



Effects of astilbic acid on airway hyperresponsiveness and inflammation in a mouse model of allergic asthma

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ARTICLE INFO

Article history:

Received 6 July 2010

Received in revised form 8 November 2010

Accepted 2 December 2010

Available online 17 December 2010

Keywords:

Astilbic acid

Asthma

Cytokine

ABSTRACT

Bronchial asthma is characterized by chronic lung inflammation, airway hyperresponsiveness (AHR), and airway remodeling. Astilbic acid, extracted from the medicinal herb *Astilbe chinensis*, is used as a headache remedy in traditional medicine and has anti-pyretic and analgesic effects. However, the effect of astilbic acid on asthma remains to be established. In the present study, we therefore examined the effect of astilbic acid in a mouse model in which asthma was established by sensitization and challenge with ovalbumin (OVA). Astilbic acid inhibited OVA-induced AHR to inhaled methacholine and significantly suppressed the levels of T-helper 2-type cytokines (including IL [interleukin]-4, IL-5, and IL-13) and inflammatory cells (including eosinophils) in bronchoalveolar lavage (BAL) fluid. Histochemical analysis revealed reduced goblet cell hyperplasia and mucus production, as well as attenuated eosinophil-rich leukocyte infiltration, in the astilbic acid-treated group, compared with OVA-challenged mice. Moreover, the compound significantly inhibited synthesis of IL-4-, IL-5-, IL-13-, IL-17-, and eotaxin-encoding mRNA following asthma induction in lung tissue, in addition to suppressing the immunoglobulin E (IgE) response to asthma in both BAL fluid and serum. Our results indicate that astilbic acid has great potential as a therapeutic candidate for the treatment of asthma.

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1. Introduction

Asthma is a multifactorial airway disease associated with significant morbidity and mortality worldwide. Asthma is generally caused by allergic responses, but can be exacerbated by viral or bacterial infections. Indeed, asthma is among the top 10 most devastating diseases caused by infectious agents in terms of loss of life and overall morbidity on a global scale [1]. An allergic disorder of the respiratory system, asthma is characterized by excessive infiltration of leukocytes (particularly eosinophils) into the lung, bronchoconstriction, increased airway hyperresponsiveness (AHR), and mucus production [2]. The inflammatory response typically involves increases in eosinophil and mast cell numbers, enhanced mucus secretion, and activation of T-cells via enhanced production of T-helper 2 (Th2) cytokines [3]. High-level release of the Th2 cytokines IL-4, IL-5, and IL-13 induces IgE switching in B cells, recruitment of mast cells and eosinophils, and production of various inflammatory mediators [4]. IL-17 is implicated in several inflammatory responses; the cytokine induces the expression of a number of mediators of inflammation [5]. Activated lymphocytes, monocytes, neutrophils, and eosinophils are recruited to the lung upon

IL-17 expression. Numerous proinflammatory chemokines, cytokines, adhesion molecules, respiratory mucins, and growth and angiogenic factors are involved in this process, and expression of chemokines and inflammatory proteins is induced in a process involving the Rel/nuclear factor- κ B (NF- κ B) family of transcription factors [6].

Astilbic acid is a natural product extracted from *Astilbe chinensis* (Maxim) Franch *et* Savat, a herb belonging to the Saxifragaceae family [7]. *A. chinensis* is a perennial found in China, Russia, Japan, and Korea. The dried roots of this plant are used in Korean traditional medicine to treat headaches and bronchitis, and as antipyretic and analgesic remedies [8]. Previous reports found that extracts of *A. chinensis* rhizomes had potential anti-inflammatory and antitumor properties [9]. However, the effect of astilbic acid on asthma remains to be established.

In the present study, we therefore examined the effect of astilbic acid in a mouse model in which asthma was established by sensitization and challenge with ovalbumin (OVA). We show that astilbic acid has anti-allergic effects on the lungs of asthmatic mice.

2. Materials and methods

2.1. Chemicals and reagents

Commercially available enzyme-linked immunosorbent assays (Biosource, Carlsbad, CA) were used to measure IL-4, IL-5, IL-13, and

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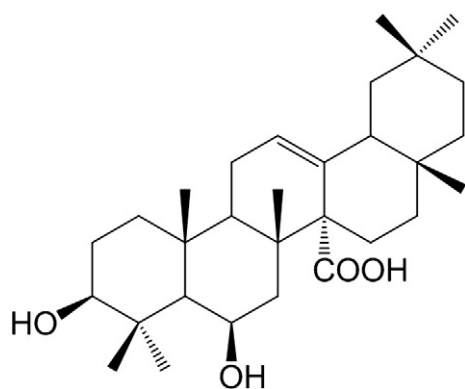


Fig. 1. Chemical structure of astilbic acid.

eotaxin levels in the BALF, according to the manufacturer's instructions. OVA, aluminum hydroxide, methacholine, and *o*-phenylenediamine dihydrochloride was purchased from Sigma-Aldrich (Sigma, St Louis, MO).

2.2. Extraction and purification of astilbic acid

A. chinensis is a perennial herb found in Korea, mainland China, Japan, and Eastern Russia. Freshly harvested *A. chinensis* was washed three times with tap water to remove salts, epiphytes, and sand, and stored at -20°C . Frozen samples were lyophilized and homogenized in a grinder before extraction. Dried *A. chinensis* powder (15 kg) was extracted with MeOH ($3 \times 20\text{ L}$) at room temperature to obtain 3 kg of solid extract. This MeOH extract was suspended in H_2O and further extracted with hexane ($3 \times 3\text{ L}$) to produce a hexane-soluble fraction (160 g). An aliquot of this fraction (155 g) was subjected to chromatography on a silica gel column eluted with a stepwise gradient of hexane and EtOAc, yielding four fractions (fractions A–D: 20 g, 10 g, 3 g, and 120 g).

As shown in Fig. 1, astilbic acid (2.1 g) was obtained from fraction B3 (5 g) using a RP C18 column, upon elution with $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (2:1, v/v).

