



Anti-asthmatic effect of schizandrin on OVA-induced airway inflammation in a murine asthma model

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ABSTRACT

Asthma comprises a triad of reversible airway obstruction, bronchial smooth muscle cell hyperreactivity to bronchoconstrictors, and chronic bronchial inflammation. Clinical and experimental findings have established eosinophilia as a sign of allergic disorders. In the present investigation, we evaluated the anti-asthmatic effects of schizandrin and its underlying mechanisms in an *in vivo* murine asthmatic model. To accomplish this, female BALB/c mice were sensitized and challenged with ovalbumin (OVA), and examined for the following typical asthmatic reactions: increased numbers of eosinophils and other inflammatory cells in bronchoalveolar lavage fluid (BALF); production of Th1 cytokines (such as tumor necrosis factor (TNF)- α in BALF); production of Th2 cytokines (such as interleukin IL-4 and IL-5) in BALF; presence of total and OVA-specific immunoglobulins (Ig)E in serum; presence of oxidative stress; hyperplasia of goblet cells in the lung; and marked influx of inflammatory cells into the lung. Our results collectively show that schizandrin exerts profound inhibitory effects on accumulation of eosinophils into the airways and reduces the levels of IL-4, IL-5, IFN- γ , and TNF- α in BALF. Additionally, schizandrin suppresses the production of reactive oxygen species (ROS) in a dose-dependent manner, and inhibits goblet cell hyperplasia and inflammatory cell infiltration in lung tissue. Thus, schizandrin has anti-asthmatic effects, which seem to be partially mediated by reduction of oxidative stress and airway inflammation, in a murine allergic asthma model. These results indicate that schizandrin may be an effective novel therapeutic agent for the treatment of allergic asthma.

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1. Introduction

Allergen-induced bronchial asthma is a complex inflammatory disorder characterized by airway hyperresponsiveness (AHR), eosinophilia, and mucus hypersecretion by goblet cells. Numerous eosinophil- and T helper 2 (Th2) lymphocyte-induced cytokines, including interleukin IL-4, IL-5, and IL-13, play key roles in AHR and serum immunoglobulin (Ig)E in animal models via Th2 cell production [1,2]. Th2-type cytokines, including IL-4, IL-5, and IL-13 produced by activated CD4⁺ T-cells, play a central role in the pathogenesis of asthma [3] by controlling the key processes of immunoglobulin E (IgE) production, growth of mast cells, and differentiation and activation of mast cells and eosinophils [4].

TNF- α is produced by various cell types in response to allergic pulmonary inflammation, including mast cells, macrophages, neutrophils, eosinophils, and epithelial cells [5]. In particular, mast cells are an important potential source of TNF- α [6].

ROS generation is not only associated with intracellular signaling but also reproduces many of the pathophysiologic features associated with asthma by altering the organization and function of cell membranes and increasing airway reactivity, airway secretions, vascular permeability, and release of chemoattractants [7,8]. Accordingly, determining protective mechanisms against ROS activity in conjunction with development of agents with the ability to protect macrophages from ROS insults are important issues of focus in asthma treatment. Several drugs for asthma therapy are currently available. However, most of these drugs are inefficient and have associated with adverse effects. Consequently, there is an urgent need for novel anti-asthma drugs associated with fewer adverse effects. Schizandrin, one of the lignan components of *Schisandra chinensis* Baill, is widely used to treat hepatitis. Schizandrin has several biological properties [9], including hepatoprotective [10], anti-inflammatory [11], antitumor [12], and anti-amnesic activities [13], a suppressive effect against lipid peroxidation, and potentiation of glutathione mediated antioxidation [14]. Several biological activities of schizandrin have been reported to date, but the mechanism underlying its anti-asthmatic activity remains to be determined. The present study was designed to determine the anti-asthmatic mechanism of schizandrin.

