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Inhibition of allergic airway inflammation through the blockage of NF- κ B activation by ellagic acid in an ovalbumin-induced mouse asthma model

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Allergic asthma is a complex inflammatory disease characterized by airway hyperresponsiveness, eosinophilic inflammation and the hypersecretion of mucus by goblet cells. Ellagic acid (EA), a natural polyphenolic compound present as ellagitannins in fruits and fruit juices, has been reported to show potent anti-inflammatory properties in various diseases. We aimed to investigate the effects of EA in an ovalbumin (OVA)-induced mouse asthma model and to explore its potential mechanism of action. Our results showed that EA resulted in a significant reduction in lung eosinophilia, increased Th2 cytokines in bronchoalveolar lavage fluid and increased OVA-induced specific IgE in serum samples. Moreover, histological examination showed that EA markedly inhibited lung eosinophilic inflammation and goblet cell hyperplasia. In addition, EA attenuated the development of airway hyperresponsiveness and blocked NF- κ B activation. These results demonstrate that EA shows obvious anti-inflammatory effects in OVA-induced asthma in a mouse model, possibly through inhibiting NF- κ B activation. Therefore it may be a potential therapeutic agent for the treatment of allergic asthma.

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Introduction

The morbidity and mortality caused by allergic asthma have been increasing in the past few decades. In China, more than 30 million people are affected by this disease.¹ Allergic asthma is an inflammatory disease of the lungs, characterized by airway hyperresponsiveness (AHR), inflammatory infiltrates containing eosinophils in the airways, increased serum immunoglobulin E (IgE), and the excessive production of mucus in the airways. It has been reported that the initiation and maintenance of allergic airway inflammation involves Th2 cytokines, including IL-4, IL-5, and IL-13.^{2,3} These cytokines are closely related to effector functions, such as stimulating B-cell growth, initiating immunoglobulin isotype switching to IgE, and inducing goblet cell hyperplasia and the associated production of mucus, eosinophilia, mastocytosis and fibrosis.⁴

NF- κ B transcription factors are vital promoters of immune system and inflammatory responses, which regulate pro-inflammatory cytokines, chemokines, anti-apoptotic proteins and growth factors. In resting cells, NF- κ B dimers are maintained in an inactive cytoplasmic complex by inhibitory proteins of the inhibitory protein kappa B (I κ B) family. Once activated, the phosphorylation of I κ B generally leads to the rapid dissociation of the complex, accompanied by the proteolytic degradation of I κ B and the release of NF- κ B, which subsequently

transmigrate from the cytoplasm into the nucleus to promote the transcription of target genes and further induce the transcription of pro-inflammatory mediators.⁵

Ellagic acid (EA) is a natural polyphenolic compound present as ellagitannins in fruits and fruit juices, including grape juice, grape wine, blueberries and raspberries.⁶ It has been reported that EA has a variety of biological activities, such as the inhibition of lipid peroxidation, and also anti-oxidation, anti-inflammation, anti-proliferation, anti-angiogenesis, anti-carcinogenesis and chemoprevention activities.^{7–9} It has been reported that EA inhibits inflammatory responses through blocking the activation of NF- κ B.^{10,11} However, there have been few studies on the effects of EA on ovalbumin (OVA)-induced asthma in a mouse model. The work reported here investigated the anti-inflammatory effects of EA on OVA-challenged asthma in mice.

Materials and methods

Materials

Ellagic acid (Fig. 1), dimethyl sulfoxide and ovalbumin (grade V) were all purchased from Sigma (St Louis, MO, USA). Mouse IL-4

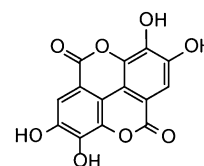


Fig. 1 Chemical structure of ellagic acid.

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and IL-5 enzyme-linked immunosorbent assay (ELISA) kits were obtained from Biolegend (USA) and the IL-13 ELISA kit was obtained from eBioscience (USA). Western blot antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies were provided by GE Healthcare (UK). All other chemicals were of analytical-reagent grade.

Animals

Female BALB/c mice (6–8 weeks old and weighing 18–20 g) were purchased from the Center of Experimental Animals of Baiqien Medical College of Jilin University (Jilin, China). Before the experiments, all mice were allowed to become accustomed to their new environment for at least 1 week and were supplied with standard rodent feed and tap water *ad libitum*. The animal room was maintained with a relative humidity of 60–80% at room temperature (22 ± 3 °C) under a 12/12 h light/dark cycle. All animal experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals established by the US National Institutes of Health.

