

Astragalus Extract Attenuates Allergic Airway Inflammation and Inhibits Nuclear Factor κ B Expression in Asthmatic Mice

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Abstract: *Background:* Astragalus membranaceus from traditional Chinese herbal medicines previously showed that it possesses a strong anti-inflammatory activity. The purpose of this study was to elucidate the effect of astragalus on allergen-induced airway inflammation and airway hyperresponsiveness and investigate its possible molecular mechanisms. *Methods:* Female BALB/c mice sensitized and challenged with ovalbumin (OVA) developed airway inflammation. Bronchoalveolar lavage fluid was assessed for total and differential cell counts and cytokine and chemokine levels. *In vivo* airway responsiveness to increasing concentrations of methacholine was measured 24 hours after the last OVA challenge using whole-body plethysmography. The expression of inhibitory κ B- α and p65 in lung tissues was measured by Western blotting. *Results:* Astragalus extract attenuated lung inflammation, goblet cell hyperplasia and airway hyperresponsiveness in OVA-induced asthma and decreased eosinophils and lymphocytes in bronchoalveolar lavage fluid. In addition, astragalus extract treatment reduced expression of the key initiators of allergic T_H2 -associated cytokines (interleukin 4, interleukin 5) ($P < 0.05$). Furthermore, astragalus extract could inhibit nuclear factor κ B (NF- κ B) expression and suppress NF- κ B translocation from the cytoplasm to the nucleus in lung tissue samples. *Conclusions:* Taken together, our current study demonstrated a potential therapeutic value of astragalus extract in the treatment of asthma and it may act by inhibiting the expression of the NF- κ B pathway.

Key Indexing Terms: Astragalus plant; Asthma; Nuclear factor κ B; Airway inflammation. [Am J Med Sci 2013;346(5):390-395.]

The morbidity and mortality of asthma have increased worldwide, and it has become a severe global public health problem.¹ Asthma is an inflammatory disease of the airways, characterized by lung eosinophilia, mucus hypersecretion by goblet cells and airway hyperresponsiveness (AHR) to inhaled allergens. Corticosteroid treatment remains the first preference of treatment; however, steroids are not always completely effective for asthma.²

The molecular regulatory pathways in induction of long-term cytokine expression and recruitment/activation of inflammatory cells in asthma remain elusive. However, there is growing recognition that these processes involve increased transcription of inflammatory genes via transcription factors.³ One such transcription factor, nuclear factor κ B (NF- κ B), is abundant of p50 (NF- κ B1)/p65 (RelA) heterodimer. In a latent

state, NF- κ B is sequestered as an inactive trimer by complexing with I κ B- α , a 37-kDa inhibitory protein, which promotes cytoplasmic retention and maintains a low basal transcriptional activity. I κ B- α consists of an N-terminal domain containing specific phosphorylation sites, 5 ankyrin repeat sequences and a C-terminal domain of Pro-Glu-Ser-Thr polypeptides.⁴ Upon stimulation, I κ B- α is phosphorylated by the I κ B kinase, ubiquitinated and degraded through the 26S proteasome pathway.⁵ Subsequently, the nuclear localization sequence of NF- κ B is unmasked to allow its translocation into the nucleus, where it binds to DNA and initiates transcription of a wide range of NF- κ B-dependent genes in association with immune and inflammatory responses.⁶

Astragalus membranaceus is a traditional Chinese herbal medicine used for the treatment of common cold, diarrhea, fatigue anorexia and cardiac diseases.^{7,8} It has also been used as an immunomodulating agent in treating immunodeficiency diseases and to alleviate the adverse effects of chemotherapeutic drugs. Our previous study demonstrated the usefulness of astragalus extract in the treatment of asthma, which can efficiently inhibit airway remodeling, relieve symptoms and reduce the frequency of asthma attacks in a mouse asthma model.⁹ However, there have been no reports regarding the role of astragalus extract on airway inflammation from asthma.

In this study, we examined the effect of astragalus extract on airway inflammation of a mouse asthma model and regulate the NF- κ B expression in ovalbumin (OVA)-sensitized mice, providing a novel mechanism for the astragalus extract inhibitory effect on airway inflammation in animal models of asthma.

