



Pinocembrin attenuates allergic airway inflammation via inhibition of NF- κ B pathway in mice

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ABSTRACT

Pinocembrin, one of the primary flavonoids in propolis, possesses many biological activities, including anti-inflammation, anti-oxidation and immunoregulation. This study aimed to evaluate whether pinocembrin could attenuate ovalbumin (OVA)-induced allergic airway inflammation in mice and to explore the possible mechanism. BALB/c mice sensitized and challenged with OVA were administered intraperitoneally with pinocembrin. Airway inflammation and airway hyperresponsiveness were examined. T-helper type (Th) 2 cytokines in bronchoalveolar lavage fluid (BALF) and OVA-specific immunoglobulin E (IgE) in serum were determined. The activation of nuclear factor kappa B (NF- κ B) p65 were also measured. Our results showed that pinocembrin resulted in significant inhibition of pathophysiological signs of allergic asthma, including increased pulmonary eosinophilia infiltration, mucus hypersecretion and airway hyperresponsiveness (AHR). Treatment with pinocembrin significantly reduced Th2 cytokines interleukin (IL)-4, IL-5 and IL-13 in BALF, and OVA-specific IgE in serum. Moreover, pinocembrin treatment suppressed phosphorylation of inhibitor- κ B α (I κ B α) and NF- κ B subunit p65 activation in lung tissue of OVA-sensitized mice. These data suggest that pinocembrin may inhibit allergic airway inflammation, and providing potential benefits in the treatment of inflammatory disease.

1. Introduction

Allergic asthma is a complex chronic pulmonary disease associated with reversible obstruction of airway hyperresponsiveness (AHR) and airway eosinophilic inflammation, mucus hypersecretion, and elevated serum immunoglobulin E (IgE) and Th2 cytokine production [1]. Th2-biased immunologic response, characterized by increased levels of Th2 cytokines IL-4, IL-5 and IL-13, is an important molecular mechanism involved in asthma.

NF- κ B is an important transcriptional factor in the pathogenesis of asthma. NF- κ B regulates many cellular pathways and processes, including the inflammation, the immune response, apoptosis, and proliferation. Persistent activation of NF- κ B has been observed in allergic airway inflammation [2–3]. Therefore, NF- κ B was emerged as a vital therapeutic molecular target and inhibition of NF- κ B activity would be a welcome method to manage this disease.

Pinocembrin (5,7-dihydroxyflavanone), is one of the primary flavonoids in propolis and certain plants. The chemical structure of this compound is shown in Fig. 1. Previous data have demonstrated that

pinocembrin has various pharmacological activities, such as anti-inflammatory, anti-oxidant and anti-cancer properties [4–6]. In several types of cells and animal models for several diseases, pinocembrin has been shown to suppress inflammatory response process through blocking NF- κ B activation [6–8]. These results indicate that pinocembrin may have a beneficial effect in asthma. In this study, we investigated the role of pinocembrin on allergic airway inflammation in a murine model of asthma. The present study demonstrates that pinocembrin ameliorates allergic airway inflammation and airway hyperresponsiveness, which may be linked to the suppression of NF- κ B activation.

2. Materials and methods

2.1. Experimental animal

A total of 32 Specific-pathogen-free (SPF) female BALB/c mice, weighing 18–22 g and aged 6 to 8 weeks each, were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). All

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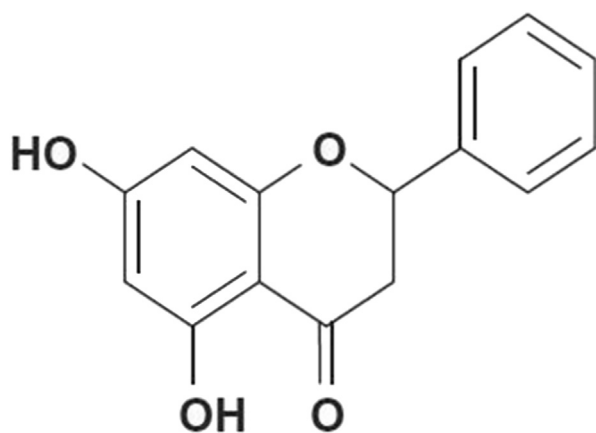


Fig. 1. Chemical structure of pinocembrin.

experimental animals were performed under a protocol approved by the Institutional Animal Care and Use Committee of the Nanjing Medical University and the experimental protocol was approved by the institutional animal ethics committee (Nanjing, China).