Experimental procedure

Mice were randomly divided into six groups ($n = 8$): the control group, sham sensitized, challenged with phosphate-buffered saline (PBS); the OVA group, sensitized, challenged with OVA; three treatment groups, sensitized, challenged with OVA plus EA (2.5, 5, or 10 mg kg⁻¹); and the positive group, sensitized, challenged with OVA plus 2 mg kg⁻¹ dexamethasone (DEX).

The OVA-induced mouse asthma model was established as described previously,¹² with minor modifications. In brief, mice were sensitized on days 1 and 14 by an intraperitoneal injection of 20 µg OVA emulsified with 2 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL, USA) in a total volume of 200 µL PBS (pH 7.4). On days 21, 22 and 23 after the initial sensitization, the mice were challenged by intranasal inhalations of 100 µg OVA in a volume of 50 µL PBS. EA (2.5, 5 or 10 mg kg⁻¹) was administered orally and DEX (used as a positive control) was given by intraperitoneal injection 1 h before the OVA challenge. All determinations were performed 24 h after the last challenge.

Measurement of AHR

On day 24 (24 h after the last challenge), the mice were anesthetized with sodium pentobarbital and their tracheas were cannulated *via* tracheostomy. Each mouse was placed in an unrestrained whole body plethysmography and exposed to nebulized PBS for 3 min. Increasing concentrations of nebulized methacholine (2.5, 5, 10, 25 and 50 mg mL⁻¹) were administered by an ultrasonic nebulizer. AHR was expressed as an enhanced minute pause (Penh), a calculated value that reflects pulmonary resistance. Penh values were averaged and expressed as absolute Penh values. The results are expressed as the percentage increase in Penh over the baseline following the challenge performed with each concentration of methacholine, where the baseline Penh (after PBS challenge) is expressed as 100%.

Collection of blood and bronchoalveolar lavage fluid

After measurement of the AHR, blood was collected by puncture of the vena cava and centrifuged. Serum was stored at -80 °C for IgE assay. Blood and bronchoalveolar lavage fluid (BALF) was obtained as follows. After the mice had been anesthetized, the tracheas were cannulated and lavaged with two 0.8 mL aliquots of cold PBS. BALF was collected and immediately centrifuged. The supernatants were stored at -80 °C for the determination of cytokines and the cell pellets were resuspended in PBS to perform total and differential cell counts. Total cells were counted using a hemocytometer and the differential cell counts were obtained with a cytocentrifuge treated with Diff-Quik solution.

Determination of OVA-specific IgE and cytokine levels

OVA-specific IgE levels were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well flat-bottomed microtiter plates were coated with 100 µL of 100 µg mL⁻¹ OVA in NaHCO₃ buffer (pH 9.6) overnight at 4 °C. After blocking with PBS-10% fetal calf serum, appropriate dilutions of serum samples in PBS-10% fetal calf serum were added into the wells and incubated for 2 h at room temperature. The wells were then washed four times with PBS-Tween. Biotinylated OVA and HRP-conjugated streptavidin were added to the wells, which were then incubated for 1 h. The plates were incubated with 3,3',5,5'-tetramethylbenzidine substrate for 30 min in the dark, followed by termination with 2 N H₂SO₄. The plates were then read at 450 nm. The levels of IL-4, IL-5 and IL-13 in BALF were also measured using ELISA kits, according to the manufacturer's protocols.

Histopathology

Lung tissues were removed, fixed in 10% neutral-buffered formalin solution for 24 h, embedded in paraffin and then cut into 3 µm sections. The sections were stained with hematoxylin and eosin (H&E) to detect inflammatory infiltrates and with periodic acid-Schiff (PAS) to identify the airway goblet cells.