2.3. Animals

Six to 8-week-old pathogen-free female BALB/c mice were obtained from Orient Co. (Seoul, Korea). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments, and provided with water and standard chow *ad libitum*. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of Korea.

2.4. Sensitization and airway challenge

Mice were divided into four groups (each of 6–7 animals), and airway inflammation was induced by ovalbumin (Grade III) in three of the groups, using the method described by Oh et al. [31]. Mice were immunized via intraperitoneal (i.p.) injection with 20 μg chicken OVA and 2 mg aluminum hydroxide in 200 μL PBS buffer (pH 7.4), on days 0 and 14. Mice were exposed to a 1% (w/v) OVA solution in PBS, for 20 min, using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) on days 28, 29, and 30 after initial sensitization. Animals were sacrificed 48 h after the last challenge (thus on day 32) to characterize the suppressive effects of astilbic acid. A schematic diagram of the treatment schedule is shown in Fig. 2.

Montelukast is a cysteinyl leukotriene (cys-LT)-1 receptor antagonist [28], and useful clinical responses to this agent have been obtained in patients with aspirin-sensitive asthma, nocturnal exacerbation of asthma, and allergic asthma. These data have resulted in successful commercialization of the drug [29]. In the present work, montelukast was used as a positive control.

2.5. Measurement of airway hyperresponsiveness (AHR)

At 24 h after the final aerosol challenge, AHR was assessed in conscious and unrestrained mice by means of whole-body plethysmography (OCP3000; Allmedicus, Seoul, Korea). Each mouse was placed in a plastic chamber and exposed to aerosolized PBS, followed by delivery of aerosols containing increasing concentrations of methacholine (6.25, 12.5, and 25 mg/mL; Sigma) in PBS, for 3 min at each exposure level. Bronchoconstriction was recorded for an additional 5 min after each methacholine dose. The highest Penh value obtained during each methacholine challenge was expressed as a proportion of the basal Penh value recorded in response to the PBS aerosol.

2.6. Collection of bronchoalveolar lavage (BAL) fluid

Mice were sacrificed using an overdose of 50 mg/kg of pentobarbital 48 h after the last challenge, and tracheotomy was performed. After instilling ice-cold PBS (0.5 mL) into a lung, BAL fluid was obtained by three successive aspirations (total volume 1.5 mL) via tracheal cannulation [2]. BAL fluid was centrifuged (250 g, 4°C , for 5 min) and each supernatant was collected and stored at -70°C . Cell pellets were assessed by counting of cells in at least five squares of a hemocytometer after exclusion of dead cells stained with trypan blue [10]. BAL fluid (100 μL) was placed on a slide and centrifuged (200 g, 10 min, at 4°C) to fix the cells, using a cytospin machine (Hanil Science Industrial, Seoul, Korea). After the slides were dried, cells were fixed and stained using Diff-Quik® Stain reagents (B4132-1A; Dade Behring Inc., Deerfield, IL), according to the manufacturer's instructions.

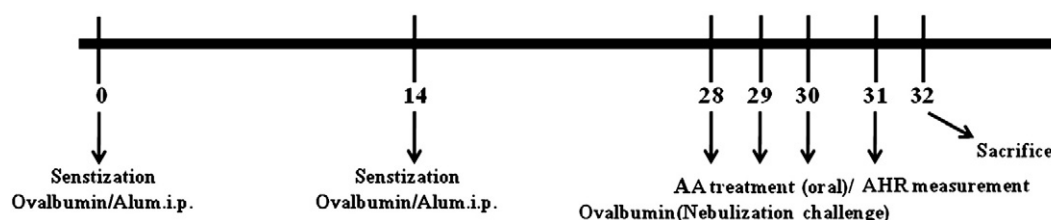


Fig. 2. Experimental protocol for induction of airway inflammation, and treatment with astilbic acid, using a mouse model. Mice were divided into four groups (with 6–7 animals/group) and sensitized via intraperitoneal injection of 20 μg OVA emulsified in 2 mg aluminum hydroxide in 100 μL PBS buffer (pH 7.4) on days 0 and 14. Each mouse was challenged through the airway with OVA (1%, w/v, in PBS) for 20 min, using an ultrasonic nebulizer, on days 28, 29, and 30 after initial sensitization. Mice were given 30 mg/kg/day of astilbic acid, orally, on each of days 28 to 30. Animals were sacrificed 48 h after the last challenge.

2.7. Analysis of bronchoalveolar lavage (BAL) fluid and serum

The concentrations of total and OVA-specific-IgE in the BAL fluid or serum were measured using a sandwich enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with 100 μ L/well IgE (2 μ g/mL; Serotec, Oxford, UK) in PBS. Antibodies were detected in the BAL fluid and serum using isotype-specific secondary antibodies (anti-mouse IgE; Serotec). After four washes, 200 μ L of *o*-phenylenediamine dihydrochloride (Sigma, St Louis, MO) was added to each well. Plates were incubated for 10 min in the dark, and the absorbance was measured at 450 nm. Total and OVA-specific-IgE concentrations were calculated from a standard curve generated using 250 ng/mL recombinant IgE (Serotec).

2.8. Measurement of Th2 cytokine levels in the BAL fluid

The concentrations of IL-4, IL-5, IL-13, and eotaxin in the BAL fluid were measured using ELISA kits, according to the manufacturer's instructions.