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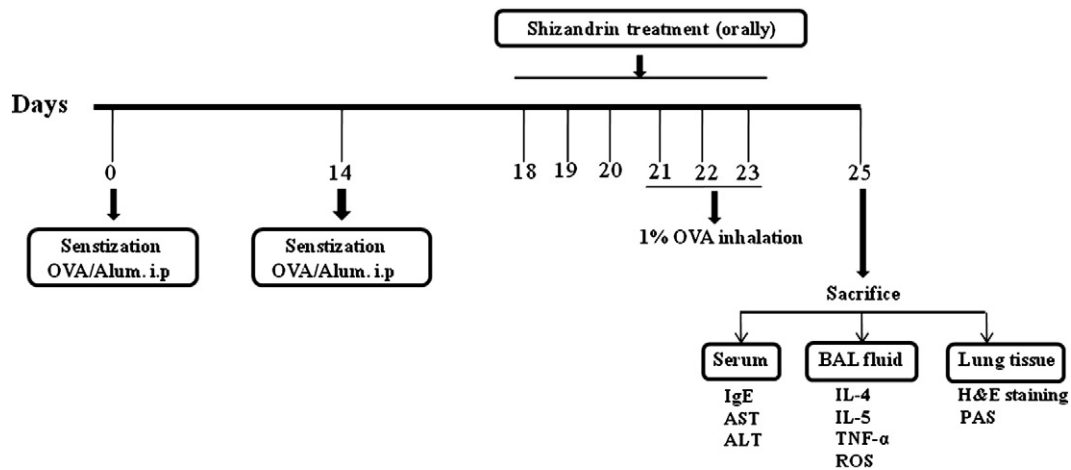


Fig. 1. Mouse model of airway inflammation and treatment with schizandrin.

2. Methods and Materials

2.1. Animals

Specific 7 week-old pathogen-free (SPF) inbred female BALB/c mice routinely screened serologically for relevant respiratory pathogens were purchased from Daehan Biolink Co. Ltd. (Seoul, Korea). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments, and provided water and standard chow *ad libitum*. All experimental procedures were performed 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.

2.2. Sensitization and airway challenge

Mice were divided into groups ($n = 5$ or 6) receiving the following treatments: (1) sham sensitization plus challenge with phosphate-buffered saline (PBS; ipNeb), (2) sensitization plus challenge with OVA (Sigma A5503; Sigma, St. Louis, MO) (ipNeb), and (3) sensitization with OVA (i.p.) plus challenge with OVA (Neb) and schizandrin (20 or 30 mg/kg) or Montelukast (30 mg/kg) p.o. Briefly, mice were sensitized with an intraperitoneal injection of 20 μ g OVA emulsified in 2 mg aluminum hydroxide in 200 μ L PBS buffer (pH 7.4) on days 0 and 14. Mice were challenged through the airway with OVA (1%, w/v, in PBS) for 30 min using an ultrasonic nebulizer (NE-U12; Omron Co., Tokyo, Japan) on days 21, 22 and 23 after initial sensitization. Animals were sacrificed 48 h after the final challenge to characterize the suppressive effects of schizandrin. Montelukast was developed as a cysteinyl leukotriene (cys-LT)-1 receptor antagonist [28], in which the clinical responses in patients with aspirin-sensitive asthma, nocturnal exacerbations of asthma and allergic asthma led to montelukast being successfully introduced into the market [29]. A schematic diagram of the treatment schedule is shown in Fig. 1.

2.3. Inflammatory cell counts in bronchoalveolar lavage fluid (BALF)

Mice were sacrificed with an overdose (50 mg/kg) of pentobarbital 48 h after the last challenge, and tracheostomy performed. Following instillation of ice-cold PBS (0.6 mL) into the lungs, BALF was obtained with three aspirations (total volume 1.8 mL) via tracheal cannulation. BALF was centrifuged at 200 g for 10 min, and the supernatant fractions collected and stored at -70°C . The total inflammatory cell number was assessed by counting cells in at least five squares of a hemocytometer after exclusion of dead cells via trypan blue staining. BALF (100 μ L) was pipetted on a slide and centrifuged (200 g , 4°C , 10 min) to fix cells using

a cytospin machine (Hanil Science Industrial, Seoul, Korea). Cell pellets was suspended in 0.5 mL PBS, and 100 μ L of each solution spun onto a slide. After slides were dried, cells were fixed and stained using Diff-Quik® Staining reagents (B4132-1A; Dade Behring Inc., Deerfield, IL), according to the manufacturer's instructions.

