chamber, and mice were placed into the chamber for 1 h in a single day. The OVA aerosol challenge was repeated for 5 days. Test agents were orally administered 1 h before OVA aerosol challenge for 5 days. Normal control was treated with saline alone, but without OVA. On the next day, the mice were sacrificed.

The level of IgE in the blood was measured by the ELISA kit (BD Biosciences, San Diego, CA, U.S.A.) according to previously reported method (Chen *et al.*, 2006).

Assay of acetic acid-induced writhing syndrome in mice

The analgesic effect of test agents was determined by measuring acetic acid-induced writhing syndrome in male ICR mice. The writhing syndrome was induced by the intraperitoneal administration of 0.7% acetic acid saline solution 30 min after the test agents were orally administered. The frequency of the writhing syndrome was measured for 10 min at 10 min after intraperitoneal injection of acetic acid saline solution. The normal and control groups

were treated with saline instead of test agents.

Assay of histamine release from rat peritoneal exudate cells induced by compound 48/80

Male Sprague-Dawley rats (200 ± 20 g) were exsanguinated and then injected intraperitoneally with 20 mL of a physiological solution (137 mM NaCl, 2.7 mM CaCl₂, 1.0 mM MgCl₂·6H₂O, 5.6 mM glucose, 1 unit of heparin/mL and 5 mM phosphate buffer at pH 7.2). The abdominal region was gently massaged for 2 min, and the peritoneal exudates cells were collected. The cell suspension was centrifuged at 300×g and 4°C for 5 min, and washed several times with the physiological solution. The cells obtained were then pooled (5×10⁴/mL cells). The prepared peritoneal exudate cell suspension (2.5 mL) was mixed with various concentrations of the test agents, in 0.5 mL of each physiological solution, and the mixtures were preincubated at 37°C for 5 min. The control and blank were treated in a similar manner. The reaction mixture (3.0 mL) was mixed with 0.5 mL of compound 48/80 (a degranulation activator), cooled to 4°C and then centrifuged at 2500×g and 4°C for 10 min. The compound 48/80-induced release of histamine from the rat peritoneal exudate cells was examined using the method reported by Choo *et al.* (2003).

Assay of degranulation of RBL-2H3 cells stimulated by IgE-antigen complex

The inhibitory activity of test agents against the release of β-hexosaminidase from RBL-2H3 cells was evaluated according to Choo *et al.* (2003).

Enzyme-linked immunosorbent assay (ELISA)

RBL-2H3 cells (5×10⁵ cells), previously cultured in DMEM, were treated with 0.5 μg/mL of mouse monoclonal IgE to sensitize the cells. The cells (1.8 mL) were exposed to 0.2 mL of the test agents for 4 h, followed by treatment with 0.2 mL DNP-HSA (1 μg/mL) for 40 min at 37°C. The supernatant (50 mL) was transferred to 96-well ELISA plates, and the concentration of TNF-α and IL-4 then determined using commercial ELISA Kits (Pierce Biotechnology, Inc., Rockford, IL, USA) (Matsuda *et al.*, 2003).

Statistics

All the data is expressed as a mean ± standard deviation, and statistical significance was analyzed using one-way ANOVA followed by Student-Newman-Keuls test.

RESULTS

To evaluate antiallergic activity of PL, inhibitory effects of was PL and its main constituents paeoniflorin and paeonol against IgE-induced PCA reaction and compound 48/80-induced scratching behaviors in mice were

measured (Table I). PCA reaction was induced by the injection of IgE-antigen complex, PL and its constituents were administered orally or intraperitoneally 60 min prior to challenge with antigen. IgE-antigen complex potently induced PCA reaction. PL and its constituents, paeoniflorin and paeonol, showed the potent inhibition against PCA reaction. Paeonol most potently inhibited PCA reaction. PL and its constituents, paeoniflorin and paeonol, also inhibited scratching behaviors induced by compound 48/80. Paeoniflorin and paeonol at a dose of 50 mg/kg reduced the scratching behavior frequency by 69% and 52%, respectively. The effect of paeoniflorin and paeonol in IgE production of asthmatic mice induced by OVA was investigated (Fig. 1). Paeonol inhibited IgE production, but paeoniflorin did not reduce it. Nevertheless, the inhibitory effect of paeonol was weaker than that of betamethasone.

