



Abietic acid attenuates allergic airway inflammation in a mouse allergic asthma model



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ARTICLE INFO

Article history:

Received 11 April 2016

Received in revised form 30 May 2016

Accepted 30 May 2016

Available online xxxx

Keywords:

Abietic acid

OVA

Asthma

NF- κ B

ABSTRACT

Abietic acid (AA), one of the terpenoids isolated from *Pimenta racemosa* var. *grisea*, has been reported to have anti-inflammatory and immunomodulatory effects. However, the anti-allergic effects of AA remain unclear. The aim of this study was to investigate the anti-allergic effects of AA in an ovalbumin (OVA)-induced asthma murine model. The model of mouse asthma was established by induction of OVA. AA (10, 20, 40 mg/kg) was administered by oral gavage 1 h after the OVA treatment on days 21 to 23. At 24 h after the last challenge, bronchoalveolar lavage fluid (BALF) and lung tissues were collected to assess pathological changes, cytokines production, and NF- κ B expression. The results showed that AA attenuated lung histopathologic changes, inflammatory cells infiltration, and bronchial hyper-responsiveness. AA also inhibited OVA-induced the nitric oxide (NO), IL-4, IL-5, IL-13, and OVA-specific IgE production, as well as NF- κ B activation. In conclusion, the current study demonstrated that AA exhibited protective effects against OVA-induced allergic asthma in mice and the possible mechanism was involved in inhibiting NF- κ B activation.

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1. Introduction

Allergic asthma is a complex and heterogeneous disease which is characterized by airway inflammation, elevated IgE level, and airway hyperreactivity (AHR) [1]. The pathogenesis of allergic asthma is thought to result from the aberrant production of the type 2 (Th2) cytokines such as IL-4, IL-5, and IL-13 [2]. Elevated IL-4, IL-5, and IL-13 levels in BALF were observed in patients of allergic asthma [3]. These cytokines induce release of IgE and the infiltration of inflammatory cells into the lungs which lead to the development of allergic asthma [4]. During the last decade, the incidence, morbidity, and mortality of allergic asthma in the world are increasing [5]. Therefore, the development of novel and efficient therapeutic drugs for the treatment of allergic asthma is urgently needed.

Abietic acid (AA), one of the terpenoids isolated from *Pimenta racemosa* var. *grisea*, has been reported to have anti-inflammatory and anti-obesity effects [6,7]. AA has been reported to exhibit anti-obesity effect in mice fed a high-fat diet [6]. AA was found to inhibit carrageenan-induced rat paw oedema and 12-O-tetradecanoylphorbol acetate-induced mouse ear oedema [8]. AA also inhibited UVB-induced MMP-1 expression in human dermal fibroblast cells [9]. Furthermore, AA has been reported to suppress LPS-induced TNF- α production in macrophages [10]. However, the protective effects of AA on OVA-induced asthma have not been reported. In the present study, we investigate the protective effects and mechanism of AA on OVA-induced asthma in mice.

2. Materials and methods

2.1. Reagents

OVA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse IL-4, IL-5, IL-13 and IgE ELISA kits were obtained from R&D Systems (Minneapolis, MN). Abietic acid was purchased from the Chinese Institute for Drug and Biological Product Control (Beijing, China). Antibodies against NF- κ B, I κ B α , and β -actin were purchased from Santa Cruz Biotechnology (Autogen, Bioclear, UK).

2.2. Animals

Female BALB/c mice (6–8 weeks) weighing 20–25 g were purchased from Guangdong Medical Lab Animal Center (Guangzhou, China). All mice were housed in a temperature-controlled room with alternating 12-h light 12-h dark cycle. All animal experimental procedures were approved by Animal Care and Use Committee of the Guangzhou Medical University.