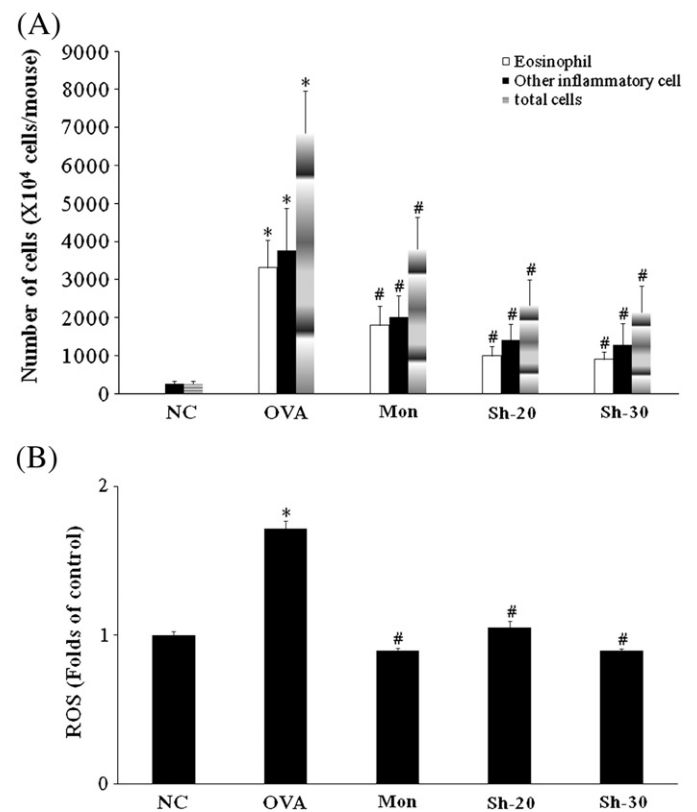


Fig. 2. Effects of schizandrin on recruitment of inflammatory cells and reactive oxygen species (ROS) in bronchoalveolar lavage fluid (BALF) of mice 48 h after the final OVA challenge. (A) Cells were isolated by centrifugation and stained with Diff-Quik® Stain reagent. Cell numbers were determined by counting within at least five squares of a hemocytometer under a light microscope. Dead cells, stained with trypan blue, were excluded from the total cell number. (B) Oxidative stress was quantified in BALF, based on DCF fluorescence. Values represent relative fluorescence units per milligram protein. NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; schizandrin-20, schizandrin (20 mg/kg) + OVA-sensitized/challenged mice; schizandrin-30, schizandrin (30 mg/kg) + OVA-sensitized/challenged mice. Schizandrin or montelukast treatment was performed 1 h before challenge. *Significant difference from NC, $P < 0.05$, and #significant difference from OVA, $P < 0.05$.

2.4. The 2',7'-dichlorofluorescein (DCF) fluorescence assay

Induction of oxidative stress was monitored using 2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR), which is converted into highly fluorescent DCF by cellular peroxides, including hydrogen peroxide. In brief, BALF was treated with 25 μ M DCF-DA for 10 min at 37 °C. Intracellular ROS activity was measured by determining fluorescence at 488 nm excitation and 525 nm emission using a fluorescence plate reader (Perkin-Elmer, Waltham, MA).

2.5. Measurement of total and OVA-specific IgE in serum

Serum were collected and stored at -70°C after centrifugation (3000 rpm, 10 min). Total and OVA-specific IgE levels were measured using enzyme-linked immunosorbent assays (ELISA). Microtiter plates were coated with 100 μ L/well IgE (anti-mouse IgE; 10 μ g/mL; Serotec, Oxford, UK) conjugated to HRP (Bethyl Laboratories, TX, USA) in PBS-Tween 20. Antibodies in serum were detected using isotype-specific secondary antibodies (anti-mouse IgE; Serotec, Oxford, UK). After washing four times, 200 μ L *o*-phenylenediamine dihydrochloride (Sigma, St Louis, MO) was added to each well. The plate was incubated for 10 min in the dark and absorbance measured at 450 nm.

2.6. Measurement of cytokine levels in BALF

The concentrations of IFN- γ , TNF- α , IL-4, and IL-5 (BioSource International, Camarillo, CA) in BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions.

2.7. Lung tissue histopathology

After BALF was obtained, lung tissue was fixed in 10% (v/v) neutral buffered formalin for 24 h at 4 °C. Tissues were embedded in paraffin, sectioned at 4 μ m thickness, and stained with H&E solution (hematoxylin, Sigma MHS-16, and eosin, Sigma HT110-1-32) and periodic acid-Schiff (PAS) (IMEB Inc., San Marcos, CA) to measure mucus production. Tissues were subsequently mounted and coverslipped using Dako mounting medium (Invitrogen corporation, Camarillo, CA).

