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Original Article

Hydroxysafflor yellow A attenuates allergic airway inflammation by suppressing the activity of nuclear factor-kappa B in ovalbumin-induced asthmatic mice

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Abstract: Objective: This study is to investigate the effects of hydroxysafflor yellow A (HSYA) on the airway inflammation in ovalbumin (OVA)-induced asthmatic mice. Methods: After drug administration, the airway hyper-responsiveness (AHR), and cell differentials and cytokine levels in bronchoalveolar lavage (BAL) fluid, were assessed. Lung tissue was detected with histological examination. Protein expression levels were determined with Western blot analysis. NF-κB/DNA binding activity was assessed with the electrophoretic mobility shift assay (EMSA). Results: The AHR, cell chemotaxis, and inflammatory responses were substantially enhanced in OVA-induced asthmatic mice, which would be prevented by HSYA or BAY 11-7085. Moreover, levels of total and OVA-specific IgE, and inflammation-related cytokines, in the BAL fluid were significantly increased in OVA-induced asthmatic mice, which would be decreased by HSYA or BAY 11-7085. On the other hand, the nuclear NF-κB p65 level was significantly increased, while the cytosolic NF-κB p65 level was significantly decreased, by the OVA challenge. Moreover, EMSA showed increased NF-κB/DNA binding activity in OVA-induced asthmatic mice. Furthermore, immunostaining confirmed the nuclear translocation of the p65 subunit. However, the treatment of HSYA or BAY 11-7085 significantly inhibited the nuclear translocation of NF-κB p65 in OVA-induced asthmatic mice. In addition, the treatment of HSYA or BAY 11-7085 significantly reduced the phosphorylation and degradation of IκB-α in lung of OVA-induced asthmatic mice. Conclusion: HSYA protects against the airway inflammation in OVA-induced asthmatic mice, through down-regulating inflammation-related cytokines and inhibiting NF-κB activity. Our findings support the utility of HSYA in the treatment of asthma in clinic.

Keywords: Asthma, hydroxysafflor yellow A (HSYA), airway hyper-responsiveness (AHR), airway inflammation, nuclear factor-kappa B (NF-κB)

Introduction

Bronchial asthma is a kind of chronic airway disease characterized by eosinophilic inflammation, reversible airway obstruction, increased mucus production, and airway hyper-responsiveness (AHR) [1, 2]. These symptoms are mainly caused by various inflammatory factors, including T-helper2 (Th2) cells, B cells, mast cells, eosinophils, and cytokines and chemokines. In particular, interleukin (IL)-4, IL-5, and IL-13 produced by Th2 cells could activate eosinophils, which are related to the AHR and airway inflammation [3]. Moreover, Th2 cell differentiation has also been associated with the pathogenesis and aggravation of asthma. Therefore, it is of great importance to investigate the Th2 cell activation for the development of disease treatment.

Ubiquitous eukaryotic transcription factor nuclear factor-kappa B (NF-κB) plays key roles in various inflammatory and immune responses, including asthma. Mammalian NF-κB family is comprised of p65 (Rel A), Rel B, c-Rel, p50/p105, and p52/p100, which exist in cytoplasm,

as homo- or heterodimers bound to the IκB inhibitory complex. In response to pro-inflammatory stimuli, IκB-α would be phosphorylated and degraded, leading to the nuclear translocation of NF-κB, which promotes the transcription of target cytokines and growth factors [4]. Allergen challenge could enhance the NF-κB activity in lung and increase the numbers of airway epithelial cells and macrophages in patients with asthma [5]. It has been shown that, the antisense NF-κB p65 pretreatment significantly inhibits the established asthmatic reaction in a murine model [6, 7]. Thus, inhibition of NF-κB in lung might be a new strategy of the management of asthma.

Safflower (*Carthamus tinctorius L.*) is a traditional Chinese medicine extensively used to promote the blood circulation in patients suffering from trauma, and cardiovascular and cerebrovascular diseases. Among over 50 compounds in safflower, hydroxysafflor yellow A (HSYA) has been recognized as the active ingredient [8]. HSYA is able to reduce platelet aggregation, suppress oxidative stress, and inhibit inflammation in animal models [9, 10]. Moreover, HSYA has been shown to alleviate the lipopolysaccharide-induced inflammatory response and attenuate the development of bleomycin-caused fibrosis in the lung tissue in murine models [11-13]. However, the effects of HSYA on the airway inflammation in asthma have rarely been evaluated.

