Paeonol attenuates airway inflammation and hyperresponsiveness in a murine model of ovalbumin-induced asthma

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Abstract: Paeonol, the main active component isolated from Moutan Cortex, possesses extensive pharmacological activities such as anti-inflammatory, anti-allergic, and immunoregulatory effects. In the present study, we examined the effects of paeonol on airway inflammation and hyperresponsiveness in a mouse model of allergic asthma. BALB/c mice sensitized and challenged with ovalbumin were administered paeonol intragastrically at a dose of 100 mg/kg daily. Paeonol significantly suppressed ovalbumin-induced airway hyperresponsiveness to acetylcholine chloride. Paeonol administration significantly inhibited the total inflammatory cell and eosinophil count in bronchoalveolar lavage fluid. Treatment with paeonol significantly enhanced IFN-γ levels and decreased interleukin-4 and interleukin-13 levels in bronchoalveolar lavage fluid and total immunoglobulin E levels in serum. Histological examination of lung tissue demonstrated that paeonol significantly attenuated allergen-induced lung eosinophilic inflammation and mucus-producing goblet cells in the airway. These data suggest that paeonol exhibits anti-inflammatory activity in allergic mice and may possess new therapeutic potential for the treatment of allergic bronchial asthma.

Key words: paeonol, asthma, airway inflammation.

Résumé : Le paeonol, principal composant actif isolé de la pivoine arbustive (Moutan Cortex), possède de nombreuses activités pharmacologiques, notamment anti-inflammatoires, anti- allergènes et immunorégulatrices. Dans la présente étude, nous avons examiné les effets du paeonol sur l'inflammation et l'hyperréactivité des voies aériennes dans un modèle d'asthme allergique chez la souris. Des souris BALB/c sensibilisées et soumises à un test de provocation à l'ovalbumine ont reçu quotidiennement une dose de 100 mg/kg de paeonol par voie intragastrique. Le paeonol a supprimé considérablement l'hyperréactivité bronchique au chlorure d'acétylcholine induite par l'ovalbumine. L'administration de paeonol a inhibé de manière significative les taux de polynucléaires éosinophiles et de cellules inflammatoires totales dans le liquide de lavage bronchoalvéolaire. Le traitement au paeonol a nettement augmenté le taux d'INF-γ et diminué les taux d'interleukine-4 et d'interleukine-13 dans le liquide de lavage bronchoalvéolaire, et les taux d'immoglobulines E totales dans le sérum. L'examen histologique du tissu pulmonaire a démontré que le paeonol a atténué de manière significative l'inflammation éosinophilique pulmonaire induite par l'allergène de même que les cellules caliciformes produisant le mucus dans les voies aériennes. Ces résultats donnent à penser que le paeonol a une activité anti-inflammatoire chez les souris allergiques et qu'il pourrait présenter un potentiel thérapeutique dans le traitement de l'asthme bronchique allergique.

Mots-clés : paeonol, asthme, inflammation des voies aériennes.

[Traduit par la Rédaction]

Introduction

Allergic asthma is a complex chronic disease of the lung characterized by airway inflammation, lung eosinophilia, mucus hypersecretion by goblet cells, elevated serum immunoglobulin E (IgE) levels, and airway hyperresponsiveness (AHR) (Elias et al. 2003). The T helper 2 (Th2) cytokines interleukins (IL)-4 and IL-13, which are generated by activated CD4+ T cells, play a central role in the pathogenesis of airway inflammation and hyperresponsiveness in asthma.

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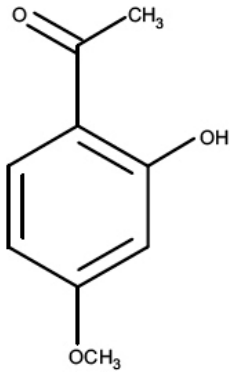
Current guidelines for asthma emphasize anti-inflammatory therapy. Glucocorticoids remain the first-line agents in asthma. However, some asthmatic patients still exhibit AHR following a prolonged treatment with glucocorticoids. Moreover, patients with chronic asthma treated with inhaled glucocorticoids, particularly in the long term, may develop serious side effects (Irwin and Richardson 2006). Thus there is a need for new or alternative approaches to manage this disease.