2.3. Experimental protocols

To induce the asthma model, seventy-two mice were randomly divided into six groups and each group contained twelve mice: control group (PBS with DMSO), OVA group, OVA + AA (10, 20, 40 mg/kg) group, and OVA + dexamethasone (DEX) (5 mg/kg) group. The asthma model was induced as previous reports [11]. Briefly, on days 1 and 14,

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the mice were sensitized with 20 µg OVA with 1 mg aluminum hydroxide adsorbed in 200 µl PBS by intraperitoneal injection. Then the mice were received 100 µg OVA adsorbed in 20 µl PBS by intranasal inhalations at days 21, 22, and 23 once a day. AA (10 mg) was diluted with 100 µl DMSO to prepare the stock solutions. Then 100 µl stock solution was further diluted with 900 µl PBS and each mouse was given 100 µl to reach 40 mg/kg. The mouse of control group was given 90 µl PBS and 10 µl DMSO. AA (10, 20, 40 mg/kg) was given by oral gavage 1 h after each OVA challenge after the initial OVA sensitization.

2.4. Histopathologic evaluation

The lung from each animal was collected and immersed in 10% neutral-buffered formalin, embedded in paraffin and sliced. The sections were stained with periodic acid-Schiff (PAS) to observe goblet cells in the epithelium. After hematoxylin and eosin (H&E) staining, pathologic changes were captured with a Nikon SMZ 1500 Microscope (Nikon, Japan). The histological changes in the lungs were scored as previously described [12]. Each histological characteristic was scored 0 to 5. PAS-stained goblet cells in the airway epithelium were counted in a double-blind manner using a numerical scoring system (0: <5% goblet cells; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: >75%) [13].

2.5. BALF collection and cell counting

For the BALF collection, the trachea was cannulated, and the lungs were lavaged three times with PBS (0.3 ml each time). Then the BALF samples were centrifuged and the cells were re-suspended in 50 µl PBS. The number of total cells was counted using a hemocytometer. The numbers of eosinophils, neutrophils, macrophages and lymphocytes in BALF were stained with the Kwik-Diff staining set (Thermo, USA) according to the manufacturer's instructions.

2.6. Measurement of airway hyperresponsiveness

Airway hyperresponsiveness (AHR) was measured using noninvasive whole-body plethysmography (Model PLY 3211; Buxco, Sharon, CT, USA) 24 h after the last OVA treatment as described previously [14]. AHR was expressed as an enhanced minute pause (Penh), a calculated value that reflects pulmonary resistance. The results are expressed as the percentage increase in Penh over the baseline following challenge performed with each concentration of methacholine (2.5, 5, 10, 25, and 50 mg/ml).

2.7. Cytokines and NO assay

The levels of IL-4, IL-5, IL-13, and IgE in the BALF and serum were analyzed using commercially ELISA kits (R&D Systems, Minneapolis, MN). The level of NO in BALF was tested by NO assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction.

2.8. Western blot analysis

Total proteins of lung tissues were extracted using Tissue Protein Extraction Reagent Kit (Shengong, Shanghai, China). The protein concentrations were detected using BCA assay (Pierce, USA). The proteins were separated via 12% SDS-PAGE gel and transferred onto PVDF membranes. The membranes were blocked with 5% bovine serum albumin at room temperature for 2 h. Then, the membranes were probed with primary antibodies against NF-κB, p-NF-κB, IκBα, p-IκBα, and β-actin overnight at 4 °C. After incubating with HRP-conjugated secondary antibody at room temperature for 1 h, the bands were visualized using the ECL kit (Thermo, USA).

2.9. Statistical analysis

The data are presented as the mean ± S.E.M. The differences between mean values of multiple groups were analyzed by ANOVA followed by Bonferroni post hoc. A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. AA inhibited OVA-induced inflammatory cells infiltration in BALF

In this study, to investigate the effects of AA on the infiltration of inflammatory cells, the numbers of inflammatory cells including neutrophils, eosinophils, macrophages, and lymphocytes in BALF were detected. The results showed that compared with the control group, the numbers of inflammatory cells, including neutrophils, eosinophils, macrophages, and lymphocytes in BALF increased significantly in OVA-challenged mice. However, treatment of AA dose-dependently inhibited OVA-induced inflammatory cells infiltration, including neutrophils, eosinophils, macrophages, and lymphocytes in BALF (Fig. 1). Furthermore, OVA-induced inflammatory cells infiltration in BALF was significantly inhibited by treatment of DEX (Fig. 1).