In this study, the effects of HSYA on the inflammatory responses in asthmatic mice were investigated. Mouse model of asthma was established by ovalbumin (OVA) induction. After drug administration, the airway inflammation and AHR in these mouse models were assessed, analyzed, and compared.

Materials and methods

Study animals

Totally 35 female BALB/c mice (specific pathogen-free), 7-w old, were purchased from the House Section of the Yanbian University Health Science Center (Yanji, Jilin, China). These mice were maintained in a facility under standard condition for 1 w before experiments, and provided with water and standard chow *ad libitum*. The animal experimental procedures were performed in compliance with the guideline approved by the Institutional Animal Care and Use Committee of Yanbian University School of Medical Sciences.

Animal modeling and grouping

Mice were intraperitoneally immunized with 10 μg OVA (Sigma, St. Louis, MO, USA) and 1.0 mg aluminum hydroxide adjuvant (Imject® Alum; Pierce, Rockford, IL, USA). After 10 d, a booster injection of 10 μg OVA and 1.0 mg aluminum hydroxide was administered. These immunized mice were challenged with 1% OVA aerosol in PBS for 20 min on days 17-19.

These mouse models were randomly divided into the following groups: (1) the control group (n = 7), in which the models were treated with aerosolized saline; (2) the HSYA-treated groups (n = 7 per group), in which the mice were subjected to intraperitoneal administration of HSYA (dissolved in saline; Santa Cruz, Santa Cruz, CA, USA) at 30 and 60 mg/kg body weight, respectively, on days 21-23, beginning at 1 h before provocation; and (3) the BAY-treated group (n = 7), in which the mice were intraperitoneally treated with the NF-κB inhibitor BAY 11-7085 (20 mg/kg body weight; dissolved in DMSO-PBS; Sigma), on days 21 and 23.

AHR assessment

Airway responsiveness was measured at 2 d after the last OVA challenge. Mice were placed in a barometric plethysmographic chamber (Buxco Electronics, Wilmington, NC, USA), and baseline reading was taken for 3 min. Aerosolized methacholine (Mch) was nebulized through the main chamber inlet, with increasing concentrations (2.5-50 mg/mL), over 3 min. Bronchopulmonary resistance was expressed as the increase in enhanced pauses (Penh) compared to baseline, where the baseline Penh (for the saline challenge) was considered as 100%.

Differential cell counting in bronchoalveolar lavage (BAL) fluid

Mice were anesthetized immediately following the airway responsiveness assessment, and the trachea was cannulated while gently massaging the thorax. The lung tissue was lavaged with 0.7 Ml PBS, and the BAL fluid sample was collected. Total cell number in a 0.05-mL aliquot was counted using a hemocytometer

(Baxter Diagnostics, Deerfield, IL, USA). After centrifugation, the cell pellets were re-suspended in PBS, and the Cytospin preparations of BAL cells were stained with Diff-Quik solution (International Reagents, Kobe, Japan). Cell differentials were enumerated based on the cellular morphology and the staining profile.

Enzyme-linked immunosorbent assay (ELISA)

The BAL fluid sample was collected and centrifuged, and the levels of total and OVA-specific IgE, tumor necrosis factor (TNF)-α, IL-1β, IL-4, IL-5, IL-13, eotaxin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) in the supernatant were determined with the ELISA kits (R & D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The lower limits of detection for the cytokines were as follows (pg/mL): TNF-α, 5.1; IL-1β, 2.0; IL-4, 3.3; IL-5, 5.0; IL-13, 1.5; eotaxin, 3.0; ICAM-1, 0.017; and VCAM-1, 20.

Histological examination

The lung tissue was extracted and fixed in 10% formalin. After embedded in paraffin, the tissue was cut into 4-μm sections, which were subjected to the hematoxylin-eosin (H & E) and periodic acid-Schiff (PAS) staining. The peribronchial and perivascular inflammation degree was evaluated on a subjective scale of 0 to 3, as previously described [14]. The airway mucus expression level was quantified by counting the numbers of PAS-positive and -negative epithelial cells in bronchioles [15].

