



The effects of ($\pm$)-Praeruptorin A on airway inflammation, remodeling and transforming growth factor- β 1/Smad signaling pathway in a murine model of allergic asthma

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ABSTRACT

($\pm$)-Praeruptorin A (PA) is a pair of coumarin enantiomers isolated from the root of *Peucedanum praeruptorum* Dunn (PPD), a common Chinese herbal medicine for the treatment of asthma. Considering its anti-inflammatory, anti-contractile and anti-hyperplasia activities, the effects of PA on airway inflammation and airway remodeling were investigated using a murine model of chronic asthma. Ovalbumin-sensitized BALB/c mice were challenged with ovalbumin to induce asthma every other day on eight successive weeks. PA was administered intragastrically before every ovalbumin challenge. Airway responsiveness was evaluated by a lung function analysis system 48 h after the last ovalbumin challenge. The total and differential leukocytes in bronchoalveolar lavage fluid (BALF) were counted using a hemocytometer and Diff-Quick-stained smears. Lung tissue samples were used for hematoxylin and eosin, periodic acid Schiff, Masson's trichrome and α -SMA immunohistochemistry staining. Levels of cytokines in BALF, immunoglobulin (Ig) E in serum as well as expression of TGF- β 1 and Smad proteins in lung tissue were measured by enzyme-linked immunosorbent assay, immunohistochemistry or western blot analysis. Compared with the model group, PA suppressed airway inflammation, airway hyperresponsive and remodeling, reduced levels of IL-4 and IL-13 in BALF, and IgE in serum, inhibited expression of TGF- β 1 and pSmad2/3, up-regulated the expression of Smad7 in lung tissue, and also increased the levels of INF- γ in BALF. These results suggested that PA significantly suppressed airway inflammation and airway remodeling induced by ovalbumin challenge, and is a potential candidate for the treatment of asthma.

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1. Introduction

Allergic asthma is an inflammatory disease, characterized by the infiltration of eosinophils, lymphocytes and macrophages in peribronchial and perivascular areas [1]. The infiltrating inflammatory cells cause inflammatory lesions that contribute to the development of airway remodeling through the secretion of inflammatory mediators, [2,3]. This remodeling is visually characterized by loss of epithelial integrity, enlargement of goblet cell and submucosal gland, hypertrophy and hyperplasia of smooth muscle cell, fibrosis of subepithelial tissue and thickening of basement membrane [4,5], which are responsible for airway hyper-responsiveness (AHR), mucus hypersecretion, and reversible airway obstruction in patients with asthma [6].

Treatment of asthma according to current guidelines is based on minimizing symptoms and optimizing lung function by suppressing airway inflammation, restoring normal lung function and preventing complications such as irreversible airway remodeling. Glucocorticoids are

the most effective anti-inflammatory agents for the treatment of asthma, but not effective in totally controlling airway hyperresponsiveness and stopping the development of tissue remodeling [7,8]. In addition, these agents also lead to some serious side effects, such as hyperglycemia, hypertension, osteopenia to patients with large dosages and/or long-term treatment [9]. These pitfalls call for highly active anti-asthma drugs with no or lower toxicity.

The roots of *Peucedanum praeruptorum* Dunn (family Umbelliferae) have been widely used to treat asthma for centuries in China and other Asian countries. Decoction of the roots could relax pulmonary arterial and tracheal preparations [10]. *P. praeruptorum* contains various angular-type pyranocoumarins, such as ($\pm$)-praeruptorin A (PA) and ($\pm$)-praeruptorin B [11]. Previous studies have demonstrated that PA has various and distinct pharmacological effects, including relaxing isolated tracheas and pulmonary arteries of rabbits [12,13], inhibiting the release of anaphylactic mediators from rat mast cells induced by conca-nalvin A [14], decreasing levels of SOD, MDA after local myocardial ischemia-reperfusion injury in rats [15], and inhibiting the activity of NF- κ B in murine macrophages stimulated by lipopolysaccharide [16]. Furthermore, PA can improve the symptom of vascular hypertrophy by reducing area of smooth muscle cells (SMCs), and diminishing the levels

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of collagen [17]. Considering the fact that asthma is an allergic airway inflammatory disease characterized by airway remodeling, and that PA has favorable anti-inflammatory, anti-contractile and anti-hyperplasia effects, we investigated the effects of PA on airway allergic inflammation and airway remodeling in BALB/c mice.

2. Materials and methods

2.1. PA preparation

The shattered roots of *P. praeruptorum* Dunn (3.0 kg) were thrice extracted with 9 L of boiling light petroleum (boiling range 60–90 °C) for 2 h, 2 h and 3 h, respectively. The combined solutions were evaporated using a rotary vapor (Eyela, Tokyo, Japan) at 40 °C under reduced pressure to get a yellow crude extract (48 g). The obtained extract was subjected to CC over silica gel (100–200 mesh), eluted with petroleum ether-EtOAc (100:1–4:1). Fractions that were eluted with petroleum ether-EtOAc (10:1) were combined, concentrated and recrystallized with petroleum ether-EtOAc (4:1) to yield 10 g PA. The molecular structure of PA was identified by ^1H NMR and ^{13}C NMR [18]. The purity of PA was determined to be over 98% by HPLC on a ODS- C_{18} column (Agilent Technologies, Santa Clara, CA), and enantiomers of PA were resolved by chiral HPLC on a Daicel Chiralpak AD-H column (Daicel Chemical Industries, Tokyo, Japan) (Fig. 1).

2.2. Mice

Forty-eight seven-week-old female BALB/c mice (Vital River Lab Animal Technology Co., Ltd., China) were reared in laboratory animal center of Nanjing Medical University. Experiments were performed following the guidelines for the care and use of experimental animals of the International Association for Assessment and Accreditation of Laboratory Animal Care.

2.3. Antigen sensitization, challenge, and treatment

The mice were assigned randomly to one of six groups of eight animals each: control, PA with three dosages (30, 60 and 120 mg/kg), model, and budesonide. Except for the control group, the mice were actively sensitized by intraperitoneal injection of 10 μg ovalbumin (grade V; Sigma, St. Louis, MO) in 0.2 ml aluminum hydroxide gel (Sigma-Aldrich, Brøndby, Denmark) on days 0 and 14. Starting on day 28, the mice were exposed to 1% ovalbumin to challenge their airways in an exposure chamber for 20 min every other day on eight successive weeks. Control animals were injected with 0.2 ml aluminum hydroxide gel and exposed to phosphate-buffered saline (PBS) in a similar manner. One hour prior to each ovalbumin exposure, the mice in the PA groups were intragastrically administered, respectively, with 30, 60 and 120 mg/kg PA dissolved in 0.5% sodium carboxymethyl cellulose solution. The budesonide group mice were nebulized with 3 mg/kg budesonide (Sigma, St. Louis, MO) 1 h before each ovalbumin exposure. The control and model groups were treated with intragastric feeding of 0.5% sodium carboxymethyl cellulose solution. The drugs used for immunopharmacological studies were all endotoxin free, determined using a commercial Limulus amoebocyte lysate (LAL) test kit (Associates of Cape Cod Incorporated, Liverpool, UK).

