



Therapeutic effects of rosmarinic acid on airway responses in a murine model of asthma



Zhengmin Liang^a, Haiying Nie^a, Yangfeng Xu^a, Jianbo Peng^b, Yun Zeng^a, Yingyi Wei^a, Xuemei Wen^a, Jiaming Qiu^c, Weiting Zhong^c, Xuming Deng^c, Jiakang He^{a,*}

^a College of Animal Science and Technology, Guangxi University, Nanning, Guangxi 530005, PR China

^b Guangxi Engineering Research Center for Veterinary Medicine, Nanning, Guangxi 530007, PR China

^c College of Animal Science and Veterinary Medicine, Jilin University, Changchun, Jilin 130062, PR China

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ABSTRACT

Rosmarinic acid (RA) is an active component of a traditional Chinese herbal medicine. Previously, we reported that RA exerted a strong anti-inflammatory effect in a mouse acute lung injury model. Therefore, we hypothesized that RA might also have potential therapeutic effects in a murine model of asthma. In this study, we aimed to evaluate the anti-asthmatic activity of RA and explored its possible molecular mechanisms of action. Female BALB/c mice that had been sensitized to and challenged with ovalbumin (Ova) were treated with RA (20 mg/kg) 1 h after challenge. The results showed that RA greatly diminished the number of inflammatory cells and the production of Th2 cytokines in the bronchoalveolar lavage fluid (BALF); significantly reduced the secretion of total IgE, Ova-specific IgE, and eotaxin; and markedly ameliorated airway hyperresponsiveness (AHR) compared with Ova-induced mice. Histological studies further revealed that RA substantially decreased inflammatory cells infiltration and mucus hypersecretion compared with Ova-induced mice. Moreover, our results suggested that the protective effects of RA were mediated by the inhibition of JNK and p38 MAPK phosphorylation and nuclear factor- κ B (NF- κ B) activation. Furthermore, RA treatment resulted in a significant reduction in the mRNA expression of AMCase, CCL11, CCR3, Ym2 and E-selectin in lung tissue. These findings suggest that RA may effectively delay the development of airway inflammation and could thus be used as a therapy for allergic asthma.

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1. Introduction

Allergic asthma is increasing in prevalence in both developed and developing countries [1]. Asthma is a serious health issue and affects people of all ages [2,3], and although great progress has been made in the diagnosis and treatment of asthma, asthma remains a severe public health problem. Moreover, there has been a substantial increase in the global prevalence, morbidity and mortality of asthma over the past two decades due to the off-target and side effects of conventionally available anti-asthma drugs, particularly in children [4].

Asthma is a chronic airway disorder characterized by a variety of symptoms, including airway inflammation, mucus hypersecretion and airway hyperresponsiveness (AHR) [5]. Dozens of studies have shown that these symptoms are part of an inflammatory reaction that is caused by Th2 cells and other inflammatory cells, such as eosinophils, macrophages and neutrophils, as well as inflammatory cytokines and chemokines [6–8]. Interleukin (IL)-4, IL-5 and IL-13 have been shown to play an important role in allergic airway inflammation [9]. IL-4 is pivotal for the initiation of Th2 inflammatory responses [10]; IL-5 is known

to play a vital role in eosinophil maturation, differentiation, recruitment and survival [11]; and IL-13 plays a dominant role in the effector phase of Th2 responses, such as AHR, eosinophilic inflammation and mucus secretion [12]. IL-4 and IL-13 are also required for IgE class switching in B cells. In particular, IL-13 is thought to play the most important and central role in allergic asthma [13]. In addition, CCL11 is a chemokine that binds to the receptor CCR3 and is involved in eosinophil, neutrophil, and macrophage recruitment and Th2 cell infiltration [14].

Currently, inhaled glucocorticoids play a main role in combating asthma. Although inhaled glucocorticoids are usually effective, their use at high doses or for a prolonged time can lead to certain side effects, such as impaired growth, adrenal inhibition, and an increased risk of fracture [15]. Recent studies have supported the development of novel asthma treatments based on anti-inflammatory strategies [15,16]. For instance, a number of previous studies have shown that sustained activation of nuclear factor κ B (NF- κ B) and suppression of the MAPK signalling pathway in the lung may have potential therapeutic value in the treatment of allergic asthma [17–19]. NF- κ B plays a vital role in the production of Th2 cytokines and the recruitment of inflammatory cells in the airways in murine models of asthma [20,21]. In addition, the MAPK signalling cascade is known to be important for the activation of various immune cells [22]. p38 MAPK and ERK have been shown to

* Corresponding author.

E-mail address: ahong18@vip.sina.com (J. He).

participate in the regulation of IL-5 and other cytokines [23]. Meanwhile, JNK has been demonstrated to be associated with IgE class switching [24]. Therefore, NF- κ B and MAPK have emerged as promising molecular targets for asthma treatment. Likewise, more and more studies have shown that many components of Chinese medicinal herbs exert notable anti-inflammatory effects via the negative regulation of the NF- κ B and MAPK signalling pathways [25–27].

Rosmarinic acid (RA) is a polyphenolic phytochemical that is found in numerous herbal plants, including rosemary (*Rosmarinus officinalis*), oregano (*Origanum vulgare*) and mint (commonly *Mentha spicata*). RA shows a broad spectrum of biological effects, such as anti-microbial, anti-inflammatory and immunomodulatory actions [28]. Our previous study revealed the anti-inflammatory effects in a mouse model of acute lung injury induced by lipopolysaccharide (LPS) via the administration of RA at the dose of 20 mg/kg [29]. These results suggested that RA may be used for the treatment of asthma. Moreover, no previous study has evaluated the effectiveness of RA in ameliorating asthmatic symptoms. Here, we evaluated the effects of RA on an ovalbumin (Ova)-induced mouse asthma model and its potential mechanism of action, attempting to provide a new underlying mechanism for the treatment of allergic asthma using traditional Chinese herbal medicine.

