



Immunopharmacology and Inflammation

Effects of ($\pm$)-praeruptorin A on airway inflammation, airway hyperresponsiveness and NF- κ B signaling pathway in a mouse model of allergic airway diseaseYouyi Xiong ^{a,1}, Junsong Wang ^{a,1}, Feihua Wu ^b, Juan Li ^b, Linfu Zhou ^c, Lingyi Kong ^{a,*}^a Department of Natural Medicinal Chemistry, China Pharmaceutical University, 24 Tongjia Xiang, Nanjing 210009, Jiangsu, China^b Department of Pharmacology of Chinese Materia Medica, China Pharmaceutical University, 639 Longmian Avenue, Jiangning District, Nanjing 211198, Jiangsu, China^c Department of Respiratory Medicine, The First Affiliated Hospital, Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, Jiangsu, China

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ABSTRACT

The root of *Peucedanum praeruptorum* Dunn is a traditional Chinese medicine commonly used to treat asthma in China. ($\pm$)-praeruptorin A (PA) is the most abundant constituent of *P. praeruptorum* Dunn, the effects of which on asthma were investigated using a murine model of allergic airway disease. BALB/c mice were sensitized and challenged by ovalbumin to induce airway inflammation. PA was administered intragastrically before every OVA challenge. Airway responsiveness was measured by a lung function analysis system. The number of total leukocytes in bronchoalveolar lavage fluid was counted using a hemocytometer, and differential cell counts were determined using Diff-Quick-stained smears. Histopathology of lung tissue was analyzed by hematoxylin–eosin and Congo red staining. Levels of inflammatory mediators in bronchoalveolar lavage fluid and immunoglobulins in serum were measured by enzyme-linked immunosorbent assay. The expression of pulmonary eotaxin was detected by immunohistochemistry and reverse transcription polymerase chain reaction. The activation of NF- κ B was evaluated by electrophoretic mobility shift assay and western blot analysis. Compared with model group, PA significantly reduced airway hyperresponsiveness and airway eosinophilic inflammation, improved pathologic lesion of the lungs, reduced levels of interleukin (IL)-4, IL-5, IL-13 and LTC₄ in bronchoalveolar lavage fluid and immunoglobulin (Ig) E in serum, and inhibited eotaxin protein and mRNA expression, I κ B α degradation, NF- κ B nuclear translocation, NF- κ B DNA-binding activity and RelA/p65 phosphorylation in lung, which suggested that PA can significantly suppress OVA-induced airway inflammation and airway hyperresponsiveness in mice, showing great therapeutic potential for the treatment of allergic asthma.

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1. Introduction

Allergic asthma is a prevalent and severe disease with ever-increasing worldwide incidence (Anandan et al., 2010). It is characterized by airway inflammation involving many different effector cells including mast cells, Th2 lymphocytes and eosinophils, and by airway remodeling in pathophysiology, associated with airway hyperresponsiveness, airway hyper secretion, and reversible airway obstruction (Kon and Kay, 1999). Studies on the mechanism of allergic asthma have identified a number of inflammatory mediators, including cytokines, chemokines, leukotrienes, adhesion molecules and prostaglandins that contribute to the development, maintenance and aggravation of airway inflammation (Housen et al., 2010).

Numerous genes that encode inflammatory mediators, such as IL-4, IL-5, IL-13, eotaxin and LTC₄, contain within their promoters an NF- κ B binding motif (Barnes and Karin, 1997), which could be bound directly

by activated NF- κ B, thus regulating the expression of their encoded inflammatory mediators. (Christman et al., 2000; Crimi et al., 1998). These mediators induce the infiltration of inflammatory cells, in particular eosinophils into the airways, and cause the smooth muscle contraction, vascular permeability, mucosal oedema and increased intra-alveolar exudation. Treatment strategies aimed at selective inhibition of NF- κ B activity would therefore present an effective immunotherapy approach with the potential of abating the allergic reaction (Desmet et al., 2004).

Corticosteroids are the most powerful and effective anti-asthma drugs up to now (Barnes, 2001). Most of the anti-inflammatory actions of corticosteroids can be accounted for by inhibiting the activity of NF- κ B that regulates inflammatory gene expression (Adcock, 2001). However, adverse events were commonly associated with the administration of corticosteroids, especially for long-term use, such as hyperglycemia, hypertension, and osteopenia (Buchman, 2001), which necessitated the search for natural products as a source of novel effective anti-asthma drugs with favourable no or low side-effects.

The dry roots of *Peucedanum praeruptorum* Dunn have long been used in traditional Chinese medicine for the treatment of certain respiratory

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diseases such as asthma, chronic bronchitis and pulmonary hypertension. The decoctions of the roots could relax pulmonary arterial and tracheal preparations (He et al., 2007). *P. praeruptorum* Dunn contains various angular-type pyranocoumarins, such as (±)-praeruptorin A (PA) and (±)-praeruptorin B (Chen et al., 1979). Previous studies indicated that PA has a multifacet pharmacological activities to effectively relax tracheal and vascular smooth muscles of rabbits *in vitro* (Aida et al., 1998; Zhao et al., 1999), to inhibit the release of anaphylactic mediator from rat mast cells induced by concanavalin A (Suzuki et al., 1985), and to lower levels of superoxide dismutase and malondialdehyde after local myocardial ischemia-reperfusion injury in rats (Jiang et al., 2002). Based on the fact that asthma is an airway inflammatory and airway hyperreactive disease, and that PA has anti-inflammatory and anti-contractile activities, we investigated effects of PA on allergic airway inflammation and airway hyperresponsiveness in BALB/c mice.