2.4. Evaluation of AHR

Airway responsiveness was evaluated by measuring the changes of acetylcholine chloride-induced airway resistance using an AniRes 2005 mouse lung function analysis system (SYNOL High-Tech, Beijing, China) as described previously [19]. Briefly, the mice were anesthetized with pentobarbital sodium (70 mg/kg) by intra-peritoneal injection 48 h after the last ovalbumin challenge. A plastic tube of 2 mm internal diameter linked to a respirator was inserted into the trachea for mechanical

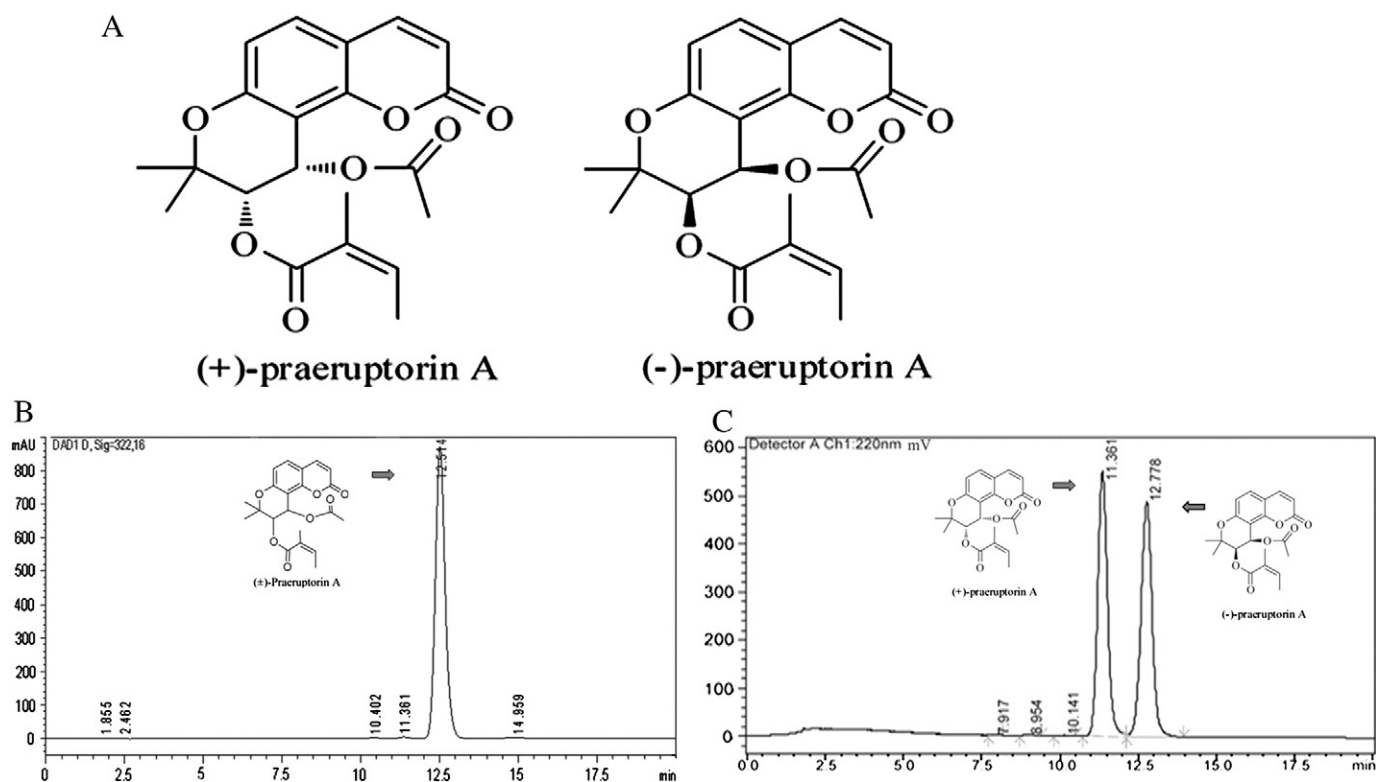


Fig. 1. Molecular structures and HPLC chromatograms of (±)-Praeruptorin A (PA). (A) Molecular structures of PA. (B) HPLC chromatogram of PA obtained by a ODS- C_{18} column. (C) Typical HPLC chromatogram of PA obtained by a Chiralpak AD-H column.

ventilation. When a stable tracing of airway pressure was achieved, acetylcholine chloride was administered intravenously via caudalis vein through a micro-infusion pump in progressive doses (10, 30, 90 and 270 $\mu\text{g/kg}$). Data were collected continuously from 5 s to 1 min after administration of each dose, and the maximal value of airway resistance (R_L) for each dose was recorded to reflect changes in murine airway function.

2.5. Bronchoalveolar lavage

After the evaluation of AHR, the airway was lavaged thrice with 0.4 ml PBS and then 85–90% of the volume inputted was recovered. The lavaged samples were centrifuged (1000 g for 10 min at 4 °C). The supernatants were transferred into tubes and stored at –70 °C before use to analyze the cytokine production. Cell pellets were resuspended in 0.2 ml PBS for total and differential cell counting.

2.6. BALF leukocyte counts

Whole blood was collected and the number of total leukocytes in BALF was counted on a hemocytometer. Leukocyte subpopulations were determined by differential counts on Diff-Quick-stained smears. At least 200 cells were counted per slide under light microscopy and they were differentiated according to standard morphologic criteria.

2.7. ELISA for IgE and cytokines

Levels of IL-4 (Jingmei Biotech, Shanghai, China), IL-13, INF- γ , eotaxin and TGF- β 1 (R&D Systems, Minneapolis, MN) in BALF, and IgE (Shibayagi Co., Shibukawa, Japan) in serum were measured by commercially available ELISA-kit, following the manufacturer's instructions.