Western blot analysis

Cytosolic and nuclear protein was extracted from the lung tissue as previously described [16]. After protein concentration determination, 30 μg protein was subjected to SDS-PAGE, and then electronically transferred onto polyvinylidene difluoride membrane. After blocking with 5% non-fat dry milk in PBS for 1 h, the membrane was incubated with rabbit anti-mouse anti-NF-κB p65 primary antibody (1:1000 dilution; Santa Cruz), rabbit anti-mouse anti-IκB-α primary antibody (1:1000 dilution; Santa Cruz), rabbit anti-mouse anti-phosphorylated (p)-IκB-α primary antibody (1:1000 dilution; Santa Cruz), and rabbit anti-mouse anti-β-actin primary antibody (1:1000 dilution; Santa Cruz), respectively, at 4°C for overnight. Then the membrane was incubated with HRP-conjugated sheep anti-rabbit secondary antibody (1:2000 dilution; Santa Cruz) at 37°C for 1 h. The protein bands were detected with the ECL detection system (Amersham Biosciences, Piscataway, NJ, USA), and the relative protein expression levels were calculated accordingly.

Electrophoretic mobility shift assay (EMSA)

For the EMSA, 1 mM PMSF was first added to the lung nuclear extract. An oligonucleotide containing the κ-chain binding site (κB, 5'-CCGGTTAACAGAGGGGGCTT-TCCGAG-3') was used as probe. Two complimentary strands were annealed and labeled with [α-32P]dCTP. The 20-μL reaction system consisted of 10,000 cpm labeled probe, 10 μg nuclear extract, and 2 μL binding buffer [10 mM Tris-HCl, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly (dI·dC), and 1 mM dithiothreitol, pH 7.6. After 30 min at room temperature, the reaction mixture was subjected to 4% SDS-PAGE in 0.5× Tris-borate buffer. DNA-protein interaction was specific for NF-κB as demonstrated in the competition EMSA using 50-fold excess unlabeled oligonucleotide.

Statistical analysis

Data were expressed as mean ± SD. Analysis of variance was performed for group comparison, with the Dunnett's post-hoc test. $P < 0.05$ was considered as statistically significant.

Results

HSYA inhibits AHR, cell chemotaxis, and inflammatory response in OVA-induced asthmatic mice

Effects of HSYA on AHR, chemotaxis, and inflammatory response in the OVA-induced asthmatic mouse models were first investigated. AHR is a functional consequence of inflammation that underlies asthma [17]. Our results showed that, compared with the control group, the AHR in response to inhaling Mch was substantially enhanced in the OVA-challenged mice (**Figure 1A**). However, the treatment of HSYA or BAY 11-7085 significantly prevented the AHR in response to inhaling Mch (**Figure 1A**), suggesting modified *in vivo* immune-mediated pathology.