MATERIALS AND METHODS

Reagents

Astragalus extracts (formononetin and calycosin) were obtained from Haerbin Shengtai Botanical Development Co, Ltd (Haerbin, Heilongjiang, China), and their chemical structures as previously reported.⁹ Chicken egg OVA was purchased from Sigma-Aldrich (St. Louis, MO). Interferon gamma (IFN- γ), interleukin (IL) 4 and IL-5 enzyme-linked immunosorbent assay (ELISA) kit were purchased from R&D Systems (Minneapolis, MN). Phospho-I κ B- α , I κ B- α , p65, glyceraldehyde-3-phosphate dehydrogenase and Histone H3 antibodies as well as secondary antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Other laboratory reagents were obtained from Sigma.

Animal Experimental Protocols

Thirty-six healthy female BABL/c mice, 6 to 8 weeks old, weighing 18 to 24 g were randomly divided into 3 groups, with 12 mice in each group: normal control group (A), asthma group (B) and astragalus extract group (C). The asthmatic models were established by OVA. The mice were sensitized on days 0, 7 and 14 by intraperitoneal injection of 20 μ g OVA emulsified in 1 mg of aluminum hydroxide in a total volume of

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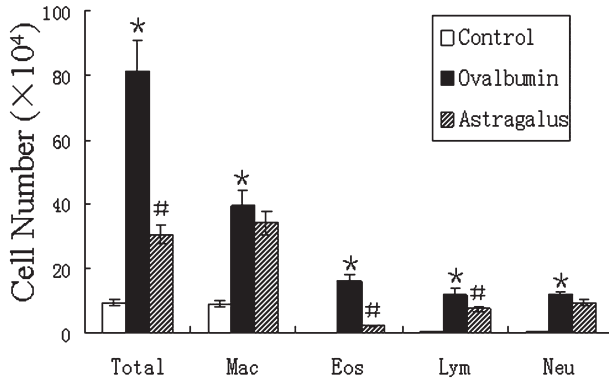


FIGURE 1. Astragalus extract suppressed accumulation of inflammatory leukocytes in the airways of OVA-challenged mice. Total cell counts and differential counts of at least 400 cells in BAL from mice subjected to various treatments were determined 24 hours after the final challenge. The data were summarized as mean $\pm$ standard error of the mean from at least 3 separate experiments. *Significant difference versus control group ($P < 0.05$). #Significant difference versus OVA group ($P < 0.05$). OVA, ovalbumin.

0.2 mL in groups B and C. Seven days after the last sensitization, the mice were exposed to 1% OVA aerosol for up to 30 minutes every other day for 7 days. The 1% OVA aerosol was generated by a compressed air atomizer driven by filling a perspex cylinder chamber (diameter 50 cm, height 50 cm) with a nebulized solution. Saline was used in group A instead of OVA. At the same time, mice in group C were treated with 0.5 g/kg astragalus extract by gavage every other day for 28 days. All the experiments described below were performed in accordance with the regulations of the Center of Animal Experiments of Qingdao University.

Bronchoalveolar Lavage Fluid Analysis

At 24 hours after the last challenge, bronchoalveolar lavage fluid (BAL) was obtained from the mice under anesthesia using 1 mL sterile isotonic saline. Lavage was performed 4 times in each mouse, and the total volume was collected separately. Cells from BAL fluid were suspended in phosphate-buffered saline and counted, and cytopspins were

prepared (2000 rpm, 10 minutes) and stained with Wright-Giemsa. Differential counts of at least 400 cells were carried out in the high-power field of a microscope, and cells were identified based on their morphologic features.

Enzyme-Linked Immunosorbent Assay

The BAL sample was collected and immediately centrifuged at 2000 rpm for 10 minutes at room temperature and stored at -80°C until use. The levels of IgE, IFN- γ , IL-4 and IL-5 in BAL were then assayed with ELISA kit according to the manufacturer's instructions.