2.8. Histological study

The low right lobe of lung from each mouse was removed for histological analysis. The lungs were inflated via the tracheal cannula with 10% phosphate-buffered formalin and fixed in the same solution, and embedded in paraffin. Lung tissues were cut into 2- μm -thick sections; stained with Hematoxylin-eosin, periodic acid Schiff, Masson's trichrome stain; and examined for airway inflammation and remodeling changes by light microscopy. The area of peribronchial Masson's trichrome staining in a paraffin-embedded lung was outlined and quantified with a light microscope attached to an Image-Pro Plus image-analysis system (Media Cybernetics, Silver Spring, MD). Results were expressed as the area of Masson's trichrome staining per micrometer length of bronchiolar basement membrane with 150–200 μm internal diameter. At least ten bronchioles were counted in each slide. To quantify the level of mucus production in the airway, epithelial cells (goblet cells), either periodic acid Schiff-negative or periodic acid Schiff-positive in bronchioles were counted as described previously [20].

2.9. Immunohistochemistry

Lung tissues were cut into 4- μm -thick sections, deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 30 min. Being blocked by 1% bovine serum albumin in PBS for 1 h, samples were incubated with mouse anti-human α -smooth muscle actin (α -SMA) antibody (M0851; DAKO, Grostrup, Denmark) and rabbit anti-mouse TGF- β 1 antibody (sc-146; Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 24 h. Washed with PBS, slides were again incubated with biotinylated goat anti-rat IgG or goat anti-rabbit IgG for 30 min at room temperature, and then stained using diaminobenzidine chromogen solution at room temperature. Sections were counterstained with haematoxylin, dehydrated and observed under a light microscope. The area of α -SMA staining was quantified

by an Image-Pro Plus image-analysis system. Data were expressed as the area of α -SMA immunostaining per μm length of basement membrane of bronchioles with an internal diameter of 150–200 μm . The number of peribronchial cells staining positive for TGF- β 1 per bronchiole with 150–200 μm of internal diameter were counted. At least ten bronchioles were counted in each slide.

2.10. RT-PCR for TGF- β 1

Cellular RNA extraction with TRIzol Reagent (Invitrogen, Carlsbad, CA) from lung tissues was performed following the manufacturer's protocol. First-strand cDNA of TGF- β 1 was generated using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA was amplified with the following primers (upstream and downstream primer, respectively): TGF- β 1 (5'-CAA CGC CAT CTA TGA GAA AAC C-3', 5'-ACT GCC GTA CAA CTC CAG TGA C-3'), β -actin (5'-GTC ACC AAC TGG GAC GAC ATG-3', 5'-GCC GTC AGG CAG CTC GTA GC -3'). The PCR conditions were as follow: initial denaturation at 95 °C for 5 min, then altogether 33 cycles composed of denaturation at 95 °C for 40 s, primer annealing at 52 °C for 30 s, extension at 72 °C for 25 s, and followed by a final extension at 72 °C for 10 min. PCR products were quantified by 1% agarose gel electrophoresis.

2.11. Western blot analysis of Smads proteins

The total proteins were extracted following the manufacturer's protocol. Equal amount of denatured sample proteins (50 μg) was separated in a 10% SDS-PAGE gel and were electroblotted onto a polyvinylidene fluoride (PVDF) membrane in a buffer containing Tris-HCl (25 mM), glycine (192 mM), and methanol (20%, volume/volume). After being incubated at room temperature for 1 h in TBST buffer (0.05 M Tris-Cl [pH 7.5], 0.85% NaCl, 0.1% Tween 20) containing 5% powdered milk, the membranes were treated with anti-phospho-Smad2/3, Smad7 (sc-11769, sc-101152; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies overnight at 4 °C and then exposed to corresponding HRP-conjugated goat anti-rabbit IgG (Beyotime Biotechnology, China) for 1 h at room temperature. Labeled band was detected using an enhanced chemiluminescence detection kit (BestBio Inc, Shanghai, China).

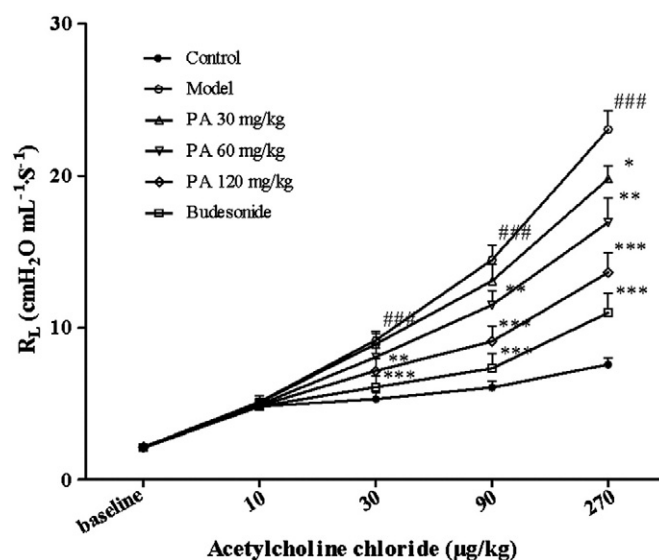


Fig. 2. Treatment with ($\pm$)-Praeruptorin A (PA) attenuated airway responsiveness induced by acetylcholine chloride in a murine model of allergic airway disease. Values are expressed as mean $\pm$ S.E.M. ($n=8$). Significant at: ### $P<0.001$ vs. control group; * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. OVA group.