To understand the mechanism of antiallergic effect of paeoniflorin and paeonol, their inhibitory effects against compound 48/80-induced degranulation of rat peritoneal mast cells and RBL-2H3 cells were investigated (Table II). Of tested agents, paeonol, which exhibited the most potent PCA reaction-inhibitory activity, showed the most potent degranulation-inhibitory effect. The inhibitory effect of paeoniflorin and paeonol on protein expression of proinflammatory cytokines TNF-α and IL-4 in RBL-2H3 cells induced by IgE-antigen complex was measured by ELISA (Fig. 2). These constituents weakly inhibited TNF-α and IL-4 expression, although paeoniflorin almost did not inhibit these cytokine expressions.

To evaluate the analgesic effect of paeoniflorin and paeonol, their inhibitory effects against acetic acid-in-

Table I. Effect of PL and its constituents on mouse passive cutaneous anaphylaxis (PCA) reaction induced by IgE-antigen complex and scratching behaviors induced by compound 48/80

Agent	Dose (mg/kg)	Inhibition ^{a)} (%)	
		PCA reaction ^{c)}	Scratching behaviors ^{d)}
PL ^{b)}	100	40 ± 10 ^{e,f}	34 ± 15 ^e
	250	56 ± 9 ^{f,g}	39 ± 4 ^e
Paeoniflorin	10	39 ± 14 ^e	61 ± 16 ^{g,h}
	50	59 ± 13 ^{f,g,h}	69 ± 8 ^{h,i}
Paeonol	10	86 ± 18 ⁱ	43 ± 13 ^{e,f}
	50	92 ± 15 ^j	52 ± 3 ^{f,g}
Azelastine	10	80 ± 16 ⁱ	76 ± 2 ^{h,i}

^{a)}Inhibition (%) values indicate mean ± S.D. (n=6).

^{b)}PL, 80% ethanol extract of root of *Paeonia lactiflora*.

^{c)}Amounts of extravasated Evan blue in the dorsal skin (1×1 cm) of control stimulated with IgE-antigen complex and vehicle-treated groups were 27 ± 3 μg and 12 ± 2 μg, respectively.

^{d)}Scratching behavior frequency numbers of normal control, which was treated with saline alone, and control group, which was treated with compound 48/80 and saline, for 1 h were 349 ± 32 and 3 ± 2, respectively.

^{e),(g,h,i)}Items with the same letter in each column were not significantly different.

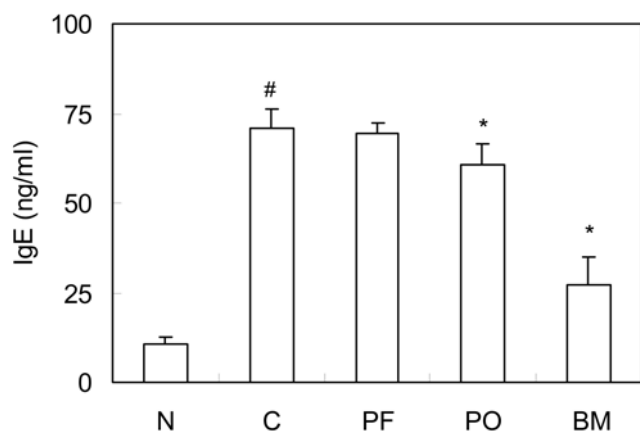


Fig. 1. Effect of paeoniflorin and paeonol on the blood IgE level of asthmatic mice induced by ovalbumin. Test agents (Con, saline alone; PF, paeoniflorin at a dose of 50 mg/kg; PO, paeonol at a dose of 50 mg/kg; and BM, betamethasone at a dose of 10 mg/kg) were orally administered 1 h before OVA aerosol challenge for 5 days. Normal control was treated with saline alone, but without OVA. The level of IgE in the blood was measured by the ELISA kit (BD Biosciences, San Diego, CA, U.S.A.). Inhibition values indicate mean $\pm$ S.D. ($n = 6$). [#]Significantly different, compared with normal group ($p < 0.05$). ^{*}Significantly different, compared to control group ($p < 0.05$).