Tissue Samples

Lungs were removed from the mice after killing 24 hours after the last challenge. The tissues from the left lung were directly obtained from the surgical suite and immediately fixed in 10% buffered formalin and then embedded in paraffin. Sections (5 μm) were prepared and stained with hematoxylin and eosin. Additionally, periodic acid-Schiff staining was performed to identify mucus production in epithelial cells, and the number of positive cells per unit length of basement membrane perimeter was determined. Quantitative analysis was performed blinded as described.¹⁰

Isolation of Thoracic Lymph Node Cells

Thoracic lymph node (TLN) cells were used to determine the immune regulatory effects of astragalus. After the asthmatic mice were killed, TLN cells were isolated and cell clumps were disaggregated into single-cell suspensions using filtration through nylon mesh (30 μm). Red blood cells were lysed by the addition of lysis buffer. The isolated TLN cells were cultured at the density of $3 \times 10^6/\text{mL}$ in 24-well plates under stimulation with 200 $\mu\text{g}/\text{mL}$ OVA for 96 hours. The culture medium was collected to detect cytokine levels by ELISA.

Measurements of AHR

Twenty-four hours after the final aerosol challenge, AHR was measured in unrestrained mice using a whole-body plethysmograph. Before recording, the chambers were calibrated with an injection of 1 mL of air. Conscious mice received aerosol challenge with methacholine at increasing concentrations (0–20 mg/mL in saline) for 3 minutes. Enhanced pause (Penh) was recorded for 3 minutes after each challenge.

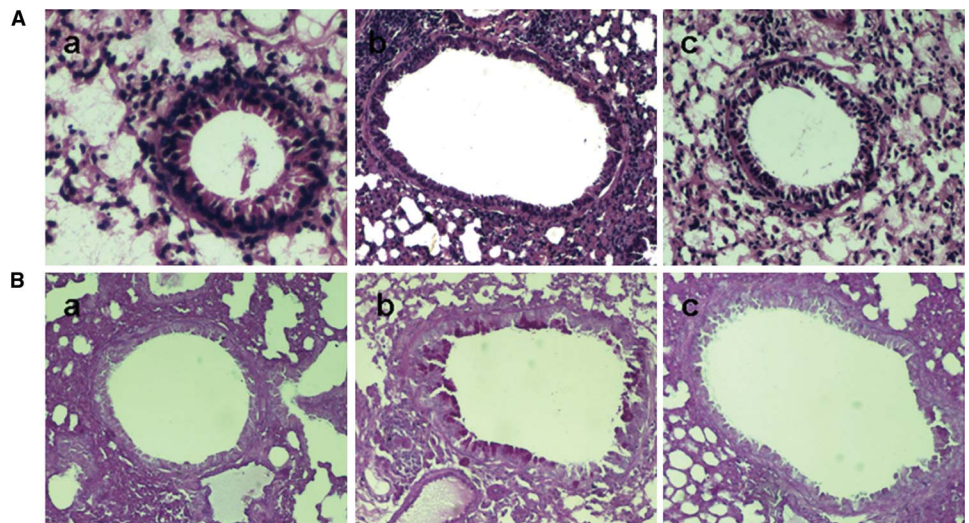


FIGURE 2. Astragalus extract suppressed OVA-induced lung inflammation and goblet cell hyperplasia. Lung tissue sections obtained from mice 24 hours after the last OVA challenge were stained with H&E (A) and PAS (B). Control group (a), asthmatic group (b) and astragalus extract-treated group (c). All photographs were captured at 100 $\times$ magnification. H&E, hematoxylin and eosin; OVA, ovalbumin; PAS, periodic acid-Schiff.

TABLE 1. Scoring of the extent of inflammation via quantitative analysis of inflammatory cell infiltration in lung sections

Groups	Mucus score
Control	0
OVA	3.91 ± 0.91^a
Astragalus	1.48 ± 0.67^b

^a Significantly different from control, $P < 0.05$.
^b Significantly different from OVA, $P < 0.05$.
OVA, ovalbumin.

Penh = pause $\times$ PIF/PEF; pause = (Te – Rt)/Rtl (PIF, peak inspiratory height; PEF, peak expiratory height; Te, expiratory time; and Rt, time to expire 65% of the volume).