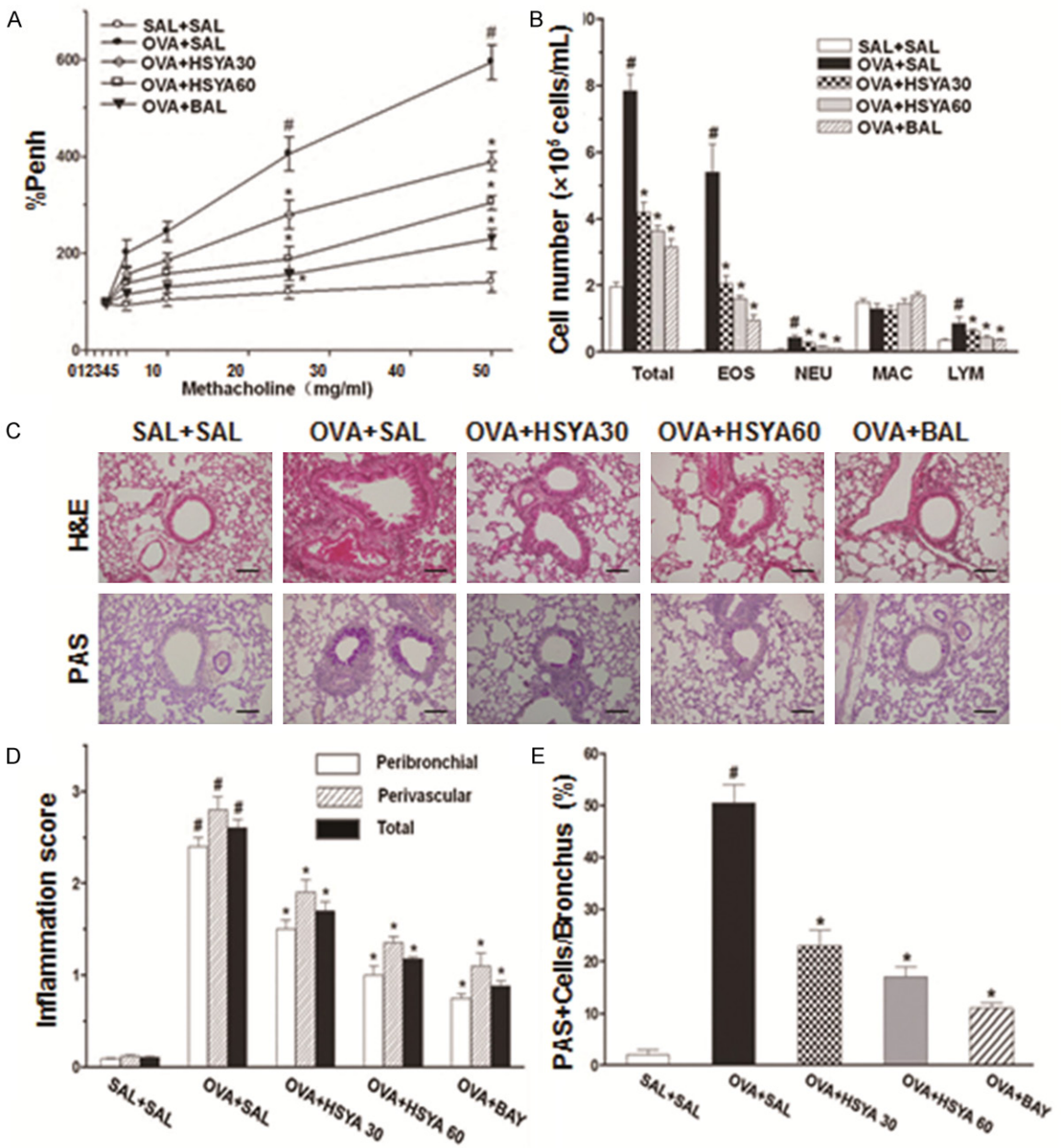

Figure 1. Effects of HSYA on AHR, cell chemotaxis in BAL fluid, and inflammatory response in OVA-induced asthmatic mice. A. The AHR in response to inhaling Mch was assessed in the saline-induced mice administered with saline (SAL+SAL), OVA-induced asthmatic mice administered with saline (OVA+SAL), and OVA-induce mice administrated with 30 mg/kg HSYA (OVA+HSYA 30), 60 mg/kg HSYA (OVA+HSYA 60), or BAY 11-7085 (OVA+BAY), respectively. Bronchopulmonary resistance was expressed as the increase in enhanced pauses (Penh) compared to baseline. Baseline Penh of the saline-treated control group was 100%. B. After drug administration with HSYA or BAY 11-7085, differential cell counts in the BAL fluid in OVA-induced asthmatic mice were analyzed. EOS, eosinophil; NEU, neutrophil; MAC, macrophage; LYM, lymphocyte. C. Lung tissue sections were subjected to the H & E and PAS staining, respectively. Scale bar, 50 μm. Representative picture from three independent experiments was shown for each group. D. Statistical analysis of the inflammation scores based on H & E staining. E. Statistical analysis of the airway mucus expression according to PAS staining. Compared with the OVA+SAL group, $^{*}P < 0.05$; compared with the SAL+SAL group, $^{\#}P < 0.05$.

Next, the number of the inflammatory cells in the BAL fluid was counted, at 48 h after the last OVA challenge. Our results showed that, compared with the control group, the OVA challenge resulted in dramatically increased eosinophils, as well as slightly increased neutrophils and