2.2. Allergen sensitization/challenge protocol

BALB/c mice were randomly divided equally into 6 groups: Control, OVA, OVA + dimethyl sulfoxide (DMSO), OVA + PIN25 (pinocembrin 25 mg/kg), OVA + PIN50 (pinocembrin, 50 mg/kg) and OVA + dexamethasone (DXM, 3 mg/kg). The mice were sensitized on days 0 and 14 by intraperitoneal injection of 20 μ g OVA (Grade V; Sigma, St Louis, MO, USA) emulsified in 2 mg aluminum hydroxide gel (InvivoGen, San Diego, USA) with a total volume of 0.2 ml. Mice were challenged for 30 min via the airway with 1% (wt/vol) OVA daily on days 22, 23 and 24. OVA challenge was performed in a plexiglas box (29 $\times$ 22 $\times$ 18 cm) using an ultrasonic nebulizer (NE-U11B, Omuron, Tokyo, Japan). Pinocembrin (25 and 50 mg/kg; purity $\geq$ 98%, Sigma–Aldrich) [8–9] or vehicle (20 μ l DMSO in a total of 200 μ l saline) was given by intraperitoneal injection from day 21 to day 24. For a positive control, DXM (3 mg/kg, Sigma–Aldrich) was given in the same manner. Control mice were sensitized and challenged using the same protocol but using only saline.

2.3. Measurement of airway responsiveness

For the measurement of airway responsiveness to acetylcholine chloride (ACh), an animal lung function analysis system (Buxco Electronics Inc., NY, USA) was used to evaluate lung resistance (RL) as previous described [10]. Briefly, 24 h after the last OVA challenge, mice were weighed and anaesthetized via intraperitoneal injection of 70 mg/kg pentobarbital sodium. A tracheal tube (2 mm internal diameter) was inserted into the trachea via a tracheotomy. Then, mice were placed in the whole-body plethysmography chamber and the tracheal tube was connected to the ventilator for mechanical ventilation with a tidal volume of 0.2 ml and a frequency of 140 breaths/min. After 3 min of equilibration on the ventilator, PBS and a series of increasing doses of ACh (3.125, 6.25, 12.5 and 25 mg/ml) were administered through the ventilator with an ultra-sonic nebulizer. RL was measured to express the change of AHR [10–11].

2.4. Analysis of bronchoalveolar lavage fluid (BALF) samples and serum

Mice were sacrificed 24 h after the last aerosol challenge, the sample of the blood were collected via retroorbital puncture using heparinized capillary tubes. Blood samples were centrifuged at 1000 $\times$ g for 15 min at 4 $^{\circ}$ C, and plasma was stored at -70° C until further analysis. Then BALF from each mouse was collected with 0.9% ice-cold

saline (0.5 ml $\times$ 3) and centrifuged at 1000 $\times$ g for 15 min at 4 $^{\circ}$ C. The total number of inflammatory cells in BALF was counted with a hemocytometer. Wright's staining was used to evaluate the inflammatory cells classification. Two independent investigators counted the total number of BALF cells in a single-blind study, and at least 200 cells each from four different random locations were analyzed using a microscope. The levels of IL-4 (Jingmei Biotech Company, Shanghai, China), IL-5 (Jingmei Biotech Company, Shanghai, China) and IL-13 (ExCell Biology, Shanghai, China) in BALF and OVA-specific IgE (Shibayagi, Gunma, Japan) were evaluated by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

2.5. Lung histology

After the mice were sacrificed, the right lungs were immersed in 4% formaldehyde overnight, and then embedded in paraffin. A series of micro sections (5 μ m) were cut on a microtome. The inflammatory cell infiltration was evaluated with hematoxylin and eosin (H & E) staining and the airway goblet cell hyperplasia was quantified with Periodic acid Schiff (PAS) staining, respectively. The degree of peribronchial and perivascular inflammation was evaluated by a subjective scale of 0–4, as described previously [12]. 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 pulmonary inflammation scores was defined as the mean of the peribronchial and perivascular inflammation scores. Scoring for mucus production was quantified as previous described [12–13]: 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. Each slide was scored at least three different fields and in a blinded fashion.