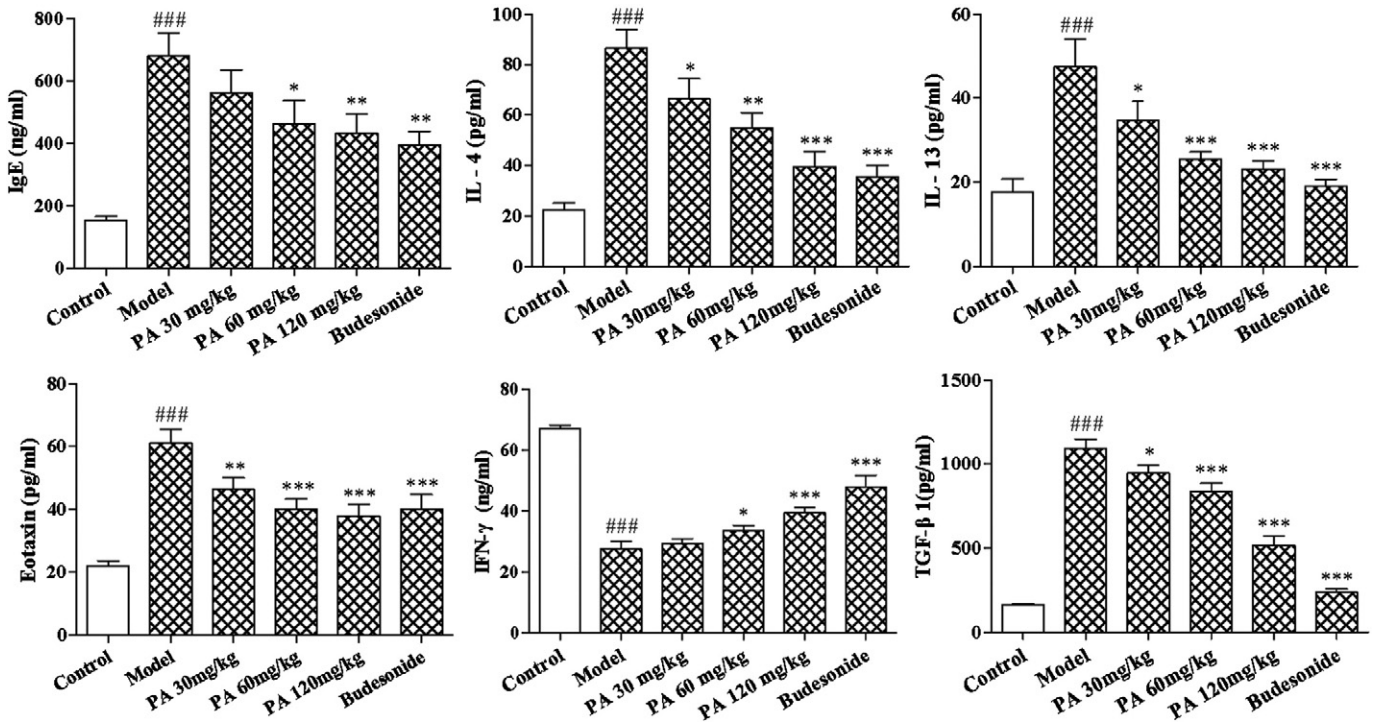


Fig. 3. Treatment with (±)-Praeruptorin A (PA) reduced levels of IgE in serum and cytokines in bronchoalveolar lavage fluid in a murine model of allergic airway disease. Values are expressed as mean ± S.E.M. (n = 8). Significant at: ###*P* < 0.001 vs. control group; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. OVA group.

2.12. Determination of cytotoxicities of PA against RAW 264.7 cells

RAW 264.7 cells were cultured in DMEM medium with 10% fetal bovine serum in 5% CO₂ at 37 °C and harvested in the exponential phase. One hundred μl of the cell suspension with a density of 1 × 10⁵ cells/ml were seeded into 96-well cell culture plates for 24 h to allow the adherence of cells, followed by exposure to PA in triplicate for 48 h at 6.25, 12.5, 25, 50 and 100 μmol/L, respectively. A volume of 20 μl MTT was then added to the plates, and 4 h later, the old medium was abandoned and 100 μl DMSO was added to dissolve the formazan product in each well. Afterwards, the optical density was measured at 570 nm with a Spectra Shell Microplate Reader to detect cell viability and calculate the IC₅₀ value.

2.13. Statistical analysis

Data are presented as the means ± SEM of *n* experiments. The SPSS software for Windows 16 was used for statistical analysis. Statistical comparisons between groups were evaluated by ANOVA, followed by Bonferroni's test. Values of *P* < 0.05 were considered statistically significant.

3. Results

3.1. Effect of PA on AHR

Airway hyper-responsiveness leads to recurrent acute attacks of asthma, airway bronchoconstriction and airway over-secretion. The lung resistance (R_L) of mice, as a result of airway obstruction induced by acetylcholine chloride, was measured by an AniRes 2005 mouse lung function analysis system. Compared with the control group, the mice in the model group developed distinct acetylcholine chloride-induced lung resistance. PA treatment significantly inhibited the effect in a dose-dependent manner (Fig. 2).