Western blot analysis

Lung tissue was homogenized with a lysis buffer (Thermo Scientific T-PER, 78510) and then centrifuged at 3000 g for 10 min at 4 °C. The supernatant was collected and the total protein concentration was measured using a BCA protein assay kit. A loading of 20 µg protein from each group sample was separated by 10% SDS-PAGE and electrotransferred onto a PVDF membrane. The membrane was blocked with 5% skimmed milk for 2 h at room temperature and then incubated with primary antibodies (1 : 1000 dilution in TBST) overnight at 4 °C. Subsequently, the membrane was washed with TBST for 3 × 5 min, incubated with HRP-labeled secondary monoclonal antibody for 1 h at room temperature and washed with TBST for 3 × 5 min. Finally, the proteins were visualized with Super-signal West Pico Chemiluminescent Substrate (Thermo Scientific, USA).

Statistical analysis

All values obtained were expressed as mean $\pm$ SEM values. Statistical evaluation of the results was performed using one-way ANOVA, followed by Duncan's multiple range tests. Results with $p < 0.05$ were considered statistically significant.

Results

Inhibition of lung inflammation by EA in OVA-induced mouse asthma

To determine the effects of EA on airway inflammation, the numbers of total and differential cells (eosinophils, neutrophils, macrophages and lymphocytes) in BALF were counted and the levels of Th2 cytokines (IL-4, IL-5 and IL-13) were measured by ELISA. The OVA challenge resulted in a dramatic increase in inflammatory cells in the BALF of mice, especially eosinophils. The levels of IL-4, IL-5 and IL-13 were also markedly increased after the OVA challenge. EA reduced the number of total cells and eosinophils (Fig. 2) and the levels of IL-4, IL-5 and IL-13 (Fig. 3) in BALF in a dose-dependent manner.

Reduction in levels of OVA-specific IgE levels in serum samples

The level of OVA-specific IgE was also measured by ELISA. As shown in Fig. 4, a significant increase in the levels of OVA-specific IgE was observed in the OVA-challenged mice. The level of OVA-specific IgE was decreased in the presence of 2.5, 5 and 10 mg kg⁻¹ of EA. The effect of EA (10 mg kg⁻¹) was nearly equivalent to that of DEX (2 mg kg⁻¹).

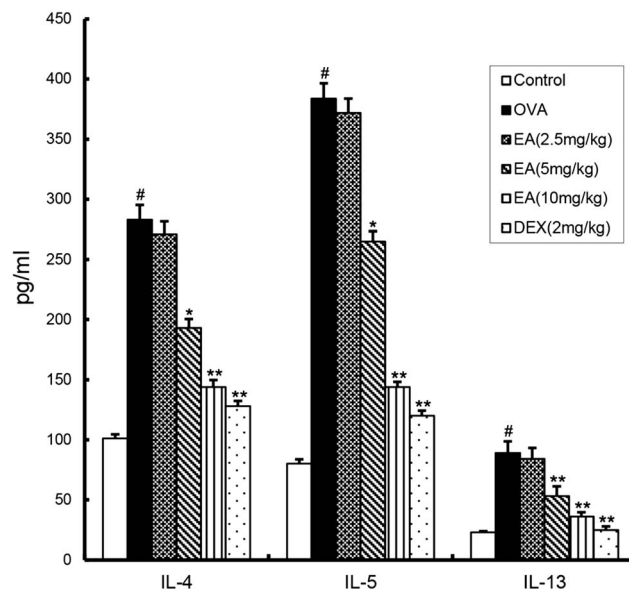


Fig. 3 Effect of EA on the levels of IL-4, IL-5 and IL-13 in BALF of BALB/c mice ($n = 8$). BALF were collected from BALB/c mice 24 h after the last challenge. The levels of these cytokines were quantified by ELISA. All data are given as mean $\pm$ SEM values. # $p < 0.05$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs. OVA-challenged group.

Reduction in OVA-induced AHR in response to methacholine

AHR is the most common symptom in asthmatic disorders. In this study, AHR was determined by the calculation of Penh values. In OVA-challenged mice, a significant increase in the Penh value was observed compared with that in control mice. Pretreatment with EA reduced the Penh value in OVA-