Western Blotting

Lung tissues were homogenized in liquid nitrogen. Cytosolic fractions were extracted using cytosolic lysis buffer, and nuclear fractions were extracted using nuclear lysis buffer. Cytosolic and nuclear protein lysates (100 μ g per lane) were suspended in 5 $\times$ sample buffer, boiled for 5 minutes, electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membranes by electroblotting. The membrane was blocked in 1% bovine serum albumin/0.05% Tween-20/phosphate-buffered saline solution overnight at 4°C, followed by incubation with the primary antibody for 24 hours. A horse-radish peroxidase–labeled IgG was used as the secondary antibody. The blots were then developed by incubation in a chemiluminescence substrate and exposed to x-ray films.

Statistical Analysis

Data are expressed as mean $\pm$ standard deviation. Statistical comparisons of the data from the various groups were

performed using the Student *t* test. Differences between groups were considered statistically significant at $P < 0.05$.

RESULTS

Astragalus Extract Inhibited OVA-Induced Inflammatory Cell Infiltration and Goblet Cell Hyperplasia

To investigate whether astragalus extract could suppress the OVA-induced infiltration of lungs by inflammatory cells, we performed BAL 24 hours after the final aerosol challenge and examined each specimen using Wright-Giemsa stain. OVA challenge significantly increased the total number of cells in BAL fluid, as well as the numbers of eosinophils, macrophages, lymphocytes and neutrophils (Figure 1). OVA-induced accumulation of inflammatory cells around the bronchioles and small vessels was detected in hematoxylin and eosin–stained paraffin-embedded lung sections. Compared with control animals, mucus overproduction was clearly observed as a violet color in bronchial airways in OVA-induced mice. In contrast, the extent of mucus staining was markedly diminished in OVA-induced mice treated with astragalus extract (Figure 2 and Table 1).

Astragalus Extract Reduced OVA-Induced T_H2 Cytokines and Asthma-Related Chemokines

Inflammation in asthma is considered as a T_H2 -predominant immune reaction. OVA inhalation in sensitized mice caused a notable increase in IL-4 and IL-5 levels into BAL fluid compared with saline aerosol control. In contrast, BAL fluid level of IFN- γ , a T_H1 cytokine, dropped slightly in OVA-challenged mice. Noticeably, astragalus extract markedly upregulated IFN- γ and downregulated of IL-4 and IL-5 levels in BAL. These finding implied that astragalus extract was able to modify the T_H2 -predominant immune activity in our OVA-induced mouse asthma model (Figure 3).

