

Inhibitory effects of astragaloside IV on ovalbumin-induced chronic experimental asthma

Qiang Du, Zhen Chen, Lin-fu Zhou, Qian Zhang, Mao Huang, and Kai-sheng Yin

Abstract: Astragaloside IV, a new cycloartane-type triterpene glycoside extract of *Astragalus membranaceus* Bunge, has been identified for its potent immunoregulatory, antiinflammatory, and antifibrotic actions. Here we investigated whether astragaloside IV could suppress the progression of airway inflammation, airway hyperresponsiveness, and airway remodeling in a murine model of chronic asthma. BALB/c mice sensitized to ovalbumin (OVA) were chronically challenged with aerosolized OVA for 8 weeks. Astragaloside IV was orally administered at a dose of 50 mg·kg⁻¹·day⁻¹ during each OVA challenge. Astragaloside IV treatment resulted in significant reduction of eosinophilic airway inflammation, airway hyperresponsiveness, interleukin (IL)-4 and IL-13 levels in bronchoalveolar lavage fluid, and total immunoglobulin E levels in serum. Furthermore, astragaloside IV treatment markedly inhibited airway remodeling, including subepithelial fibrosis, smooth muscle hypertrophy, and goblet cell hyperplasia. In addition, the expression of transforming growth factor- β 1 in the lung was also reduced by astragaloside IV. These data indicate that astragaloside IV may mitigate the development of characteristic features in chronic experimental asthma.

Key words: *Astragalus membranaceus*, astragaloside IV, airway remodeling, chronic asthma.

Résumé : L'astragaloside IV, un nouvel extrait glycoside triterpénique de type cycloartane de *Astragalus membranaceus* Bunge, est connu pour ses puissantes actions immunorégulatrices, anti-inflammatoires et antifibrosantes. Ici, nous avons examiné si l'astragaloside IV peut supprimer la progression de l'inflammation bronchique, l'hyperréactivité bronchique et le remodelage bronchique dans un modèle murin d'asthme chronique. Des souris BALB/c sensibilisées à l'ovalbumine (OVA) ont été soumises à une provocation chronique à l'OVA en aérosol pendant 8 semaines. L'astragaloside IV a été administré par voie orale à une dose de 50 mg·kg⁻¹·jour⁻¹ durant chaque provocation. Le traitement à l'astragaloside IV a entraîné une réduction significative de l'inflammation bronchique éosinophilique, de l'HB, des taux d'interleukine (IL)-4 et IL-13 dans le liquide de lavage bronchoalvéolaire et des taux d'immunoglobuline E totale dans le sérum. De plus, le traitement a inhibé significativement le remodelage bronchique, y compris la fibrose sous-épithéliale, l'hypertrophie des muscles lisses et l'hyperplasie des cellules caliciformes. L'astragaloside IV a aussi réduit l'expression du facteur de croissance transformant- β 1 dans le poumon. Ces résultats indiquent que l'astragaloside IV pourrait atténuer l'apparition d'éléments caractéristiques dans l'asthme expérimental chronique.

Mots-clés : *Astragalus membranaceus*, astragaloside IV, remodelage bronchique, asthme chronique.

[Traduit par la Rédaction]

Introduction

Bronchial asthma is an allergic inflammatory disease marked by chronic airway inflammation, airway hyperresponsiveness, reversible airway obstruction, mucus hypersecretion, and airway remodeling (Bousquet et al. 2000; Zhou et al. 2006). In turn, airway remodeling is characterized by structural and morphometric changes, such as subepithelial fibrosis, hyperplasia of mucus glands, myofibroblast and smooth muscle proliferation, and vascular changes (Elias et al. 1999). These structural changes in the airway, occurring in response to persistent inflammation, are responsible for the airway thickening,

airway flow limitation, and airway hyperresponsiveness seen in patients with severe asthma (Benayoun et al. 2003).

Current treatment guidelines for asthma emphasize anti-inflammatory therapy, and glucocorticoids remain the first-line therapeutic agents. Indeed, glucocorticoids are effective in preventing airway inflammation, but not in eliminating the progression of airway remodeling changes (Tang et al. 2006). Patients with chronic asthma who use inhaled glucocorticoids may develop serious side effects, particularly over the long term (Irwin and Richardson 2006). Moreover, few asthmatic patients achieve a normal bronchial reactivity, even after years of steroid therapy. Thus, new or alternative immunotherapeutic agents are clearly needed to manage this disease.