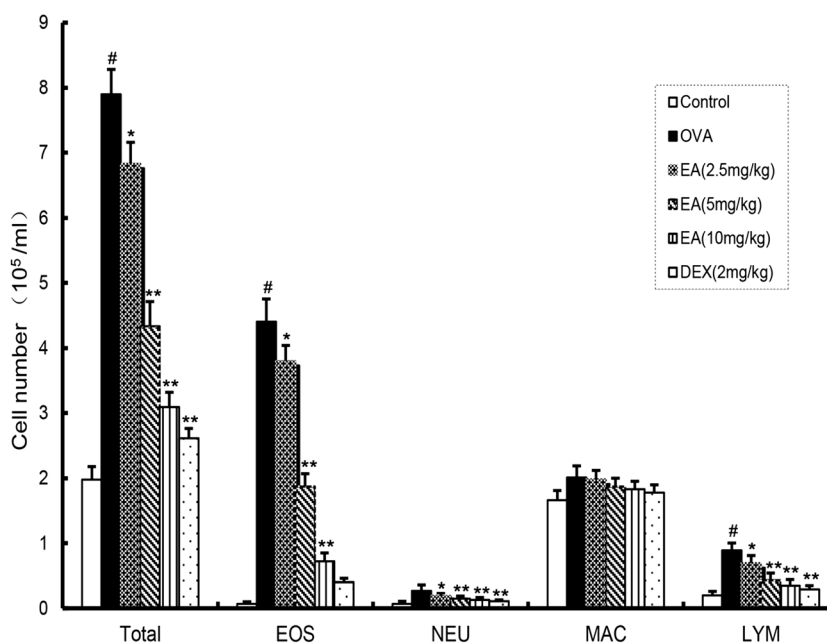


Fig. 2 Effect of EA on recruitment of inflammatory cells in BALF of BALB/c mice ($n = 8$). Cells collected from the BALF of BALB/c mice were counted 24 h after the last challenge. All data are given as mean $\pm$ SEM values. # $p < 0.05$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs. OVA-challenged group.

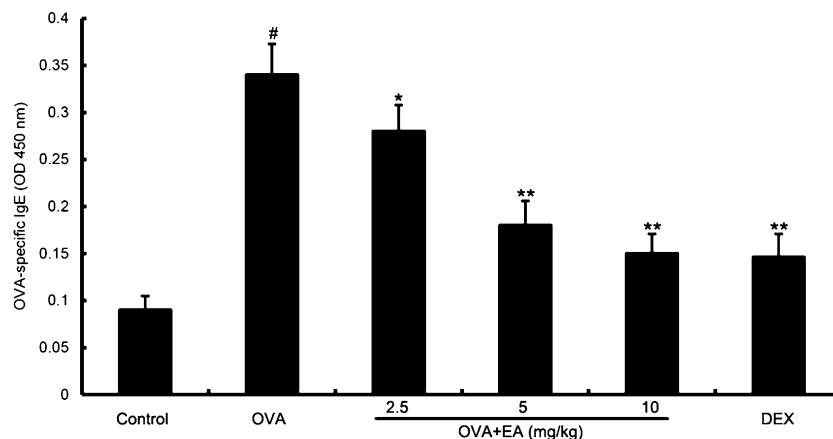


Fig. 4 Effect of EA on OVA-specific IgE levels in serum. Serum was collected from BALB/c mice 24 h after the last challenge ($n = 8$). The levels of OVA-specific IgE were analyzed by ELISA. All data are given as mean $\pm$ SEM values. # $p < 0.05$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs. OVA-challenged group.

challenged mice in a dose-dependent manner (Fig. 5). EA (10 mg kg⁻¹) resulted in a similar decrease in AHR to that of the DEX group.

Amelioration of the histopathological changes in lung tissues

To observe the effects of EA on lung tissues in the OVA-induced mouse asthma model, lung sections were stained with H&E. The OVA challenge caused a massive inflammatory infiltration into the peribronchial tissue compared with that in control mice. Pretreatment with EA led to a significant reduction in inflammatory infiltrates in a dose-dependent manner (Fig. 6).

To evaluate the hypersecretion of mucus and goblet cell hyperplasia in the airways of the mice, lung sections were

stained with PAS. In OVA-challenged mice, an obvious increase in goblet cells and mucus in the airway was observed compared with the control mice. Pretreatment with EA significantly inhibited goblet cell hyperplasia in the lung tissues of mice with OVA-induced asthma in a dose-dependent manner (Fig. 6).