Paeonol (2′-hydroxy-4′-methoxyacetophenone, Fig. 1), a major phenolic component of Moutan Cortex, possesses extensive pharmacological activities such as anti-inflammatory, sedative, hypnotic, anti-pyretic, analgesic, anti-allergic, immunoregulatory, anti-oxidative, anti-bacterial, and anti-tumoral effects (Kim et al. 2004; Sun et al. 2004; Nizamutdinova et al. 2007; Chae et al. 2009). However, paeonol has not yet been demonstrated to have an inhibitory effect on allergic eosinophilic airway inflammation in vivo. The aim of the present study was to investigate the

Fig. 1. Chemical structure of paeonol.



effect of paeonol on the airway inflammation in a mouse model of allergic asthma.

Materials and methods

Chemicals

Paeonol (purity >99%) was purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China).

Antigen sensitization, challenge, and treatment

Forty-eight 6-week-old female BALB/c mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China). All experimental animals used in this study were maintained under conditions approved by the Institutional Animal Care and Use Committee of Nanjing Medical University, and the experimental protocol was approved by the institutional animal ethics committee. The 48 BALB/c mice were randomly assigned to a control group, an ovalbumin (OVA) group, a paeonol group, and budesonide group. Every group contained 12 mice, 6 for pulmonary resistance and 6 for bronchoalveolar lavage (BAL). Mice in the OVA, paeonol, and budesonide groups were sensitized on days 0 and 14 by intraperitoneal injection of 100 μg OVA (grade V; Sigma, St. Louis, Mo., USA) emulsified in 1 mg aluminum hydroxide (Pierce Chemical Co., Rockford, Ill., USA) in a total volume of 0.2 mL. Mice were challenged via the airway with 1% OVA daily on days 22, 23, and 24. OVA challenge was performed for 30 min by placing the mice in a Plexiglas box (29 × 22 × 18 cm) and aerosolizing OVA using an ultrasonic nebulizer (NE-U11B, Omuron, Tokyo, Japan). Mice in the paeonol group were also administered paeonol intragastrically at a dose of 100 mg/kg (Sun et al. 2008; Zhong et al. 2009) daily from days 15 to 24 on consecutive days. Mice in the budesonide group were exposed to aerosolized budesonide (2 mg, 1 mg/2 mL; Astra Zeneca, Luton, UK) for 30 min/day from days 22 to 24 one hour before challenge. Mice in the control group received the same schedule for sensitization and challenge with an equivalent amount of 0.9% sterile saline instead of OVA.

Evaluation of airway responsiveness

Airway responsiveness to acetylcholine chloride (ACh) was measured in mice 24 h after the last challenge with an AniRes animal lung function analysis system (SYNOL High-Tech, Beijing, China). The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg). A plastic tube (2 mm i.d.) was inserted into the trachea via tracheotomy for mechanical ventilation, and a 27-gauge needle was inserted into the caudalis vein for drug administration. The mice were then placed in a whole-body plethysmography chamber and ventilated mechanically at a rate of 90 breaths/min with a tidal volume of 6 mL/kg. After establishment of stable airway pressure recording, ACh was administered intravenously with a microinfusion pump at a rate of 36 mL/h in progressively increasing doses (10, 30, 90, and 270 μg/kg). After the administration of each dose, data were collected continuously from 5 s to 1 min, and maximum values of lung resistance were taken to express the changes in airway function of the mice (Du et al. 2008).

Analysis of bronchoalveolar lavage fluid samples and serum

Blood samples were collected 24 h after the last aerosol challenge by retroorbital puncture using heparinized capillary tubes. Blood samples were centrifuged (10 min, 4 °C, 1000*g*), and plasma was stored at –70 °C until further use. The lungs were washed 3 times with 0.5 mL saline to collect bronchoalveolar lavage fluid (BALF). The BALF was centrifuged (10 min, 4 °C, 1000*g*), and the total number of inflammatory cells in BALF was counted with a hemocytometer. Smears of BAL cells were stained with Wright's stain for performing differential cell counts. The cells in the BALF were counted by 2 independent investigators in a single-blind study that analyzed at least 200 cells each of 4 different random locations using a microscope. The levels of IL-4, IFN-γ (Jingmei Biotech Company, Shanghai, China), and IL-13 (R&D Systems, Minneapolis, Minn., USA) in BALF and total serum IgE (Bionewtrans Pharmaceutical Biotechnology Co. Ltd., Franklin, Mass., USA) were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol. The limits of detection were 7 pg/mL for IL-4, 7 pg/mL for IFN-γ, 4 pg/mL for IL-13, and 1 ng/mL for total IgE.