3.2. Effects of PA on IgE and cytokines

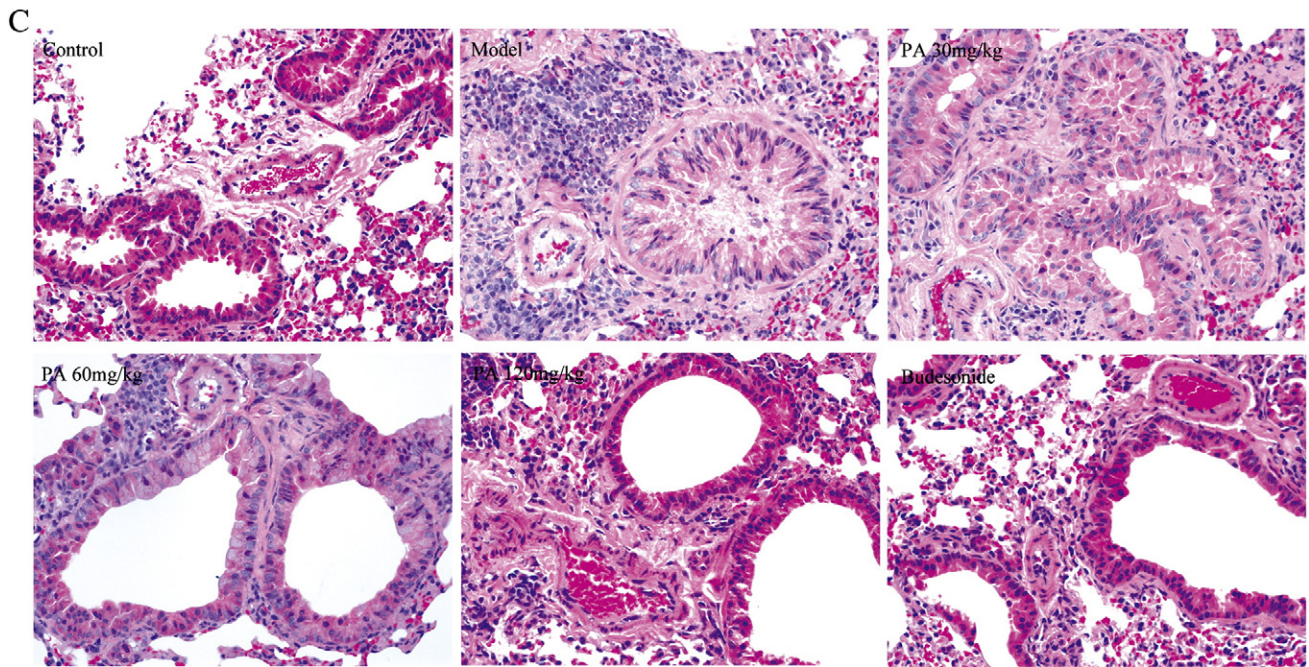
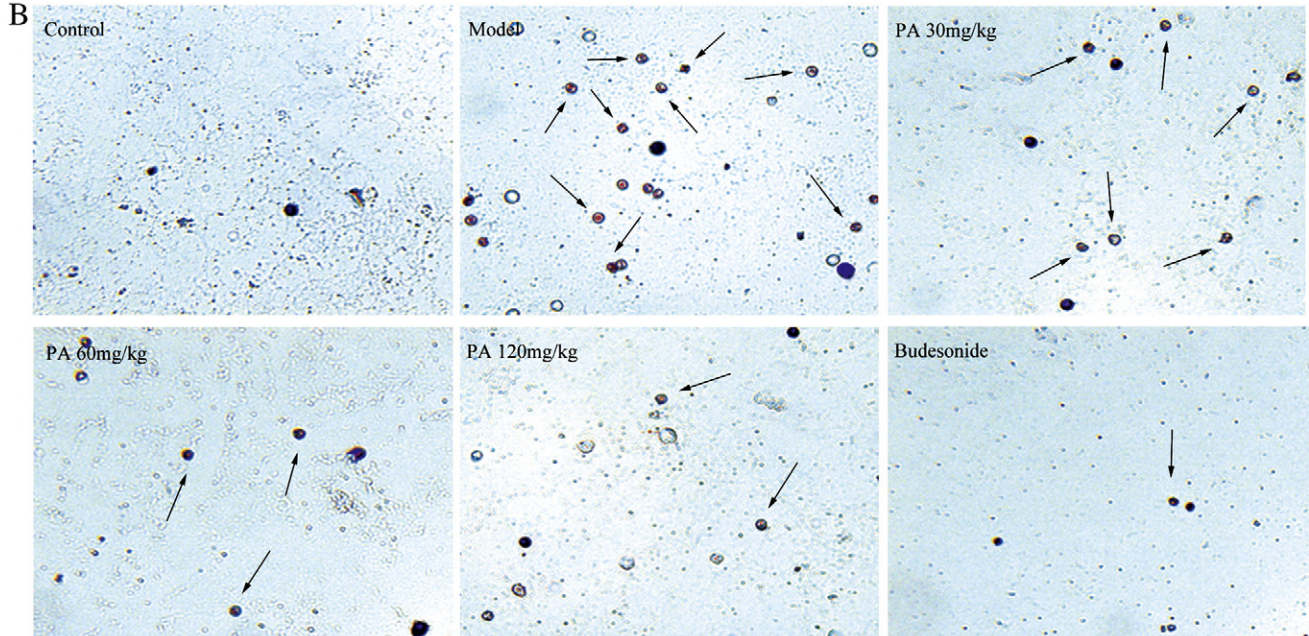
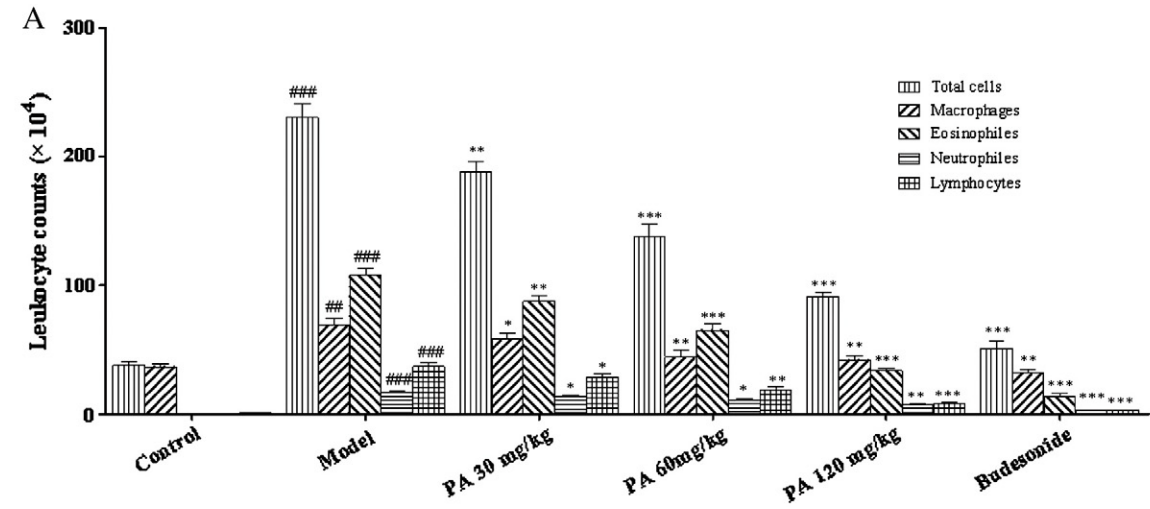
Production of numerous cytokines is an important facet of asthmatic inflammation. The effects of PA on the levels of IL-4, IL-13 and eotaxin in BALF, and IgE in serum were investigated. Compared with the mice of the control group, levels of IL-4, IL-13, eotaxin and IgE were sharply increased and those of IFN-γ were greatly decreased in the mice of the model group (Fig. 3). However, compared with the mice of the model group, levels of IL-4, IL-13, eotaxin and IgE were sharply decreased and those of IFN-γ were greatly increased in the mice of the PA groups, which also holds true for the mice of budesonide group (Fig. 3).

3.3. Effect of PA on airway inflammation

The development of airway inflammation was evaluated by leukocyte counts of BALF and histopathological analysis of lung lesions. Compared with the control mice, the number of total inflammatory cells, eosinophils and lymphocytes in BALF were markedly elevated in the model group mice (Fig. 4A, B). However, these increased inflammatory cells could be significantly decreased by the treatment of PA and budesonide (Fig. 4A, B). In addition, histopathological analysis of lung lesions was evaluated by hematoxylin and eosin staining (Fig. 4C). In the model group, the mice exhibited a significant peribronchial and perivascular inflammatory cell infiltration, intra-alveolar exudation, bronchi and interstitial edema compared with those in the control group (Fig. 4B). In the PA treated mice, the inflammatory cell infiltration, exudation and edema within the peribronchial and perivascular regions were markedly inhibited compared with those in the ovalbumin-challenged mice (Fig. 4C).