Blockage of the activation of the NF- κ B signaling pathway

NF- κ B activation plays a key role in many diseases, including allergic asthma. We examined the effect of EA on NF- κ B activation. I κ B- α and NF- κ B p65 were determined by Western blot analysis. As shown in Fig. 7, the OVA-challenged mice showed a notably increased level of NF- κ B p65 phosphorylation compared with the control mice. EA treatment reduced the level of

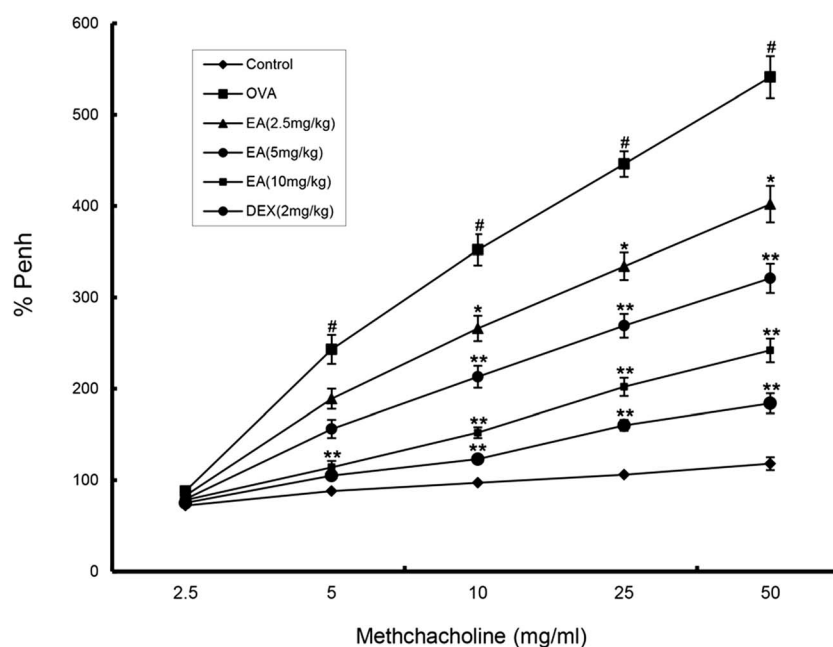


Fig. 5 Effect of EA on airway hyperresponsiveness in OVA-induced asthma in allergic BALB/c mice ($n = 8$). Airway hyperresponsiveness was assessed 24 h after the last challenge using unrestrained whole body plethysmography. All data are expressed as mean $\pm$ SEM values. # $p < 0.05$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs. OVA-challenged group.