2. Materials and methods

2.1. Preparation of PA

The dried roots of *P. praeruptorum* Dunn (1.5 kg) were shattered to powder (about 30 mesh) and extracted with 4500 ml of boiling light petroleum (boiling range 60–90 °C) three times with extraction time of 2 h, 2 h and 1 h, respectively. The combined extracts were evaporated using a rotary evaporator (Eyela, Tokyo, Japan) at 40 °C under reduced pressure to get a yellow crude extract (25 g), which was subjected to CC over silica gel (100–200 mesh) eluted with a mixture of petroleum ether-EtOAc (100:1–4:1) to give fractions. Fraction that was eluted by petroleum ether-EtOAc (10:1) was recrystallized with petroleum ether-EtOAc (4:1) to yield 6 g PA. The molecule structures of PA were identified by ¹H NMR and ¹³C NMR as described previously (Xu et al., 2010). The purity of PA was determined to be over 98% by HPLC on a ODS-C₁₈ column (Agilent Technologies, Santa Clara, CA), with 70:30 ratio of methanol/H₂O solvent system, and enantiomers of PA were isolated by HPLC on a Daicel Chiralpak AD-H column (Daicel Chemical Industries, Tokyo, Japan), with 90:10 ratio of hexane/isopropanol solvent system (Fig. 1).

2.2. Mice

Specified pathogen-free female BALB/c mice, aged 5 to 7 weeks, were purchased from Vital River Lab Animal Technology Co., Ltd. (Beijing, China), and were reared in laboratory animals of Nanjing Medical University. The guidelines for the care and use of laboratory animals of Nanjing Medical University were followed, and the experimental protocol was approved by the Association for Assessment and Accreditation of Laboratory Animal Care, International. All mice were provided with water and standard chow *ad lib* for 2 weeks prior to any experimental performances to make them accommodate their new surroundings.

2.3. Antigen sensitization, challenge, and treatment

At the start of the protocol, forty-eight mice were assigned randomly to one of the following six experimental groups: control, three different doses of PA (30, 60 and 120 mg/kg), model and dexamethasone. All mice, except for those in the control group, were sensitized on days 0 and 14 by intra-peritoneal injection of 10 µg ovalbumin (grade V; Sigma, St. Louis, Mo.) dissolved in saline containing 200 µg aluminium hydroxide gel (alum; Sigma-Aldrich, Brøndby, Denmark) in a total volume of 0.2 ml; mice in control group received alum only. On day 28, all ovalbumin-sensitized mice were aerosol challenged with 1% ovalbumin in phosphate-buffered saline in an exposure chamber (55 cm × 30 cm × 30 cm) by a nebulizer (NE-U11B, Omuron Co., Tokyo, Japan) for 20 min per day for 5

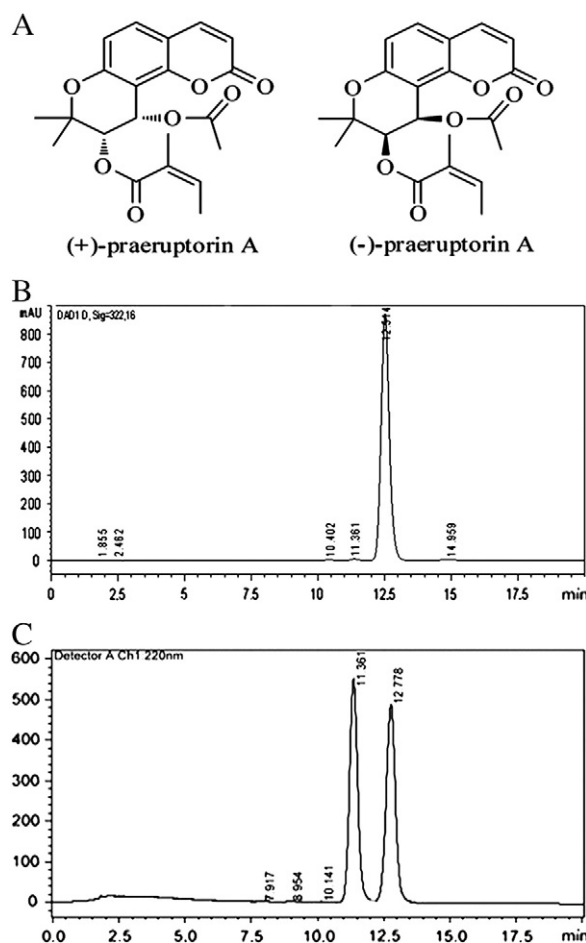


Fig. 1. Molecular structures and HPLC chromatograms of (±)-praeruptorin A (PA). (A) Molecular structures of PA. (B) HPLC chromatogram of PA obtained by an ODS-C₁₈ column. (C) Typical HPLC chromatogram of PA obtained by a Chiralpak AD-H column: retention times for (+)-praeruptorin A and (–)-praeruptorin A were 11.361 and 12.778 min, respectively.

consecutive days; mice in control group received only phosphate-buffered saline aerosol. One hour before every airway challenge, mice in PA groups were administered intragastrically with PA dissolved in 0.5% sodium carboxymethyl cellulose solution at a dose of 30, 60 and 120 mg/kg, respectively; mice in dexamethasone group were injected with dexamethasone (Sigma, St. Louis, MO) dissolved in 0.9% sodium chloride at a dose of 3 mg/kg; mice in control and model group were intragastrically administered with 0.5% sodium carboxymethyl cellulose solution for parallel manipulation. The endotoxin levels in all reagents used for immunopharmacologic studies are endotoxin free, which were determined using a commercially available Limulus amoebocyte lysate (LAL) test kit (Associates of Cape Cod Incorporated, Liverpool, UK).