Histological examination

The left lungs taken from sacrificed mice were immersed in 10% formaldehyde overnight, then embedded in paraffin and cut into sections. Each section (5 μm thickness) was stained with either hematoxylin and eosin to assess the inflammatory cell infiltration or periodic acid – Schiff (PAS) to quantify airway global cells. A light microscope attached to an image analysis system (Image-Pro Plus; Media Cybernetics, Minneapolis, Minn., USA) was used to analyze histopathology. The degree of peribronchial and perivascular inflammation was evaluated by a subjective scale of 0–4, as described elsewhere (Lee et al. 2007; Shen et al. 2008). Briefly, the scoring system was as follows: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells >4 cells deep. Total lung inflammation was defined as the mean of the peribronchial and perivascular inflammation scores. Scoring for mucus production in the PAS-stained sections was as follows: 0, no goblet cells; 1, <25% of the epithelium; 2, 25%–50% of the epithelium; 3, 50%–75% of the epithelium; 4, >75% of the epithelium. At least 5 bronchioles in each slide that were randomly distributed throughout the left lung were an-

alyzed. Scoring was undertaken by 2 individuals blinded to the experimental protocol.

Statistical analysis

Data are expressed as means ± SE. Statistical analysis was performed using one-way analysis of variance (ANOVA) and post hoc least significant difference (LSD) test with SPSS software for Windows version 12 (SPSS, Inc. 2003). Results with $P < 0.05$ were considered statistically significant.

Results

Effects of paeonol on AHR

To determine whether paeonol administration improved pulmonary function of allergic mice after challenge with OVA, invasive whole-body plethysmography was performed to examine AHR in response to ACh (Fig. 2*a*). No significant differences were found in baseline airway resistance among the 4 groups. The airway resistance generated by administration of ACh at doses from 30 to 270 μg/kg increased significantly in the OVA-sensitized and -challenged mice compared with airway resistance in the control mice. The OVA group had significantly greater airway resistance than the control group. Treatment with paeonol and budesonide resulted in a significant decrease of enhanced lung resistance in allergic mice in response to ACh.

Effect of paeonol on cellular changes in BALF

Apart from macrophages, very few inflammatory cells were detected in control BALB/C mice. Sensitization and challenge with OVA resulted in a marked increase in the number of total leukocytes in the BALF. Total inflammatory cells and eosinophils counts in the BALF were significantly decreased in paeonol- or budesonide-treated mice compared with counts in OVA-challenged mice (Fig. 2*b*).