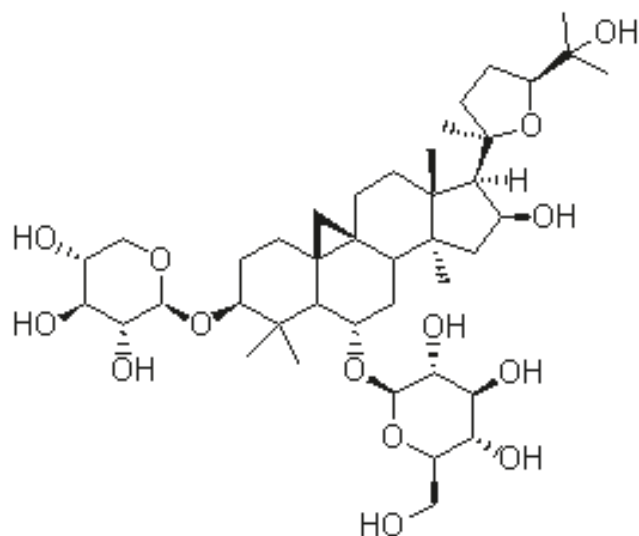
Astragalus membranaceus Bunge, a member of the family Fabaceae and a traditional Chinese medicinal herb, has been widely used for centuries to treat asthma in China. Astragaloside IV (3-O- β -D-xylopyranosyl-6-O- β -D-glucopyranosyl-cycloastragenol) (Fig. 1), the primary pure saponin isolated from the root of *A. membranaceus*, is an effective compound with distinct pharmacological effects,

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Fig. 1. Chemical structure of astragaloside IV, a cycloartane-type triterpene glycoside extract from the roots of *Astragalus membranaceus* Bunge.



including antiinflammation, immunoregulation (Wang et al. 2002; Zhang et al. 2003), and antifibrosis (Yu et al. 2004; Zhang et al. 2007; Gui et al. 2006). Previous studies have shown that astragaloside IV could be used for the treatment of diabetes (Yu et al. 2006), cardiovascular disease (Xu et al. 2007; Zhang et al. 2006), and ischemic brain injury (Luo et al. 2004). It is unclear, however, whether astragaloside IV can improve the airway inflammation, hyperresponsiveness, and remodeling observed in asthma. Here we sought to evaluate the potential effects of astragaloside IV on allergen-induced chronic experimental asthma.

Materials and methods

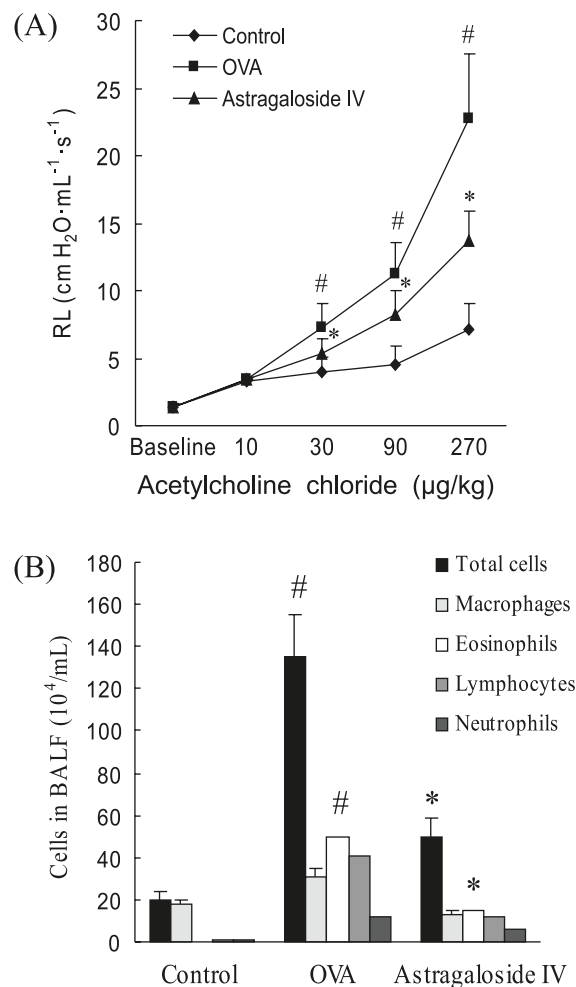
Chemicals

Astragaloside IV, extracted from *A. membranaceus*, was provided by the Department of Pharmacology, Nanjing Medical University (purity >98% by HPLC method) (Yu et al. 2006). Astragaloside IV was suspended in a 1% carboxymethylcellulose (CMC) solution at a concentration of 0.5% and administered orally.

Antigen sensitization, challenge, and treatment

Eighteen female BALB/c mice (6–8 weeks old) were purchased from Shanghai Laboratory Animals (Shanghai, China). The guidelines for the care and use of laboratory animals of Nanjing Medical University were followed, and the experimental protocol was approved by the institutional animal ethics committee. Mice were randomly divided into 3 groups of 6 animals each: control, ovalbumin (OVA), and astragaloside IV. Mice in the OVA and astragaloside IV groups were sensitized on days 1 and 14 by intraperitoneal injection of 100 µg OVA (grade V) (Sigma, St. Louis, Mo.) emulsified in 1 mg aluminum hydroxide in a total volume of 0.2 mL. One week after the last sensitization, mice were exposed to aerosolized 1% OVA for 30 min/day, 3 days/week, for 8 weeks. Mice in the astragaloside IV group were also administered intragastrically with 0.5% astragaloside IV at a

Fig. 2. Treatment with astragaloside IV decreased the development of airway hyperreactivity and the accumulation of inflammatory cells in BALF in a murine model of chronic asthma. Airway responsiveness (A) and inflammatory cell accumulation (B) in BALF were determined as described in the Methods. Data are means $\pm$ SE, $n = 6$ per group. Significant at *, $p < 0.05$ vs. OVA group. #, $p < 0.01$ vs. control group. RL, lung resistance; BALF, bronchoalveolar lavage fluid; OVA, ovalbumin.