3.4. Effects of PA on airway remodeling

The left lower lungs of the mice were removed to evaluate the pathologic changes. Sections were stained with periodic acid Schiff (Fig. 5A) or Masson's trichrome staining (Fig. 5B), or immunostained



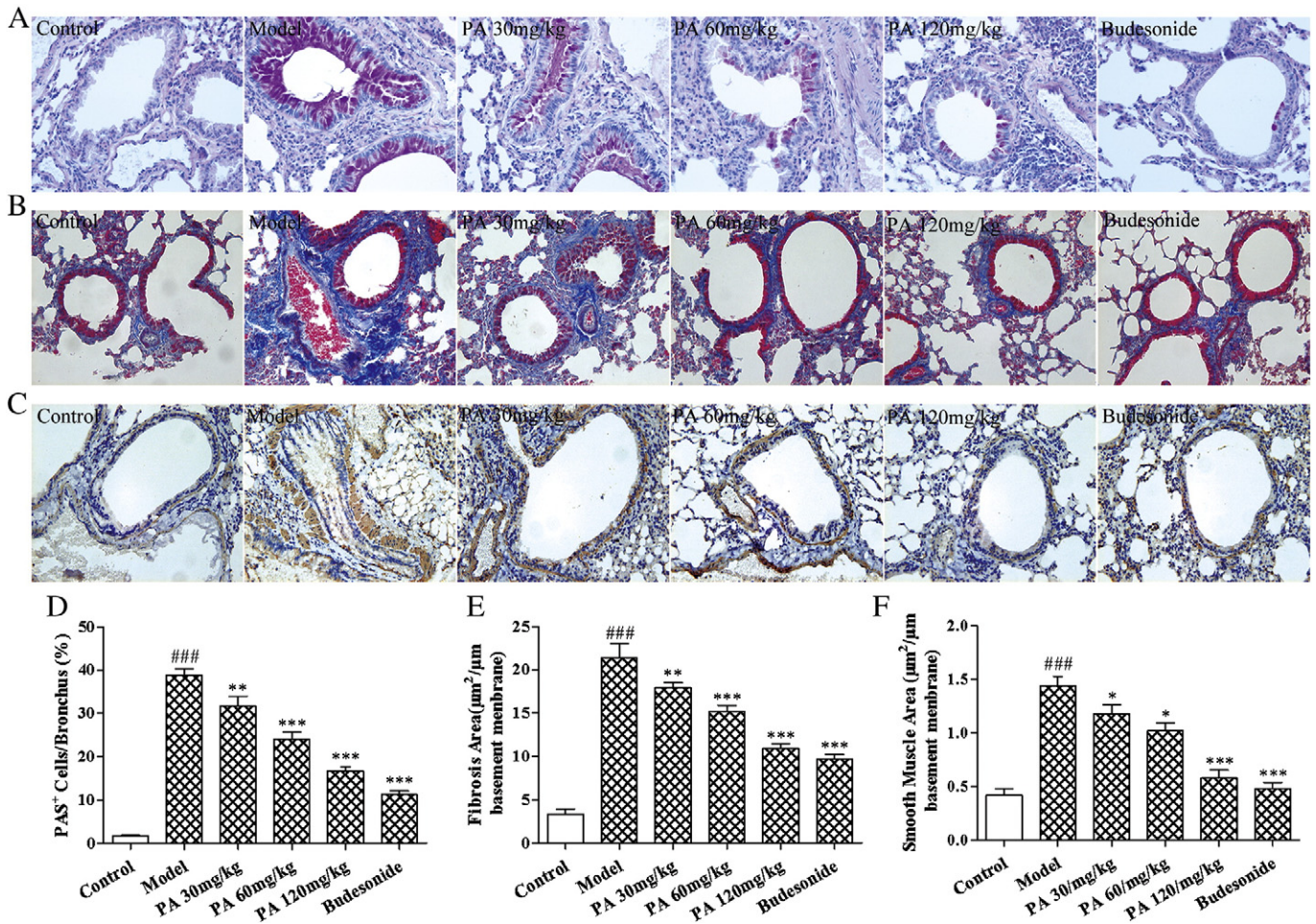


Fig. 5. Treatment with PA attenuates airway remodeling in a murine model of chronic asthma. (A) The production of mucus and hyperplasia of goblet cells analyzed by periodic acid Schiff staining. (B) The fibrosis of airway subepithelial tissues analyzed by Masson's trichrome staining. (C) The hyperplasia of smooth muscle cells analyzed by α -SMA immunostaining. (D) The percentage of periodic acid Schiff-positive cells. (E) area of airway fibrosis and (F) area of peribronchial α -SMA per bronchiole was determined as described in section 2. Values are expressed as mean $\pm$ S.E.M. (n = 8). Significant at: ^{###} $P < 0.001$ vs. control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$ vs. OVA group.

for α -SMA (Fig. 5C). The mice in the model group exhibited obvious enlargement of goblet cell (Fig. 5D), fibrosis of subepithelial tissue (Fig. 5E), and proliferation of smooth muscle cell compared with the mice in the control group (Fig. 5F), which could be significantly inhibited by the treatment of PA and budesonide (Fig. 5).

3.5. Effect of PA on the expression of lung TGF- β 1 protein and mRNA

For its capability to induce epithelial hyperplasia, levels of TGF- β 1 in BALF were measured by ELISA. Compared with the control group, levels of TGF- β 1 in BALF were sharply increased in the model mice (Fig. 3). Daily administration of PA significantly decreased levels of BALF TGF- β 1 compared with those in the ovalbumin-challenged mice (Fig. 3).

Immunohistochemical analysis of the TGF- β 1 expression in the airway epithelial cells was performed. TGF- β 1 positive cells stained brown were found in various lung tissue cells including airway epithelial cells, smooth muscle cells, macrophages and inflammatory cells, which were coincident with the previous reports [21]. In the present study, the number of peribronchial cells immunostaining positively for TGF- β 1 was counted (Fig. 6A), which was significantly increased in the model group (Fig. 6C) as compared with the control

group, such increase being attenuated significantly by the treatment with PA (Fig. 6C) or budesonide. There was no significant difference of TGF- β 1 expression in the mice treated with high dose of PA and budesonide.

The expression of TGF- β 1 mRNA in the lung was measured by RT-PCR to evaluate the expression pattern of cytokine proteins (Fig. 6B). Data were normalized to the levels of β -actin mRNA. As expected, TGF- β 1 mRNA expression in the model group was significantly increased compared with that in the control group (Fig. 6D), whereas the increase was significantly decreased by treatment with PA or budesonide (Fig. 6D).

3.6. Effect of PA on the expression of lung Smad proteins

Smad proteins are the downstream signal regulatory proteins of TGF- β receptor complex. Smad2, Smad3 and Smad7 are important members of the Smad proteins, playing an essential role in TGF- β 1-mediated airway remodeling. The expression patterns of phosphor-Smad2/3 (pSmad2/3) and Smad7 in the lung specimens were investigated. Data were normalized to the levels of GAPDH (Fig. 7A). An increase in the expression of pSmad2/3 was observed during prolonged allergen challenge, whereas treatment with PA considerably decreased pSmad2/3

Fig. 4. ($\pm$)-Praeruptorin A (PA) attenuated airway inflammation in a murine model of allergic airway disease. (A) The leukocyte counts of bronchoalveolar lavage fluid. (B) Diff-Quick staining for microscopic detection of eosinophils dyed in orangeophil red with cytoplasmic acidophil granules (arrows) (magnification $\times 400$). (C) Lung histopathologic change analyzed by Hematoxylin and Eosin staining (magnification $\times 400$). Values are expressed as mean $\pm$ S.E.M. (n = 8). Significant at: ^{**} $P < 0.01$, ^{###} $P < 0.001$ vs. control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$ vs. OVA group.