Table II. Effect of PL, paeoniflorin and paeonol on degranulation of rat peritoneal mast cells and RBL-2H3 cells

Agent	IC ₅₀ ^a (μ M)	
	Rat peritoneal mast cells	RBL-2H3 cells
PL ^b	>100 (27) ^c	>100 (21)
Paeoniflorin	>100 (12)	>100 (9)
Paeonol	90.3	>100 (41)
Azelastine	25.8	35.4

^a)IC₅₀, 50% inhibitory concentration against degranulation.

^b)The concentration of PL, which is 80% ethanol extract of the root of *Paeonia lactiflora* is mg/mL.

^c)Values in parenthesis indicate inhibition percents at a dose of 100 μ M against mast cells.

duced writhing syndrome in mice were measured (Table III). Paeoniflorin and paeonol potently inhibited the writhing syndrome. Paeonol exhibited more potent inhibition than paeoniflorin.

DISCUSSION

Azelastine, a representative antiallergic drug (Akagi *et al.*, 1987), is an H1-receptor antagonist and decreases the mediator release from mast cells and basophils. Quercetin, a non-steroidal anti-inflammatory flavonoid, inhibits PCA reaction (Ito *et al.* 1998). In the present study, PL and its constituents, paeoniflorin and paeonol, inhibited mouse PCA reaction induced by IgE-antigen complex as well as

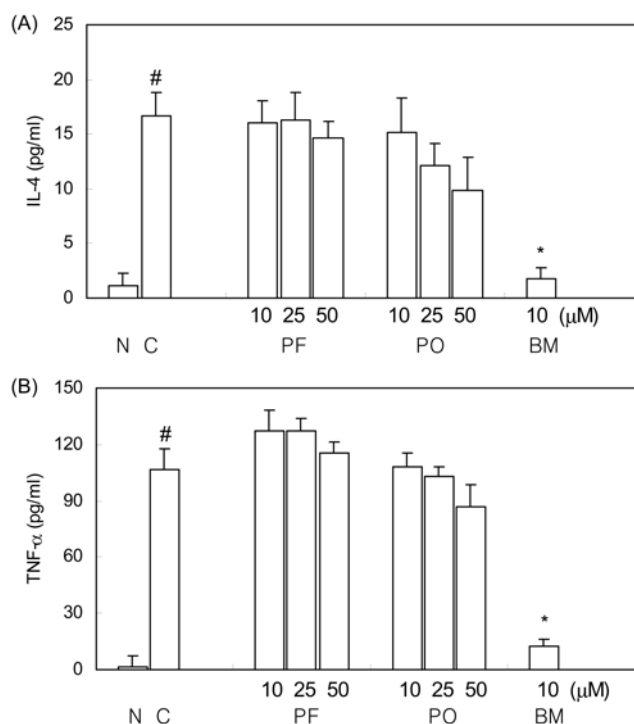


Fig. 2. Effect of paeoniflorin and paeonol on the protein expressions of IL-4 (A) and TNF- α (B) in RBL-2H3 cells induced by the IgE-antigen complex. RBL-2H3 cells (5×10^5 cells) were treated with 0.5 μ g/mL of mouse monoclonal IgE, exposed to 0.2 mL of test agents (N, normal; C, vehicle alone; PF, paeoniflorin at concentrations of 10, 25, and 50 μ M; PO, paeonol at concentration 10, 25, and 50 μ M; and BM, betamethasone at a concentration of 10 μ M) for 4 h, followed by treatment with 0.2 mL of dinitrophenol-human serum albumin (DNP-HSA, 1 mg/mL) for 40 min at 37°C, and ELISA were then performed. The normal control was treated with vehicle alone instead of the agents and IgE-antigen. Values represent the mean $\pm$ S.D. ($N=2$). [#]Significantly different from the normal group ($^{\#}P < 0.05$). ^{*}Significantly different from the control group ($^*P < 0.05$).