2. Materials and methods

2.1. Animals

Female BALB/c mice weighing approximately 18–20 g were purchased from the Centre of Experimental Animals of Guangdong (Certificate: SCXK2013-0002; Foshan, China). The animals were housed in micro-isolator cages and received food and water ad libitum. The laboratory temperature was maintained at $24 \pm 1^\circ\text{C}$, with a relative humidity of 40–80%. The mice were acclimated for 7 days before the experiments. The experiments were approved by the Ethical Committee on Animal Research of Guangxi University. All animal experiments were conducted according to The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2. Reagents

IL-4, IL-5 and IL-13 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Neobioscience (Beijing, China). The total IgE and anti-Ova IgE ELISA kits were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). The Mouse Eotaxin Platinum ELISA kit was purchased from Affymetrix (eBioscience, Vienna, Austria), and Ova (Grade V) was purchased from Sigma-Aldrich (St. Louis, MO, USA). RA (purity >98%) and dexamethasone (Dex, purity: N 99.7%) were purchased from the National Institute for Food and Drug Control (Beijing, China). The MAPK Family Antibody Sampler Kit, Phospho-MAPK Family Antibody Sampler Kit and NF- κ B Pathway Sampler Kit

were obtained from Cell Signalling Technologies Inc. (Beverly, MA, USA). The purity of all chemical reagents was at least analytical grade.

2.3. Sensitization, challenge and treatment of mice

The mice were randomly divided into five groups ($n = 12$ in each group): (1) Cont group; (2) RA 20 group; (3) Ova group; (4) Ova + RA 20 group; and (5) Ova + Dex group. The mice were sensitized via an intraperitoneal injection with 0.2 mL of sensitization fluid containing Ova (20 μg) adsorbed to Imject Alum (100 $\mu\text{g}/\text{mL}$, Pierce, Rockford, USA) on days 0, 7 and 14. On days 22–27, the mice were anesthetized and intranasally challenged with Ova (100 μg) in PBS (50 μL) [27]. The mice in Cont group and RA 20 group were sham-sensitized and challenged with PBS using the same protocol. RA (20 mg/kg, based on the results of our previous study [29]) and Dex (2 mg/kg) were dissolved in normal saline and administered intraperitoneally 1 h after each corresponding Ova challenge on days 25–27. The control mice were administered equivalent volumes of normal saline. The schematic diagram of the treatment schedule is presented in Fig. 1.

2.4. Collection of blood and bronchoalveolar lavage fluid (BALF)

The mice were euthanized on day 28. Blood was withdrawn and centrifuged at 3000 rpm for 10 min at 4°C , and the serum was stored at -80°C prior to the IgE and eotaxin measurements. The BALF was processed to separate the cell pellets and supernatants. Briefly, the BALF was lavaged three times using a tracheal cannula and 0.5 mL of ice-cold PBS [27]. The fluid recovered from each sample was centrifuged (4°C , 3000 rpm, 10 min) to pellet the cells, and the supernatant was immediately stored at -80°C prior to the cytokine, Ova-IgE and eotaxin assessments. The cell pellets were resuspended in PBS and stained with Wright-Giemsa, and the numbers of different inflammatory cells were counted. At least 200 cells were counted per slide.

2.5. Measurements of Th2 cytokines, eotaxin and IgE production

The production of IL-4, IL-5, IL-13 and eotaxin was measured with sandwich ELISA kits according to the manufacturer's instructions. In addition, the total IgE and Ova-specific IgE concentrations were determined with an ELISA according to the manufacturer's instructions.

2.6. Lung histology

Twenty-four hours after the last inhalation exposure, the lungs were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 3- μm sections, and stained with haematoxylin and eosin (H&E) to observe the general morphology or alcian blue-periodic acid-Schiff (AB-PAS) to observe mucus production and identify the goblet cells in lung tissues. Semi-quantitative analyses of inflammatory cell accumulation and

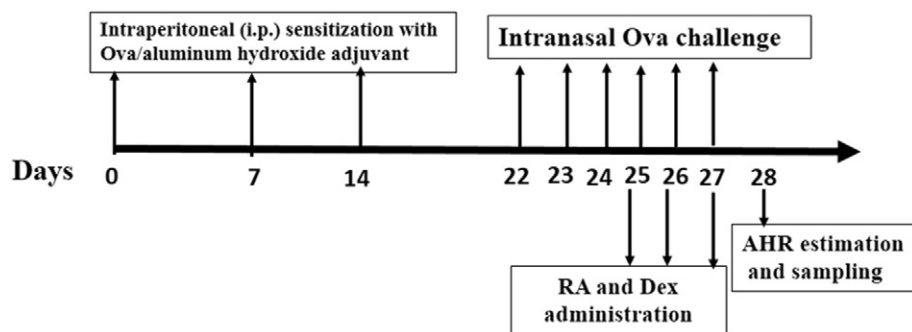


Fig. 1. Experimental protocol for the development of allergic asthma and treatment with RA or Dex. The mice were divided into five groups ($n = 12$ in each group) and sensitized to Ova on days 0, 7 and 14. Subsequently, the mice were given an intranasal instillation of 100 μg Ova in 50 μL of PBS on days 22–27. The mice were intraperitoneally injected with RA (20 mg/kg) or Dex (2 mg/kg) 1 h after each corresponding Ova or PBS challenge from days 25 to 27.

Table 1

Primer sets for the reverse transcriptase-polymerase chain reaction analysis.

Targets	Forward	Reverse
Ym2	TCCACTTTGAACACATTCCAAGGC	CGAGAGACTGAGACAGTTCAGGGA
AMCase	TGGACACACCTTCATCCTGA	CCTCAGTGGCTCCACTTCTC
CCL11	AAACCATAAACACCTCCTC	CAATAATCCCACATCTCCTT
CCR3	TCTGCTGAGATGTCCAATA	TCACCAACAAAGGCGTAG
E-selectin	CCCTTCCACAGAACCTACCA	TCAGCAGACATTGCTTCACC
β -actin	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTC

mucus secretion in the lung sections were performed as previously described [30,31]. Briefly, to estimate the severity of inflammatory cells recruitment and mucus secretion, the numbers of inflammatory cells and goblet cells were evaluated using a 5-point scoring system.