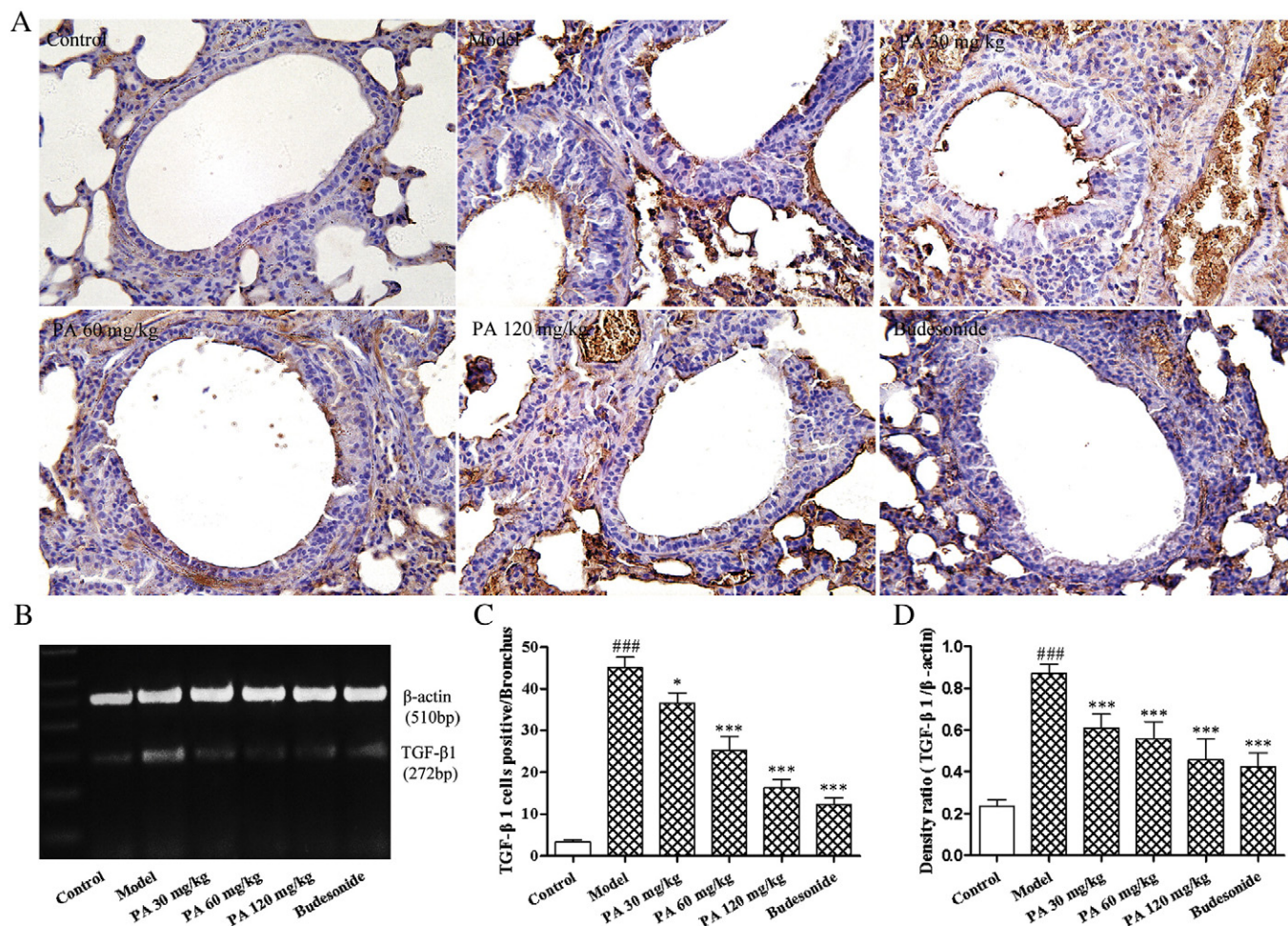


Fig. 6. PA decreased peribronchial TGF- β 1 immunostaining and pulmonary TGF- β 1 mRNA expression in a murine model of chronic asthma. (A) Pulmonary TGF- β 1 expression analyzed by immunohistochemistry. (B) Pulmonary TGF- β 1 mRNA expression analyzed by RT-PCR. (C) The immunostaining area of peribronchial TGF- β 1 analyzed by densitometry. (D) TGF- β 1 mRNA bands analyzed by the density ratio of TGF- β 1 and β -actin. Values are expressed as mean $\pm$ S.E.M. (n = 8). Significant at: ### P < 0.001 vs. control group; * P < 0.05, *** P < 0.001 vs. OVA group.

expression (Fig. 7B). In contrast with pSmad2/3, Smad7 was obviously decreased in the model group, and was markedly up-regulated by the treatment of PA compared with the model group (Fig. 7C). Treatment with budesonide also led to a decrease in the expression of pSmad2/3, and an increase in the expression of Smad7 as compared with the model group (Fig. 7A and B).

3.7. Cytotoxic activities of PA on RAW 264.7 cells

The inhibitory effect of PA on the growth of RAW 264.7 cells was also studied, revealing no cytotoxicity of PA at the tested concentrations: up to the maximum concentration of 100 μ mol/L, with an inhibition rate of 4%; at other concentrations, with inhibition rates less than 1%.