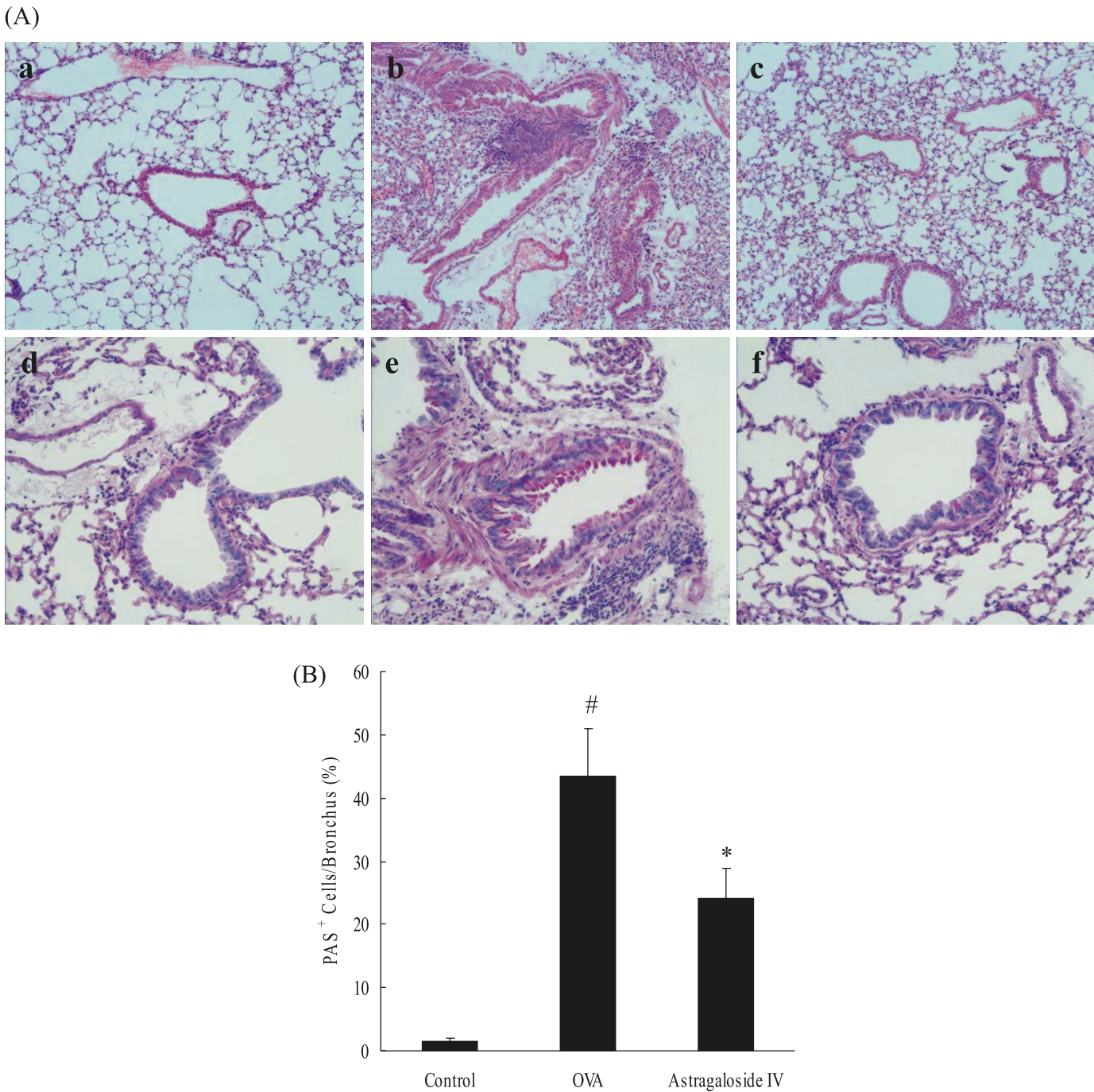


dose of 50 mg/kg (Wang et al. 2002; Luo et al. 2004) daily for 8 consecutive weeks after each airway challenge. For aerosolized challenges, mice were placed in a Plexiglas box (29 cm $\times$ 22 cm $\times$ 8 cm) and subjected to aerosolized OVA with an ultrasonic nebulizer (NE-U11B, Omuron, Tokyo, Japan). Mice in the control group were administered intragastrically with 1% CMC (vehicle) in a volume of 10 mL/kg.