2.9. Quantification of mRNA in lung tissue using reverse transcriptase polymerase chain reaction

Total RNA was extracted from lung tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. To prepare cDNA, total RNA was incubated at 37 °C for 90 min with the reagents of the first-strand cDNA synthesis kit (Promega, Madison, WI). RT-PCR was performed in a final volume of 20 μ L. Primer sequences were as follows: *IL-4* sense TCT CTA GAT CAT GGG CAT TTT GAA CGA GGT C and antisense TGC ATG ATG CTC TTT AGG CTT TCC; *IL-5* sense ATG ACT GTG CCT CTG TGC CTG GAG C and antisense CTG TTT TTC CTG GAG TAA ACT GGG G; *IL-13* sense CTC CCT CTG ACC CTT AAG GAG and antisense GAA GGG GCC GTG GCG AAA CAG; *IL-17* sense TCT CAT CCA GCA AGA CAT CC and antisense AGT TTG GGA CCC CTT TAC AC; and *eotaxin* sense AGA GGC TGA GAT CCA AGC AG and antisense CAG ATC TCT TTG CCC AAC CT. Amplification reactions were performed in an automated thermal cycler (GeneAmp PCR system 9700; PerkinElmer, Norwalk, CT). The RT-PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s; annealing at 60 °C (*IL-4*, *IL-5*, *IL-13*, *eotaxin*, and β -actin) or 50 °C (*IL-17*) for 30 s; extension at 72 °C for 30 s; and a final extension at 72 °C for 7 min. β -Actin was used as the internal control for each PCR reaction. Products were separated on 1.5% (w/v) agarose gels and visualized by ethidium bromide staining.

2.10. Lung tissue histopathology

Lung tissue was fixed in 10% (v/v) neutral buffered formalin for 24 h. Tissues were subsequently embedded in paraffin, sectioned at 4 μ m thickness, and stained with H & E solution (hematoxylin; Sigma, MHS-16; and eosin; Sigma, HT1100-1-32) and the periodic acid-Schiff (PAS) stain (IMEB Inc., San Marcos, CA), to estimate mucus production. Tissues were subsequently mounted and coverslipped using Dako mounting medium (Dakocytomation, Denmark, CA). Peribronchial cell counts were based on a five-point scoring system, as described previously, to estimate the severity of leukocyte infiltration [11]. The extent of cell infiltration into the airway was scored in a double-blind manner by two independent investigators [12]. Peribronchiole and perivascular inflammation was evaluated using scores ranging from 0 to 5: 0, no cells; 1, a few cells; 2, a ring of cells one cell-layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells; and 5, a deep layer of cells. Photomicrographs were obtained using a Photometric Quantix digital camera running a Windows program, and the montages were assembled in Adobe Photoshop 7.0. Images were cropped and corrected for brightness and contrast, but were not otherwise manipulated.

2.11. Immunoblotting

We also investigated whether astilbic acid prevented translocation of the p65 subunit of NF- κ B from the cytosol to the nucleus. Nuclear and cytosolic extracts were prepared and subjected to immunoblot analysis. β -actin was used as a control to confirm absence of contamination during tissue extraction. Lung tissue was homogenized in lysis buffer containing protease inhibitors [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) NP-40, 0.1% (w/v) SDS, 1 mM EGTA, 100 μ g/mL PMSF, 10 μ g/mL pepstatin A, and 100 μ M Na_3VO_3]. The homogenates were centrifuged at 12,000 g at 4 °C for 25 min, and protein concentrations in supernatants were determined using the Bradford reagent (Bio-Rad, Hercules, CA). Twenty-five micrograms of protein were loaded into each well of an SDS-polyacrylamide gel prior to electrophoresis at 100 V for 90 min, and separated proteins were transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked with 5% (w/v) nonfat dry milk dissolved in TBST buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% (v/v) Tween-20] overnight at 4 °C and next incubated with anti-NF- κ B (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and anti- β -actin (1:200 dilution) antibodies, again overnight at 4 °C. After removal of primary antibodies, the membranes were washed three times with TBST buffer at room temperature and next incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 dilution; Santa Cruz Biotechnology), for 1 h at room temperature. The membranes were next washed again with TBST buffer, and immunoreactive bands were visualized using the ECL reagent (Amersham Pharmacia Biotech, Uppsala, Sweden). Densitometric band values were determined using UN-SCAN-IT Gel™ image analysis software (Silk Scientific, Orem, UT), and statistically analyzed.

2.12. Statistical analysis

Data are expressed as means $\pm$ standard deviations (SDs). Statistical significance was determined using Student's two-tailed *t*-test to compare independent means. The critical level for significance was set at $P < 0.05$.

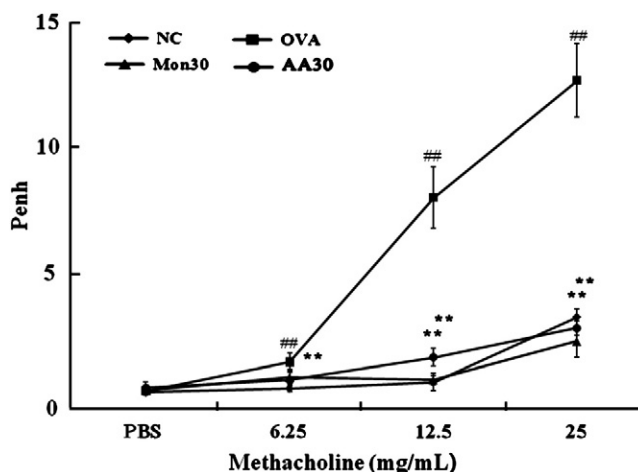


Fig. 3. Effect of astilbic acid on airway hyperresponsiveness (AHR). AHR was measured 24 h after the last OVA challenge in mice exposed to incrementally greater doses of methacholine (6.25–25 mg/mL). NC, normal control (PBS only); OVA, OVA-sensitized/-challenged mice; AA30, astilbic acid (30 mg/kg) + OVA-sensitized/-challenged animals; and Mon30, Montelukast (30 mg/kg) + OVA-sensitized/-challenged mice. Values are expressed as means $\pm$ SDs. # $P < 0.05$ and ## $P < 0.01$, significantly different from NC. * $P < 0.05$ and ** $P < 0.01$, significantly different from OVA.