2.7. Measurements of AHR

Twenty-four hours after the final Ova challenge, the mice were anesthetized and subjected to a tracheotomy, as previously described [32]. The internal jugular vein was cannulated and connected to a microsyringe for intravenous methacholine (Mch) administration. The lung compliance (C_{dy}) and airway resistance (RI) in response to increasing concentrations of Mch were recorded using a whole-body plethysmograph chamber (Buxco, Sharon, CT, USA), as previously described [32]. The results were expressed as the relative change in AHR over the basal values in response to saline.

2.8. Western blot analysis of MAPK and NF- κ B

The lungs were removed at 24 h after the last Ova challenge. The lung tissues were homogenized in liquid nitrogen and incubated in lysis buffer containing protease and phosphatase inhibitors (Roche, Basel, Switzerland) to obtain lung proteins. The protein concentrations were measured using a BCA protein assay kit (Beyotime, Nanjing, China), according to the manufacturer's protocol. Equivalent amounts of 60–100 μ g protein were denatured at 100 °C for 5 min in Tris–Glycine SDS sample loading buffer (Beyotime, Nanjing, China), loaded into each lane of a gel, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% fat-free dry milk dissolved in TBST for 2 h at room temperature. The blots were incubated with specific antibodies against ERK, JNK and p38 (CST, Beverly, MA, USA) or phospho-specific antibodies against P-ERK, P-JNK, P-p38, P-NF- κ B p65 and P-I κ B α (CST, Beverly, MA, USA) in 5% fat-free dry milk at 4 °C overnight. Then, the membrane was washed three times for 10 min each with TBST. The blots were incubated with

secondary antibodies probed with an HRP-conjugated goat anti-rabbit (1:1000) or anti-mouse (1:1000) at 37 °C for 1 h. Subsequently, the membrane was washed three times in TBST and processed with ECL plus (Millipore, Billerica, MA, USA). A β -actin (CST, Beverly, MA, USA) western blot was performed as an internal control for protein loading.

2.9. RNA preparation and quantitative RT-PCR

The lung tissues were harvested 24 h after the final Ova challenge, and the total RNA was isolated from lungs using RNAiso Plus (Takara Bio Inc., Otsu, Japan). Reverse transcription was performed using the first-strand cDNA synthesis kit (Takara Bio Inc., Otsu, Japan). Real-time PCR was performed on the cDNA samples using the SYBR Green system (Bio-Rad; Richmond, CA, USA). The primers used are shown in Table 1. The cycling conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 58–60 °C for 30–60 s, and extension at 72 °C for 60 s. Analysis was implemented using sequence detection software. A melting curve analysis was performed to control for the specificity of the amplification products. The gene-specific threshold cycles (C_t) of the respective samples were internally normalized using the average C_t value of β -actin. The Δ C_t values for all experimental samples were subtracted from the Δ C_t values of the control samples ($\Delta\Delta$ C_t). The fold change in the levels of test gene mRNAs was expressed as $2^{-\Delta\Delta C_t}$ [33].

2.10. Statistical analysis

All values were expressed as the mean $\pm$ standard error of the mean (SEM). Differences between the mean values of the normally distributed data were assessed using one-way analysis of variance (ANOVA; Dunnett's *t*-test) and Student's *t*-test. Significant differences were considered significant if *p* < 0.05.

3. Results

3.1. RA decreases the number of inflammatory cells and the production of Th2 cytokines

The BALF was obtained 24 h after the last Ova challenge, and the number of inflammatory cells in the BALF was significantly increased in the Ova-induced mice compared with the PBS control mice. However, the RA and Dex treatments reduced the number of total cells, neutrophils and eosinophils (Fig. 2A). Meanwhile, the Ova-challenged mice, compared with the PBS control mice, showed increased levels of IL-4, IL-5 and IL-13 in the BALF. However, RA strikingly reduced the levels of IL-4, IL-5 and IL-13 compared with those in the Ova-treated mice

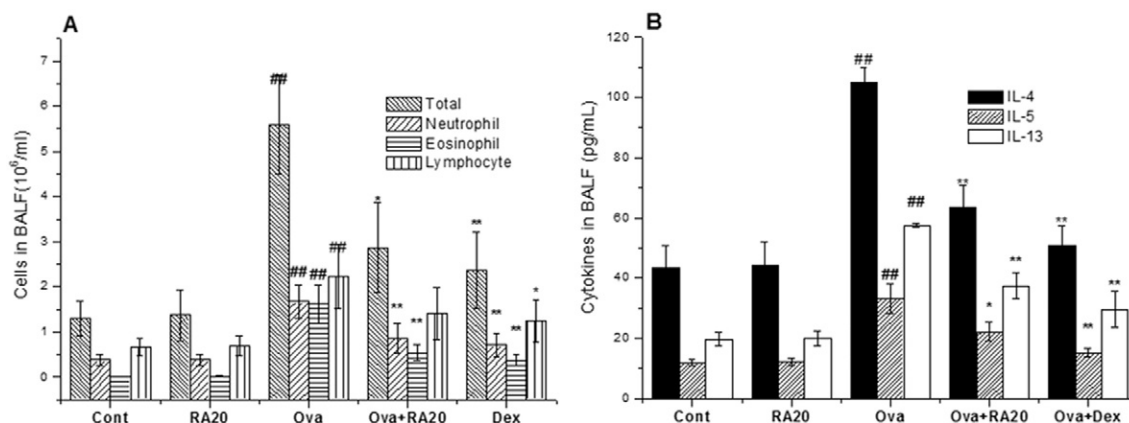


Fig. 2. Effects of RA on the recruitment of inflammatory cells and the production of Th2 cytokines in the BALF. The BALF was collected 24 h after the last Ova challenge. The numbers of neutrophils, eosinophils and lymphocytes were counted (A). The Th2 cytokine levels (B) were analysed via ELISA. The values represent the mean $\pm$ SEM of three independent experiments, #*p* < 0.05, ##*p* < 0.01 versus the control group, **p* < 0.05, ***p* < 0.01 versus the Ova group.