3.2. AA inhibited OVA-induced serum IgE, Th2 cytokines and NO in BALF

OVA-induced asthma is characterized by the elevated of serum IgE and NO, IL-4, IL-5, and IL-13 in BALF. In the present study, the effects of AA on OVA-induced serum IgE and NO, IL-4, IL-5, and IL-13 in BALF were detected by ELISA. As shown in Fig. 2, the levels of IgE, NO, IL-4, IL-5, and IL-13 in OVA-challenged mice increased significantly than that of the control group. However, treatment of AA inhibited OVA-induced IgE, NO, IL-4, IL-5, and IL-13 production in a dose-dependent manner. In addition, DEX significantly inhibited OVA-induced IgE, NO, IL-4, IL-5, and IL-13 production.

3.3. Effects of AA on OVA-mediated lung histopathologic changes

Lung histological changes were used to assess the protective effects of AA on OVA-induced asthma. As shown in Fig. 3, OVA-challenged mice showed marked inflammatory cell infiltration in the peribronchial, perivascular areas and airway epithelial thickening. However, treatment of AA or DEX significantly attenuated OVA-induced inflammatory

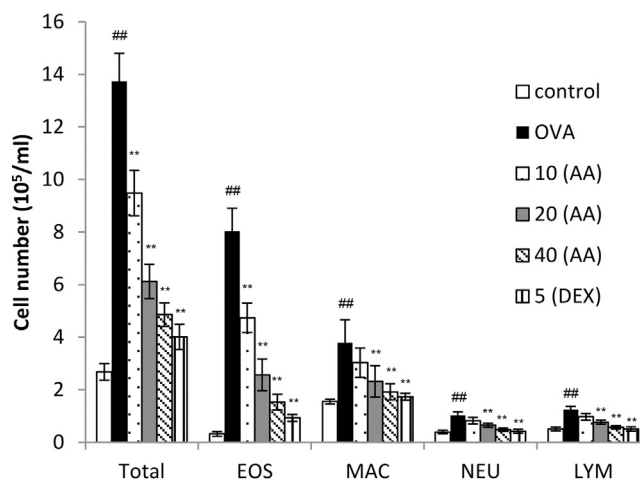


Fig. 1. Effects of AA on OVA-induced inflammatory cell count in the BALF. The values presented are the means ± S.E.M. of three independent experiments. ##p < 0.01 vs. control group; **p < 0.01 vs. OVA group.