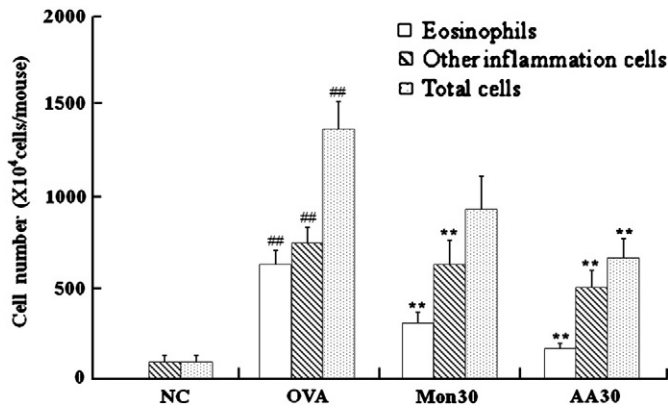


Fig. 4. Effect of astilbic acid on recruitment of inflammatory cells into BAL fluid. Cell numbers were assessed by direct counting under a light microscope in at least five squares of a hemocytometer after exclusion of dead cells stained with trypan blue. NC, normal control (PBS only); OVA, OVA-sensitized/-challenged mice; AA30, astilbic acid (30 mg/kg) + OVA-sensitized/-challenged mice; and Mon30, Montelukast (30 mg/kg) + OVA-sensitized/-challenged mice. Values are expressed as means $\pm$ SDs. # P < 0.05 and ## P < 0.01, significantly different from NC. * P < 0.05 and ** P < 0.01, significantly different from OVA.

3. Results

3.1. Effect of astilbic acid on AHR

AHR was evaluated by calculating Penh values (enhanced pauses) 24 h after the final OVA challenge. The Penh value of the OVA-challenged group was significantly higher than that of the PBS control group (P < 0.05) when concentrations of 12.5–25 mg/mL methacholine were delivered in aerosols. OVA-sensitized and -challenged mice treated with astilbic acid displayed a reduced mean Penh value upon exposure to 25 mg/mL methacholine, compared with the response of the OVA-sensitized and -challenged group. In OVA-challenged mice treated with astilbic acid, the Penh value was significantly reduced, to about 77.2% that of the OVA-challenged group, upon exposure to 25 mg/mL methacholine (P < 0.05). Positive control mice given with

montelukast showed an AHR decrease similar to that achieved using astilbic acid (Fig. 3).

3.2. Effect of astilbic acid on OVA-induced eosinophilia and BAL fluid composition

We examined changes in total cell levels in the BAL fluid to determine the effects of astilbic acid on asthma. Suppression of eosinophilia by astilbic acid in OVA-challenged mice was estimated by counting of cells recruited to the BAL fluid 48 h after the last challenge. As shown in Fig. 4, OVA induced a marked influx of leukocytes into BAL fluid in the PBS control group. Eosinophils constituted less than 1% of the total cell content of PBS-treated mice. However, eosinophil levels markedly increased, to levels greater than those of total leukocytes, in the BAL fluid of OVA-challenged mice. OVA-sensitized and -challenged mice treated with astilbic acid displayed significantly reduced leukocytosis, a fall in absolute eosinophilia, and decreased lymphocytosis, upon asthma induction. We recorded a 51.6% decrease in total cell numbers (P < 0.05) and 73.8% suppression of eosinophil levels (P < 0.05), compared to those in the OVA-treated control group. The positive drug control, montelukast, exerted similar suppressive effects on leukocyte influx into BAL fluid (Fig. 4).

3.3. Effect of astilbic acid on the levels of total and OVA-specific IgE in the serum and BAL fluid

Next, we examined whether astilbic acid influenced OVA-specific IgE production in the allergic asthma mouse model, in view of the finding that antigen-specific Th2 responses trigger IgE antibody production. The concentrations of total and OVA-specific IgE in the serum and BAL fluid were determined by ELISA in each experimental group 48 h after the last OVA challenge. The total and OVA-specific IgE levels in the serum and BAL fluid were markedly increased in OVA-treated mice compared with PBS-treated animals. Notably, astilbic acid suppressed both total and OVA-specific IgE levels in the serum and BAL fluid, compared to the levels seen in the OVA-challenged group. The positive drug control, montelukast, had a similar suppressive effect (Fig. 5).