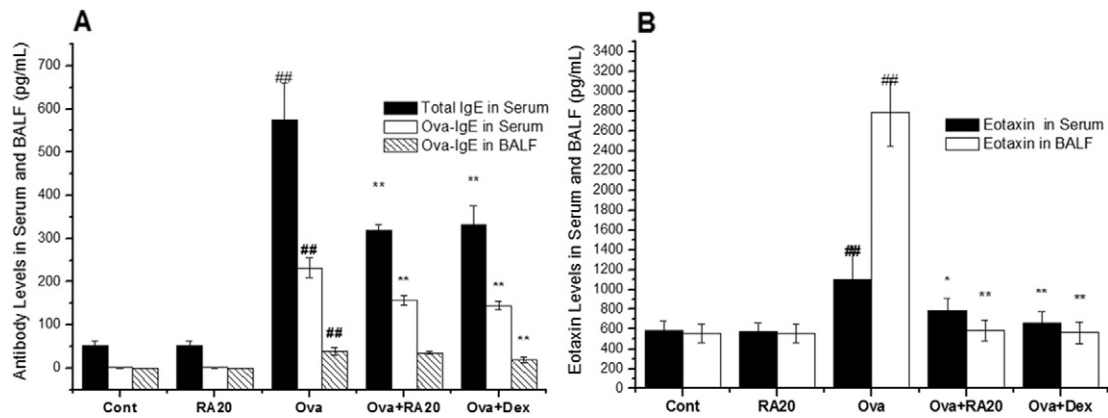


Fig. 3. Effects of RA on the secretion of Ova-specific IgE (A), eotaxin (B) and total IgE (C) in the serum and BALF. The BALF and serum were analysed via ELISA. The values represent the mean $\pm$ SEM of three independent experiments, ^{*} $p < 0.05$, ^{##} $p < 0.01$ versus the control group, ^{*} $p < 0.05$, ^{**} $p < 0.01$ versus the Ova group.

(Fig. 2B). However, control group treated with RA did not show any significant effect on airway inflammation (Fig. 2A–B).

3.2. RA reduces the concentrations of total IgE, Ova-specific IgE and eotaxin

To evaluate the effects of RA on total IgE, Ova-specific IgE and eotaxin production in Ova-induced mice, ELISAs were performed on the serum and BALF samples. As shown in Fig. 3, the total IgE, Ova-specific IgE and eotaxin concentrations were significantly elevated in the Ova-induced mice compared with the PBS control mice. However, substantial reductions in the total IgE and Ova-specific IgE concentrations were observed in the RA- and Dex-treated mice. However, there were no dramatic differences in the Ova-specific IgE concentrations in the BALF. In addition, RA and Dex markedly reduced the secretion of eotaxin in both the serum and the BALF compared with that in the Ova-induced mice. RA had no effect on IgE and eotaxin levels in control mice (Fig. 3).

3.3. RA reduces protein secretion in the BALF

To estimate the effects of RA on the secretion of total protein-containing airway mucus in the BALF, total protein secretion was measured using a BCA protein assay kit (Beyotime, Nanjing, China). A dramatic increase in total protein secretion was observed in the Ova-induced mice compared with the PBS control mice. However, RA treatment clearly reduced this increased total protein secretion in the BALF (Fig. 4).

3.4. RA ameliorates the histopathological alterations in the lung

In contrast to the histological features of the lungs observed in PBS-induced mice, a notable increase in the number of inflammatory cells surrounding the bronchi and alveoli was observed in the Ova-treated mice using H&E staining. However, RA treatment significantly inhibited the accumulation of inflammatory cells. Furthermore, the results of the AB-PAS staining clearly showed that the Ova challenge induced the over-production of mucus and goblet cells hyperplasia. However, treatment with RA or Dex markedly alleviated these histological alterations, including epithelial thickening, mucus hypersecretion, and increased the number of goblet cells in the epithelium (Fig. 5). The strong inflammatory cell infiltration around the bronchi evaluated by H&E staining is shown in Fig. 6A, and the degree of mucin secretion estimated based on AB-PAS staining is shown in Fig. 6B. The histological images in lungs of control mice treated with RA did not reveal significant effect compared with control mice (Figs. 5 and 6).

3.5. RA alleviates AHR in Ova-induced mouse asthma

Asthma is characterized by AHR, which can be estimated by determining the Cdyn and RI values in response to increasing doses of Mch in mechanically ventilated mice. Cdyn refers to the compliance of the lung, and RI refers to the pressure driving respiration divided by the flow. Strikingly, an increase in AHR was observed in the Ova-treated mice, which was typically reflected as high RI and low Cdyn values. However, RA (20 mg/kg) and Dex (2 mg/kg) treatments remarkably up-regulated the Cdyn values (Fig. 7A) and down-regulated the RI values (Fig. 7B) in the Ova-induced mice in response to Mch.

3.6. RA regulates the MAPK and NF- κ B signalling pathways

The MAPK signalling pathway controls the synthesis and release of pro-inflammatory mediators from activated macrophages during the inflammatory process [34]. Our data showed that RA clearly decreased the ratio of P-JNK/JNK and P-p38/p38 compared with Ova-induced mice. However, there was no notable change in the ratio of P-ERK/ERK between the Ova-induced mice and the RA-treated mice (Fig. 8).