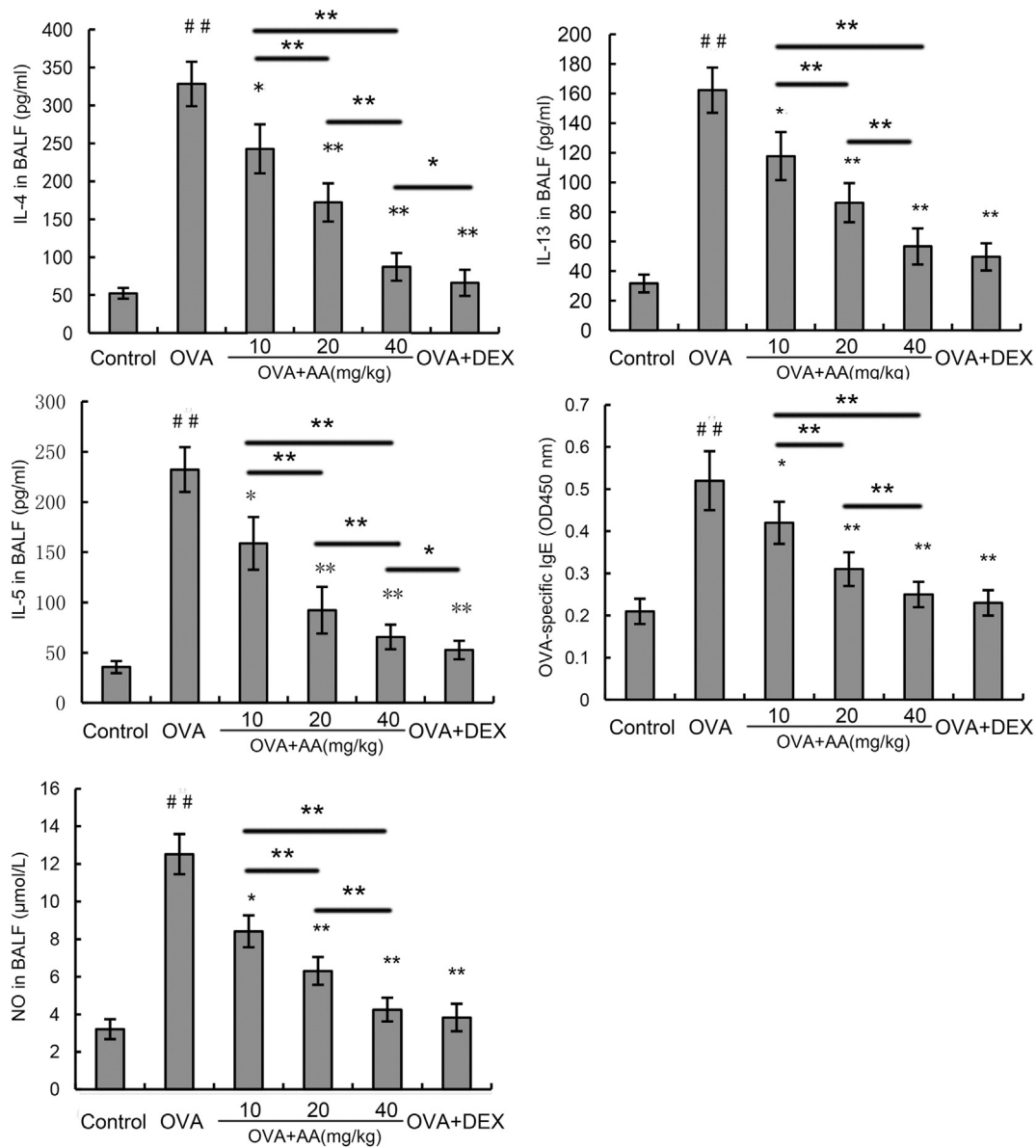


Fig. 2. Effects of AA on OVA-induced NO, IL-4, IL-5, IL-13, and IgE productions in BALF were detected by ELISA. The values presented are the means $\pm$ S.E.M. of three independent experiments. ^{##} $p < 0.01$ vs. control group; ^{*} $p < 0.05$ and ^{**} $p < 0.01$ vs. OVA group.

cell infiltration in the peribronchial, perivascular areas and airway epithelial thickening (Fig. 3). We also used PAS staining to evaluate airway hypersecretion of mucus and goblet cell hyperplasia. The results showed that OVA-challenged mice showed marked goblet cell hyperplasia in the airway. However, treatment of AA markedly inhibited mucus hypersecretion and goblet cell hyperplasia induced by OVA (Fig. 3).

3.4. AA reduced OVA-induced airway hyperresponsiveness

Airway responsiveness (AHR) was determined by Penh in response to increasing doses of methacholine. The effects of AA on OVA-induced AHR were detected in this study. The results showed that Penh value of OVA-challenged mice was significantly increased in response to methacholine inhalation than that of the control group. Treatment of AA or DEX significantly reduced AHR to inhaled methacholine in OVA-challenged mice as indicated by Penh (Fig. 4).

3.5. Effects of AA on OVA-induced NF- κ B activation

NF- κ B has been reported to play an important role in allergic inflammation. Activation of NF- κ B could regulate the expression of inflammatory mediator production. To investigate the anti-inflammatory mechanism of AA, the effects of AA on NF- κ B signaling pathway were detected in this study. The results showed that the levels of p-p65 and p-I κ B α were significantly increased in OVA-challenged mice. However, treatment of AA dose-dependently inhibited OVA-induced p-p65 and p-I κ B α expression. These results suggested that treatment of AA significantly inhibited OVA-induced NF- κ B activation and I κ B α degradation (Fig. 5). Furthermore, OVA-induced NF- κ B activation was significantly inhibited by treatment of AA (Fig. 5).