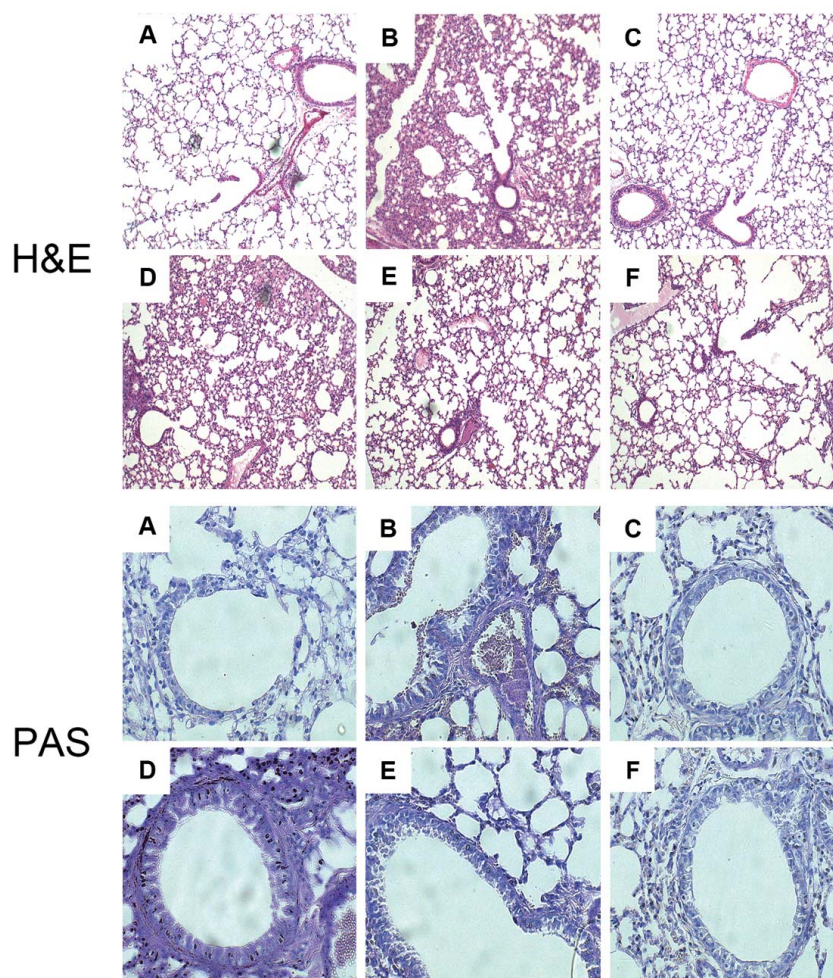


Fig. 6 Effect of EA on histopathological changes in the lung tissues of OVA-challenged asthma in allergic BALB/c mice ($n = 8$). Lung tissues were collected 24 h after the final challenge. Inflammation was evaluated in the lung tissues by analyzing inflammatory cell infiltration (H&E) and goblet cell hyperplasia (PAS) staining. Representative histological changes in mammary tissues from each group: (A) PBS-challenged mice; (B) OVA-challenged mice; (C) OVA-challenged mice treated with DEX (2 mg kg^{-1}); (D) OVA-challenged mice treated with EA (2.5 mg kg^{-1}); (E) OVA-challenged mice treated with EA (5 mg kg^{-1}); and (F) OVA-challenged mice treated with EA (10 mg kg^{-1}). H&E stained sections (red) are magnified $\times 100$ and APS stained sections (blue) are magnified $\times 200$.

phosphorylation of NF- κ B p65 in a dose-dependent manner in OVA-challenged mice. Moreover, the phosphorylation level of I κ B- α was significantly decreased in the presence of 2.5, 5 and 10 mg kg^{-1} EA.

Discussion

EA has a variety of biological properties, including anti-oxidant and anti-inflammatory activities. It has been reported that EA has potent effects on allergic responses.^{13,14} However, the underlying mechanism of its effect on allergic asthma is still not clear. In this study, a mouse model of asthma was established successfully and used to assess the effects of EA on OVA-induced mouse allergic asthma.

IgE plays a central role in the pathogenesis of allergic diseases. The development of IgE antibodies is a hallmark of allergic disease.^{15,16} After sensitization, atopic patients respond to allergen exposure through a number of IgE-dependent

mechanisms.¹⁷ Allergic inflammation is reduced by the suppression of antigen-specific IgE antibodies.¹⁸ This study found that the levels of serum OVA-specific IgE were reduced by treatment with EA in a dose-dependent manner. The early airway response in asthma is IgE dependent and is caused by the activation and degranulation of pulmonary mast cells by the allergen-mediated cross-linking of IgE antibodies bound to the high affinity IgE receptor (Fc ϵ RI).¹⁹ A higher production of IgE is more favorable for the degranulation of mast cells and airway constriction. Studies have shown that EA attenuates the IgE-mediated allergic response in mast cells and *in vivo*.^{20,21}

The increase in AHR caused by exposure to allergens is associated with the influx of eosinophils into the airways in allergic asthma.²² In this study, AHR in OVA-challenged mice was markedly reduced by treatment with EA at a dose of 10 mg kg^{-1} . Moreover, the number of total cells and eosinophils in BALF also decreased on treatment with EA. These results