NF- κ B is a transcription factor that plays a crucial role in asthma [35]. Thus, we investigated whether RA attenuated the NF- κ B signalling pathway, which is closely associated with the transcriptional modulation of inflammatory mediators in Ova-induced asthma. Our data showed that

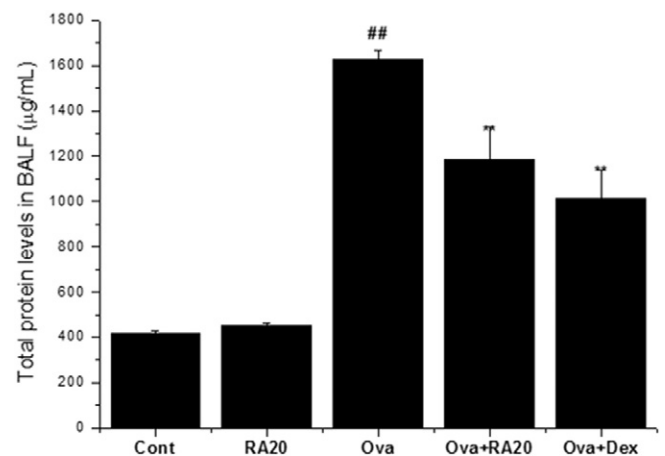


Fig. 4. Effects of RA on total protein secretion in the BALF in Ova-challenged mice. The BALF was collected 24 h after the final Ova challenge. Each sample was analysed using the BCA protein assay kit. The values represent the mean $\pm$ SEM of three independent experiments. ^{*} $p < 0.05$, ^{##} $p < 0.01$ versus the control group, ^{*} $p < 0.05$, ^{**} $p < 0.01$ versus the Ova group.

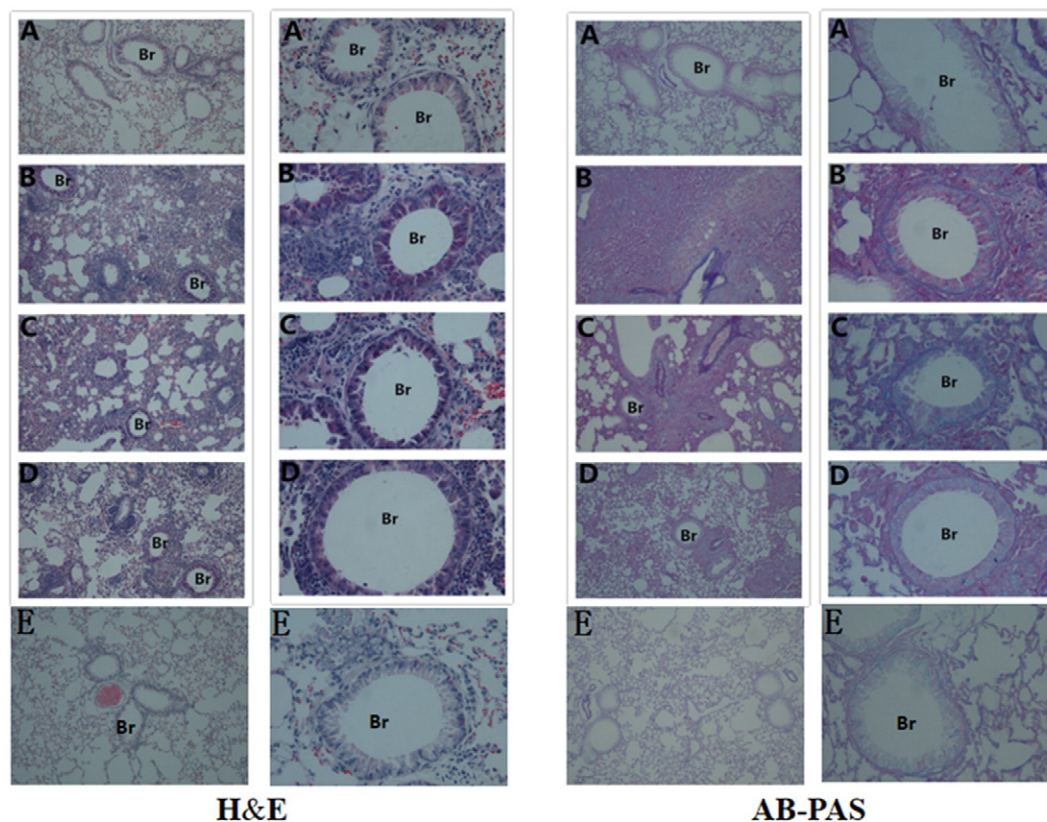


Fig. 5. Effects of RA on airway inflammation and mucus production. Representative H&E- and AB-PAS-stained sections of the lungs from (A) PBS-challenged mice; (B) Ova-challenged mice; (C) Ova-challenged mice treated with RA; and (D) Ova-challenged mice treated with Dex; (E) PBS-challenged mice treated with RA. The left panel shows images at 100 × magnification; the right panel shows images at 400 × magnification.

Ova challenge induced I κ B α and NF- κ B p65 phosphorylation. However, RA substantially attenuated the phosphorylation of I κ B α and NF- κ B p65 (Fig. 9).

3.7. RA inhibits the expression of inflammatory genes

The whole-lung mRNA expression levels were measured using a quantitative RNA analysis. The expression levels of the chemokine CCL11 and its receptor CCR3 were significantly elevated in the Ova-treated mice compared with the control PBS-induced mice. However, RA treatment greatly reduced the mRNA expression of CCL11 and CCR3. Based on the above results showing that RA suppressed the recruitment of inflammatory cells and reduced Th2 cytokine production and mucus hypersecretion, we further evaluated the mRNA expression of E-selectin, AMCase and Ym2. The results shown that RA treatment

also strongly inhibited the expression of E-selectin, AMCase and Ym2 mRNAs in lung tissue (Fig. 10).