Evaluation of airway responsiveness

Airway responsiveness to acetylcholine chloride was measured with AniRes 2005 animal lung function analysis systems (SYNOL High-Tech, Beijing, China). The mice were anaesthetised by intraperitoneal injection of pentobarbital sodium (70 mg/kg) 24 h after the last OVA challenge. A plastic tube of 2 mm internal diameter was inserted into the trachea via tracheotomy for mechanical ventilation, and a 27-gauge needle was inserted into the cau-

Fig. 3. Astragaloside IV attenuated chronic airway inflammation and antigen-induced mucus production in a murine model of chronic asthma. (A) Top row: hematoxylin and eosin staining (original magnification $\times 100$): (a) control group, (b) ovalbumin (OVA) group, and (c) astragaloside IV group. Bottom row: periodic acid–Schiff (PAS) staining (original magnification $\times 200$): (d) control group, (e) OVA group, and (f) astragaloside IV group. (B) The percentage of PAS-positive cells per bronchiole was determined as described in the Methods, $n = 6$ in each group. Significant at *, $p < 0.05$ vs. OVA group. #, $p < 0.01$ vs. control group.



dalys vein for drug administration. The mice were then placed in a whole-body plethysmography chamber and ventilated mechanically at a rate of 90 breaths/min with a tidal volume of 6 mL/kg. After establishment of a stable airway pressure recording, acetylcholine chloride was administered intravenously with a microinfusion pump in progressive doses (10, 30, 90, and 270 μ g/kg). After each dose, the data

were collected continuously from 5 s to 1 min, and maximal values of lung resistance (RL) were taken to express changes in murine airway function (Rui et al. 2006; Xu et al. 2005).

Analysis of BALF and serum

After measurement of airway hyperresponsiveness, the

Table 1. Levels of IL-4 and IL-13 in BALF and total serum IgE in 3 groups of mice.

Groups	BALF, pg/mL		Serum, ng/mL
	IL-4	IL-13	Total IgE
Control	21.97±4.29	11.02±2.84	120.75±33.15
OVA	123.81±28.38 [#]	45.43±7.04 [#]	656.84±73.14 [#]
Astragaloside IV	49.87±8.42 [*]	23.67±5.03 [*]	223.67±69.87 [*]

Note: Treatment with astragaloside IV reduced the levels of Th2-type cytokine IL-4 and IL-13 in BALF and lowered the total serum IgE in a murine model of chronic asthma. Data are means ± SE, *n* = 6 per group. Significant at ^{*}, *p* < 0.05 vs. OVA group. [#], *p* < 0.01 vs. control group. Th2, T helper type 2 cells; IL, interleukin; BALF, bronchoalveolar lavage fluid; IgE, immunoglobulin E; OVA, ovalbumin.

mice were bled by retroorbital puncture using heparinized capillary tubes. Blood samples were centrifuged, and plasma was stored at -70 °C until use. The lungs were washed 3 times with 0.4 mL saline to collect bronchoalveolar lavage fluid (BALF). The BALF was centrifuged (10 min, 4 °C, 1000g), and the total number of inflammatory cells in BALF was counted with a haemocytometer. Differential cell counts were conducted by using cytospin techniques and Wright's staining, and by counting at least 200 cells per slide. Interleukin (IL)-4 and IL-13 levels in BALF and total immunoglobulin E (IgE) in serum were determined by ELISA according to the manufacturer's protocol. The limits of detection were 4 pg/mL, 7 pg/mL, and 1 ng/mL for IL-13 (R&D Systems, Minneapolis, Minn.), IL-4 (Jingmei Biotech, Shanghai, China), and total serum IgE (Bionewtrans Pharmaceutical Biotechnology, Franklin, Mass.), respectively.

Histological examination

The left lungs from euthanized mice were immersed in 10% formaldehyde overnight, then embedded in paraffin. Serial sections 5 µm thick were either stained with hematoxylin and eosin, periodic acid-Schiff (PAS), or Masson's trichrome, or they were processed for immunohistochemistry. Histological analyses were performed by pathologists blinded to the treatment groups. The area of peribronchial Masson's trichrome staining in a paraffin-embedded lung was outlined and quantified with a light microscope attached to an Image-Pro Plus image-analysis system (Media Cybernetics, Silver Spring, Md.). Results were expressed as the area of trichrome staining per micrometre length of bronchiolar basement membrane with 150–200 µm internal diameter by counting at least 10 bronchioles in each slide. To quantitate the level of mucus expression in airway, the number of PAS-negative and PAS-positive epithelial cells (goblet cells) in individual bronchioles was counted as described (Cho et al. 2004). At least 10 bronchioles were counted in each slide. Results were expressed as the percentage of PAS-positive cells per bronchiole, which was calculated from the number of PAS-positive epithelial cells per bronchus divided by the total number of epithelial cells of each bronchiole.