2.6. Western blot analysis

Fresh lung tissues were collected and homogenized in lysis buffer using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Protein concentrations were measured using the BCA protein assay kit (Thermo Scientific). Equal amounts of protein were loaded on a 10% SDS–PAGE gels and were electrotransferred to nitrocellulose membranes. The blots (the membranes) were incubated with anti-I κ B α , p-I κ B α , NF- κ B p65, p-NF- κ B p65, and anti- β -actin antibodies (Invitrogen, USA) for 2 h. After washing, the blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Invitrogen, USA) for 1 h at room temperature. The intensity of each band was quantitatively determined using Gel-Pro Analyzer Software (Media Cybernetics Inc., Rockville, MD, USA). The density ratio represents the relative intensity of each band against those of the controls in each experiment.

2.7. Statistical analysis

Data were shown as the means $\pm$ standard error of the mean (SEM). Results were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc least significant difference (LSD) test with SPSS software 19 (SPSS, Chicago, IL, USA). Significant levels were set at $P < 0.05$.

3. Results

3.1. Pinocembrin suppresses airway hyperreactivity in OVA-sensitized mice

To evaluate the effect of pinocembrin on AHR in response to ACh, RL was measured in an invasive whole-body plethysmography chamber in anaesthetized mice. There were no significant differences in baseline airway resistance among the four groups. Administration of ACh at doses from 3.125 to 25 mg/ml leads to increased dramatically RL in the

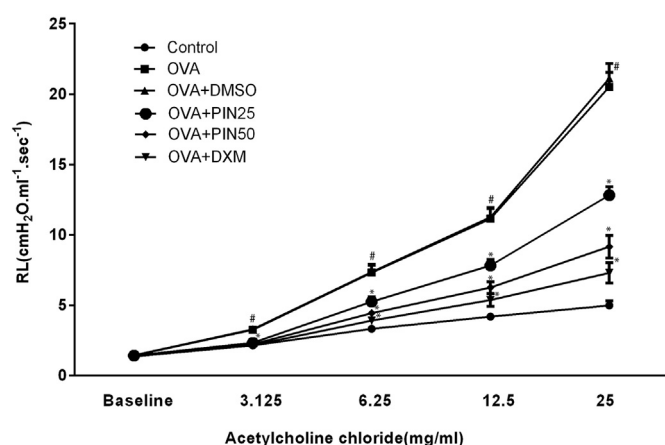


Fig. 2. Pinocembrin suppresses airway hyperactivity in OVA-sensitized mice. Data represent the mean $\pm$ SEM ($n = 6$ per group). # $P < 0.05$ compared with the control group, * $P < 0.05$ compared with the OVA group.

OVA group compared with the control group. The OVA-sensitized/challenged mice had significantly higher airway resistance than the control mice. Treatment with pinocembrin resulted in a significant decrease in RL compared with the OVA group (Fig. 2).

3.2. Pinocembrin inhibits allergic airway inflammation in OVA-sensitized mice

To assess the effect of pinocembrin on airway inflammation in asthma, we examined changes of the total number and classification of inflammatory cells in BALF in allergic mice. Comparison with control group, the number of total inflammatory cells, especially eosinophils, and neutrophils were markedly increased in the OVA group. The percentage of eosinophils and neutrophils were decreased dose-dependently after treatment with pinocembrin (Fig. 3). The anti-inflammatory effect of pinocembrin was also investigated with lung sections stained by H&E. we found an significant peribronchial and perivascular inflammatory cell infiltration in OVA-sensitized/challenged mice, but not in the control mice. The majority of the infiltrated inflammatory cells were eosinophils. Treatment with pinocembrin alleviated eosinophil-rich inflammatory cells infiltration when compared with OVA group (Fig. 4).