2.8. Measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum

ALT and AST levels were determined to assess liver and kidney function using commercial kits (BECKMAN coulter, Inc., Fullerton, CA, USA) and an auto-analyzer (BECKMAN CX4, CA, USA).

2.9. Image capture and photomicrography

Photomicrographs were obtained using a Photometric Quantix digital camera running a Windows program, and montages assembled in Adobe Photoshop 7.0. Images were cropped and corrected for brightness and contrast, but not otherwise manipulated.

2.10. Statistical analysis

Data are expressed as means $\pm$ standard deviations (SD). Statistical comparisons were made with one-way analysis of variance (ANOVA). *P* values <0.05 were considered statistically significant.

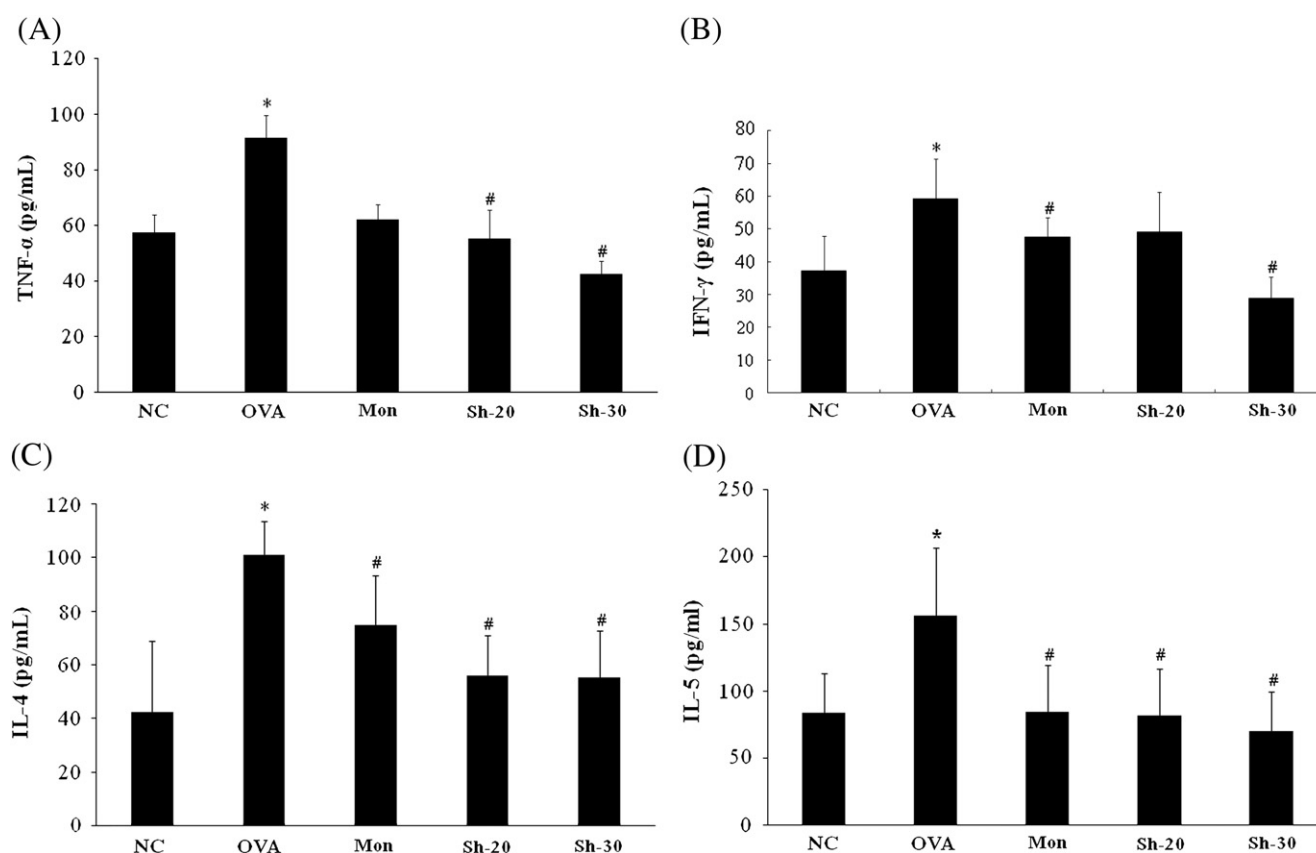


Fig. 3. Effects of schizandrin on cytokine levels in BALF and lung tissue. BALF and lung tissue were collected 48 h after the last OVA challenge of mice. Individual samples were analyzed using ELISA. (A) TNF- α level in BALF; (B) IFN- γ level in BALF; (C) IL-4 level in BALF; and (D) IL-5 level in BALF. NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; schizandrin-20, schizandrin (20 mg/kg) + OVA-sensitized/challenged mice; schizandrin-30, schizandrin (30 mg/kg) + OVA-sensitized/challenged mice. Schizandrin or montelukast treatment was performed 1 h before challenge. *Significant difference from NC, $P < 0.05$, and #significant difference from OVA, $P < 0.05$.

3. Results

3.1. Effects of schizandrin on eosinophilia and ROS generation in BALF