4. Discussion

Our previous research found that RA exerted significant anti-inflammatory effects in models of LPS-induced lung injury [29]. Therefore, we aimed to investigate the effects of RA treatment in a mouse model of asthma. In this study, we demonstrated that RA ameliorated asthma features by reducing the number of inflammatory cells, interfering with Th2 cytokine production, blocking the phosphorylation of MAPK and NF- κ B and decreasing the mRNA expression of inflammatory genes.

In recent years, a growing number of studies have demonstrated the utility of drugs targeting Th2 cytokines or Th2-specific transcription factors for asthma treatment [36]. Moreover, anti-IL-5 antibody therapy

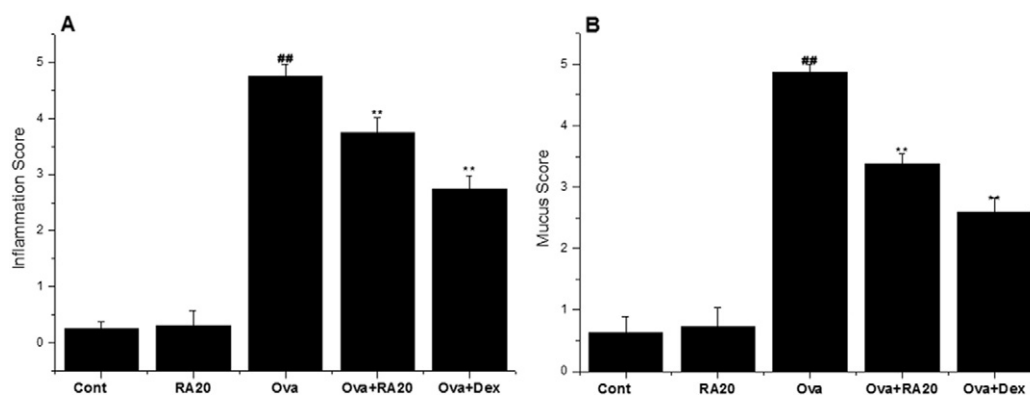


Fig. 6. Semi-quantitative analyses of inflammatory cell infiltration (A) and mucus production (B) in the lung sections were performed as previously described [30,31],

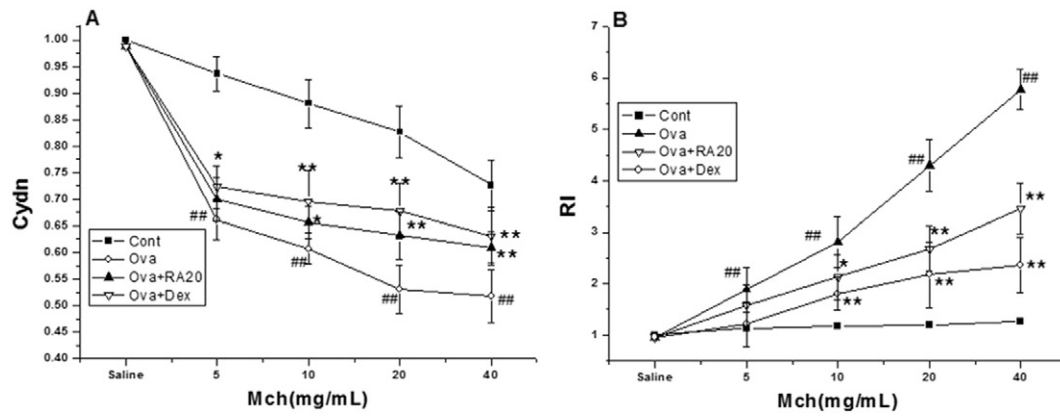


Fig. 7. Effects of RA (20 mg/kg) and Dex (2 mg/kg) on Ova-induced AHR in mice. AHR was assessed by determining the percent change from the baseline (A) dynamic compliance (Cdyn) and (B) lung resistance (RI) values, #*p* < 0.05, ##*p* < 0.01 versus the control group, **p* < 0.05, ***p* < 0.01 versus the Ova group.

has shown clinical benefit in severe asthma [37]. In addition, anti-IL-13-targeting therapies have shown an improvement in lung function [38]. Accordingly, the effects of RA on airway inflammation are mainly mediated through the inhibition of cytokine secretion. In our study, the concentrations of IL-4, IL-5 and IL-13 were strikingly increased in Ova-treated mice, while the production of these Th2 cytokines was significantly reduced after RA treatment (Fig. 2B). Importantly, these results were consistent with the reduced number of inflammatory cells in the BALF of RA-treated animals (Fig. 2A).

Histopathological changes, such as the accumulation of inflammatory cells, mucus hypersecretion and goblet cell hyperplasia in the lung, are the major characteristics of asthma [39]. Accordingly, our results using H&E staining revealed inflammatory cell infiltration in the peribronchial and perivascular areas of Ova-challenged mice (Figs. 5 and 6A). However, RA treatment markedly reduced the number of inflammatory cells. Moreover, the results of AB-PAS staining showed goblet cell hyperplasia and oversecretion of mucus in the bronchial airways of Ova-induced mice (Figs. 5 and 6B). It is worth mentioning that these data are in accordance with the protein secretion measurements in BALF (Fig. 4). In addition, because histopathological alterations are loosely associated with Th2 cytokine levels [40], the pathological

changes in the lung tissue that were ameliorated by RA may be the result of reduced Th2 cytokine levels. Moreover, an increase in the Ova-specific IgE level is regarded as a hallmark of the Th2-driven immune response to Ova exposure [18]. Our results suggested that the level of Ova-specific IgE was substantially inhibited by RA in Ova-induced mice (Fig. 3A). These data are consistent with our measurements of the Th2 cytokine levels.