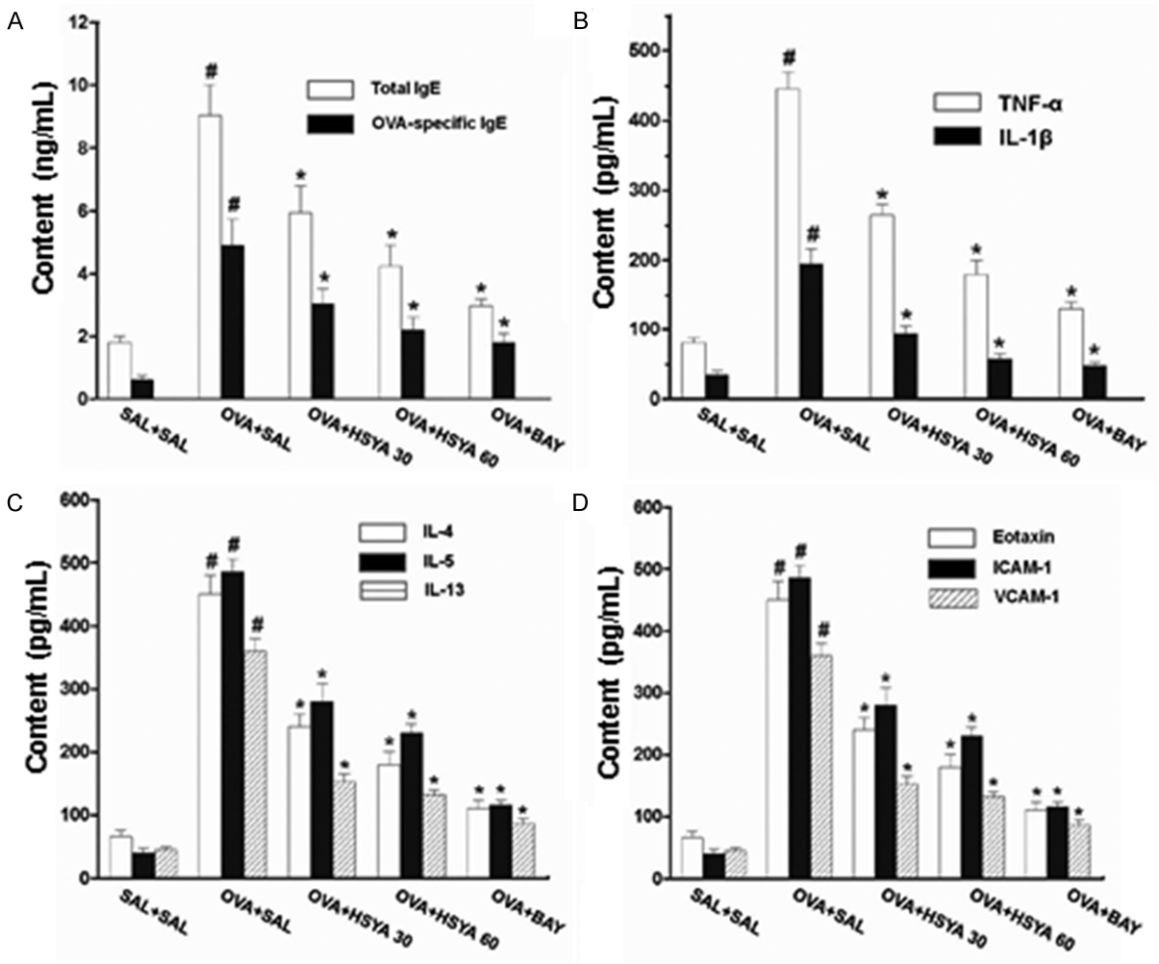

Figure 2. Effects of HSYA on levels of IgE, pro-inflammatory and Th2 cytokines, and adhesion molecules in the BAL fluid in OVA-induced asthmatic mice. Levels of total and OVA-specific IgE (A), pro-inflammatory cytokines (B), Th2 cytokines (C), and adhesion molecules (D) in the BAL fluid were detected with ELISA in the saline-induced mice administered with saline (SAL+SAL), OVA-induced asthmatic mice administered with saline (OVA+SAL), and OVA-induce mice administrated with 30 mg/kg HSYA (OVA+HSYA 30), 60 mg/kg HSYA (OVA+HSYA 60), or BAY 11-7085 (OVA+BAY), respectively. Compared with the OVA+SAL group, $^{*}P < 0.05$; compared with the SAL+SAL group, $^{\#}P < 0.05$.

lymphocytes, in the BAL fluid (**Figure 1B**). However, treatment of HSYA or BAY 11-7085 significantly attenuated the OVA-induced recruitment of eosinophils in the BAL fluid ($P < 0.05$), indicating reduced cell chemotaxis into the airway.