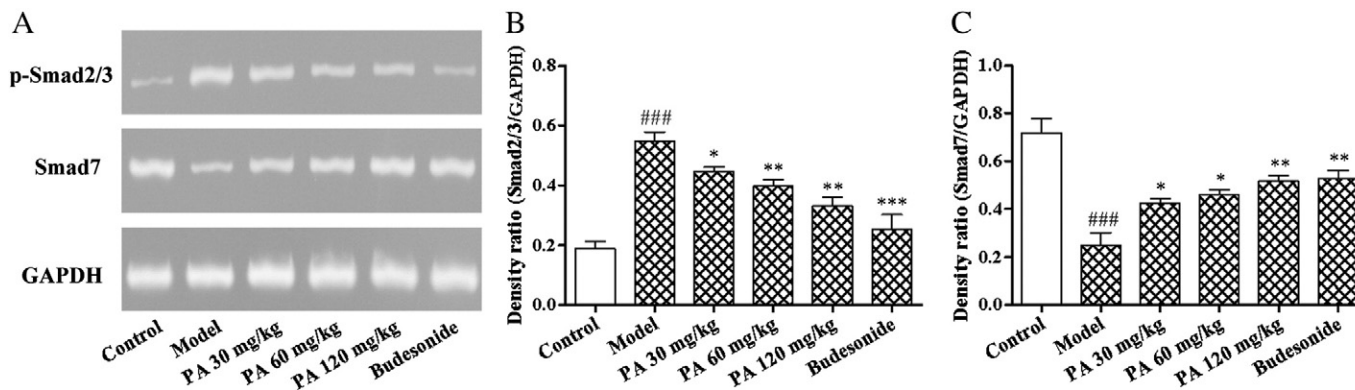


Fig. 7. PA inhibited pulmonary Smad protein expression in a murine model of chronic asthma. (A) Levels of Smad7 and Smad2/3 protein in the lung analyzed by western blot. The band of Smad2/3 protein (B) and band of Smad7 protein (C) assayed by the densitometry. Values are expressed as mean $\pm$ S.E.M. (n = 8). Significant at: ### P < 0.001 vs. control group; * P < 0.05, ** P < 0.01, *** P < 0.001 vs. OVA group.

4. Discussion

Human asthma is associated with chronic inflammatory disorders accompanied by airway wall remodeling [22]. In this study, the BALB/c mice were chronically challenged to ovalbumin (OVA) to produce airway remodeling in the mice to mimic features of chronic human asthma [23]. Effects of PA on the progression of airway inflammation and airway remodeling were investigated using this model. Treatment of the mice with PA led to a marked attenuation in airway inflammation, bronchial wall thickness, smooth muscle proliferation, mucous gland hypertrophy, goblet cell hyperplasia, and collagen deposition in ovalbumin-challenged mice. Interestingly, all of these ameliorated features are associated with the improvement of allergen-induced airway remodeling, which contributes greatly to airway hyper-responsiveness and reversibility of air-flow obstruction in patients suffering from asthma [24].

Numerous investigations have clarified the crucial role of the overproduction of T helper type 2 (Th2) cytokines in orchestrating the allergic inflammatory response [25]. Particularly, IL-4 and IL-13 are considered the key mediators that cause phenotypic or functional changes of bronchial asthma such as eosinophil infiltration, airway hyperresponsiveness, and mucus overproduction in the model mice [26–28]. In the present study, treatment of the mice with PA led to a significant reduction in these cytokines as compared with the model group. Elevated level of serum IgE, vital important sponsor of the Th2 immune response, could also be reduced by PA administration [29]. These findings suggested that PA can inhibit antigen-induced airway inflammation by interfering with the synthesis of some Th2 cytokines.

The development of airway inflammation was also involved in the expression of chemokines, which are important for selective leukocyte recruitment [30]. Eotaxin, produced by epithelial cells, endothelial cells and lung fibroblasts represent one of the most efficient eosinophil chemoattractants [31]. The expression of eotaxin is increased in atopic asthma and in the murine model of asthma induced by antigens [32,33]. In the present study, treatment of mice with PA significantly prevented the increase of eotaxin levels in BALF induced by ovalbumin challenge, which indicated that PA may inhibit the recruitment of eosinophils into the airway by interfering with eotaxin expression.

The effect of PA on the TGF- β 1/Smad signal pathway was investigated to clarify the anti-remodeling mechanism of PA on allergic asthma. Expressed by eosinophils, macrophages, Th2 lymphocytes, airway epithelial cells, TGF- β 1 is a multifunctional cytokine that contributes to the pathophysiology of asthma, such as subepithelial fibrosis and smooth muscle cell proliferation in airways [34–36]. The correlation between the TGF- β 1 level and basement membrane thickness has been demonstrated in some clinical studies using biopsies from asthmatic patients [37], and the increased levels of TGF- β 1 were reported in patients with severe asthma, and in animals induced by antigen [38,39]. Therefore, inhibiting the production of TGF- β 1 and/or its ability to suppress the airway remodeling is considered as one of the potential way to treat chronic allergic asthma [40]. Our results showed that treatment with PA could inhibit the expression of TGF- β 1 induced by OVA in lung tissues, which might be due to the inhibitory effects of PA on the recruitment of eosinophils, macrophages, and Th2 lymphocytes into the airways.

The translocation of intracellular effectors, Smad2 and Smad3, was reported to play an important role in the TGF- β 1-mediated airway remodeling. They are directly phosphorylated and activated by the TGF- β type I receptor kinases and undergo heterodimerization to form heteromeric complexes with Co-Smads (Smad 4). The activated Smad complexes are translocated into the nucleus and, in conjunction with other nuclear cofactors, regulating the transcription of target genes [41,42]. Another related factor, Smad7, is a member of the inhibitory Smad subfamily that could antagonize the TGF signaling by directly associating with the activated TGF- β receptors and interfering with the activation of Smad2 and Smad3 [43]. The decreased

Smad7 expression in the airways of patients with chronic asthma was reported [44]. Interestingly, in our study, the increased pSmad2/3 levels in OVA-challenged mice were significantly decreased by the treatment of PA, and the decreased levels of the inhibitory Smad7 protein was significantly increased. Therefore, these results demonstrated that PA could inhibit the airway remodeling in asthmatic mice by interfering with the TGF- β 1 signal pathway. In addition, the effects of TGF- β 1 could be antagonized by Smad7 through the IFN- γ /STAT pathway, the expression of which was greatly upregulated by IFN- γ [45]. In our study, the levels of IFN- γ in BALF were increased in the mice treated with PA as compared with those in the model group, which indicated that PA inhibits the TGF- β 1 signal activity at least in part in an IFN- γ -Smad7-independent manner. Moreover, the increased IFN- γ levels also contributed to the attenuation of T helper cell type 2 responses in the mice treated with PA [46].

In summary, our results demonstrated that PA could inhibit airway inflammation and airway remodeling in a murine model of chronic asthma. Interestingly, recent research reported that the major distribution tissues of PA in rats were lung, heart and spleen [47], which was in accord with its traditional usage for the treatment of chronic bronchitis and asthma. The median lethal dose (LD50) of PA in the mice was determined to be over 3.0 g/kg by acute toxicity testing with no observed signs of toxicity shortly before the present study. PA was tested for its cytotoxicity on macrophage RAW cells, showing almost no such effect with an inhibitory rate of 4% up to the concentration of 100 μ mol/L. In conclusion, PA may be a favorable candidate for the long-treatment of patients with a severe asthma.

Acknowledgments

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