AHR has been used in epidemiological studies as a useful marker of airway abnormalities in asthma, and it is a predictor of the subsequent course of the disease [41,42]. IL-4, IL-5 and IL-13 play critical roles in the development of AHR [43–45]. In our study, we demonstrated that RA significantly ameliorated AHR (Fig. 7). These data are in accordance with our hypothesis that RA reduces Th2 cytokine production. One possible explanation for this finding may be that RA ameliorates AHR by suppressing Th2 cytokine production.

To confirm the mechanism by which the Th2 cytokine levels and eosinophil aggregation in the lungs are reduced, we evaluated the inhibitory effect of RA on the activation of NF- κ B and MAPK in the mouse asthma model. NF- κ B induces the expression of multiple pro-inflammatory genes that contribute to allergic asthma [20,46]. Selective ablation of NF- κ B function in the airway epithelium was shown to diminish

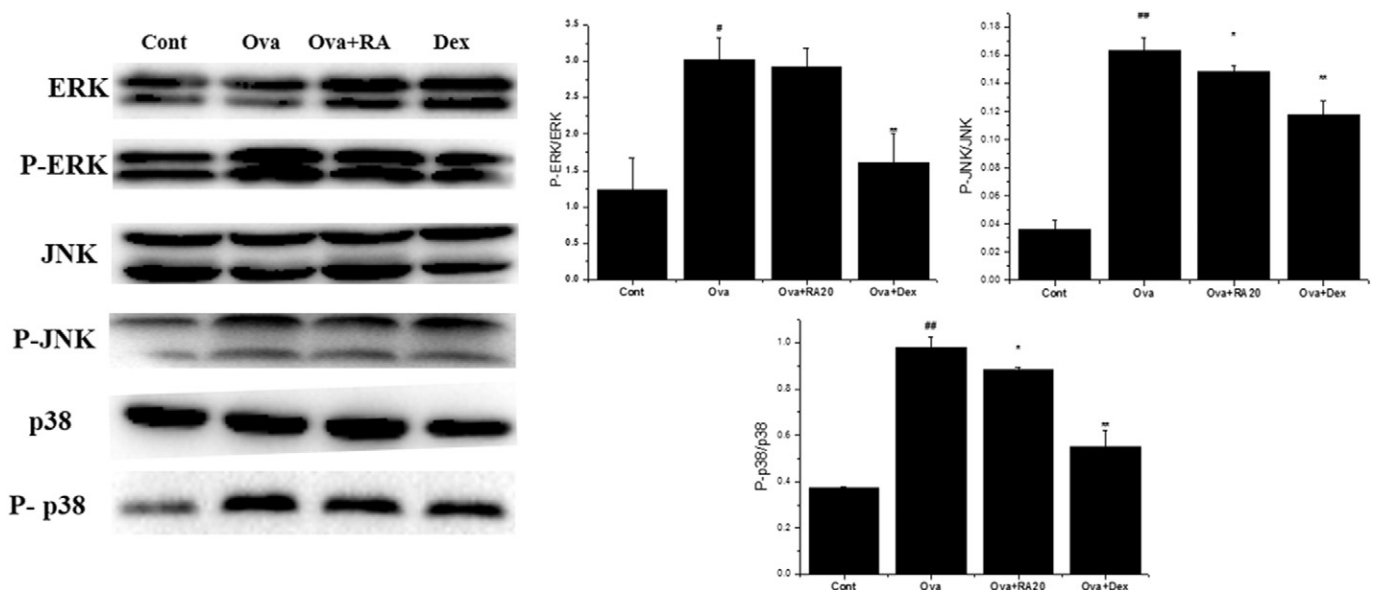


Fig. 8. Effects of RA on Ova-induced phosphorylation of MAPK molecules in the lungs. The cellular proteins from the lungs were used to detect the phosphorylation of three MAPK molecules, ERK, p38 MAPK and JNK, by western blotting. The values represent the mean $\pm$ SEM of three independent experiments. #*p* < 0.05, ##*p* < 0.01 versus the control group, **p* < 0.05, ***p* < 0.01 versus the Ova group.