4. Discussion

AA, one of the terpenoids isolated from *Pimenta racemosa* var. *grisea*, has been reported to have anti-inflammatory effects. In this

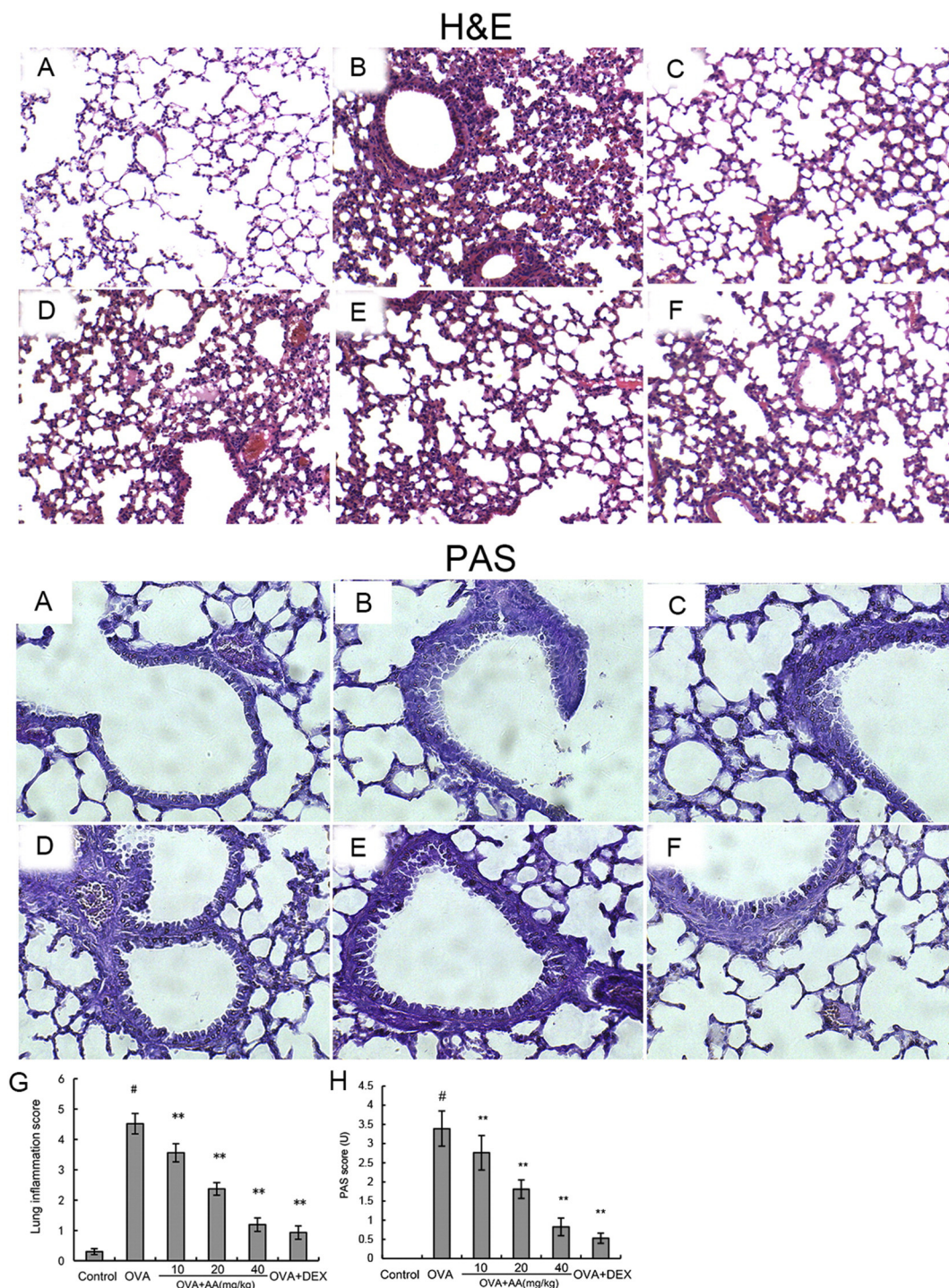


Fig. 3. Effects of AA on histopathological changes in lung tissues in OVA-induced asthma. Inflammation was evaluated in the lung tissues by analyzing inflammatory cell infiltration using H&E and goblet cell hyperplasia using PAS staining. Representative histological changes of lung obtained from mice of different groups. A: Control group, B: OVA group, C: OVA + DEX group, D: OVA + AA (10 mg/kg) group, E: OVA + AA (20 mg/kg) group, and F: OVA + AA (40 mg/kg) group. H&E sections are magnified 100 $\times$ and PAS staining sections are magnified 200 $\times$. G: Lung inflammatory scores were calculated according to the sum of the levels of cell infiltration and damage levels as assessed from the lung sections. H: PAS scores. The values presented are the means $\pm$ S.E.M. of three independent experiments. [#] $p < 0.01$ vs. control group; ^{**} $p < 0.01$ vs. OVA group.

study, we found that AA could protect against OVA-induced asthma in mice by inhibiting NF- κ B activation. AA has potential as a therapeutic drug in the treatment of asthma.

OVA-induced asthma is characterized by the infiltration of inflammatory cells, elevated IgE level, and airway hyperreactivity (AHR) [15]. AHR is a clinical hallmark of asthma [16]. The development of AHR was associated with inflammatory cytokines. Inflammation has