Immunohistochemistry for α-SMA and TGF-β1

The sections of the left lung were deparaffinized in xylene and rehydrated in graduated alcohol solutions. Endo-

genous peroxidase activity was blocked by incubation with 3% H₂O₂. To prevent nonspecific absorption of immunoglobulin, specimens were flooded with 5% normal goat serum. Thereafter, specimens were incubated for 90 min with mouse anti-human α-smooth muscle actin (α-SMA) monoclonal antibody (DAKO, Grostrup, Denmark) at a dilution of 1:50 and rabbit anti-mouse transforming growth factor (TGF)-β1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) at a dilution 1:50. Substitution of each primary antibody with PBS was used as a negative control. The slides were incubated overnight at 4 °C, rinsed 3 times with PBS, then incubated with peroxidase-labeled goat anti-mouse IgG or goat anti-rabbit IgG for 30 min at 37 °C, and then washed again with PBS followed by diaminobenzidine (DAB) stain. The sections were counterstained with hematoxylin, dehydrated, and observed under light microscope. The area of α-SMA staining in a paraffin-embedded lung was outlined and quantified with a light microscope attached to the Image-Pro Plus image-analysis system. Results were expressed as the area of α-SMA immunostaining per micrometre length of bronchiolar basement membrane with 150–200 µm internal diameter by counting at least 10 bronchioles in each slide. The number of peribronchial cells expressing TGF-β1 was evaluated as described previously (Miller et al. 2006).

Measurement of hydroxyproline content

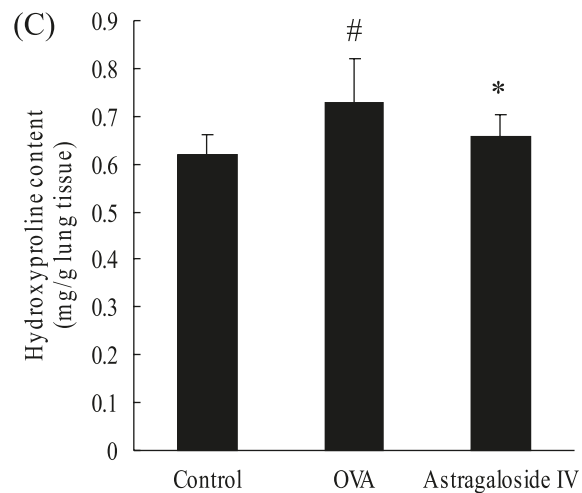
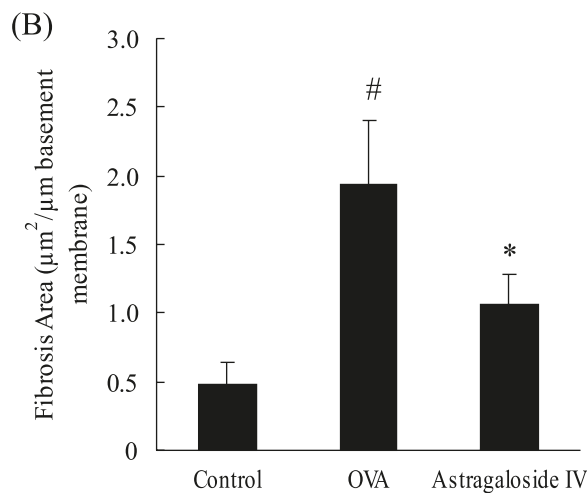
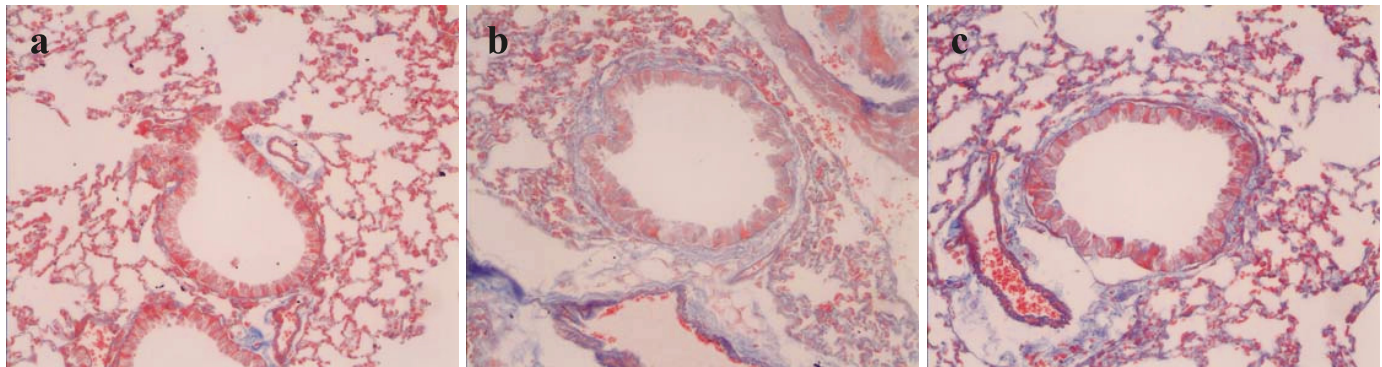
Total collagen content of the right lung was determined by hydroxyproline assay. The hydroxyproline content was detected according to the protocol of a commercial hydroxyproline detection kit (Jiancheng Biotechnology, Nanjing, China). Briefly, frozen lung tissues were homogenized in saline containing 0.1 mol/L phenylmethylsulfonyl fluoride with a Polytron tissue homogenizer. The homogenized sample was hydrolyzed in 6 mol/L HCl, and the hydroxyproline concentration was determined as described previously (Yao et al. 2006).