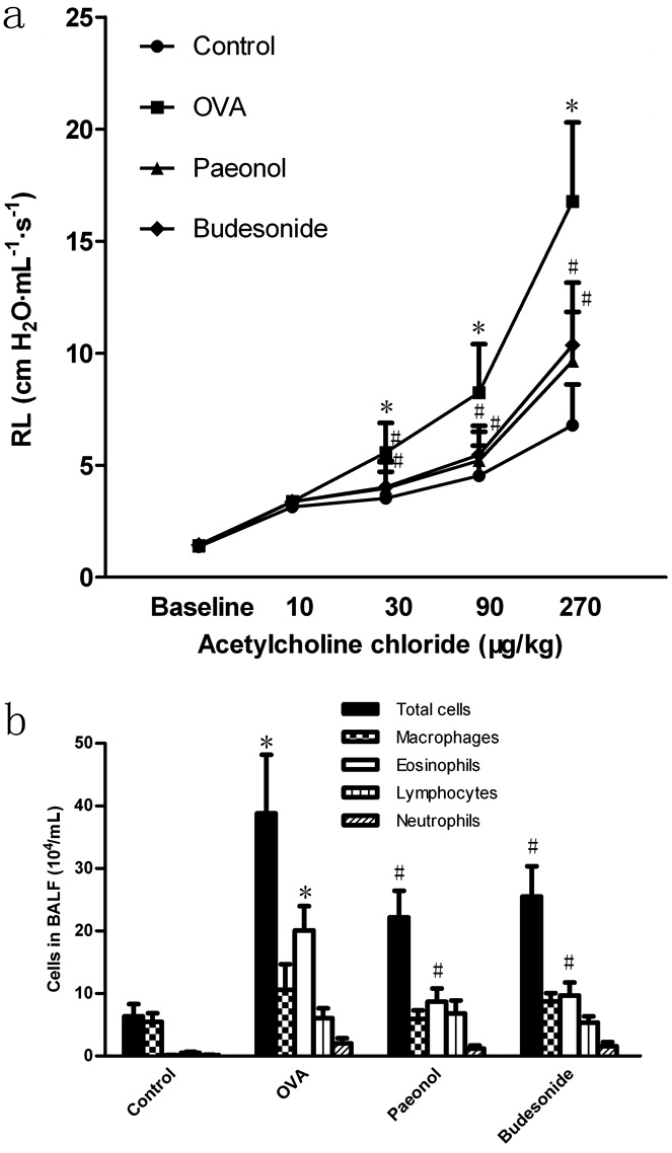
Effect of paeonol on inflammatory infiltration in lung tissue

To assess the anti-inflammatory effect of paeonol, histopathological studies were performed. A significant change in inflammatory cells in the airway and around the blood vessels was observed in OVA-sensitized and -challenged mice, but not in the saline-treated control mice. The majority of the infiltrated inflammatory cells were eosinophils. Paeonol significantly attenuated eosinophil-rich inflammatory leukocyte infiltration compared with infiltration in OVA-challenged mice (Fig. 3*a*–3*d*). The inflammation scores of peribronchial and perivascular regions were significantly decreased in the paeonol and budesonide groups compared with those in the OVA group (Fig. 3*e*).

Effect of paeonol on airway goblet cell hyperplasia and mucus production

To evaluate goblet cell hyperplasia, lung sections were stained with PAS to identify mucus-containing cells in the airway epithelium. Overproduction of mucus and goblet cell hyperplasia were observed in the bronchial airways of OVA-sensitized and -challenged mice, but not in the saline-treated control mice (Fig. 4*a*–4*d*). PAS staining scores were calculated for each experimental group, as described in the Materials and methods. As shown in Fig. 4*e*, mucus secretion was markedly increased in mice in the OVA group with enhanced staining scores. Paeonol inhibited OVA-induced overproduction of mucus and goblet cell hyperplasia.