To evaluate the effects of schizandrin on eosinophilia in OVA-challenged mice, cells recruited to BALF were counted 48 h after the final challenge. In PBS-challenged mice, total cell and eosinophil numbers in BALF remained relatively unchanged. Interestingly, the numbers of total cells, eosinophils and other inflammatory cells in BALF were significantly increased in OVA-challenged mice compared with PBS-challenged mice. Administration of schizandrin at doses of 20 and 30 mg/kg 1 h before OVA challenge significantly suppressed the eosinophil and total inflammatory cell numbers, compared with those in OVA-challenged mice in BALF, in a dose-dependent manner (Fig. 1A). ROS levels in BALF were markedly increased in OVA-

challenged mice, as shown in Fig. 2B. However, pre-administration of schizandrin led to a significant dose-dependent decrease in the ROS level (Fig. 2B).

3.2. Effects of schizandrin on cytokine levels in BALF

To determine the possible inhibitory mechanisms of schizandrin action in airway inflammation, we examined the Th1 and Th2 cytokine levels associated with asthma in BALF. TNF- α , IL-4 and IL-5 levels in BALF were markedly increased in OVA-sensitized/challenged mice compared with those in PBS-challenged mice. Administration of schizandrin (20 and 30 mg/kg) led to significant suppression of the levels of IFN- γ , TNF- α (Fig. 3A and B) and Th2 cytokines (IL-4 and IL-5) (Fig. 3C and D) in BALF in relation to those in the OVA-sensitized/challenged group.