Statistical analysis

Data were expressed as means ± standard error of the mean (SE). Statistical analysis was carried out using one-way analysis of variance (ANOVA) and least significant difference (LSD) test with SPSS software (SPSS, Chicago, Ill.). A *p* value less than 0.05 was considered statistically significant.

Fig. 4. Astragaloside IV inhibited peribronchial fibrosis in chronic experimental asthma in mice. (A) Masson's trichrome stain-positive peribronchiolar collagen layer (original magnification $\times 200$) of (a) control group, (b) OVA group, (c) astragaloside IV group. (B) Area of airway fibrosis and (C) total lung hydroxyproline content in the right lung were determined as described in the Methods ($n = 6$ per group). Data are means $\pm$ SE. Significant at *, $p < 0.05$ vs. OVA group. #, $p < 0.01$ vs. control group.

(A)



Results

Astragaloside IV decreases airway hyperresponsiveness in chronic experimental asthma

To examine the effect of astragaloside IV on airway hyperreactivity, we measured the airway resistance in anaesthetised mice using invasive whole-body plethysmography. There were no significant differences in baseline airway resistance among the 3 study groups. As shown in Fig. 2A, the airway resistance in the OVA group and astragaloside IV group was obviously increased in a concentration-dependent manner by acetylcholine chloride administration, whereas only a slight increase could be detected in the control group. The OVA group had significantly greater airway resistance than that in the control group ($p < 0.01$), and treatment with astragaloside IV led to a sharp decrease in airway resistance compared with the OVA group ($p < 0.05$).

Astragaloside IV attenuates chronic airway inflammation and antigen-induced mucus production

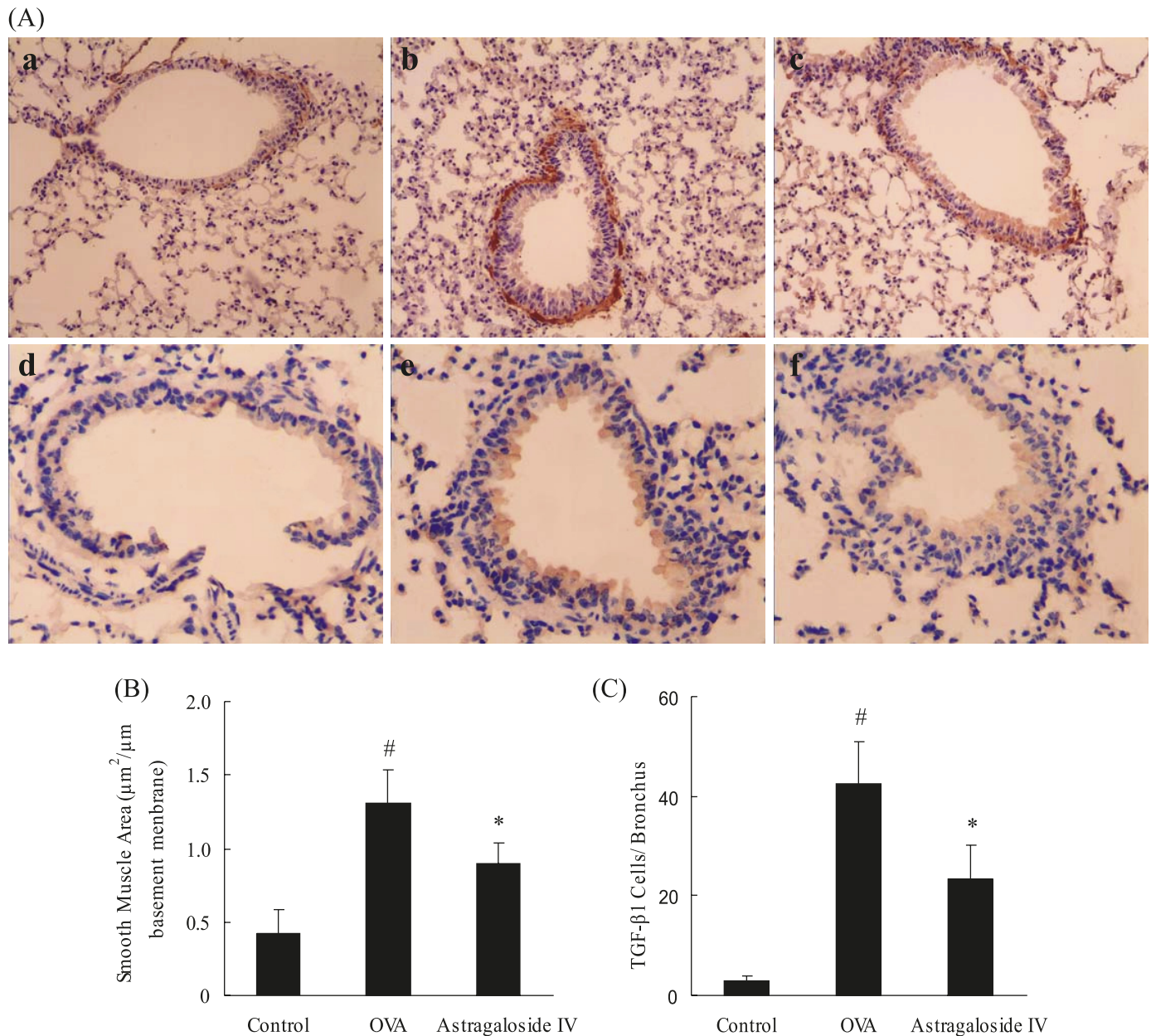
Apart from macrophages, few inflammatory cells were detected in the control mice. Sensitization and challenge