2.4. Evaluation of airway responsiveness

Airway responsiveness to acetylcholine chloride was evaluated using an AniRes 2005 mouse lung function analysis system (SYNOL High-Tech) 24 h after the last ovalbumin challenge as described previously (Caceres et al., 2009). Mice were anesthetized with pentobarbital sodium (70 mg/kg) by intra-peritoneal injection, intratracheally intubated, placed in a rodent plethysmograph capable and mechanically ventilated at a tidal volume of 6 ml/kg and 90 breaths/min. After reaching a stable tracing, lung resistance was measured at baseline and following intravenous administration of increasing doses of acetylcholine chloride via caudalis vein through a micro-infusion pump

in progressive doses (10, 30, 90 and 270 $\mu\text{g/kg}$) every other 5 min. The signals of lung resistance (R_L) were collected continuously from 5 s to 1 min for each dose, and the maximal response was recorded to reflect the change in airway function. Lung resistance was expressed by continuously quantitating the ratio of ΔP to ΔV (where ΔP is the change in tracheal pressure, ΔV is the change in flow) using a pulmonary mechanics analyzer.

2.5. Collection of blood and bronchoalveolar lavage fluid

Mice were bled from the retro-orbital venous plexus under light anesthesia. Collected blood samples were centrifuged at 1000 g for 10 min at 4 °C and then the obtained serum was stored at –70 °C before use to estimate the IgE production. The airway was treated thrice by intratracheal instillation of phosphate-buffered saline, 0.4 ml each. Bronchoalveolar lavage fluid was collected and centrifuged at 1000 g for 10 min at 4 °C. The supernatant was transferred into tubes and stored at –70 °C before use to analyze the cytokine production. Cell pellets were resuspended in phosphate-buffered saline to a final volume of 0.5 ml for total and differential cell counting.

2.6. Leukocyte counts

The total number of leukocytes in bronchoalveolar lavage fluid was counted using a hemocytometer, and the differential cell counts were conducted on the Diff-Quik stained cytospin preparations. Differential counting was performed on Diff-Quik stained cytospins and routinely at least 200 cells were counted on each cytospin according to standard morphologic criteria under light microscopy.

2.7. Measurement levels of IgE, LTC_4 and cytokines

Levels of IgE in serum, expressed in ng/ml of serum, were measured by commercially available enzyme-linked immunosorbent assay kits, following the manufacturer's instructions (Shibayagi Co., Shibukawa, Japan); levels of IL-4, IL-5, IL-13 and LTC_4 in the bronchoalveolar lavage fluid, reported in pg/ml, were also determined by corresponding enzyme-linked immunosorbent assay kits according to the manufacturer's protocol (Bender MedSystems, Vienna, Austria).

2.8. Histopathological analysis

Lung tissues were collected and fixed in 10% phosphate-buffered formalin, and embedded in paraffin for histological analysis. Tissues were cut into 2 μm sections and stained with hematoxylin–eosin for analysis of histological changes. Since eosinophils are not easily distinguished from other inflammatory cells by hematoxylin–eosin staining in the lung of BALB/c mice, Congo red staining was used to detect them. All images were taken with a camera (Olympus DP50, Olympus Sverige AB, UK) mounted on a light microscope (Olympus BX51, Olympus Sverige AB, USA).

2.9. Immunohistochemistry

Lung tissues embedded in paraffin were cut into 4 μm sections and deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase was deactivated by incubation with 3% hydrogen peroxide for 30 min. After blocking with 5% normal goat serum for 10 min, the supernatant was discarded. The monoclonal primary antibody to mouse eotaxin (R&D Systems, Minneapolis, MN, USA) was then applied and incubated at 4 °C for 24 h at a dilution ratio of 1: 50. Sections were rinsed 3 times with phosphate-buffered saline after overnight incubation, and then incubated with biotinylated goat anti-rat IgG (Envision system; DAKO) for 30 min at room temperature, and then washed again with phosphate-buffered saline and

stained using diaminobenzidine chromogen solution (DAB; Santa Cruz Biotechnology) at room temperature. Sections were counterstained with haematoxylin, dehydrated and observed under a light microscope. The immunostaining area of eotaxin-positive cells was quantified by an Image-Pro Plus image-analysis system (Media Cybernetics, Silver Spring, MD). Results were expressed as the area of eotaxin staining per micrometre length of bronchiolar basement membrane with 150–200 μm internal diameter by counting at least 10 bronchioles in each slide.

2.10. Reverse transcription polymerase chain reaction

RNA was extracted from lung tissues, using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. First-strand cDNA of eotaxin was generated using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA was amplified with the following primers (upstream and downstream primer, respectively): eotaxin (5'- GCTCACGGTCACTTCCTTCAC-3', 5'-CTCTTTGCC AACCTGGTCTT-3'), β -actin (5'-GTCACCAACTGGGAC-GACATG-3', 5'-GCCGT CAGGACGCTCGTAGC-3'). Polymerase chain reaction conditions were an initial denaturation at 94 °C for 5 min, followed by denaturation at 94 °C for 40 s and primer annealing at 56 °C for 30 s for 33 cycles, and a final extension at 72 °C for 10 min. Polymerase chain reaction products were quantified by 1% agarose gel electrophoresis.

2.11. Extraction of cytoplasmic and nuclear proteins

Cytoplasmic and nuclear proteins from lung tissues were extracted as described previously (Heijink et al., 2007). Briefly, cytoplasmic proteins were extracted from aliquots of liquid nitrogen-frozen tissue in 400 μl of a low-salt buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 $\mu\text{g/ml}$ each of antipain, aprotinin, leupeptin and pepstatin) using a homogenizer, and allowed to swell on ice for 10 min. Then, 10% NP-40 was added and nuclei were pelleted by centrifugation at 15000g for 1 min. The supernatant containing cytoplasmic proteins was transferred to a new tube and stored at –70 °C until used. The obtained nuclear pellet was further resuspended in 50 μl of hypertonic lysis buffer (20 mM HEPES of pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM

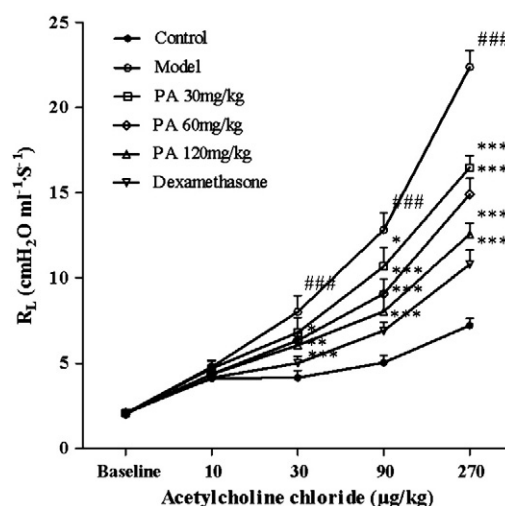


Fig. 2. Treatment of mice with ($\pm$)-praeurptorin A (PA) decreased the lung resistance (R_L) in a murine model of allergic airway disease. Values are expressed as mean $\pm$ S.E.M. (n = 8). Significant at: ### $P < 0.001$ vs. control group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. OVA group.

DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 μ g/ml each of anti-pain, aprotinin, leupeptin and pepstatin) on ice for 30 min. After centrifugation (12500g for 10 min) at 4 °C, the supernatant containing nuclear proteins was also transferred to a new tube and stored at –70 °C until used.

2.12. Western blot analysis

Denatured proteins (70 μ g) were fractionated by 10% SDS–PAGE and transferred to polyvinylidene fluoride membrane by electroblotting. Membranes were blocked for 1 h in TBST buffer (0.05 M Tris–Cl

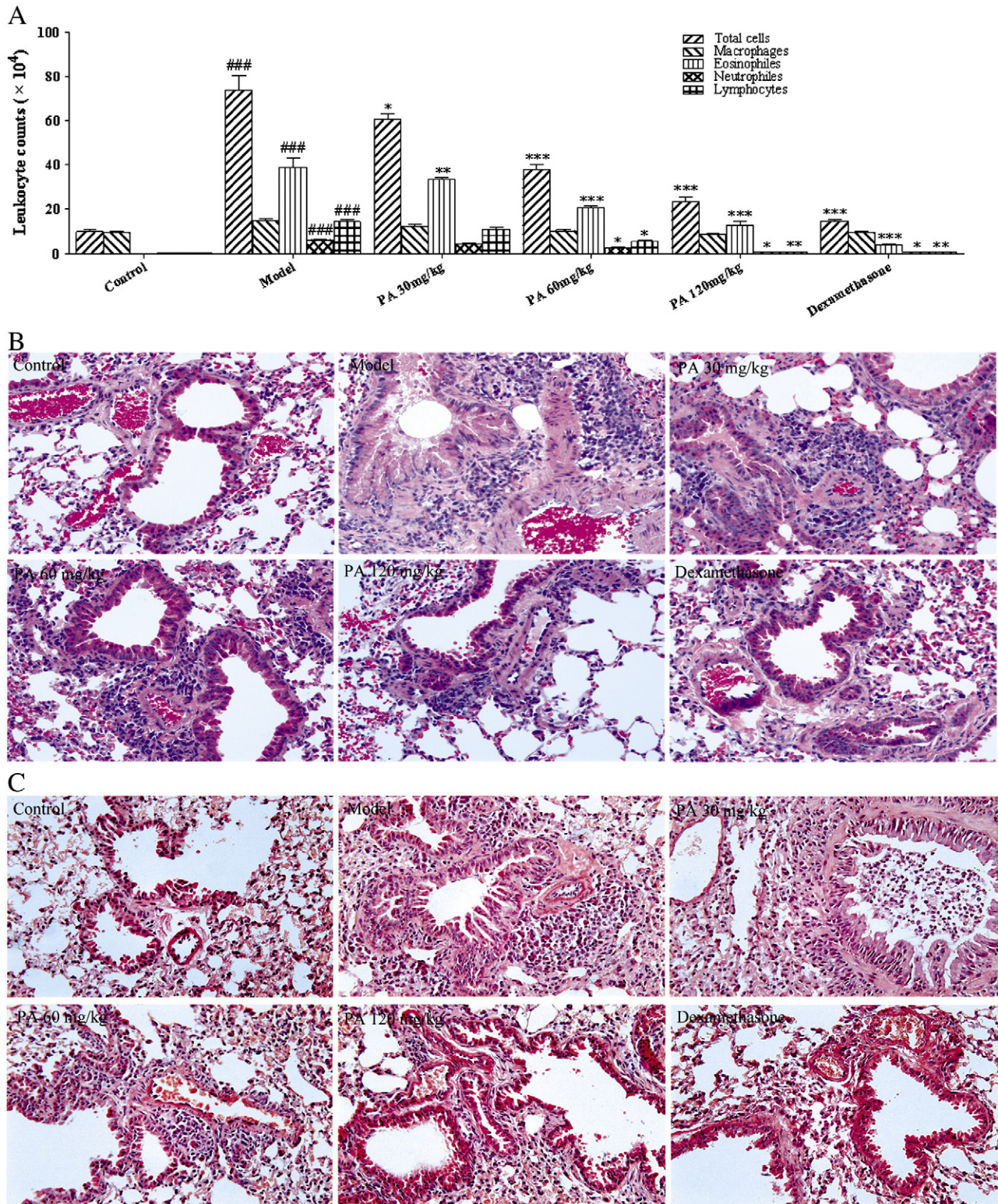


Fig. 3. Treatment of mice with (±)-praeurptorin A (PA) attenuates airway inflammation in a murine model of allergic airway disease. (A) The leukocyte counts of bronchoalveolar lavage fluid. (B) Lung histopathologic changes analyzed by hematoxylin–eosin staining (original magnification×400). (C) Lung eosinophils detected by Congo red staining (red cytoplasm and blue nucleus; original magnification×400). Values are expressed as mean±S.E.M. (n=8). Significant at: #*P*<0.05, ###*P*<0.001 vs. control group; **P*<0.05, ***P*<0.01, ****P*<0.001 vs. OVA group.

pH 7.5, 0.85% NaCl, 0.1% Tween 20) containing 5% skim milk, and subsequently incubated with either anti-phospho-p65 (Ser⁵³⁶), anti-p65 (Beyotime Biotechnology, China) or anti-I κ B α antibody (Cell Signaling Technology, Beverly, MA) overnight at 4 °C, and then exposed to corresponding HRP-conjugated goat anti-rabbit IgG (Beyotime Biotechnology, China) for 1 h at room temperature. The labeled band was visualized using enhanced chemiluminescent (ECL) solution (BestBio Inc, Shanghai, China).