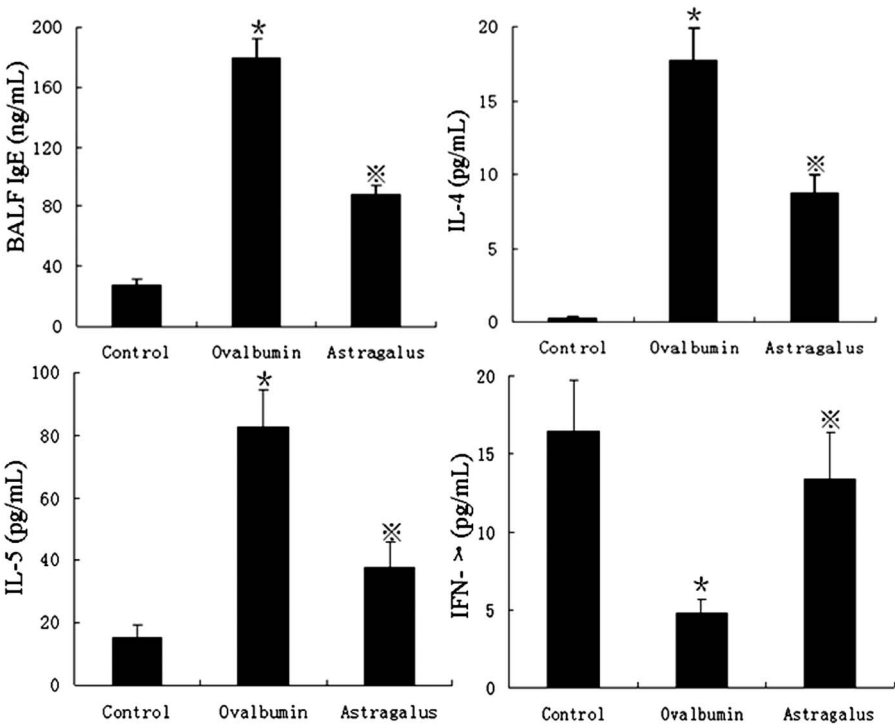


FIGURE 3. Astragalus extract reduced levels of T_H2 cytokines and asthma-related chemokines in BAL. BAL was collected from mice subjected to various treatments 24 hours after the final challenge. IgE, IL-4, IL-5 and IFN- γ levels were determined by ELISA. The data were summarized as mean $\pm$ standard error of the mean from at least 3 separate experiments. *Significant difference versus control group ($P < 0.05$). **Significant difference versus OVA group ($P < 0.05$). OVA, ovalbumin.

Astragalus Extract Suppressed OVA-Induced T_H2 Regulators in Cultured TLN Cells

To investigate whether astragalus extract treatment directly suppressed T_H2 -related responses in immune cells, we cultured TLN cells isolated from the mice under various treatments. IL-4 and IL-5 levels secreted by TLN cells were both higher from OVA-challenged mice than from control mice (Figure 4), and astragalus extract reduced OVA-induced IL-4 and IL-5 production.

Astragalus Extract Suppressed OVA-Induced AHR

Because astragalus extract suppressed T_H2 -related inflammation and goblet cell hyperplasia after OVA challenge, we then investigated whether astragalus extract attenuates AHR. We measured Penh using whole-body plethysmograph

in free moving mice after exposure to increasing concentrations of methacholine (5, 10, 15 and 20 mg/mL), an airway constrictor (Figure 5). The degree of Penh increase was notably higher in OVA-challenged mice, and astragalus extract can partially inhibit these effects.

Influence of Astragalus Extract on NF- κ B Expression in Mouse Lung Tissue

To determine whether the therapeutic effect of astragalus extract on OVA-induced asthma is through NF- κ B inhibition, we examined the expression of phosphorylated I κ B- α and the nuclear localization of p65 subunit of NF- κ B in lung tissues of mice subjected to OVA challenge. Astragalus extract suppressed phosphorylation of I κ B- α in lung parenchyma, whereas total I κ B- α expression levels remain unchanged. OVA-sensitized and OVA-challenged mice displayed increased concentrations of NF- κ B p65 in nuclear protein extracts from lung tissues compared with the control group. Conversely, the NF- κ B p65 level was significantly lower in the astragalus extract group (Figure 6).

DISCUSSION

In the current study, we investigated that astragalus extract significantly reduced the number of infiltrating leukocytes in the airways of OVA-challenged mice, especially eosinophils and lymphocyte. Accordingly, OVA-induced T_H2 -associated cytokines (IL-4 and IL-5) were significantly suppressed by astragalus extract treatment. Furthermore, we confirmed that the astragalus extract could modulate the expression of signaling molecules of the NF- κ B pathway, which may be involved in modulating airway inflammation.

Astragalus membranaceus contains many isoflavones and isoflavonoids, such as formononetin, calycosin and ononin. Formononetin significantly reduces arachidonic acid release and production of nitric oxide in lipopolysaccharide-activated macrophages. In addition, formononetin and calycosin can inhibit glutamate-induced cell damage by increasing endogenous antioxidant and stabilizing cell membrane. It has also been used as an immunomodulating agent in treating