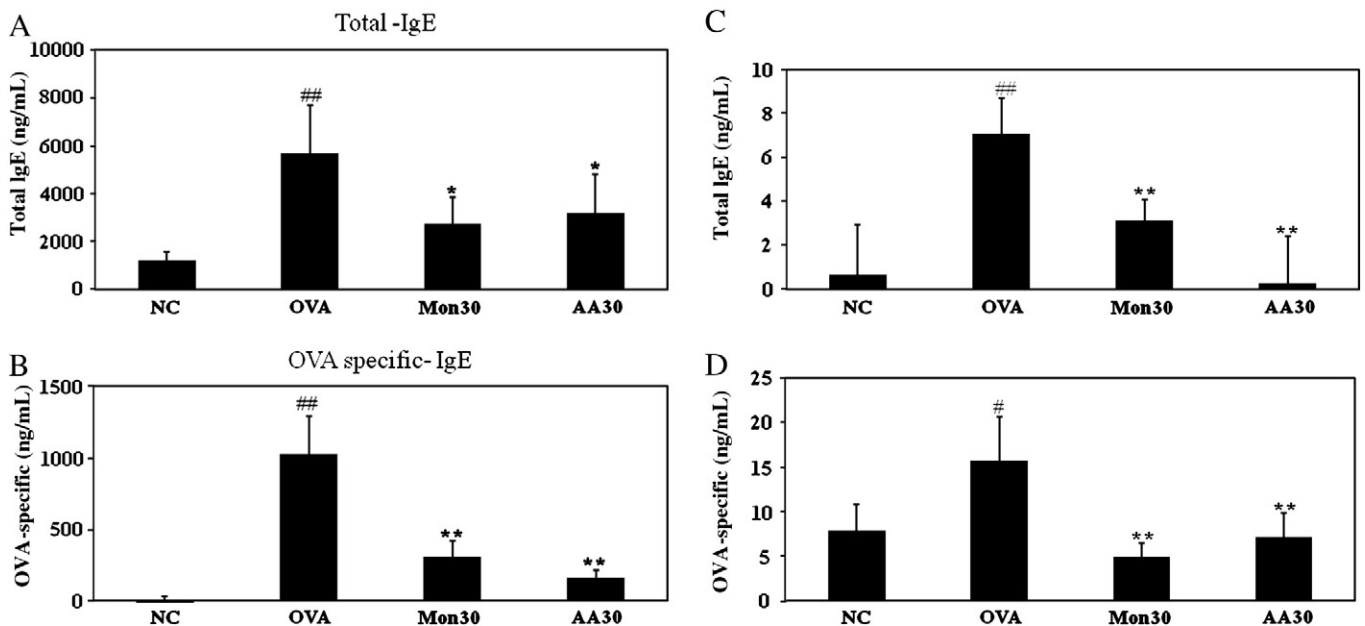


Fig. 5. Effects of astilbic acid on the total and OVA-specific IgE levels in the serum and BAL fluid. Serum was collected 48 h after the last OVA challenge of mice. Total and OVA-specific IgE levels in the serum (A, B) and BAL fluid (C, D) were analyzed by ELISA (n = 6 or 7 animals). NC, normal control (PBS only); OVA, OVA-sensitized/-challenged mice; AA30, astilbic acid (30 mg/kg) + OVA-sensitized/-challenged mice; and Mon30, Montelukast (30 mg/kg) + OVA-sensitized/-challenged mice. Values are expressed as means $\pm$ SDs. # P < 0.05 and ## P < 0.01, significantly different from NC. * P < 0.05 and ** P < 0.01, significantly different from OVA.

Table 1

The effects of AA on cytokine and chemokine production in BALF.

Group	Dose (mg/kg)	IL-4 (pg/mL)	IL-5 (pg/mL)	IL-13 (pg/mL)	Eotaxin (pg/mL)
NC		42.69 ± 14.14	78.21 ± 11.27	15.38 ± 4.21	16.24 ± 1.21
OVA		78.18 ± 16.21 [#]	109.24 ± 5.47 [#]	24.89 ± 3.75 [#]	29.45 ± 5.42 [#]
Montelukast	30	45.23 ± 13.18 [*]	56.17 ± 10.32 ^{**}	13.21 ± 3.89 ^{**}	15.20 ± 3.12 [*]
Astilbic acid	30	37.85 ± 15.78 [*]	58.12 ± 12.54 ^{**}	13.28 ± 2.79 ^{**}	13.28 ± 2.12 [*]

Significant difference from NC, [#]*P*<0.05 and ^{##}*P*<0.01, and significant difference from OVA, ^{*}*P*<0.05 and ^{**}*P*<0.01.

3.4. Effect of astilbic acid on cytokine and chemokine expression levels in lung tissue

To determine whether astilbic acid influenced cytokine secretion into BAL fluid, IL-4, IL-5, IL-13, and eotaxin levels in the BAL fluid were measured by ELISA 48 h after the final challenge. As shown in Table 1, OVA challenge triggered significant increases in cytokine levels in the BAL fluid, compared with control fluid. In OVA-sensitized and -challenged mice treated with astilbic acid, the rises in cytokine levels were significantly suppressed, compared with the concentrations seen in the OVA-challenged group. Specifically, a 37.3 ± 12.5 pg/mL decrease in IL-4 (*P*<0.05), a 54.8 ± 19.4 pg/mL fall in IL-5 (*P*<0.05), and a 10.9 ± 2.7 pg/mL decrease in IL-5 (*P*<0.05), were observed. Our results thus clearly show that astilbic acid reduces IL-4, IL-5, IL-13, and eotaxin concentrations in the BAL fluid of asthmatic mice. To further determine whether astilbic acid modulated cytokine expression, the levels of relevant mRNAs were estimated using RT-PCR. Total RNA was extracted from lung tissue and PCR of cDNA was performed using specific primers amplifying cDNA encoding IL-4, IL-5, IL-13, IL-17, and eotaxin; the products were separated on 1.5% (w/v) agarose gels. Notably, astilbic acid significantly suppressed the levels of mRNAs encoding all of IL-4, IL-13, IL-17, and eotaxin, following asthma induction, as shown in Fig. 6A and B. However, IL-5 expression was more weakly suppressed than was that of IL-4 or IL-13.

3.5. Effect of astilbic acid on OVA-induced eosinophilia in lung tissue

Because astilbic acid inhibits inflammatory cell recruitment into the BAL fluid, we next examined the anti-asthmatic and anti-remodeling effects of the drug. Lung tissue was collected 48 h after the final OVA challenge. In OVA-induced asthmatic lung tissue, we observed marked infiltration of inflammatory cells into perivascular and peribronchial connective tissues, compared with the normal tissue. Moreover, most leukocytes were eosinophils. The PBS control group displayed no change in the extent of inflammatory cell infiltration, similar to what was seen in montelukast-treated positive control mice. Eosinophilia in OVA-sensitized and challenged mice treated with astilbic acid was significantly attenuated compared with the level seen in OVA-challenged mice (Fig. 7).

3.6. Effect of astilbic acid on airway goblet cell hyperplasia and mucus production

Mucus hypersecretion is a characteristic of airway remodeling. The number of goblet cells containing mucus was increased in OVA-challenged mice. To determine whether astilbic acid suppressed mucus overproduction caused by goblet cell hyperplasia, we stained the lung sections with PAS. In OVA-challenged mice, compared with the PBS-treated animals, mucus overproduction was clearly observed as a violet color in bronchial airways. In contrast, the extent of mucus staining was markedly diminished in OVA-sensitized and -challenged mice treated with astilbic acid (Fig. 8). Our data thus confirm that astilbic acid significantly reduces the goblet cell hyperplasia and mucus hypersecretion characteristic of the airway remodeling process.

3.7. Effect of astilbic acid on NF-κB p65 protein levels in lung tissue

We assessed the effect of astilbic acid on NF-κB p65 levels during OVA-induced allergic airway inflammation. OVA-sensitized and -challenged mice displayed increased concentrations of NF-κB p65 in nuclear protein extracts from lung tissues, compared with the PBS control group (Fig. 9). Conversely, the NF-κB p65 level was significantly lower in the astilbic acid-treated group.