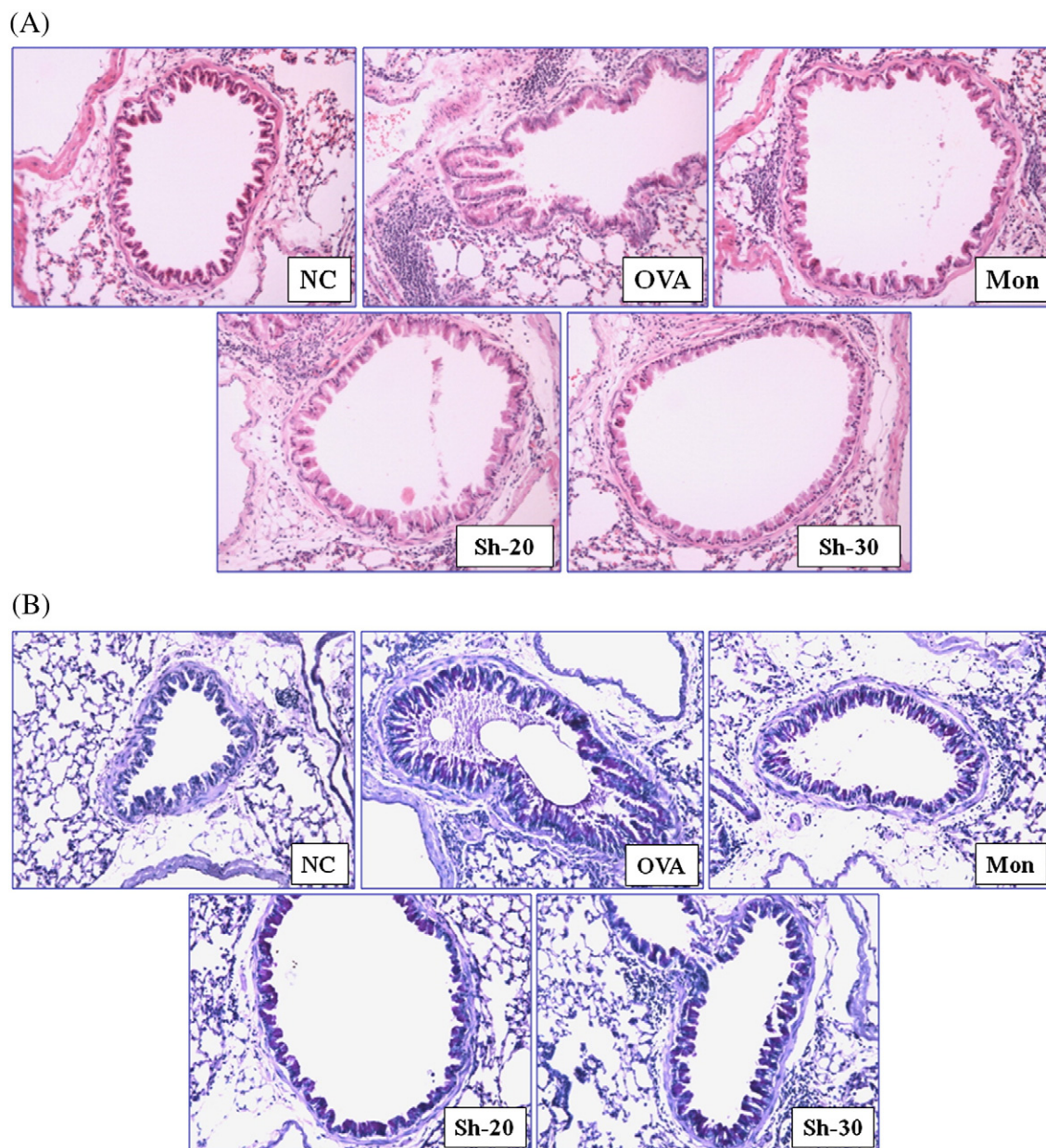


Fig. 4. Effects of schizandrin on recruitment of leukocytes and mucus production in lung tissue. Histological examination of lung tissue was performed 48 h after the final OVA challenge. Lung tissues were fixed, sectioned at 4 μ m thickness, and stained with H&E solution (A) and periodic acid-Schiff (PAS) (B). (Magnification $\times 200$). NC, normal control mice (PBS only); OVA, OVA-sensitized/challenged mice; schizandrin-20, schizandrin (20 mg/kg) + OVA-sensitized/challenged mice; schizandrin-30, schizandrin (30 mg/kg) + OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + OVA-sensitized/challenged mice. Schizandrin or montelukast treatment was performed 1 h before challenge.

3.3. Effects of schizandrin on inflammatory cell accumulation and goblet cell hyperplasia in lung tissue

The anti-inflammatory effects of schizandrin were illustrated by histological examination. Inflammatory cells were not detected in the peribronchial/peribronchiolar lung areas of PBS-challenged mice. In contrast, OVA-challenged mice displayed severe infiltration of leukocytes in the peribronchiole and perivascular connective tissue. Treatment with 20 or 30 mg/kg schizandrin led to a significant reduction in the inflammatory cell number (Fig. 4A). To evaluate airway hypersecretion of mucus and goblet cell hyperplasia, we stained lung sections with PAS. OVA-challenged mice induced significant goblet cell hyperplasia in the airway (mucus overproduction was clearly observed as a violet color) compared with PBS-challenged mice. Treatment with schizandrin suppressed mucus hypersecretion in lung tissue in a dose-dependent manner. This finding is consistent with the results presented in Fig. 4A, which indicate that schizandrin inhibits airway inflammation in asthma (Fig. 4B).

3.4. Effects of schizandrin on IgE levels

Levels of total IgE were determined using ELISA. We observed substantial increases in total and OVA-specific IgE in serum from OVA-sensitized and -challenged mice compared with PBS-challenged mice. Schizandrin significantly reduced the total and OVA-specific IgE level in a dose-dependent manner in serum (Fig. 5).