A Cytosol

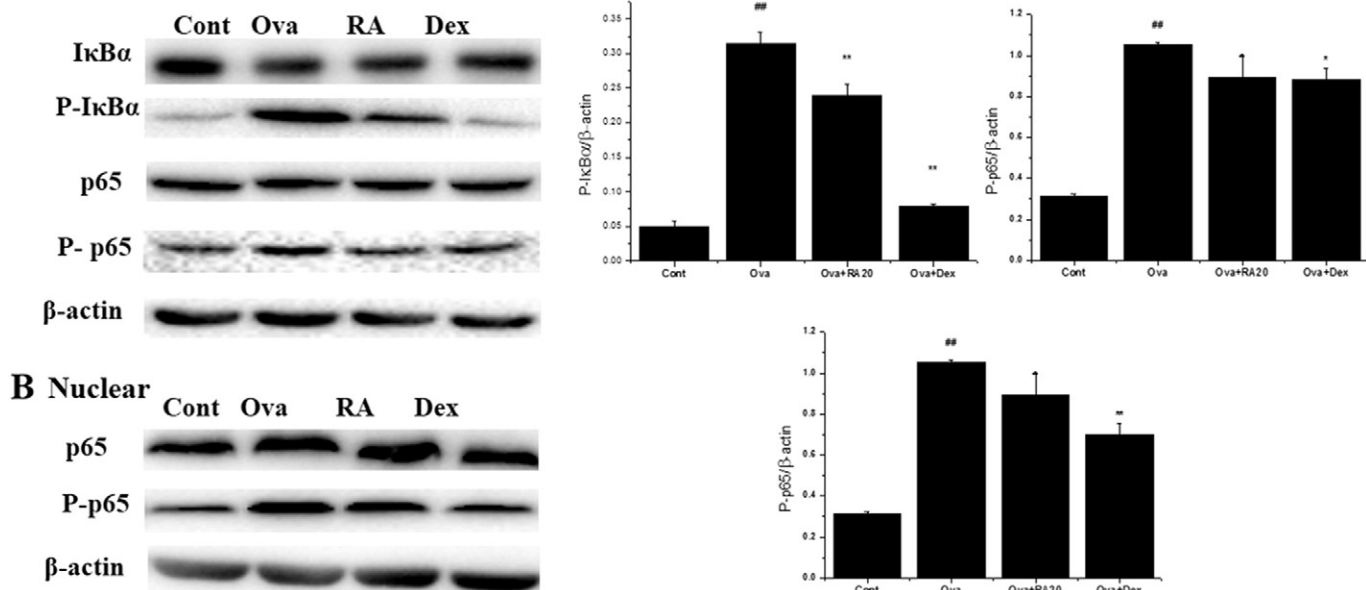


Fig. 9. Effects of RA on the Ova-induced phosphorylation of IκBα and NF-κB p65 in the lungs. The phosphorylation IκBα and NF-κB p65 was measured by western blotting. The values represent the mean ± SEM of three independent experiments. [#]*p* < 0.05, ^{##}*p* < 0.01 versus the control group, ^{*}*p* < 0.05, ^{**}*p* < 0.01 versus the OVA group.

mucus production in Ova-induced mice [47]. Our data illustrated that IκBα phosphorylation and p-NF-κB p65 translocation from the cytoplasm to the nucleus were remarkably inhibited by RA in Ova-challenged mice (Fig. 9). These data indicate that the anti-inflammatory effect of RA is at least partially modulated by suppression of the NF-κB pathway.

MAPK signalling predominantly regulates the synthesis and production of pro-inflammatory mediators during inflammation [34]. Our data indicated that MAPK activity was increased in response to Ova challenge. However, RA treatment inhibited the release of inflammatory cytokines, which likely provides the mechanism for its anti-asthmatic activity. Accordingly, treatment with a p38 inhibitor was shown to significantly inhibit eosinophilic inflammation in the lungs of Ova-induced mice and guinea pigs [48]. Therefore, MAPK signalling is a key mediator of allergic diseases. Our results showed that RA could inhibit p38 and JNK phosphorylation (Fig. 8), which suggests that RA may exert a beneficial effect on asthma by suppressing p38 and JNK phosphorylation.

Accumulating evidence indicates that chemokines play a critical role in the sequential, local migration of inflammatory cells. AMCase and Ym2 expression was recently shown to be significantly increased during

allergic airway inflammation in humans and mouse models of asthma [49–51]. In addition, the CC chemokines in the lung are known to orchestrate allergic inflammation in murine models of asthma [52]. Our results showed that RA markedly down-regulated the mRNA expression of AMCase, Ym2, CCL11 and CCR3 in the lungs of Ova-induced mice (Fig. 10). These results may be a consequence of the substantial decrease in the production of IL-4, IL-5 and IL-13 observed in the airways following RA treatment. E-selectin is important for leukocyte transmigration into the airways [53]. In our study, we demonstrated that RA suppressed the expression of E-selectin mRNA in the lungs of Ova-challenged mice (Fig. 10). These data are consistent with our evaluations of the inflammatory cells and Th2 cytokines in the BALF. Together, these findings suggest that the therapeutic effect of RA is associated with dampened inflammatory responses and the prevention of AHR.

5. Conclusions

Our study demonstrated that RA effectively inhibited inflammatory cell accumulation, Th2 cytokine production, Ova-specific IgE levels in the BALF and alleviated AHR development in Ova-induced mice. Our

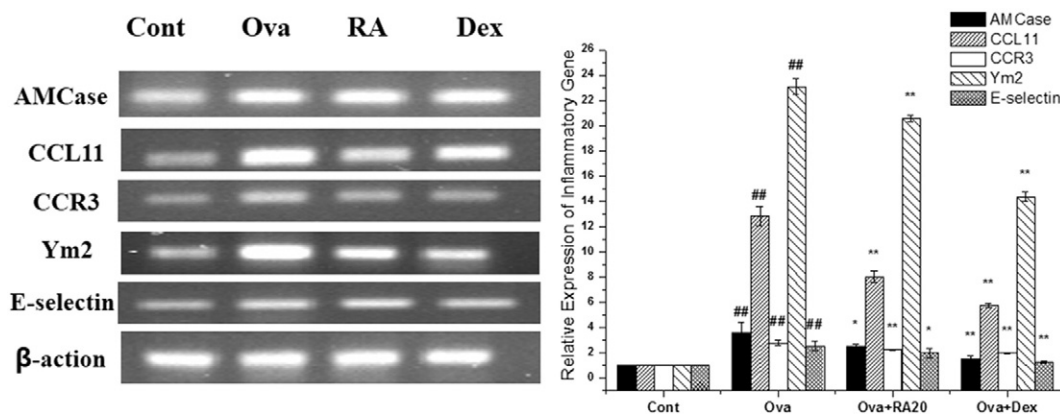


Fig. 10. Effects of RA on Ova-induced expression of inflammatory genes in Ova-challenged mice. The lung tissues were collected 24 h after the last Ova challenge. The mRNA levels in the whole-lung extracts were measured by real-time PCR. The values represent the mean ± SEM of three independent experiments. [#]*p* < 0.05, ^{##}*p* < 0.01 versus the control group, ^{*}*p* < 0.05, ^{**}*p* < 0.01 versus the Ova group.

histological studies showed that RA substantially inhibited Ova-induced eosinophilia in the lung tissue and mucus hypersecretion by goblet cells in the airways. Moreover, RA substantially diminished the mRNA expression of AMCase, Ym2, CCL11, CCR3 and E-selectin in the lung tissue. Taken together, our findings suggest that RA may effectively inhibit the MAPK and NF- κ B signalling pathways. These preclinical findings suggest that RA may serve as a promising therapeutic agent for the treatment of patients with allergic airway inflammation. However, additional, more comprehensive studies are required before the clinical application of RA can proceed.

Disclosure

All authors declare that they have no conflicts of interest.

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