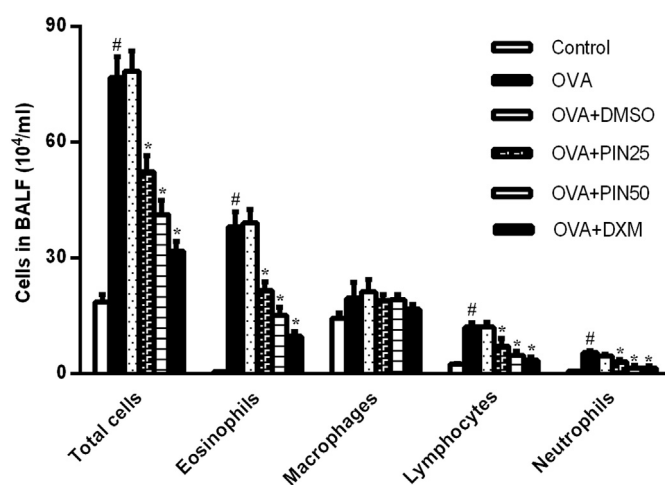


Fig. 3. Pinocembrin inhibits the inflammatory cell accumulation in bronchoalveolar lavage fluid (BALF) in OVA-sensitized mice. Data represent the mean $\pm$ SEM ($n = 6$ per group). # $P < 0.05$ compared with the control group, * $P < 0.05$ compared with the OVA group.

3.3. Pinocembrin attenuates airway goblet cell hyperplasia and mucus production in OVA-sensitized mice

PAS staining was used to evaluate airway global cells hyperplasia and mucus production. Marked goblet cell hyperplasia and mucus hypersecretion in the lumen of the bronchioles were observed in the OVA-sensitized/challenged mice. Treatment with pinocembrin showed a significant reduction in the overproduction of mucus and goblet cell hyperplasia (Fig. 5A–D). PAS staining scores were calculated for each experimental group, as described in Materials and methods section. Mucus secretion was markedly increased in the OVA-sensitized/challenged mice with enhanced staining scores. Treatment with Pinocembrin significantly reduced the proportion of PAS-stained goblet cells in the airway compared with the control mice (Fig. 5E).

3.4. Pinocembrin inhibits the levels of Th2 cytokines in BALF

To determine whether pinocembrin influenced cytokines levels in BALF, the levels of Th2 cytokines IL-4, IL-5 and IL-13 in BALF were measured by ELISA. 24 h after the final challenge, the secretion of IL-4, IL-5 and IL-13 in BALF were notably enhanced in the OVA group, compared with the control group ($P < 0.05$; Fig. 6). Treatment with pinocembrin inhibited the levels of these Th2 cytokine IL-4, IL-5 and IL-13 in BALF compared with the OVA group ($P < 0.05$; Fig. 6). This finding indicated the Pinocembrin could modify Th2-predominant immune activity in OVA-induced allergic airway inflammation in mice.

3.5. Pinocembrin decreases the level of OVA-specific IgE in serum

Elevated IgE level is a key feature in bronchial asthma. To assess the anti-asthmatic effect of Pinocembrin, OVA-specific IgE levels in serum was examined by ELISA. After sensitisation and challenges with OVA, the level of OVA-specific IgE in serum was significantly elevated in the OVA group compared with the control group, whereas this elevation was abolished by Pinocembrin ($P < 0.05$; Fig. 6).

3.6. Pinocembrin inhibits NF- κ B activation in OVA-sensitized mice

In the OVA sensitized and challenged mice, NF- κ B signaling pathways were significantly activated. As shown in Fig. 7, treatment with pinocembrin suppressed the level of phosphorylation of I κ B- α and reversed the tendency that I κ B- α decreased in the cytoplasm in a dose-dependent manner in OVA-challenged mice. The level of phosphorylation of NF- κ B p65 were inhibited by pinocembrin treatment in the presence of 25 and 50 mg/kg. These data indicate that the activation of NF- κ B signaling pathways was effectively blocked by treatment with pinocembrin in this murine model of asthma.