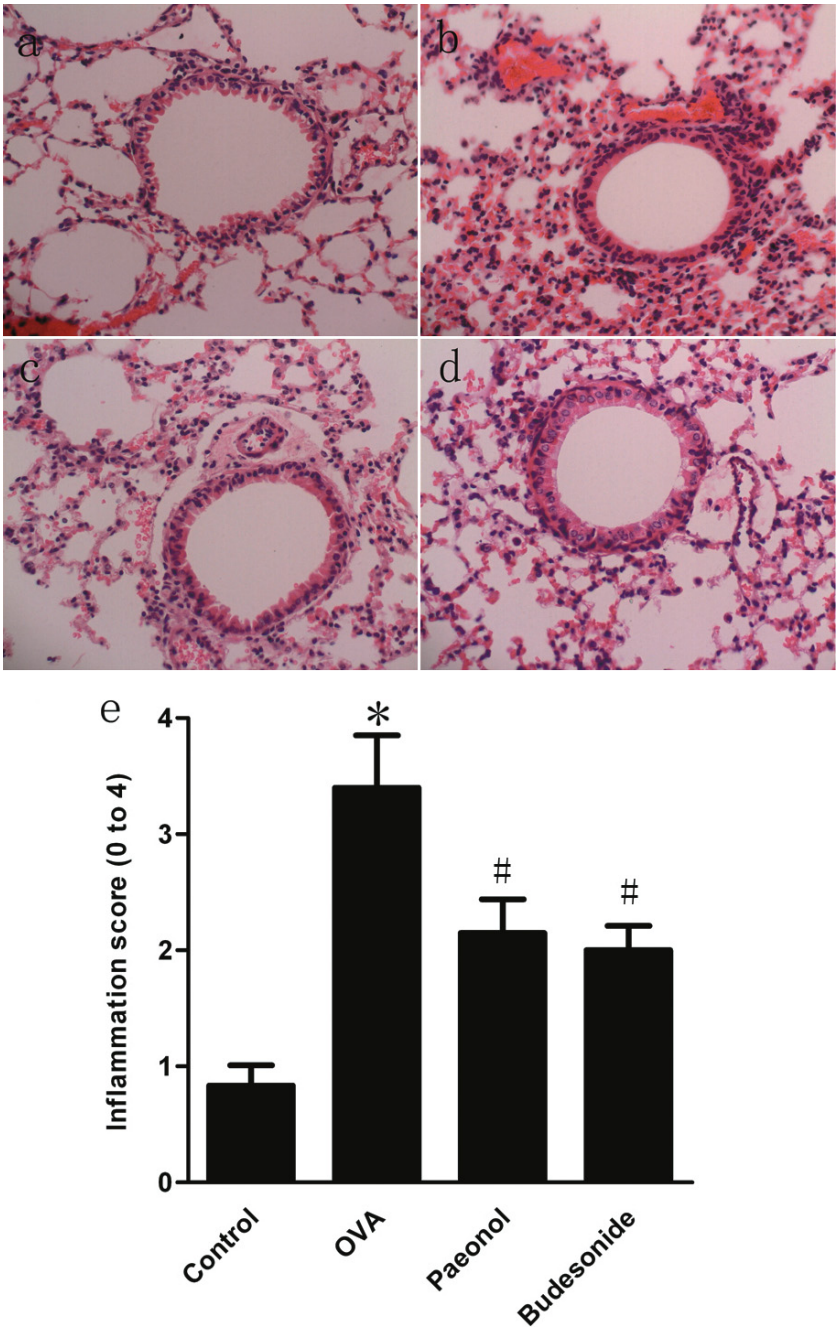
Fig. 2. Treatment with paeonol decreased the development of airway hyperresponsiveness and inflammatory cell accumulation in bronchoalveolar lavage fluid (BALF) in a murine model of asthma. Airway responsiveness (*a*) and inflammatory cell accumulation (*b*) in BALF were determined as described in the Materials and methods. Data represent means ± SE ($n = 6$ per group). *, significant difference from control ($P < 0.05$); #, significant difference from ovalbumin (OVA) ($P < 0.05$).



Effect of paeonol on Th1/Th2 cytokines in BALF and total serum IgE

BALF was collected 24 h after the last OVA challenge, and the concentration of IFN-γ, IL-4, and IL-13 were assessed using ELISA. OVA sensitization and challenge significantly induced the production of IL-4 and IL-13 in BALF. Daily administration of paeonol reduced the levels of these Th2 cytokines in BALF compared with those in the

Fig. 3. Treatment with paeonol attenuates airway inflammation in a murine model of asthma. Hematoxylin and eosin staining (original magnification 100×): (*a*) control, (*b*) ovalbumin (OVA), (*c*) paeonol, and (*d*) budesonide. (*e*) Quantitative analysis of inflammatory cell infiltration in lung sections were performed as described in the Materials and methods. Data represent means ± SE (n = 6 per group). *, significant difference from control ($P < 0.05$); #, significant difference from OVA ($P < 0.05$).



OVA group ($P < 0.05$; Table 1). IFN-γ level in the OVA group was less than that in the control group. Paeonol administration significantly promoted the IFN-γ level in the BALF of allergic mice compared with that in the OVA group ($P < 0.05$; Table 1). Total serum IgE levels were significantly elevated in the OVA group compared with those in the control group. Total serum IgE levels were reduced in the paeonol-treated group compared with those in the OVA group ($P < 0.05$; Table 1).