with OVA resulted in a marked increase of eosinophils in BALF. Treatment with astragaloside IV significantly suppressed the number of eosinophils and total inflammatory cells in BALF ($p < 0.05$) (Fig. 2B). The antiinflammatory effects of astragaloside IV were further illustrated by histological examination with hematoxylin and eosin staining (Fig. 3A, *a-c*). OVA-sensitized and OVA-challenged mice developed extensive infiltration of inflammatory cells, especially eosinophils, in the peribronchial and perivascular areas, but the control mice did not. Still, treatment with astragaloside IV notably reduced inflammatory cell infiltration within the peribronchial and perivascular regions. Lung sections were stained with PAS to identify mucus-containing cells in the airway epithelium (Fig. 3A, *d-f*). An obvious increase in the number of PAS-positive epithelial cells was found in the OVA group compared with the control group ($p < 0.01$) (Fig. 3B). Treatment with astragaloside IV significantly reduced the number of PAS-positive cells ($p < 0.05$).

Astragaloside IV reduces the levels of Th2-type cytokine IL-4 and IL-13 in BALF and total serum IgE

The levels of Th2-type (T helper type 2) cytokine IL-4

Fig. 5. Astragaloside IV decreased peribronchial α -SMA and TGF- β 1 immunostaining. (A) The immunostaining area of peribronchial α -SMA (immunohistochemistry, original magnification $\times 200$) of (a) control group, (b) OVA group, (c) astragaloside IV group. (B) The number of peribronchial cells expressing TGF- β 1 in the lungs (immunohistochemistry, original magnification $\times 400$) of (d) control group, (e) OVA group, (f) astragaloside IV group and (C). Data are means $\pm$ SE, $n = 6$ in each group. Significant at *, $p < 0.05$ vs. OVA group. #, $p < 0.01$ vs. control group. α -SMA, α -smooth muscle actin; TGF- β 1, transforming growth factor- β 1; OVA, ovalbumin.



and IL-13 in BALF and total IgE in serum were greatly increased in the OVA group relative to the control group ($p < 0.01$) (Table 1). Daily administration of astragaloside IV reduced the levels of these Th2 cytokine in BALF and total IgE in serum compared with the OVA group ($p < 0.05$).

Astragaloside IV inhibits peribronchial fibrosis in chronic experimental asthma

Peribronchial trichrome staining

Masson's trichrome staining revealed dense collagen dep-

osition or fibrosis throughout the lung interstitium in airways and blood vessels of tissues from OVA-sensitized and OVA-challenged mice (Fig. 4A). The mean area of airway fibrosis in the OVA group was profoundly enhanced compared with that of the control group ($p < 0.01$) (Fig. 4B). Administration of astragaloside IV caused overt reduction of the collagen deposition in lung interstitium including the perivascular collagen deposition ($p < 0.05$).

Lung collagen assay

Total lung hydroxyproline content in the OVA group was significantly greater than that in the control mice ($p <$

0.01) (Fig. 4C). In contrast, there was a significant decrease in total lung hydroxyproline content in the astragaloside IV group compared with the OVA group ($p < 0.05$).

Astragaloside IV decreases peribronchial α -SMA and TGF- β 1 immunostaining

The immunostaining area of peribronchial α -SMA (Fig. 5A, *a-c*) in the OVA group was more than that in the control group ($p < 0.01$) (Fig. 5B). Administration of astragaloside IV decreased the immunostaining area of α -SMA compared with the OVA group ($p < 0.05$). In contrast to minor constitutive staining for TGF- β 1 in the lungs of control mice, staining for TGF- β 1 was markedly elevated in the lungs of OVA-sensitized and OVA-challenged mice (Fig. 5A, *d-f*). Strong cytoplasmic staining was mainly localized to bronchial epithelial cells. The expression of TGF- β 1 in the astragaloside IV group was significantly less than that in the OVA group ($p < 0.05$) (Fig. 5C).