Besides, compared with the control group, the lung tissue from the OVA-challenged mice showed widespread perivascular and peribronchiolar inflammatory cell infiltration (**Figure 1C** and **1D**). Moreover, PAS staining showed that, the percentage of mucus-producing goblet cells in OVA-exposed mice was significantly higher than the control group (**Figure 1C** and **1E**). However, administration of HSYA or BAY 11-7085 significantly decreased the number of infiltrating inflammatory cells and the degree of goblet cell hyperplasia in these OVA-induced asthmatic mice (**Figure 1C-E**). Taken together, these results indicate that HSYA could efficiently attenuate the allergic airway inflammation and mucus hypersecretion in OVA-induced asthmatic mice.

HSYA attenuates IgE release into BAL fluid in OVA-induced asthmatic mice

To investigate the effects of HSYA on the release of total and OVA-specific IgE into the BAL fluid in OVA-induced asthmatic mice, ELISA was performed. Our results showed that, compared with the control mice, the IgE levels in

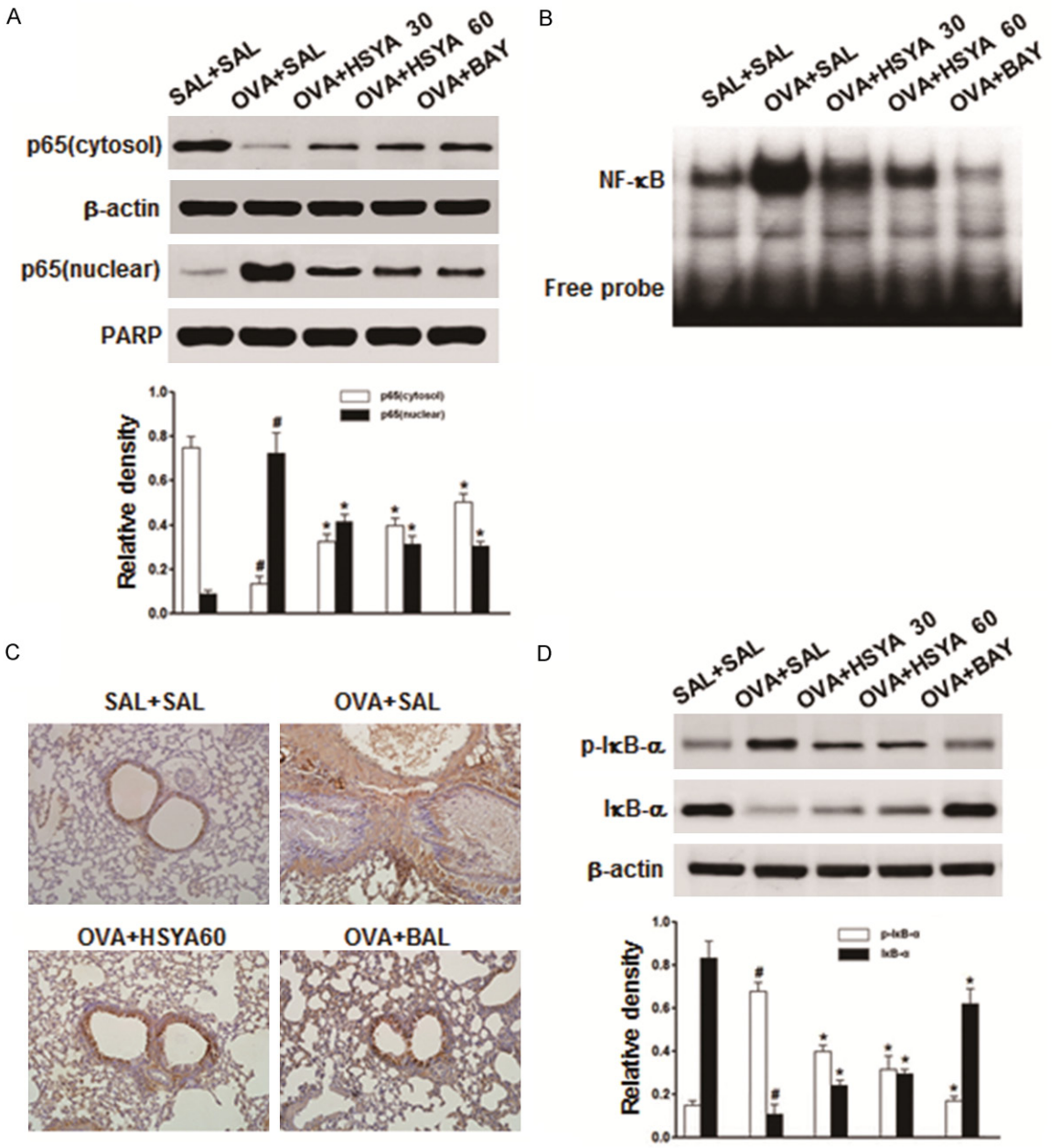

Figure 3. Effects of HSYA on NF-κB activation and IκB-α phosphorylation in OVA-induced asthmatic mice. A. Levels of the NF-κB p65 subunit in cytosol and nucleus were assessed with the Western blot analysis, for the saline-induced mice administered with saline (SAL+SAL), OVA-induced asthmatic mice administered with saline (OVA+SAL), and OVA-induce mice administrated with 30 mg/kg HSYA (OVA+HSYA 30), 60 mg/kg HSYA (OVA+HSYA 60), or BAY 11-7085 (OVA+BAY), respectively. B. NF-κB/DNA binding activity was assessed with EMSA. C. Immunohistochemical staining was performed to detect the nuclear translocation of p65. D. IκB-α phosphorylation and degradation were evaluated by the Western blot analysis. Compared with the OVA+SAL group, $^{*}P < 0.05$; compared with the SAL+SAL group, $^{\#}P < 0.05$.

the BAL fluid were significantly increased in the OVA-challenged mice ($P < 0.05$) (**Figure 2A**). However, administration of HSYA or BAY 11-7085 significantly reduced the total and OVA-specific IgE levels in the BAL fluid in the OVA-exposed mice (**Figure 2A**). These results suggest that, the HSYA treatment could attenuate the release of total and OVA-specific IgE into the BAL fluid in OVA-induced asthmatic mice.

HSYA reduces inflammation-related cytokine levels in BAL fluid in OVA-induced asthmatic mice