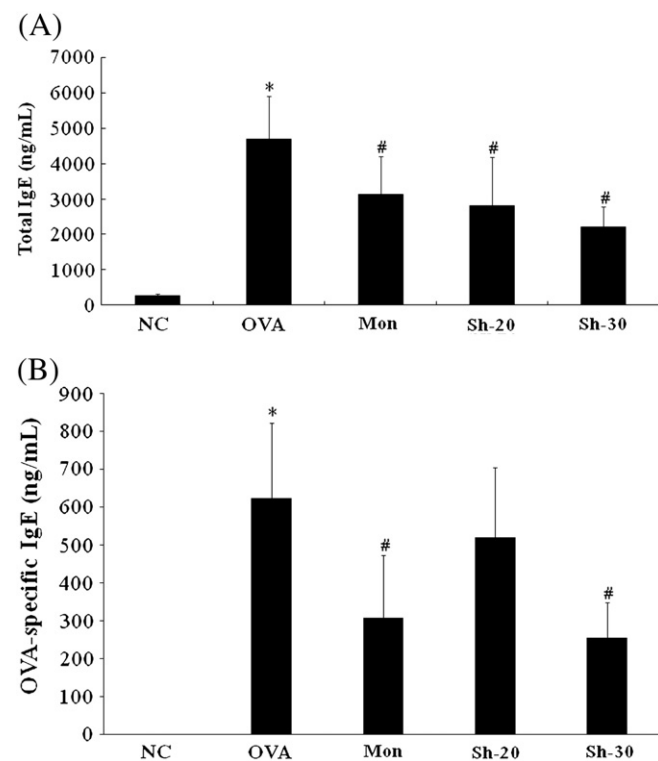


Fig. 5. Effects of schizandrin on total and OVA-specific IgE levels in serum. Serum was collected 48 h after the final OVA challenge in mice. Each sample was analyzed using ELISA. (A) Total IgE level (B) OVA-specific IgE level. NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; schizandrin-20, schizandrin (20 mg/kg) + OVA-sensitized/challenged mice; schizandrin-30, schizandrin (30 mg/kg) + OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + OVA-sensitized/challenged mice. Schizandrin or montelukast treatment was performed 1 h before challenge. *Significant difference from NC, $P < 0.05$, and #significant difference from OVA, $P < 0.05$.

3.5. Cytotoxicity of schizandrin in a mouse model of allergic asthma

Schizandrin (20 and 30 mg/kg) did not promote activities of the serum toxicity marker enzymes (ALT and AST), indicative of normal liver function (Fig. 6).

4. Discussion

Asthma is a chronic disorder of the airways characterized by reversible and intermittent airway obstruction, airway inflammation, and hyperreactivity of the airways in response to a variety of stimuli, including dust, animal hair, smoke, and airborne pollutants. Despite important advances in diagnosis and treatment, asthma remains one of the most prevalent chronic respiratory disorders, affecting 7–10% of the world's population. In the present study, we show that schizandrin significantly inhibits increases in total inflammatory cell and eosinophil numbers in BALF induced by OVA, reduces the levels of total and OVA-specific IgE, IL-4, and IL-5 in serum and BALF, and substantially inhibits both OVA-challenged eosinophilia in lung tissue and mucus hypersecretion by airway goblet cells. In addition, schizandrin suppresses ROS activity in BALF. Inflammatory cells recruited to asthmatic airways have exceptional ROS-producing capability.

At the sites of inflammation, inflammatory cells, including eosinophils, neutrophils, and macrophages, are capable of generating ROS, which participate in the development of a variety of diseases, including allergic asthma [15,16]. ROS may thus contribute to tissue injury and inflammatory reactions. Consistent with these findings, our present data show that ROS generation in BALF, which mainly consists of recruited inflammatory cells, is significantly increased in the OVA-challenged group. Moreover, this increase in ROS generation is substantially suppressed by schizandrin.