Table III. Inhibitory effect of PL, paeoniflorin and paeonol against mouse writhing syndrome induced by acetic acid

Agent	Dose (mg/kg)	Inhibition ^a (%)
PL ^b	100	9 $\pm$ 1 ^b
	250	39 $\pm$ 5 ^d
Paeoniflorin	10	17 $\pm$ 2 ^c
	50	44 $\pm$ 7 ^{d,e}
Paeonol	10	42 $\pm$ 7 ^{d,e}
	50	61 $\pm$ 12 ^{e,f}
Azelastine	10	51 $\pm$ 10 ^e
Aminopyrine	100	73 $\pm$ 11 ^{f,g}

Each experiment consisted of six observations. All inhibitory values indicate Mean $\pm$ S.D.

^a)The frequency numbers of the writhing syndrome numbers of normal control, which was treated with saline alone, and control group, which was treated with acetic acid saline solution for 10 min were 49 $\pm$ 5 and 1 $\pm$ 1, respectively.

^{b,c,d,e,f,g})Those with the same letter in each column are not significantly different ($P < 0.05$).

scratching behaviors caused by compound 48/80. To understand these findings, we evaluated the effect of PL and its constituents in degranulation of rat peritoneal mast cells. Paeonol and paeoniflorin inhibited the degranulation of rat peritoneal mast cells induced by compound 48/80 and RBL-2H3 cells induced by IgE-antigen complex. Paeonol exhibited more potent inhibition than paeoniflorin. Paeonol, not paeoniflorin, weakly reduced the IgE level in the blood in asthmatic mice induced by OVA and IgE-inducing cytokine IL-4 expression in RBL-2H3 cells induced by IgE. However, Kim *et al.* (2004) reported that paeonol potentially inhibited systemic anaphylaxis induced by compound 48/80 and biosynthesis of proinflammatory cytokines in mast cells. However, in the present study, paeoniflorin and paeonol did not show potent inhibition against proinflammatory cytokine TNF- α and IgE-inducing cytokine IL-4 in RBL-2H3 cells, although Kim *et al.* (2004) reported that paeonol potentially inhibited proinflammatory cytokine TNF- α in rat peritoneal mast cells. These findings suggest that paeonol may inhibit PCA reaction due to the inhibition against degranulation of mast cells, but can not repress the proinflammatory cytokine expression.

Nevertheless, their inhibitory effect against degranulation of mast cells and RBL-2H3 cells were in proportion to those against PCA reaction, although it is not sufficient to demonstrate the PCA-inhibitory potency of paeoniflorin and paeonol due to the degranulation-inhibitory activity.

Paeoniflorin and paeonol also inhibited the scratching behaviors induced by compound 48/80. Paeonol more potentially inhibited scratching behaviors than paeoniflorin. Their inhibitory potencies were not in proportion to their analgesic effects. These findings suggest that anti-scratching behavior activity of paeoniflorin paeonol may be due to unknown mechanism as well as its allelative effect for pain. For example, the metabolites of paeoniflorin by intestinal microflora like the previously reported (Heikal *et al.*, 1997) may be involved in the anti-scratching behavior effect.

Tsai *et al.* (2005) reported that paeoniflorin inhibited Ca^{++} influx in neuronal cells. Inangaki *et al.* (2000) reported that scratching behavior in mice was inhibited by μ -opioid antagonists. Azelastine, which was shown to inhibit the histamine release by interfering with Ca^{++} influx in rat peritoneal mast cell (Chand *et al.*, 1983), also potentially inhibited scratching behaviors. In the present study, paeoniflorin, paeonol and azelastine showed the potent analgesic effect against acetic acid-induced writhing syndrome (pain behavior) in mice. These findings suggest that anti-scratching behavior activity of paeoniflorin and paeonol may be originated from inhibition of Ca^{++} influx.

Based on these findings, PL and its constituents, paeoniflorin and paeonol, can improve IgE-induced anaphylaxis and scratching behaviors, and these effects

may be originated from inhibition of Ca^{++} influx and membrane stabilizing.

ACKNOWLEDGEMENTS

This study was financially supported by the research fund of Studies on the Identification of the Efficacy of Biological Active Components from Oriental Herbal Medicines from Korean Food and Drug Administration (2005).

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