been reported to play a critical role in the development of asthma [17]. Asthma is characterized by a Th2-type immune response, with increases in the number of eosinophils and increased amounts of Th2 cytokines [18]. Studies showed that T helper type 2 lymphocytes played an important role in the development of allergic asthma by producing cytokines IL-4, IL-5, and IL-13 [19]. IL-4 is one of the most important cytokines in airway inflammation. It induces B cell maturation and

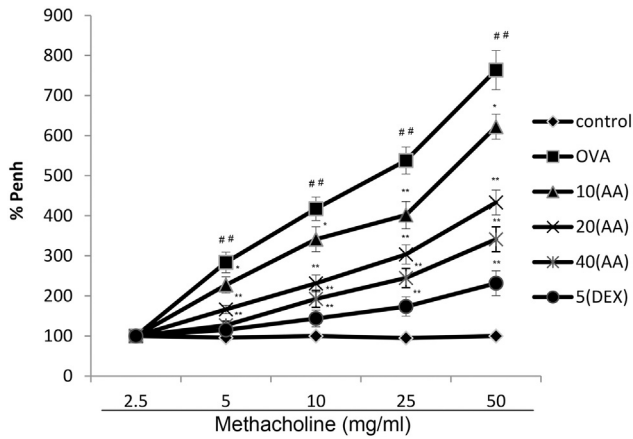


Fig. 4. AA reduced OVA-induced airway hyperresponsiveness. The effects of AA on AHR were measured using noninvasive whole-body plethysmography. The values presented are the means $\pm$ S.E.M. of three independent experiments. $^{##}p < 0.01$ vs. control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. OVA group.

synthesizes IgE [17]. IL-5 is essential for the formation and differentiation of eosinophils and could enhance IL-4-induced IgE production [20]. IL-13 has the ability to induce airway inflammation and AHR [21]. Recent studies suggested that inhibition of these Th2 cytokines might be a potential therapeutic strategy for the treatment of allergic asthma [22]. In this study, we found that treatment of AA significantly inhibited OVA-induced IL-4, IL-5, and IL-13 production in a dose-dependent manner. OVA-induced IL-4, IL-5, and IL-13 production were also significantly inhibited by treatment of DEX. NO is synthesized from L-arginine and plays an important role in the development of OVA-induced airway inflammation [23]. In this study, OVA-induced NO production was also inhibited by treatment of AA or DEX. In addition, the levels of IgE, inflammatory cells infiltration, and AHR were also inhibited by treatment of AA. The inhibitory effects were dose-dependently. These results suggested that AA had protective effects against OVA-induced asthma and the protective effects of AA at 40 mg/kg were similar to DEX.