4. Discussion

It has been reported that pinocembrin could suppress oxidative stress and inflammation reaction [4–6]. In the present study, we demonstrated that treatment with pinocembrin significantly inhibited the allergic airway inflammation, including infiltration of inflammatory cells, and mucus hypersecretion, as well as AHR in OVA induced mice. In addition, treatment with pinocembrin attenuates Th2 cytokine level in BALF. Furthermore, pinocembrin could suppress NF- κ B activity in a murine model of asthma, indicating that pinocembrin supplementation might be beneficial to the asthmatic patients.

This study used OVA antigen induced typical Th2 type airway inflammation in murine asthma model. Elevated levels of the Th2 cytokine IL-4, IL-5 and IL-13 play key roles in allergic airway inflammation. Reducing Th2 type cytokines levels will be beneficial in the treatment of asthma. IL-4 and IL-13 could lead to eosinophil-rich inflammation in lungs, enhanced IgE production [14]. IL-5 has been demonstrated to play an important role for activation and survival of eosinophils [15].

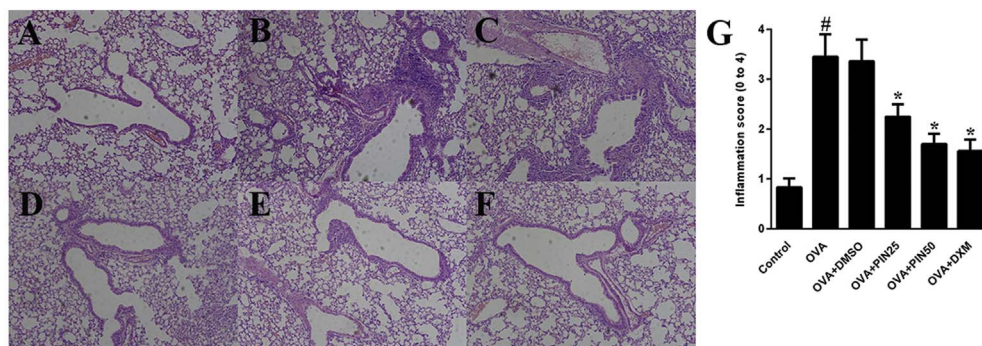


Fig. 4. Pinocembrin attenuates airway inflammation in a murine model of asthma. Hematoxylin and eosin staining (original magnification $\times 100$): (A) Control, (B) OVA, (C) OVA + DMSO, (D) OVA + PIN25, (E) OVA + PIN50, (F) OVA + DXM. (G) quantitative analyses of inflammation scores in lung sections were performed as described in the [Materials and methods](#) section. Data represent the mean $\pm$ SEM ($n = 6$ per group). [#] $P < 0.05$ compared with the control group, ^{*} $P < 0.05$ compared with the OVA group.

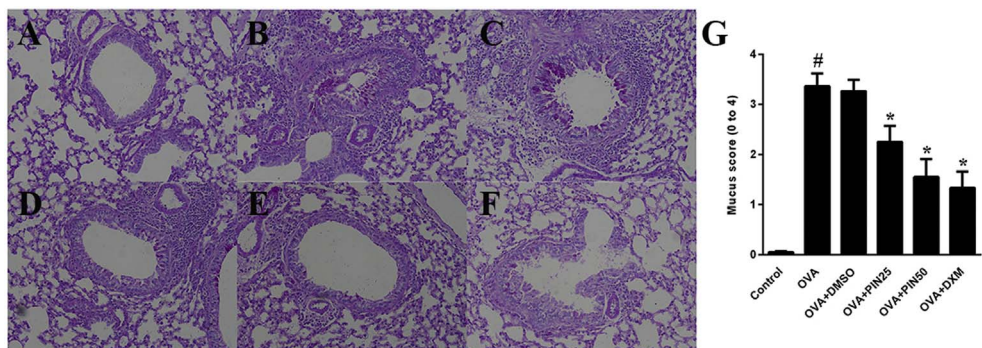


Fig. 5. Pinocembrin attenuates airway goblet cell hyperplasia and mucus production in OVA-sensitized mice. Periodic Acid Schiff (PAS) staining (original magnification $\times 200$): (A) Control, (B) OVA, (C) OVA + DMSO, (D) OVA + PIN25, (E) OVA + PIN50, (F) OVA + DXM. (G) quantitative analyses of mucus production in lung sections were performed as described in the [Materials and methods](#) section. Data represent the mean $\pm$ SEM ($n = 6$ per group). [#] $P < 0.05$ compared with the control group, ^{*} $P < 0.05$ compared with the OVA group.