2.13. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was performed using a commercial kit (Pierce, Rockford, IL, USA). Biotin-labeled NF- κ B-specific oligonucleotides were prepared as the labeled probe according to the manufacturer's instructions. Nuclear extracts (5 μ g) were mixed with 2 μ g of poly(dI-dC), 1 μ l of labeled probe, 2 μ l of binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM MgCl₂, 0.3 mM EDTA, 10% glycerol, 1 mM PMSF, 1 μ g/ μ l aprotinin and 1 μ g/ μ l leupeptin) for 20 min incubation at room temperature. Ten microliters of protein-DNA complex was subsequently fractionated on a gel electrophoresis at 150 V for 45 min with 6.5% (w/v) polyacrylamide gels in 0.5 $\times$ TBE buffer, and then transferred to a nylon membrane. The biotin end-labeled DNA is detected using a streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate. In addition, a mutated oligonucleotide sequence of probes was used to examine the specificity of binding of NF- κ B to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide.

2.14. Statistical analysis

Values are expressed as mean $\pm$ S.E.M. 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 airway hyperresponsiveness

The effect of PA on airway responsiveness induced by acetylcholine chloride was evaluated in experimental mice using a mouse lung function analysis system. The lung resistance (*R*_L) induced by acetylcholine chloride at each level of dose ranging from 30 to 270 μ g/kg in the model group was significantly higher than that in the control group (Fig. 2). Treatment of mice with PA resulted in a sharp reduction in lung resistance (*R*_L) compared with the model group (Fig. 2). Noteworthy, the high dose of PA (120 mg/kg) had comparable lung resistance inhibitory activity to dexamethasone.

3.2. Effects of PA on airway inflammation

Leukocyte counts in bronchoalveolar lavage fluid were evaluated for the development of airway inflammation in the present study. Comparison with control group mice, the numbers of total leukocytes, eosinophils, neutrophils and lymphocytes were markedly elevated in mice of the model group. Treatment of mice with PA before ovalbumin aerosol significantly prevented this increase (Fig. 3A).

Lung sections stained by hematoxylin-eosin and Congo red staining were also evaluated for the development of airway inflammation. The model group mice exhibited an obvious peribronchial and perivascular inflammation, intra-alveolar exudation, mucosal oedema as compared with the control group mice (Fig. 3B), and most of infiltrating inflammatory cells in peribronchial and perivascular inflammatory sites were eosinophils (Fig. 3C). In the PA groups, airway inflammation, intra-alveolar exudation, bronchi wall thickening and