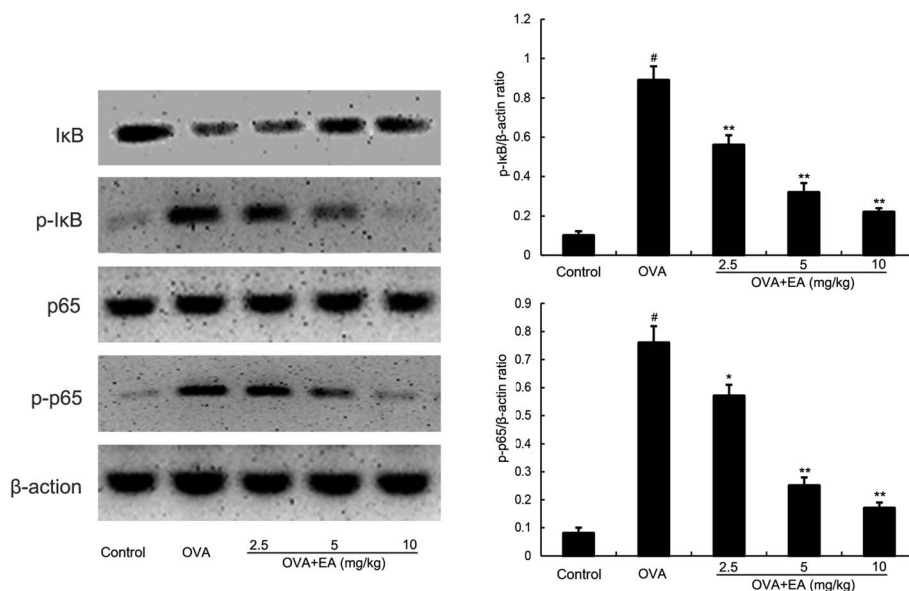


Fig. 7 Effect of EA on NF- κ B activation in OVA-induced asthma in BALB/c mice ($n = 8$). Lung homogenates were prepared 24 h after the last OVA challenge. The translocation of p65 to the nucleus and I κ B degradation were assessed by Western blot analysis. Line 1, control group; line 2, OVA group; and lines 3, 4 and 5, groups treated with 2.5, 5 and 10 mg kg⁻¹ EA, respectively; β -actin was used as an internal control. The data are expressed as mean $\pm$ SEM values. $\#p < 0.05$ vs. control; $*p < 0.05$, $**p < 0.01$ vs. OVA-challenged group.

indicate that the reduction in AHR might be due to a decrease in eosinophil infiltration into the airways.

Both the inflammatory infiltrates of lung tissues and goblet cell hyperplasia in the airway epithelium are characteristic features of asthma.²³ To observe the effects of EA on OVA-induced asthma in mice, lung sections were stained with H&E and PAS. The results showed that treatment with EA significantly inhibited inflammatory infiltrates into the peribronchial tissue and goblet cell hyperplasia in the airway.

Th2 cytokines such as IL-4, IL-5 and IL-13 are key factors in the initiation and progression of allergic disorders.²⁴ IL-4 and IL-13 play critical roles in the infiltration of eosinophils into lung tissues and cause mucus hypersecretion and AHR.²⁵ IL-5 is essential for the formation of eosinophils, which are thought to have a major role in the pathogenesis of asthma and other allergic diseases.²⁶ The reduction of Th2 cytokines attenuate allergic inflammation and AHR.^{27,28} Our results showed that EA led to a significant decrease in IL-4, IL-5 and IL-13 levels in the BALF of mice with OVA-induced asthma in a dose-dependent manner. Therefore, EA reduced OVA-induced airway inflammation, possibly through down-regulating the levels of Th2 cytokines.

NF- κ B is a critical factor and plays an essential role in regulating immune responses, including the expression of genes for many inflammatory cytokines, such as IL-4, IL-5 and IL-13.²⁹ Studies have shown that NF- κ B plays a critical role in allergic asthma.^{30,31} In a rat model of Crohn's disease, EA has been shown to return pro-inflammatory proteins to basal levels, probably through the NF- κ B and MAPK signaling pathway.³² To further clarify the anti-inflammatory mechanism of EA, the protein levels of the NF- κ B p65 subunit and I κ B- α in the NF- κ B signaling pathway were determined by Western blotting. Our

results showed that EA inhibited the activation of NF- κ B through suppressing the phosphorylation and degradation of I κ B- α and by blocking the subsequent translocation of the NF- κ B subunit p65 from the cytosol to the nucleus in lung tissues in OVA-induced mouse asthma. Therefore, EA reduced allergic airway inflammation, possibly through regulating NF- κ B activation in OVA-challenged mice.

In summary, our results showed that EA attenuated airway inflammation, the release of Th2 cytokines into the airway, goblet cell hyperplasia in the airway, OVA-specific IgE levels in serum, and airway hyperresponsiveness in a mouse model of OVA-induced asthma. In addition, NF- κ B activation was inhibited by EA. These findings indicate that the anti-inflammatory mechanisms of EA are associated with the inhibition of NF- κ B activation. Therefore EA may be a potential therapeutic agent for the treatment of allergic asthma. However, further work should be carried out before EA is put into clinical practice.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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