Discussion

Human asthma is a chronic inflammatory disorder characterized by significant airway structural changes or airway remodeling. Murine models of acute asthma are mainly indicative of the initiating phase, whereas those of chronic asthma are particularly similar to human asthma showing airway remodeling and subsequent loss of lung function. In the present study, we established an allergen-induced chronic experimental asthma and illustrated the inhibitory effects of astragaloside IV on the airway inflammation, airway hyperresponsiveness, and airway remodeling.

This chronic animal model displayed a significant increase in total serum IgE levels and eosinophilia in BALF after repeated OVA challenge. IgE mediates the allergic response in humans and rodents through triggering degranulation of mast cells. Furthermore, elevated IgE levels and eosinophilia are associated with the incidence or severity of asthma (Sears et al. 1991). Here we demonstrated that administration of astragaloside IV profoundly reduced total serum IgE levels and total inflammatory cells and eosinophilia in BALF. Lung histology validated an astragaloside IV-mediated ablation of airway inflammation in the lung. These findings suggest that astragaloside IV is an immunoregulatory or antiinflammatory herbal extract that appears beneficial for the treatment of allergic diseases.

Mucus hypersecretion and mucus plugging of the airways are characteristic features of human asthma (Aikawa et al. 1992). Adoptive transfer of Th2 cells induces airway mucus expression (Cohn et al. 1997). When overexpressed in vivo, each Th2 cytokine (IL-4, IL-9, IL-13) induces goblet-cell metaplasia in the murine lung (Wills-Karp 2000). Although the mucus-producing goblet cells are absent in small airways of healthy individuals (Jeffery and Zhu 2002), they were observed in chronic airway inflammatory conditions contributing to bronchiolar obstruction (Rogers 2002). Interestingly, in our study, astragaloside IV robustly abolished the number of mucus-producing goblet cells within the bronchiolar epithelium, in accordance with the concomitant reduction of IL-4 and IL-13 levels in BALF.

Remarkably, the increased subepithelial collagen was induced in the chronic experimental asthma, as we determined

by morphometric and hydroxyproline analyses. Collagen deposition in airways is a key feature of airway remodeling in human asthma in association with increasing disease severity and the decline of pulmonary function (Roche et al. 1989). IL-4 and IL-13 are regarded as key mediators of tissue fibrosis in the pathophysiology of asthma (Zhou et al. 2008; Richter et al. 2001). The profibrotic cytokine TGF- β 1 was suggested to play an important role in asthma. TGF- β 1 induces proliferation of fibroblast cells and their differentiation into myofibroblasts and extracellular matrix protein synthesis during the development of subepithelial fibrosis of asthmatics (Makinde et al. 2007). TGF- β 1 expression correlates with the degree of subepithelial fibrosis, and levels of TGF- β 1 are significantly increased in patients with severe asthma who have prominent airway eosinophilic inflammation (Vignola et al. 1997; Redington et al. 1997). Clearly, astragaloside IV treatment decreased the collagen deposition. Astragaloside IV-mediated reduction of IL-4 and IL-13 levels in BALF and inhibition of lung TGF- β 1 expression may conceivably address its antifibrotic action.

The pathophysiology of airway hyperresponsiveness is complex because many factors contribute to its development. Although both airway inflammation and airway fibrosis may contribute to reactivity, airway fibrosis plays a predominant role in this phenomenon (Locke et al. 2007). IL-4 and IL-13 are critical in allergic inflammation and the development of airway hyperresponsiveness (Wills-Karp et al. 1998). When these factors are combined, the astragaloside IV-induced inhibition of hyperresponsiveness may correlate with the reduction in airway inflammation and airway fibrosis.

Airway remodeling has been linked to asthma severity and may contribute to the development of airway hyperresponsiveness due to the increase of smooth muscle mass in asthmatic airways (Benayoun et al. 2003). We observed that chronic exposure to OVA led to a significant increase in α -SMA expression, consistent with previous studies in animal models of chronic asthma (Leigh et al. 2002; Lee et al. 2006). An allergen-induced increase in airway smooth muscle was greatly reduced by astragaloside IV treatment, indicating that astragaloside IV is a potential remedy that can be used to inhibit the progression of airway remodeling.

In conclusion, astragaloside IV inhibits the airway eosinophilic inflammation, airway hyperresponsiveness, and airway remodeling observed in an allergen-induced chronic experimental asthma. This murine model also showed that astragaloside IV is associated with suppression of the Th2-type cytokine IL-4 and IL-13 levels in BALF and TGF- β 1 expression in the lung. We speculate that astragaloside IV is promising to be a beneficial medication for asthma therapy.

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