Allergic airway inflammation is inhibited by the suppression of IL-5 antibodies in asthma models and severe asthmatics [16–18]. The enhanced IgE antibodies level is a hallmark of allergic disease, especially in asthma. The activation and degranulation of airway mast cells is IgE dependent via the allergen-mediated cross-linking of IgE antibodies bound to the high affinity IgE receptor (Fc3RI), which resulted in allergic airway inflammation aggravation. Our present results showed that pinocembrin treatment significantly reduced levels of IL-4, IL-5 and IL-13 in BALF in OVA sensitized/challenged mice. In addition, the enhanced OVA-specific IgE level in serum was reduced in pinocembrin treated mice. These findings indicate that pinocembrin possess significant immunoregulatory characteristic and anti-allergy effect in an OVA-induced murine asthma model.

Increased mucus production by airway epithelium goblet cells is another pathologic characteristic features of asthma. Many evidences support that mucus hypersecretion is associated with Th2 cytokines that regulate mucus-producing goblet cells function [19–20]. IL-4 and IL-13 play critical roles in the infiltration of eosinophils into lung tissues and cause mucus hypersecretion [21–22]. Pinocembrin treatment significantly attenuated goblet cell hyperplasia in the airway in allergic mice.

The enhanced AHR caused by allergens exposure, is one of the hallmark features of allergic asthma. The eosinophils infiltration into

the airways may leads to increased AHR in allergic asthma [23]. In this study, the enhanced AHR in allergic mice was inhibited by pinocembrin treatment at dose of 25 to 50 mg/kg. We also demonstrated that pinocembrin treatment suppressed the inflammatory cells such as eosinophils and neutrophils infiltration into the lung. In addition, lung histology also validated the fact that pinocembrin suppressed the inflammatory cell infiltration in the airway. These results suggest that the suppression of AHR may be attributed to reduction of eosinophilic airway inflammation in mice.

NF- κ B is considered a key regulator in immune and inflammatory responses. Inhibitor- κ B α (I κ B α), a kind of inhibitory protein called inhibitor of NF- κ B, keeps NF- κ B inactive in the cytoplasm in non-stimulated cells [24]. Exposure to many stimuli, including bacterial lipopolysaccharide, proinflammatory cytokines, NF- κ B become be activated due to the phosphorylation and subsequent ubiquitination and proteasomal degradation of the I κ B inhibitors. Activated NF- κ B transfers from cytoplasm to the nucleus and regulates the transcription of > 160 target genes [25–27]. It has been demonstrated that NF- κ B play a critical role in allergic asthma [28]. NF- κ B regulates the CD4 + T cells differentiation, and the transcriptional process of Th2 cytokines IL-4 and IL-13 [29]. Targeted inhibition of the NF- κ B signaling pathway could suppressed the airway inflammation and the production of Th2 cytokines in ova-sensitized/challenged mice [30–31]. Our preliminary