It is well-known that NF- κ B plays an important role in the development of asthma [24]. NF- κ B activation was involved in asthma both in patients or animal model [25]. TCR-mediated Th2 differentiation

process and the TCR-induced Th2 cytokine express process are critical for the induction of Th2 response. Studies showed that some signaling pathways were played critical roles in the two processes, such as the Ras/ERK MAPK cascade, LAT, and PKC/NF- κ B pathway. NF- κ B has been reported to be an important transcription factor that regulates Th2 cell differentiation and cytokine such as IL-4, IL-5, and IL-13 expression [26]. Previous studies showed that inhibition of NF- κ B could attenuate OVA-induced asthma [27]. To investigate the underlying mechanism of AA treatment in the amelioration of airway allergic diseases, the effects of AA on NF- κ B activation and I κ B α degradation were measured. The results showed that treatment of AA dose-dependently inhibited OVA-induced NF- κ B activation and I κ B α degradation. Previous study showed that AA inhibited LPS-induced NO and TNF- α production in RAW264.7 cells by inhibiting NF- κ B activation [10] and the results were consistent with our study. These results indicated AA inhibited OVA-induced asthma by inhibiting NF- κ B signaling pathway.

In conclusion, AA exerted its protective effects on OVA-induced asthma by inhibiting OVA-induced lung injury, airway hyperresponsiveness, and the levels of OVA-specific IgE, IL-4, IL-5, and IL-13. These data suggested that AA might be a therapeutic drug in the prevention and treatment of asthma.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

This work was supported by a grant from Department of Education of Guangdong Province (No. 2015001), and Outstanding Young Teachers of Guangdong Province (No. B158030).

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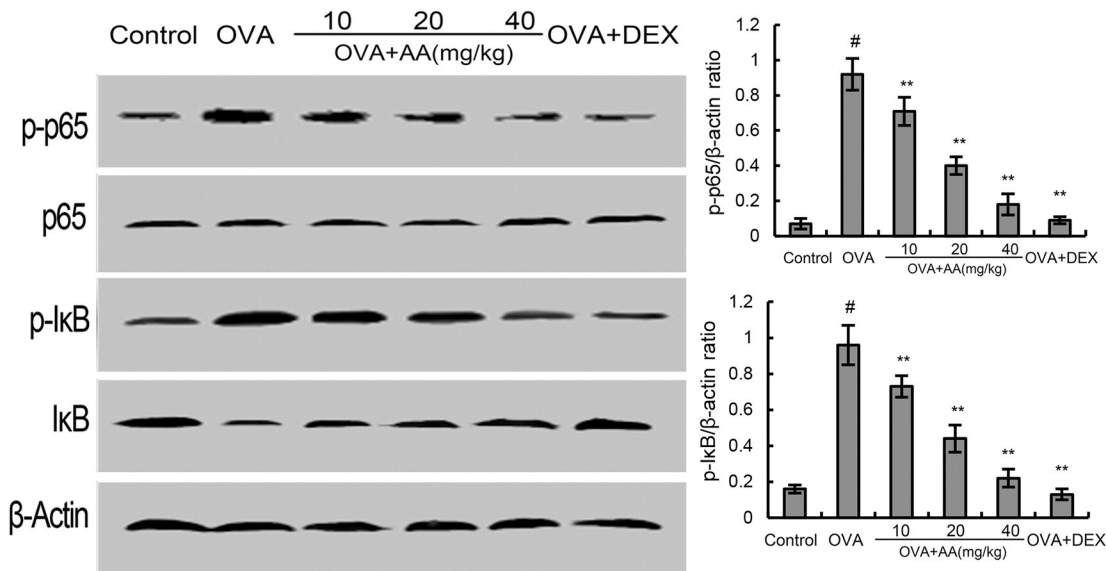


Fig. 5. Effects of AA on OVA-induced NF- κ B activation. The expression of NF- κ B and I κ B α were detected by western blotting. The values presented are the means $\pm$ S.E.M. of three independent experiments. $^{##}p < 0.01$ vs. control group; $^{*}p < 0.01$ vs. OVA group.

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