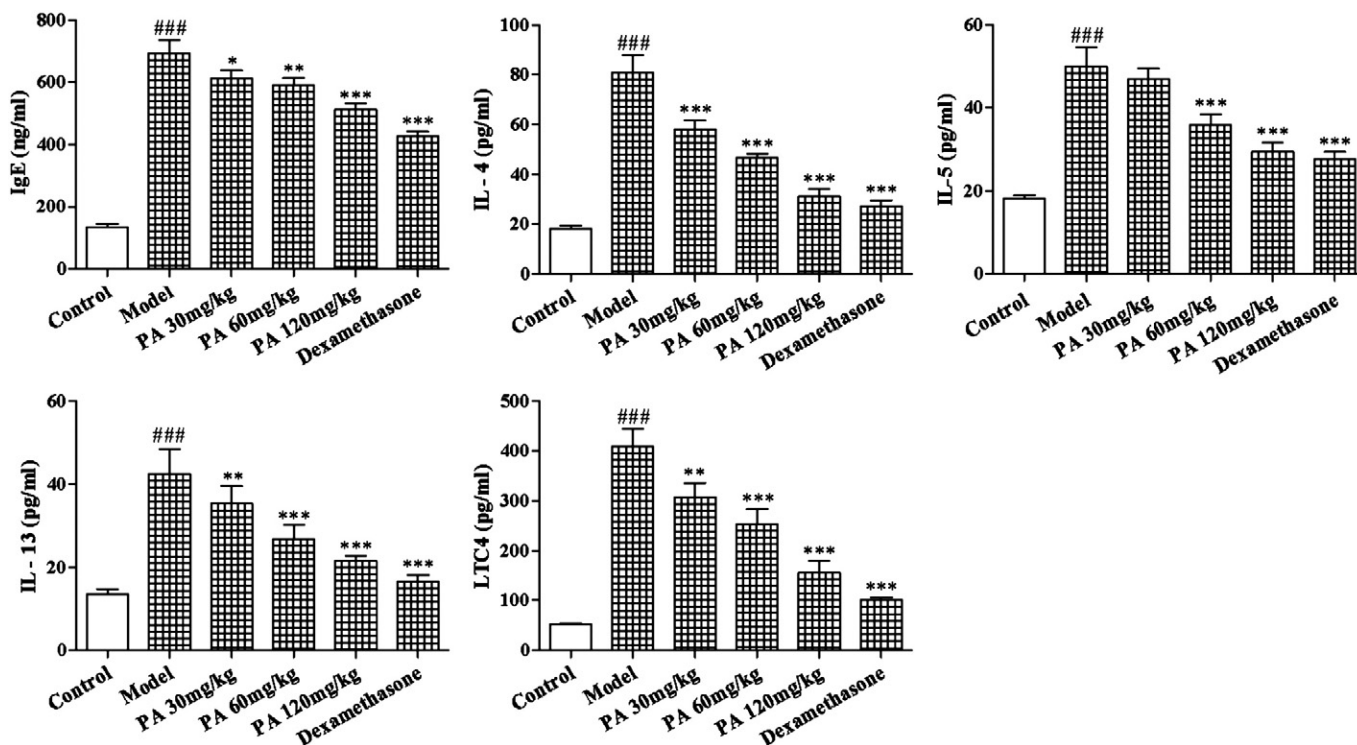


Fig. 4. Treatment of mice with ($\pm$)-praeuroptorin A (PA) reduced levels of IL-4, IL-5, IL-13 and LTC₄ in bronchoalveolar lavage fluid and IgE in serum in a murine model of allergic airway disease. Values are expressed as mean $\pm$ S.E.M. (*n* = 8). Significant at: ###*P* < 0.001 vs. control group; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. OVA group.

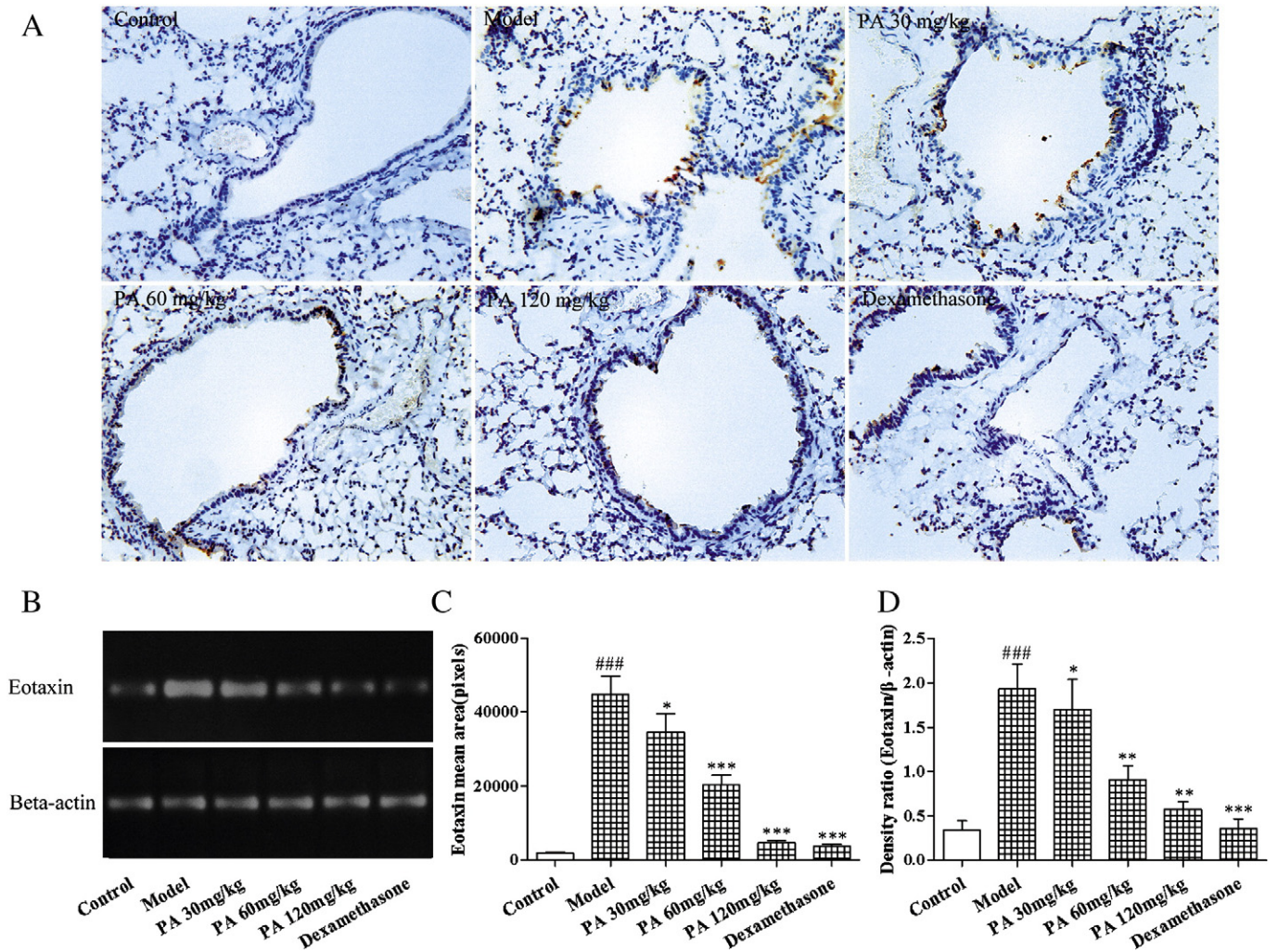


Fig. 5. Mice treated with (±)-praeprutorin A (PA) exhibited a significant reduction in lung eotaxin expression in a murine model of allergic airway disease. (A) Peribronchial eotaxin protein detected by immunohistochemistry (yellowish-brown; original magnification $\times 400$). (B) Lung eotaxin mRNA expression measured by reverse transcription polymerase chain reaction (up band: eotaxin; down band: β -actin). (C) The immunostaining area of peribronchial eotaxin protein analysed by densitometry. Data are mean $\pm$ S.E.M. ($n=8$). (D) Eotaxin mRNA expression measured by densitometry of autoradiographic films. 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.

interstitial oedema were significantly alleviated as compared with those in the model group (Fig. 3B and C).

3.3. Effects of PA on levels of IgE, LTC₄ and cytokines

To investigate the effect of PA on inflammatory mediators and immunoglobulins, levels of IL-4, IL-5, IL-13 and LTC₄ in bronchoalveolar lavage fluid and IgE in serum were detected. Compared with the control group, levels of IL-4, IL-5, IL-13, LTC₄ and IgE were sharply increased in the model group (Fig. 4). Compared with the model group, levels of these inflammatory mediators and IgE in mice of PA groups were significantly decreased by the treatment of PA in a dose-dependent manner (Fig. 4).