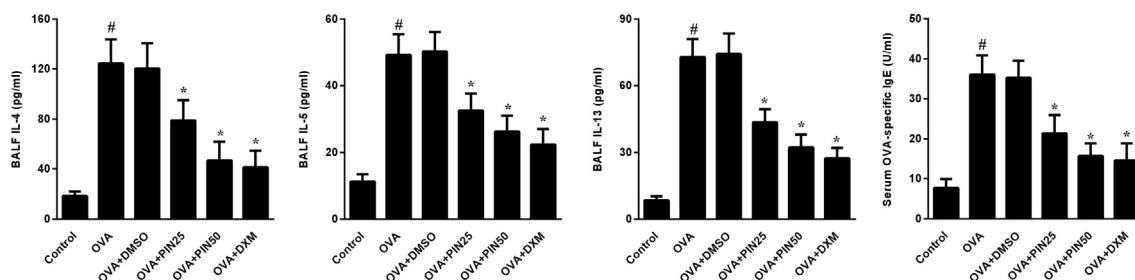


Fig. 6. Pinocembrin decreases the levels of Th2 cytokines in BALF and OVA-specific IgE in serum. Pinocembrin inhibits the levels of Th2-type cytokine IL-4 IL-5 and IL-13 in BALF and OVA-specific. IgE in a murine model of asthma. Data are the mean $\pm$ SEM ($n = 6$ per group). [#] $P < 0.05$ compared with the control group, ^{*} $P < 0.05$ compared with the OVA group.

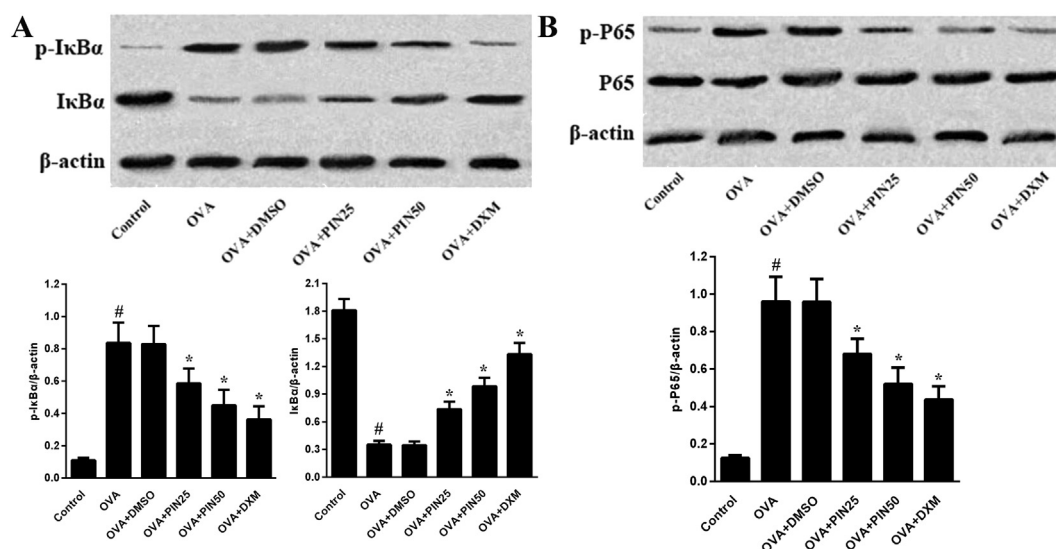


Fig. 7. Pinocembrin inhibits NF-κB activation in OVA-sensitized mice. The level of p-P65, P65, p-IκBα and IκBα were measured by western blot. Data are expressed as means $\pm$ SEM ($n = 6$ per group). [#] $P < 0.05$ compared with the control group; ^{*} $P < 0.05$ compared with the OVA group.

studies show that significant increased phosphorylation levels of NF-κB and IκBα were found in lung tissues induced by OVA. Administration of pinocembrin markedly suppressed the phosphorylation and degradation of IκBα, inhibited NF-κB P65 activation, which indicated that P65 translocation from the cytoplasm into the nucleus was suppressed in lung tissues of asthmatic mice. The above data indicate that the anti-inflammatory effect of pinocembrin may be due to suppression of NF-κB activation in the inflammatory cells.

In conclusion, our data demonstrates that pinocembrin supplementation inhibited airway hyperreactivity, eosinophilic airway inflammation, airway goblet cell hyperplasia and mucus production in OVA-induced allergic mice. In addition, treatment with pinocembrin reduced the levels of Th2 cytokines in BALF in a murine model of asthma. Furthermore, pinocembrin reduced NF-κB activation in lung tissues of OVA-sensitized mice. Therefore, we speculate that pinocembrin possesses potential therapeutic action in asthma.

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Conflict of interests

The authors claim no conflict of interests.

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