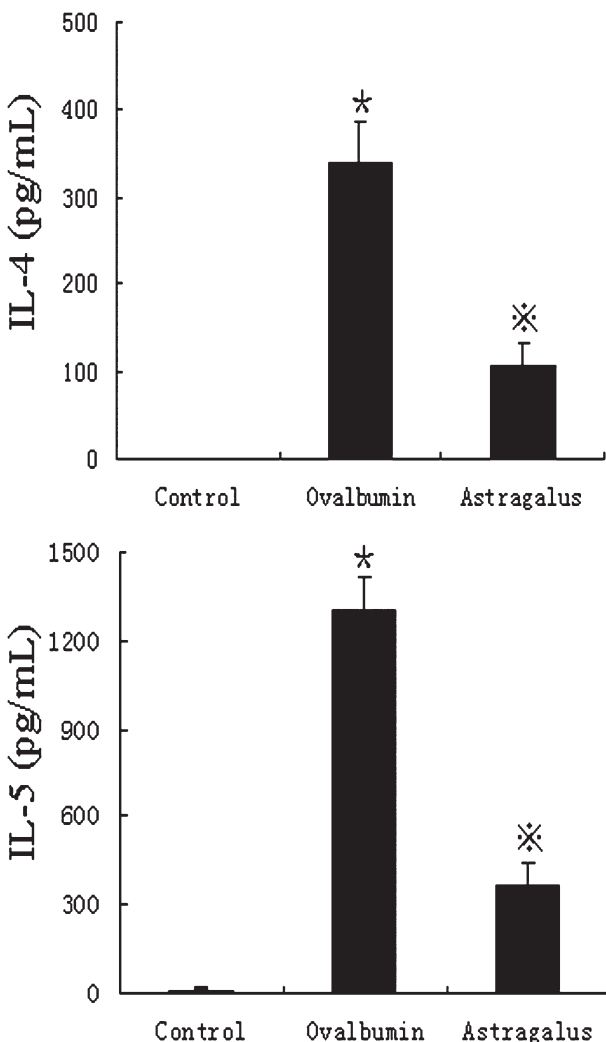


FIGURE 4. Astragalus extract inhibited the expression of the cytokines (IL-4 and IL-5) in cultured TLN cells under OVA stimulation. Twenty-four hours after the final OVA challenge, isolated TLN cells were cultured in the presence of OVA (200 μ g/mL) for 96 hours. Culture media from TLN cells of mice treated under the indicated conditions were collected, and the levels of IL-4 and IL-5 were measured using ELISA. *Significant difference versus control group ($P < 0.05$). *Significant difference versus OVA group ($P < 0.05$). OVA, ovalbumin; TLN, thoracic lymph node.

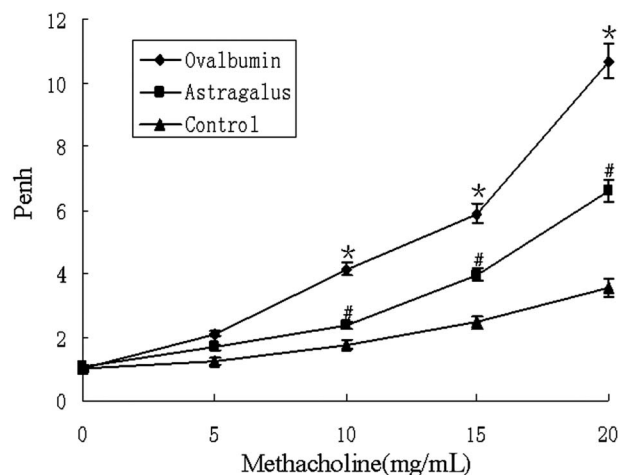


FIGURE 5. Astragalus extract suppressed OVA-induced AHR. AHR was determined using whole-body plethysmograph 24 hours after the final challenge. The data were summarized as mean $\pm$ standard error of the mean from at least 3 separate experiments. *Significant difference versus control group ($P < 0.05$). #Significant difference versus OVA group ($P < 0.05$). AHR, airway hyperresponsiveness; OVA, ovalbumin.