Several studies over the past decade have shown that Th2-driven cytokines are involved in the development of asthma. Moreover, CD4⁺ T-cells in asthma patients produce Th2 cytokines, such as IL-4, IL-5, IL-9 and IL-13, identified in BALF and biopsies [17]. Blockade of the IL-4 receptor in mice sensitized and challenged with antigen markedly decreases airway goblet cell metaplasia. Specifically, in the absence of IL-4R alpha in recipient mice, no goblet cell metaplasia or mucus hypersecretion is evident in response to OVA, even in the presence of Th2 cells and substantial eosinophilic infiltration [18]. However, IL-4 plays an indirect role in mucus production through recruitment of Th2 cells to the lungs and induction of inflammation. In addition to its function in the activation of mature eosinophils, IL-5 is the main Th2 cytokine involved in the terminal differentiation and proliferation of eosinophil precursors [19].

TNF- α , initially identified in macrophages and monocytes [20], is an important cytokine in the innate immune response that plays a key

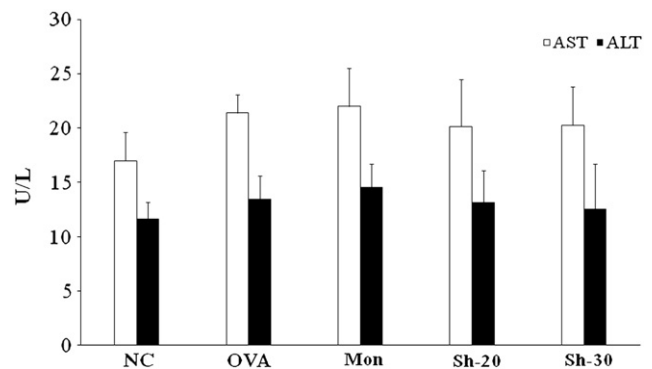


Fig. 6. Levels of AST and ALT in sera. Serum was collected 48 h after the final OVA challenge of mice. Each sample was analyzed using commercial kits (Beckman Coulter, Inc., Fullerton, CA). Schizandrin or montelukast treatment was performed 1 h before challenge.

role in the immediate host defense against invading microorganisms before activation of the adaptive immune system [21]. TNF- α has also been implicated in the pathophysiologic mechanisms of several chronic inflammatory diseases, including Crohn's disease, rheumatoid arthritis, and psoriatic arthritis [22]. Thus, in this study, blockade of TNF- α by a schizandrin is an accepted strategy used to treat human diseases involving chronic inflammation.

The presence of increased numbers of eosinophils in BALF is a characteristic of asthma. Compared with PBS-treated mice, schizandrin-treated mice displayed significantly reduced eosinophilia in BALF and lung tissue. This effect of schizandrin may result from a decrease in the levels of Th2 cytokines responsible for eosinophil recruitment into the lung in asthma. Th2 lymphocytes play an important role in the initiation and progression of allergic diseases, including asthma, by releasing IL-4, IL-5, and IL-13 [23]. These Th2 cytokines induce inflammatory responses, such as airway infiltration, eosinophil activation, IgE production, and mucus secretion [24]. Histopathological findings reveal that infiltrated inflammatory cells or mucus generation of goblet cells induced by airway challenge with OVA is markedly attenuated upon schizandrin administration. Our findings indicate that mucus hypersecretion is attenuated by the suppressive effects of schizandrin on cytokine production and eosinophilia. Although Th2 cytokines, including IL-4, IL-5 and IL-13, are important in asthma [25], TNF- α has been implicated in the inflammatory response seen in asthma [26]. TNF- α is a multifunctional proinflammatory cytokine and a chemoattractant for neutrophils and eosinophils [27]. In our experiments, schizandrin induced a significant decrease in the levels of total IgE, IFN- γ , TNF- α , IL-4 and IL-5 in serum and BALF in an OVA-induced asthmatic mouse model. IgE is closely associated with Th2-type responses. Oral administration of schizandrin to OVA-sensitized/challenged mice led to a marked decrease in the serum IgE level. These effects may be related to inhibition of Th2-related IL-4 and IL-5 synthesis, and thus a decrease in the levels of Th2 cytokines responsible for eosinophil recruitment into the lung in asthma.

In conclusion, schizandrin significantly inhibits asthmatic reactions, such as leukocyte recruitment and Th1- and Th2-type cytokine levels in BALF, and reduces total IgE levels in the serum. Furthermore, schizandrin suppresses ROS generation in the OVA-induced airway inflammation reaction. These results provide further evidence that oral administration of schizandrin is suitable for the treatment of allergic diseases such as asthma.

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