Allergic asthmatic inflammation is caused by the pro-inflammatory cytokines (such as TNF-α and IL-1β) and Th2 cytokines (such as IL-4, IL-5, and IL-13) [18]. To assess the effects of HSYA on the pulmonary inflammation in the asthmatic mice, the cytokine levels in the BAL fluid were measured with ELISA. Our results showed that, compared with the control mice, the levels of TNF-α, IL-1β, IL-4, IL-5, and IL-13 in the BAL fluid were significantly increased in the OVA-challenged mice (**Figure 2B** and **2C**). However, the cytokine levels in the BAL fluid were significantly decreased by the administration of HSYA or BAY 11-7085 (**Figure 2B** and **2C**).

Chemokines and leukocyte-endothelial adhesion molecules are important for the recruitment and migration of leukocytes to the inflammatory sites [19]. The levels of eotaxin, ICAM-1, and VCAM-1 in the BAL fluid were then measured. Our results showed that, compared with the control mice, the levels of eotaxin, ICAM-1, and VCAM-1 in the BAL fluid were significantly increased in the OVA-challenged mice, which could be significantly reduced by the treatment of HSYA or BAY 11-7085 (**Figure 2D**). These results suggest that, the HSYA treatment could significantly reduce the BAL fluid levels of inflammation-related cytokines, indicating attenuated pulmonary inflammation, in the OVA-induced asthmatic mice.

HSYA inhibits nuclear translocation of NF-κB and suppresses IκB-α phosphorylation in OVA-induced asthmatic mice

NF-κB plays a key role in the allergic lung inflammation, which activates the transcription of various pro-inflammatory mediators [4]. Whether the attenuating effects of HSYA on pulmonary inflammation in the asthmatic mice involved NF-κB was next investigated. Our results from the Western blot analysis showed that, the OVA challenge significantly increased the nuclear NF-κB p65 level, while significantly decreased the cytosolic NF-κB p65 level, in the cells from the extracted lung tissue (**Figure 3A**). Moreover, the EMSA results showed an increased binding activity of the lung nuclear extract to the NF-κB consensus sequence for the OVA-induced mice, compared with the control group (**Figure 3B**). Furthermore, the immunostaining results confirmed the nuclear translocation of the p65 subunit (**Figure 3C**). However, the treatment of HSYA or BAY 11-7085 significantly inhibited the nuclear translocation of NF-κB p65 in the OVA-induced asthmatic mice.