3.4. Effect of PA on pulmonary eotaxin expression

To assess the effect of PA on chemokines, eotaxin protein and mRNA expressed in the lung were analyzed by immunohistochemistry and reverse transcription polymerase chain reaction. The immunostaining area of bronchial epithelial eotaxin in the model group was more than that in the control group (Fig. 5A and C). Treatment of mice with PA significantly decreased the immunostaining area of eotaxin compared with the model group (Fig. 5A and C). The immunostaining

area of eotaxin in the dexamethasone group was also decreased by the treatment of dexamethasone compared with that in the model group (Fig. 5A and C).

Eotaxin gene expression has been shown to be up-regulated in allergen-challenged guinea-pig lung by northern blot analysis (Jose et al., 1994). We detected the expression of lung eotaxin mRNA by reverse transcription polymerase chain reaction in the current study. Indeed, the eotaxin mRNA expressions were found to be elevated in the model group versus control group (Fig. 5B and D), which could be inhibited by treatment of mice with PA (Fig. 5B and D).

3.5. Effect of PA on pulmonary NF- κ B activation

To evaluate the effect of PA on pulmonary NF- κ B activation in allergic asthma, DNA binding activity of NF- κ B was detected in the present study. As compared with the control group, the NF- κ B DNA binding activity in the model group was sharply increased (Fig. 6A and D). In the PA groups, NF- κ B DNA binding activity was significantly decreased as compared with that in the model group (Fig. 6A and D).

Proteins of nuclear phospho-p65 and p65 and cytoplasmic p65 and I κ B α were also detected respectively by western blot. Levels of nuclear p65 protein in the model group were strikingly enhanced as compared with the control group (Fig. 6B and E), and significantly

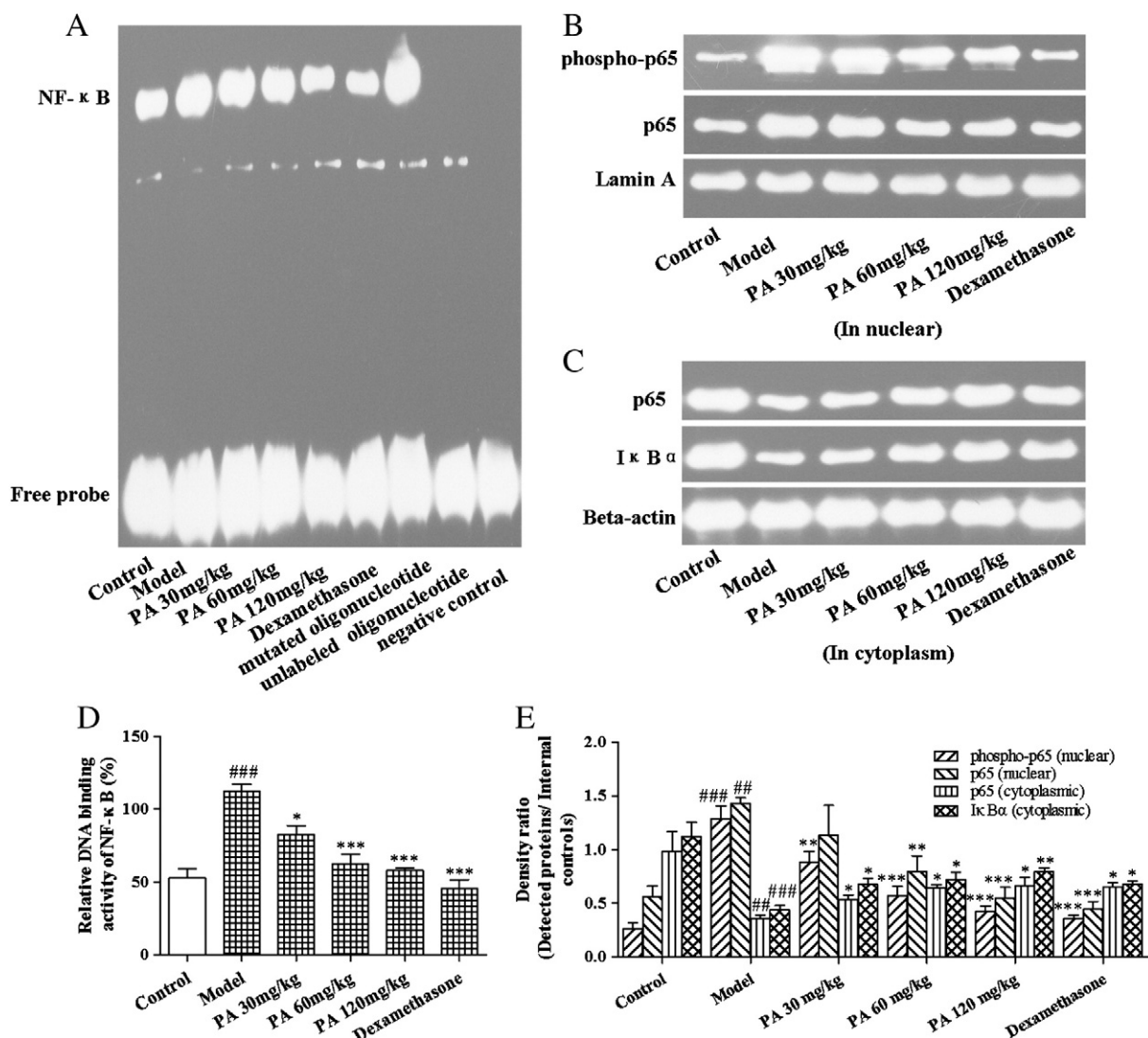


Fig. 6. Mice treated with (±)-praeurptorin A (PA) exhibited a significant inhibition in pulmonary NF-κB activation in a murine model of allergic airway disease. (A) NF-κB DNA-binding activity measured by electrophoretic mobility shift assay. (B) The levels of p65 and phospho-NF-κB p65 in nucleus measured by western blot. (C) The levels of p65 and IκBα in cytoplasm measured by western blot. (D) Relative NF-κB binding activity evaluated by photographic densitometry. (E) Relative p65, phospho-p65 and IκBα protein abundances evaluated by the photographic densitometry. Values are expressed as mean ± 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.