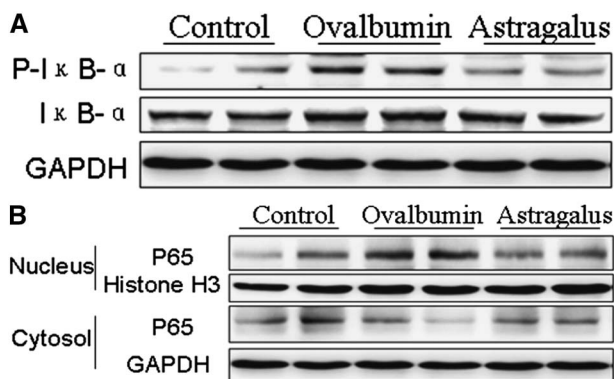


FIGURE 6. Astragalus extract suppressed OVA-induced IκB-α phosphorylation and p65 translocation in lung tissues. **A,** Phosphorylated IκB-α (P-IκB-α) and total IκB-α in cytosolic lysates of lung tissues were detected by Western blot, and expression of glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. **B,** Immunoblotting of p65 in cytosolic and nuclear extracts of lung tissues was carried out, expression of histone H3 and glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. OVA, ovalbumin.

immunodeficiency diseases,⁷ although Wong et al¹¹ studied a herbal formula containing astragalus and did not find a beneficial effect for the treatment of asthma in children. We have noticed that the dose of astragalus used in the study of Wong et al¹¹ was 0.74 g·person⁻¹·d⁻¹. As we know, the effective dose of astragalus was 5 to 60 g·person⁻¹·d⁻¹ clinically. The dose may affect the results, and we have previously reported that astragalus extract can efficiently inhibit airway remodeling, relieve symptoms and reduce the frequency of asthma attacks in a mouse asthma model.⁹

In the immunopathogenesis of asthma, the main antigen-presenting cells for activating T_H2 cells are dendritic cells.¹² However, recent data indicate that eosinophils not only can function as effector cells but also can act as antigen-presenting cells for activating and recruiting T_H2 cells in asthma. Therefore, the eosinophil is a major contributor to the pathogenesis of allergic asthma.¹³ Our present findings showed that astragalus extract prevented OVA-induced inflammatory cell infiltration into the airways as shown by a significant drop in total cell counts and eosinophil and lymphocyte counts in BAL and in tissue eosinophilia in lung sections.

T_H2 cytokines play an essential role in the pathogenesis of the allergic airway inflammation,^{14,15} and NF-κB is a critical transcription factor for T_H2 cell differentiation.¹⁶ IL-4 and IL-5 can be produced by various lung resident cells, such as bronchial epithelial cells, tissue mast cells and alveolar macrophages as well as infiltrated inflammatory cells, such as lymphocytes and eosinophils.¹⁷ Our present results show that astragalus extract significantly reduced the levels of IL-4 and IL-5 in BAL and TLN cells from OVA-challenged mice. These data showed that the anti-inflammatory effect of astragalus extract is at least in part mediated through a suppressive action on T lymphocytes. Certainly, the exact mechanisms by which astragalus extract affects eosinophils merit further investigation.

The pivotal importance of NF-κB activation in bronchial epithelial cells and immune cells has been demonstrated both in murine models of asthma and in asthma patients.^{18,19} Therefore, NF-κB has emerged as a promising molecular target for the treatment of asthma. NF-κB activation within airway epithelium is necessary to fully induce the recruitment of inflammatory cells.²⁰ NF-κB activation in airways drives a majority of

the Ag-induced inflammation and is a significant contributor to adaptive immune responses and structural remodeling of the airway wall.²¹ Its increased activation has been demonstrated in lungs after allergen challenge and in airway epithelial cells and macrophages.²² In this study, we showed that astragalus extract could inhibit NF-κB activity in lung tissues. Notably, phosphorylation of IκB-α and nuclear translocation of p65 in lung tissues were reduced upon astragalus extract treatment. Collectively, we hypothesized that astragalus extract suppressed OVA-induced asthma by inhibiting NF-κB activity. Our results further elucidated the molecular mechanisms underlying the antiasthmatic effect of astragalus extract.

CONCLUSIONS

In summary, this study demonstrated that astragalus extract from traditional Chinese herbal medicines could inhibit extensive infiltration of inflammatory cells in lung and decrease airway hyperreactivity. In addition, we confirmed that the astragalus extract could modulate the expression of signaling molecules of the NF-κB pathway, which may be involved in modulating airway inflammation. Our results support the utility of astragalus extract as a herbal medicine for asthma treatment and may have application in the development of anti-inflammatory and antiasthmatic drugs.

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