Discussion

In the present study, we have assessed the effects of paeonol on allergy-induced airway inflammatory disease. Our

Fig. 4. Treatment with paeonol inhibits antigen-induced mucus production in a murine model of asthma. Periodic acid – Schiff (PAS) staining (original magnification 200×): (*a*) control, (*b*) ovalbumin (OVA), (*c*) paeonol, and (*d*) budesonide. (*e*) Quantitative analyses of mucus production in lung sections were performed as described in the Materials and methods. Data represent means ± SE (n = 6 per group). *, significant difference from control ($P < 0.05$); #, significant difference from OVA ($P < 0.05$).

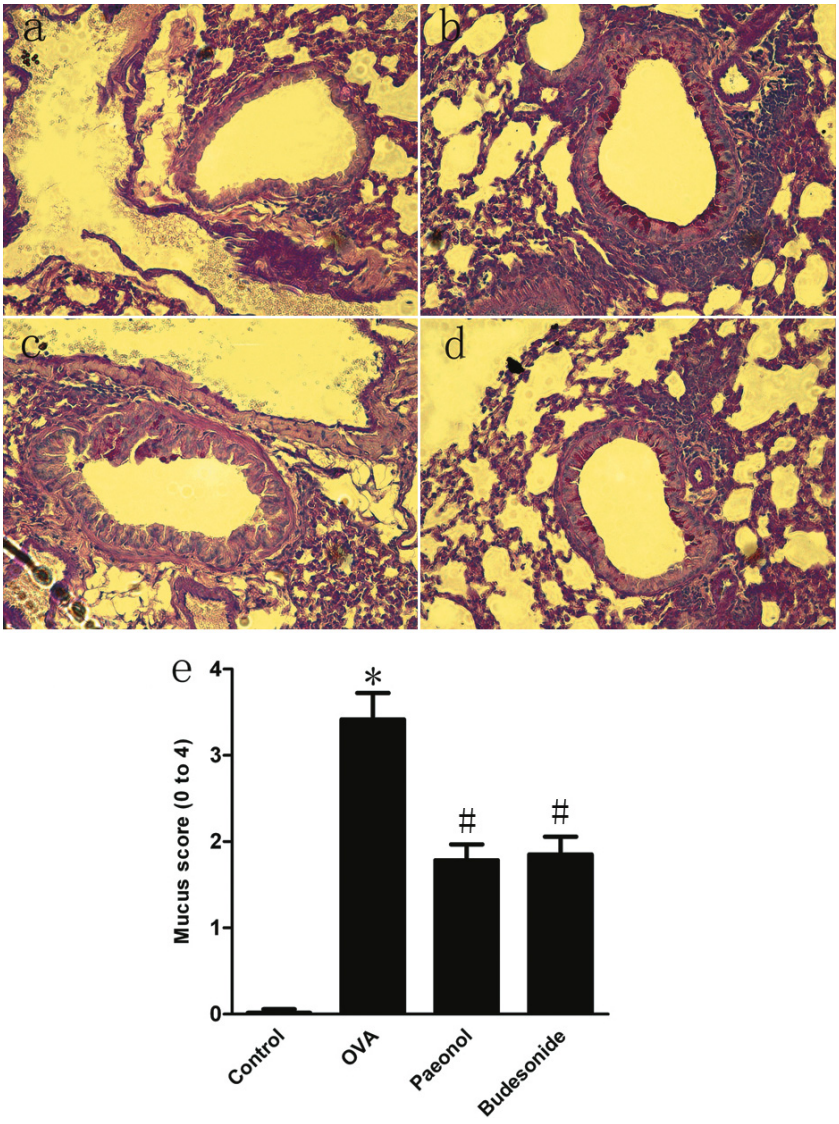


Table 1. Serum total IgE and cytokine levels in bronchoalveolar lavage fluid (BALF) were measured by ELISA.