suppressed by treatment of mice with PA, so did the phospho-p65 protein (Fig. 6B and E). Levels of cytoplasmic p65 protein in the model group were strikingly decreased as compared with the control group (Fig. 6C and E). Treatment of mice with PA significantly inhibited this decrease (Fig. 6C and E). Interestingly, levels of cytoplasmic IκBα protein in the model group were also strikingly decreased as compared with the control group (Fig. 6C and E), and significantly inhibited by the treatment of mice with PA (Fig. 6C and E).

4. Discussion

In this investigation, it was elucidated for the first time that PA has the effect of anti-inflammation activity in a murine model of allergic airway disease. The median lethal dose of PA in 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. Previous studies also found that PA did not provoke undesirable behavioural effects in mice even administered with higher doses (Lu et al., 2001). The dried root of *P. praeurptor* Dunn has long been used in traditional Chinese medicine which also ensured the potential safety use of its components. In this study, the suppressive effects of PA on airway inflammation in mice of the PA groups

were showed by a marked decrease in airway responsiveness, in the recruitment of inflammatory cells into airway tissues, in the level of IL-4, IL-5, IL-13 and LTC₄ in bronchoalveolar lavage fluid and IgE in serum, and in the expression of eotaxin in lung tissue compared with those in the model group. These results demonstrated the potent anti-inflammatory effect of PA in mice challenged by ovalbumin.

Asthma is an airway inflammatory disease associated with airway hyperresponsiveness and hypersecretion involving many molecular regulatory pathways (Pouliot and Olivier, 2009). In order to explore the mechanism underlying the inhibitory effect of PA on allergic airway inflammation, the NF-κB signal pathway was concerned. Nuclear factor-κB (NF-κB) has been considered as a potential target for anti-inflammatory treatment (Roth and Black, 2006), playing a seminal role in allergic asthma by regulating the transcriptional process of NF-κB-dependent genes encoding proteins such as IL-4, IL-13, TNF-α, IL-1β, eotaxin, iNOS and COX-2 (Baldwin, 2001; Makarov, 2000) through its binding to specific DNA elements (κB motifs: 5'-GGGpuNNPyPyCC-3') in promoter/enhancer region. Increasingly more stimulants were found present in asthmatic airways that could result in the activation of NF-κB (Wan and Lenardo, 2010), detected in bronchial biopsy specimens

of asthmatic patients with predominant localization by immunocytochemistry to epithelial cells and macrophages (Hart et al., 1998).

In a latent phase, the usual active form of NF- κ B, complexed with I κ B α protein, p50 (NF- κ B1)/p65 (RelA) transcription factors, is sequestered in the cytoplasm (Baldwin, 1996; Karin and Ben-Neriah, 2000). The stimulation of cells by different inducers results in the I κ B α phosphorylation of the p50/p65/I κ B α complex by the I κ B kinase complex (IKK) and the subsequent ubiquitination and degradation of the I κ B α protein by the 26S proteasome, which are some key steps in p50 (NF- κ B1)/p65 (RelA) activation (Baldwin, 1996). In the present study, we showed that treatment with PA inhibited the I κ B α degradation, NF- κ B p65 subunit nuclear translocation and NF- κ B DNA-binding activity in lung tissue in the ovalbumin-challenged mice. It might be inferred that PA could prevent the production of many inflammatory mediators that contribute to the airway inflammation in lungs exposed to ovalbumin, most likely by inhibiting the activation of NF- κ B.

Previous research also suggested that phosphorylation of NF- κ B is crucial for the regulation of NF- κ B activity and NF- κ B-mediated gene transcription (Zhou et al., 2000). Cells from mice deficient in GSK-3 β and TBK/TBK/NAK, which are key enzymes of p65/p50 heterodimer phosphorylation, showed normal NF- κ B activation in response to a variety of stimuli. However, in both cells, NF- κ B was unable to drive gene transcription (Hoeflich et al., 2000; Sakurai et al., 1999; Tojima et al., 2000). In the present study, treatment of mice with PA significantly inhibited RelA/p65 phosphorylation in ovalbumin-challenged mice. PA might exert potent anti-inflammatory effects in lung exposed to ovalbumin by inhibiting NF- κ B-mediated gene transcription involved in the phosphorylation of RelA/p65.

In summary, the inhibitory effect of PA on allergic airway inflammation in the ovalbumin-challenged mice may act through its interference with the NF- κ B signaling pathway. This at least partly demonstrated the inhibitory mechanism of PA on the inflammatory mediators such as IgE, IL-4, IL-13, LTC4 and eotaxin, which were increased by ovalbumin challenge in experimental mice. Interestingly, this is partially in accordance with a previous research of PA-mediated NF- κ B repression in lipopolysaccharide-induced macrophage RAW264.7 cells (Yu et al., 2011). The activation of NF- κ B was also inhibited by the administration of dexamethasone via suppressing I κ B degradation, NF- κ B DNA binding activity and RelA/p65 phosphorylation in this study, which was also consistent with previous researches (Rogerio et al., 2010; Zhou et al., 2006). Our study demonstrated the suppressive effects of PA on airway inflammation and airway hyperresponsiveness through regulating NF- κ B pathway, which supported the possible use of PA for the medication of asthma. Further studies should be performed for overall evaluation of its potency for the treatment of chronic asthma, such as airway remodeling.

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