Next, the effects of HSYA on IκB-α phosphorylation were evaluated in the OVA-induced asthmatic mice. Our results showed that, the treatment of HSYA or BAY 11-7085 significantly reduced the phosphorylation and degradation of IκB-α in the lung tissue in OVA-induced asthmatic mice (**Figure 3D**). Taken together, these findings indicate that the HSYA treatment could limit the transcriptional activity of NF-κB by inhibiting the nuclear translocation of the p65 subunit and stabilizing the IκB-α in the lung tissue in OVA-challenged mice.

Discussion

Allergic airway inflammation is a major factor in the pathogenesis of asthma, which is characterized by increased infiltrating leukocytes and mucus secretion. In particular, eosinophils are the principal effector cells in the disease pathogenesis, which release cytotoxic granule proteins [20]. In the present study, our results showed that the HSYA treatment prevented the airway infiltration of eosinophils. A significant drop in the total cell and eosinophil counts in the BAL fluid was observed. Similarly, tissue eosinophilia was also inhibited by HSYA, as revealed by the reduced infiltration of inflammatory cells.

Eosinophilic transmigration into the airway is a multistep process modulated by the pro-inflammatory cytokines (such as TNF-α and IL-1β) and Th2 cytokines (such as IL-4, IL-5, and IL-13). This process is also coordinated by the chemotactic cytokines (such as eotaxin) and adhesion molecules (such as ICAM-1 and VCAM-1) [21, 22]. IL-4 is required for the maturation of B cells and the synthesis of IgE, which participates in the initiation of Th2 inflammatory response. IL-5 is pivotal for the growth, differentiation, recruitment, and survival of eosinophils. IL-13 could potently induce mucus hypersecretion, eotaxin expression, airway inflammation, and AHR [23, 24]. Moreover, TNF-α and IL-1β have been shown to contribute to the up-regulation of eosinophil chemoattractants and adhesion

molecules, elevated eosinophil recruitment, increased cytokine release, and enhanced AHR [18]. According to our results, HSYA attenuated the enhanced release of pro-inflammatory and Th2 cytokines, eotaxin, and adhesion molecules into the airway of OVA-induced asthmatic-mice. These findings suggest that HSYA prevents the allergic airway inflammation by diminishing the secretion of related cytokines into the lung tissue.AHR is defined as the abnormal increase in the airflow limitation in response to stimuli. Development of AHR has been associated with various mediators released during the allergic inflammation [25, 26]. For example, IL-5 mobilizes and activates eosinophils to release the pro-inflammatory products (such as major basic proteins and cysteinyl-leukotrienes), which are closely associated with AHR [27]. In addition, IL-4 and IL-13 have been shown to induce AHR in a murine asthmatic model, which also involves cysteinyl-leukotrienes [28]. Moreover, AHR has been associated with the direct effect of TNF-α on the airway smooth muscle [29]. Therefore, the AHR-reducing effects of HSYA may be related to the decreased Th2 cytokine production, tissue eosinophilia, and TNF-α level.

Transcription factor NF-κB regulates a wide variety of asthma-related cytokines, including TNF-α, IL-1β, IL-4, IL-5, IL-13, ICAM-1, and VCAM-1 [4]. Some studies have suggested that HSYA modulates the NF-κB activity in a variety of cell-land animal models [30-32]. In line with these findings, our results indicated that HSYA exerted NF-κB activity-reducing effect in the lung tissue of OVA-challenged mice. Furthermore, suppression of NF-κB by BAY 11-7085 reduced the cytokine levels and ameliorated the eosinophilic airway inflammation and AHR in OVA-induced asthmatic mice. Taken together, the anti-asthmatic effects of HSYA may be attributed to the inhibited transcriptional activity of NF-κB and the subsequently reduced pro-inflammatory chemical mediators.

In conclusion, our results indicated that the HSYA treatment could efficiently attenuate the allergic airway inflammation and mucus hypersecretion in OVA-induced asthmatic mice. Moreover, HSYA significantly reduced the BAL fluid levels of IgE and inflammation-related cytokines and chemokines. Furthermore, the HSYA treatment inhibited the transcriptional activity of NF-κB by inhibiting the nuclear translocation of the p65 subunit and stabilizing the IκB-α in the lung tissue in OVA-challenged mice. These findings suggest that HSYA might be a potential anti-inflammatory agent to treat asthma in clinic.

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Disclosure of conflict of interest

None.

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