4. Discussion

Asthma, a chronic inflammatory disease of the airways, affects many individuals worldwide. The disease may cause severe morbidity, and even mortality if exacerbated. To date, a variety of drugs have been developed to treat asthma; these include steroids, leukotriene inhibitors, mast cell stabilizers, and β2 adrenergic agonists. Herbal medicines, including an *A. chinensis* extract, are commonly used to this end in the Orient, but the therapeutic efficacies and modes of

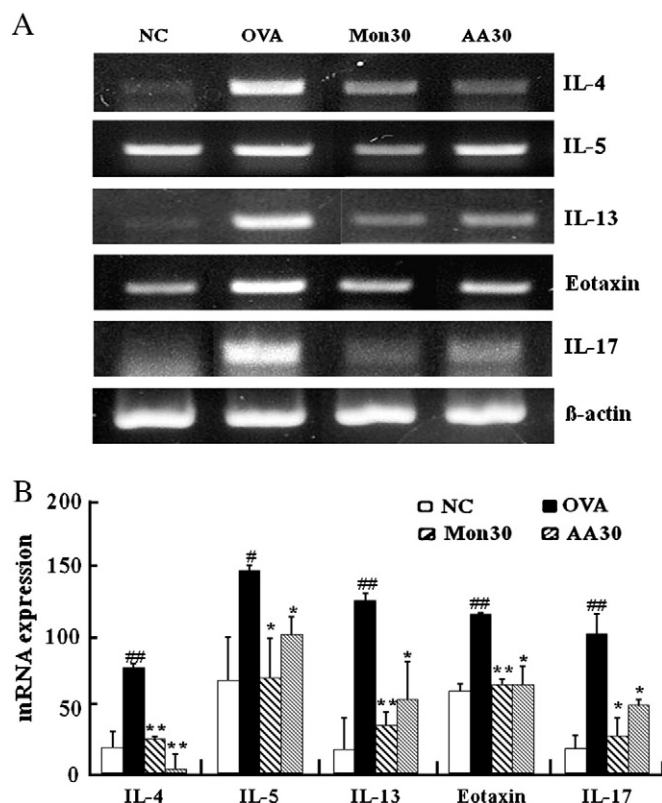


Fig. 6. Effects of astilbic acid on cytokine and chemokine expression levels in lung tissue. BAL fluid was collected 48 h after the last OVA challenge of mice (*n* = 5 or 6 animals). (A) Lung tissue was collected and total RNA was extracted. NC, normal control (PBS only, open bars); OVA, OVA-sensitized/-challenged mice (closed bars); AA30, astilbic acid (30 mg/kg) + OVA-sensitized/-challenged mice (thin hatched bars); and Mon30, Montelukast (30 mg/kg) + OVA-sensitized/-challenged mice (thick hatched bars). IL-4, IL-5, IL-13, IL-17, and eotaxin mRNA levels were analyzed using RT-PCR. (B) Densitometric data are expressed as means ± SDs. [#]*P*<0.05 and ^{##}*P*<0.01, significantly different from NC. ^{*}*P*<0.05 and ^{**}*P*<0.01, significantly different from OVA.