Group	No. of mice	IL-4 (pg/mL)	IL-13 (pg/mL)	IFN-γ (pg/mL)	IgE (ng/mL)
Control	6	19.02±6.65	9.35±3.06	28.72±5.28	53.8±14.21
OVA	6	117.15±32.43*	55.60±10.67*	17.52±3.25*	226.0±26.83*
Paeonol	6	58.53±10.33#	22.5±5.85#	24.22±4.26#	97.0±22.81#
Budesonide	6	53.70±11.55#	26.50±6.83#	23.35±5.14#	113.7±19.22#

Note: Treatment with paeonol enhances the level of IFN-γ and reduces the levels of Th2-type cytokines IL-4 and IL-13 in BALF and total serum IgE in a murine model of asthma. Data are means ± SE (n = 6 per group). OVA, ovalbumin. *, significant difference from control ($P < 0.05$); #, significant difference from OVA ($P < 0.05$).

findings showed that paeonol attenuated OVA-induced inflammatory infiltration and mucus secretion in the lung tissues of allergic mice. Paeonol administration enhanced IFN-γ levels and decreased Th2 cytokine IL-4 and IL-13 levels in BALF and total IgE levels in serum. We also showed paeonol markedly suppressed AHR in a murine model of asthma.

In many allergic inflammatory diseases, IgE mediates the allergic response in humans as well as in animal models of allergic disease by inducing the degranulation of mast cells. Elevated IgE levels and eosinophilia may correlate with incidence or severity of asthma (Sears et al. 1991). IL-4, a key cytokine in the development of allergic airway inflamma-

tion, promotes isotype switching of B cells to IgE synthesis and regulates chemokines required for eosinophil migration (Finkelman et al. 1998). IL-13 also regulates IgE isotype switching in B cells and eosinophil function (Van der Pouw Kraan et al. 1998). Previous studies have shown that paeonol could inhibit IgE production in B cells activated by IgE inducers and decrease the serum IgE level in allergic mice (Kim et al. 2004; Lee et al. 2008). In this study, we observed that paeonol reduced not only total serum IgE level, but also eosinophilic airway inflammation, which may be associated with a decrease in the levels of IL-4 and IL-13 in the airways of sensitized and challenged mice.

The pathophysiology of AHR is complex, and many factors contribute to its development. In most cases, AHR is strongly associated with airway inflammation. Th2 cells, through the release of cytokines and chemokines, especially IL-4, IL-5, and IL-13, regulate inflammatory cell recruitment to the lung, leading to AHR (Kips et al. 2001). Previous study have shown that inhibition of IL-13 significantly suppresses AHR, through eosinophil infiltration in the airways of mice (Yang et al. 2004). Eosinophils also play an important role in the development of AHR, because AHR is absent in mice whose eosinophils are completely ablated (Lee et al. 2004). Our data demonstrated that paeonol inhibited OVA-induced AHR to inhaled ACh. The inhibition of AHR may be attributed to the reduction of IL-13 production and tissue eosinophilia in allergic mice.

Mucus hypersecretion and plugging in airways are characteristic features of human asthma. Excessive mucus secretion from hyperplastic goblet cells is an important cause of morbidity and mortality in asthma (Kim 1997). Goblet cells, which are known to produce mucus and are absent in the small airways of normal individuals (Jeffery and Zhu 2002), are observed in chronic airway inflammatory conditions and contribute to bronchial obstruction (Rogers et al. 2002). TH2 cytokines IL-4 and IL-13 have potent effects on mucus secretion (Temann et al. 1997; Grünig et al. 1998). In the present study, administration of paeonol significantly reduced the number of mucus-producing goblet cells within the bronchial epithelium. Thus, the observed reduction in mucus production in paeonol-treated lung tissue may be due to the inhibition of Th2 cytokines IL-4 and IL-13 in BALF.

It is widely accepted that the immune imbalance of Th1/Th2 plays an important role in the asthmatic morbidity mechanism. Resetting the Th1/Th2 imbalance may have an important therapeutic role for asthma. Our data also showed that paeonol not only inhibited Th2 response, but also shifted the response toward Th1 type, by inducing IFN-γ. Administration of IFN-γ can effectively attenuate established allergy-induced airway inflammation and AHR (Behera et al. 2002). So it is possible that IFN-γ induced by paeonol in this study could play a protective role in ameliorating the inflammatory response seen in OVA-induced mice.

In summary, this study demonstrates that treatment with paeonol reduced airway inflammation, AHR, and mucus hypersecretion by goblet cells in the airway in a murine model of asthma. In addition, paeonol restored the Th1/Th2 imbalance in OVA-induced allergic mice. Therefore, the use of paeonol may be a promising approach towards a novel asthma therapy.

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