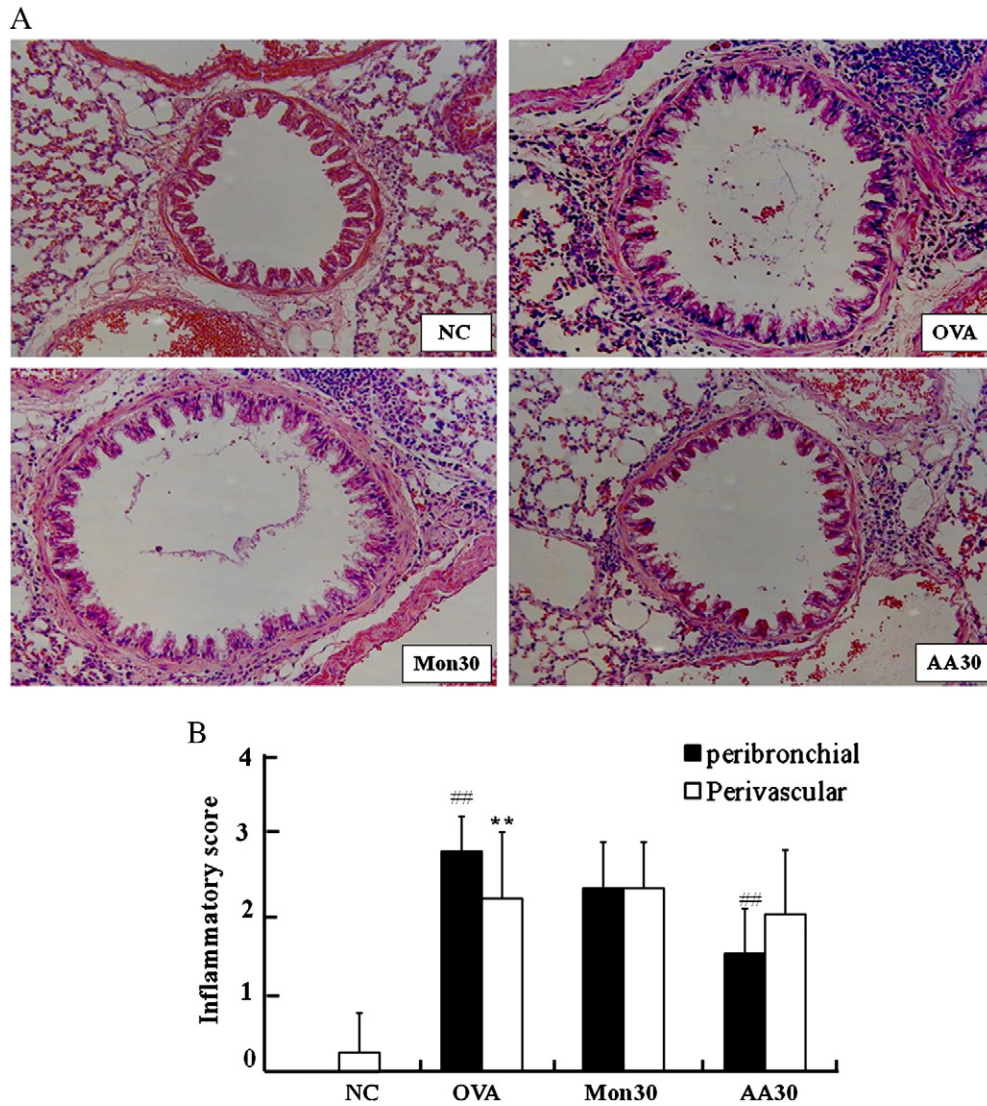


Fig. 7. Effect of astilbic acid on leukocyte recruitment into the lung tissue. (A) Histological examination of lung tissue was performed 48 h after the final OVA challenge. Lung tissues were fixed, sectioned at 4 μ m thickness, and stained with H&E solution (magnification $\times 200$). (B) Scoring of the extent of inflammation via quantitative analysis of inflammatory cell infiltration in lung sections was performed as described in the [Methods](#) section. NC, normal control (PBS only); OVA, OVA-sensitized/-challenged mice; AA30, astilbic acid (30 mg/kg) + OVA-sensitized/-challenged mice; and Mon30, Montelukast (30 mg/kg) + OVA-sensitized/-challenged mice. Values are expressed as means $\pm$ SDs. [#] $P < 0.05$ and ^{##} $P < 0.01$, significantly different from NC. ^{*} $P < 0.05$ and ^{**} $P < 0.01$, significantly different from OVA.

action of such medicines are currently unclear. Here, using an OVA-induced mouse model of asthma, we show for the first time that astilbic acid, a natural extract of *A. chinensis*, can be effective as a therapeutic drug for the treatment of allergic asthma.

Asthma is defined as a variable level of airway obstruction usually accompanied by AHR. Bronchoconstriction attributable to contraction or hypertrophy of airway smooth muscle (ASM), and inflammation within the airway, leads to decreased lung function [13]. AHR is a measure of the bronchial constriction commonly observed in individuals with asthma. Before investigating the effects of astilbic acid (30 mg/kg) in the mouse model of allergic asthma, we conducted a preliminary study to determine how the drug (20, 30, and 50 mg/kg) affected IgE and IgG2a levels in the serum, to define an optimal dose (data not shown). Astilbic acid at 30 mg/kg and 50 mg/kg showed similar effects; hence employed a dose of 30 mg/kg in the present work. We monitored the effects of astilbic acid on AHR by measuring enhanced pause times (Penh values) over 150 s. These experiments disclosed that astilbic acid inhibited the OVA-induced AHR in response to inhaled methacholine.

The mechanisms of recruitment of inflammatory cells associated with and presumably causing AHR have been well-studied. Migration

of inflammatory cells, specifically eosinophils and lymphocytes, into the lung is a major contributor to the development of allergic airway inflammation [14]. A rise in the number of eosinophils in the BAL fluid is a characteristic of asthma. The present results clearly demonstrate that astilbic acid significantly reduces eosinophil numbers in the BAL fluid and lung tissue.

Asthma and inflammation are associated with enhanced production of IgE and Th2 cytokines. IgE-mediated allergic responses are the most common inducers of AHR [15]. Both IL-4 and IL-13 contribute to AHR and inflammation, as was evident in experiments using IL-4- and IL-13-deficient mice [16]. Our data show that astilbic acid significantly suppresses both IgE and Th2 cytokine levels (Figs. 5 and 6). Th2 cytokines play essential roles in the pathogenesis of allergic airway inflammation [17,18]; these cytokines are produced by different types of resident cells (bronchial epithelial cells, tissue mast cells, and alveolar macrophages) as well as by infiltrated inflammatory cells including lymphocytes and eosinophils [19]. Specifically, IL-4 and IL-13 play critical roles in IgE switching within B cells, in eosinophil infiltration into the airway, and in mucus hypersecretion, whereas IL-5 is essential for activation and survival of eosinophils, and in development of AHR [20]. As reported previously, astilbic acid suppresses Th2 cytokine levels and

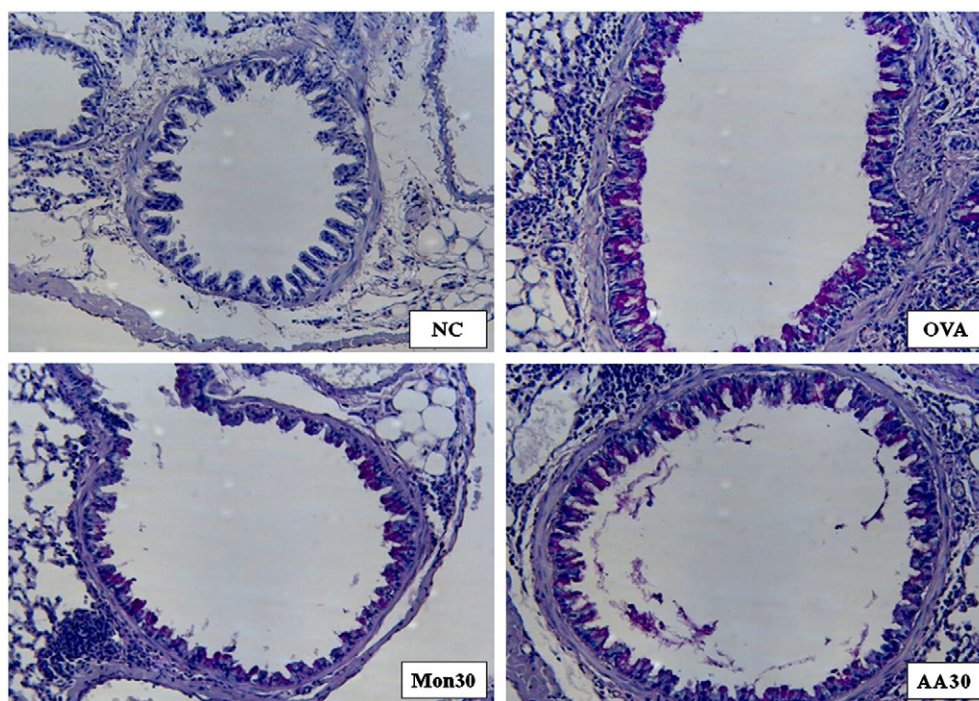


Fig. 8. Effect of astilbic acid on mucus production. Histological examination of mucus secretion in lung tissue was performed 48 h after the last OVA challenge in mice. Lung tissues were fixed, sectioned at 4 μ m thickness, and stained with periodic acid-Schiff (PAS) reagent to assess mucus production (magnification $\times 200$). NC, normal control (PBS only); OVA, OVA-sensitized/-challenged mice; AA30, astilbic acid (30 mg/kg) + OVA-sensitized/-challenged mice; and Mon30, Montelukast (30 mg/kg) + OVA-sensitized/-challenged mice.

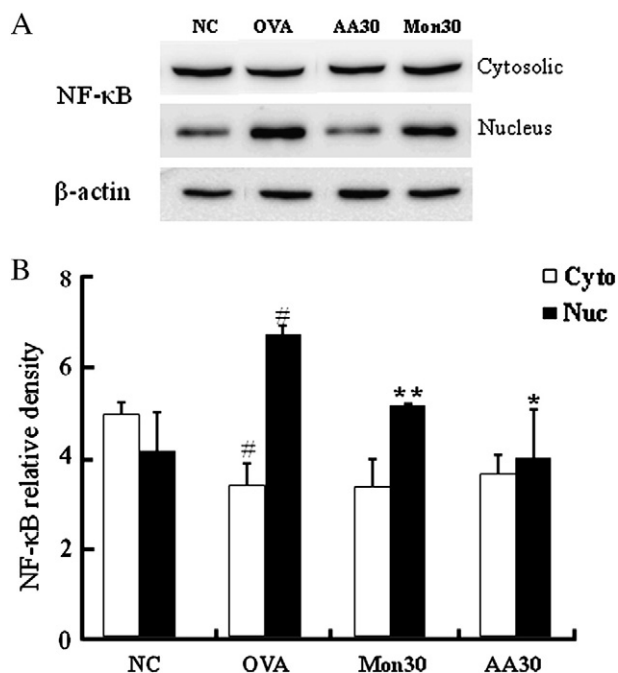


Fig. 9. Effect of astilbic acid on NF- κ B p65 protein levels in lung tissue. (A) NF- κ B p65 protein levels were measured 48 h after the last challenge in NC, normal control mice (PBS only); OVA, OVA-sensitized/-challenged mice; AA30, astilbic acid (30 mg/kg) + OVA-sensitized/-challenged mice; and Mon30, Montelukast (30 mg/kg) + OVA-sensitized/-challenged mice. (B) Densitometric data are presented as the relative ratios of NF- κ B p65 levels in NC, normal control mice (PBS only); OVA, OVA-sensitized/-challenged mice; AA30, astilbic acid (30 mg/kg) + OVA-sensitized/-challenged mice; Mon30, and Montelukast (30 mg/kg) + OVA-sensitized/-challenged mice. Values are expressed as means $\pm$ SDs. [#] $P < 0.05$ and ^{##} $P < 0.01$, significantly different from NC. ^{*} $P < 0.05$ and ^{**} $P < 0.01$, significantly different from OVA.

expression of the mRNAs encoding these factors (Table 1 and Fig. 6). IL-17, a recently identified cytokine expressed in eosinophils, neutrophils, and monocytes [23], has attracted considerable interest because it is involved in both the immune and inflammatory responses. The biological functions of IL-17 include regulation of neutrophil-dominated inflammation; the cytokine promotes granulopoiesis, neutrophil accumulation, and neutrophil activation in the lung [21,22]. Our results show that IL-17 transcription is increased in an OVA-induced mouse model, and inhibited upon treatment with astilbic acid (Fig. 6A and B).

NF- κ B plays a pivotal role in the immune and inflammatory responses, including those of asthma. Several lines of evidence indicate that NF- κ B pathway activation is enhanced in asthmatic tissues. Peripheral blood mononuclear cells (PBMCs) of adults with uncontrolled, moderate, and severe asthma have elevated concentrations of NF- κ B p65 [24]. In the OVA-sensitized and -challenged model, total lung extracts from Brown-Norway rats showed enhanced NF- κ B activity [25]. In a murine model, the bronchial epithelium displayed robust and rapid NF- κ B p65 nuclear translocation and IKK- α/β activity after asthma induction, compared with the controls [26]. NF- κ B levels in nuclear protein extracts from lung tissues are substantially increased in OVA-induced mouse models of asthma. Activated NF- κ B induces transcription of a variety of inflammatory genes that are abnormally expressed in asthma models [27]. Our results confirm that astilbic acid inhibits NF- κ B activity, thus preventing OVA-induced translocation of transcription factors into the nucleus. Additionally, astilbic acid suppresses the expression of inflammatory cytokines (IL-4, IL-5, and IL-13) and a chemokine (eotaxin), in a process mediated by NF- κ B. These cytokines and the chemokine are important for recruitment and activation of eosinophils and leukocytes [4,30]. We thus suggest that observed decrease in NF- κ B activity may explain the therapeutic effect of astilbic acid.

In summary, astilbic acid effectively suppressed cytokine and IgE expression, and inhibited both the recruitment of eosinophils and mucus overproduction, in mice with OVA-induced asthma. Moreover, airway inflammation and bronchial hyperresponsiveness were attenuated in

the presence of astilbic acid. The compound inhibits NF- κ B activity, in turn reducing inflammatory cytokine and chemokine levels. Our findings thus support the possible use of astilbic acid as a therapeutic drug for patients with allergic asthma.

Acknowledgments

This work was supported by a grant (no. KGM5231011) from the Ministry of Education, Science and Technology of the Republic of Korea.

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