

Chrysin attenuates allergic airway inflammation by modulating the transcription factors T-bet and GATA-3 in mice

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Abstract. Chrysin, a flavonoid obtained from various natural sources, has been reported to possess anti-inflammatory, anti-tumor, antioxidant and anti-allergic activities. However, its anti-inflammatory and immunoregulatory activities in asthma animal models are poorly understood. In the present study, we examined the effects of chrysin on airway inflammation and the possible mechanisms through which it acts in a murine model of allergic asthma. BALB/c mice sensitized and challenged to ovalbumin (OVA) were administered intragastrically with chrysin at a dose of 50 mg/kg daily. Chrysin significantly suppressed OVA-induced airway hyperresponsiveness (AHR) to acetylcholine chloride (Ach). Chrysin administration significantly inhibited the total inflammatory cell and eosinophil counts in bronchoalveolar lavage fluid (BALF) and total immunoglobulin E (IgE) levels in serum. Histological examination of lung tissue demonstrated that chrysin significantly attenuated allergen-induced lung eosinophilic inflammation and mucus-producing goblet cells in the airway. In addition, chrysin triggered a switch of the immune response to allergens towards a T-helper type 1 (Th1) profile by modulating the transcription factors T-bet and GATA-3 in allergic mice. These data suggest that chrysin exhibits anti-inflammatory and immunoregulatory properties and provides new insights into the immunopharmacological role of chrysin in terms of its effects in a murine model of asthma.

Introduction

Allergic asthma is a chronic pulmonary disease associated with airway hyperresponsiveness (AHR) and chronic inflam-

mation of the airways (1). A previous study showed that allergic airway inflammation is associated with increased T-helper type 2 (Th2) cytokine production and decreased T-helper type 1 (Th1) cytokine production (2). The transcription factors GATA-3 and T-bet are important in Th cytokine production; GATA-3 increased Th2 cytokine production in asthma and the loss of T-bet may be associated with asthma (3-4).

Current treatment guidelines for asthma emphasize anti-inflammatory therapy, and glucocorticoids remain the first-line agents. Even though glucocorticoids are effective in preventing allergic inflammation, few asthmatic patients are able to obtain normal bronchial reactivity, even after years of steroid therapy. Moreover, patients with chronic asthma treated with inhaled glucocorticoids, particularly in the long-term, may develop serious side effects (5-6). Thus there is a need for a new or alternative approach to manage this disease.

Chrysin (5,7-dihydroxyflavone), a natural flavonoid obtained from many plants, honey and propolis, possesses extensive pharmacological activities such as anti-inflammatory, anti-allergy and immunoregulatory effects via the suppression of inducible nitric oxide synthase and nuclear factor- κ B (7-10). Since chrysin affects the immune system, it may be able to be used to treat asthma. The aim of the present study was to investigate the role of chrysin on the allergic airway inflammation and Th1/Th2 cytokine production modulated by T-bet and GATA-3 in a murine model of allergic asthma.

Materials and methods

Experimental animal. A total of 48 six-week-old female BALB/c mice, weighing 18-22 g each, were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). All experimental animals used in this study were maintained under a protocol approved by the Institutional Animal Care and Use Committee of the Nanjing Medical University and the experimental protocol was approved by the institutional animal ethics committee.

Allergen sensitization/challenge protocol. Mice were randomly divided equally into 4 groups: Control, ovalbumin (OVA), chrysin and budesonide. Each group contained 12 mice, 6 for pulmonary resistance and 6 for bronchoalveolar lavage

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(BAL). Mice in the last three groups were sensitized on days 0 and 14 by intraperitoneal injection of 100 μg OVA (Grade V; Sigma, St Louis, MO, USA) emulsified in 1 mg aluminum hydroxide (Pierce Chemical Co., Rockford, IL, USA) with a total volume of 0.2 ml. Mice were challenged via the airway with 1% OVA daily on days 22, 23 and 24. The OVA challenge was performed for 30 min by placing the mice in a Plexiglas box (29x22x18 cm) and aerosolizing OVA using an ultrasonic nebulizer (NE-U11B, Omuron, Tokyo, Japan). Mice in the chrysin group were also administered intragastrically with chrysin (Sigma) at a dose of 50 mg/kg (11-13) daily from days 15 to 24 on consecutive days. Mice in the budesonide group were exposed to aerosolized budesonide (2 mg, 1 mg/2 ml, Astra Zeneca) for 30 min per day from days 22 to 24, 1 h prior to challenge. Mice in the control group received the same schedule for sensitization and challenge with an equivalent amount of 0.9% sterile saline instead of OVA.

Measurement of airway responsiveness to acetylcholine chloride (ACh). For the measurement of airway responsiveness to ACh, the mice were anaesthetized by intraperitoneal injection of pentobarbital sodium (70 mg/kg) 24 h after the last challenge. A plastic tube of 2 mm in internal diameter was inserted into the trachea via tracheotomy and a 27 gauge needle was inserted into the caudalis 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 with an AniRes animal lung function analysis system (Synol High-Tech, Beijing, China). Following the establishment of stable airway pressure recording, ACh was administered intravenously with a microinfusion pump at a rate of 36 ml/h in progressively increasing doses (10, 30, 90 and 270 $\mu\text{g}/\text{kg}$). Following the administration of each dose, data were continuously collected from 5 sec to 1 min and maximum values of lung resistance (RL) were taken to express the changes in the airway function of the mice (14-15).

Analysis of bronchoalveolar lavage fluid (BALF) samples and serum. Blood samples were collected 24 h after the last aerosol challenge by retroorbital puncture using heparinized capillary tubes. Blood samples were centrifuged (10 min, 4°C, 1000 x g), and plasma was stored at -70°C until further use. The lungs were washed three times with 0.5 ml saline to collect BALF. The BALF was centrifuged (10 min, 4°C, 1000 x g), and the total number of inflammatory cells in BALF was counted with a hemocytometer. Smears of BAL cells were stained with Wright's staining for the differential cell count. The cells in the BALF were counted by two independent investigators in a single-blind study, which analyzed at least 200 cells each from four different random locations using a microscope. The levels of IL-4 and IFN- γ (Jingmei Biotech Company, Shanghai, China) in BALF and total serum IgE (Bionewtrans Pharmaceutical Biotechnology Co. Ltd., Franklin, MA, USA) were determined by ELISA according to the manufacturer's instructions. The limits of detection were 7 pg/ml for IL-4, 7 pg/ml for IFN- γ and 1 ng/ml for total IgE.

Lung histology. For the histological evaluation of lung tissue, the right lungs obtained from sacrificed mice were immersed in

10% formaldehyde overnight, and then embedded in paraffin. A series of micro sections (5 μm) were cut on a microtome and stained with hematoxylin and eosin (H&E) to assess the inflammatory cell infiltration, and Periodic acid Schiff (PAS) to quantify airway goblet cells. Scoring for mucus production in the PAS-stained sections was as follows (16): 0, no goblet cells; 1, <25% of the epithelium; 2, 25-50% of the epithelium; 3, 50-75% of the epithelium; 4, >75% of the epithelium. At least 5 bronchioles in each slide that were randomly distributed throughout the left lung were analyzed. Scoring was undertaken by two individuals blinded to the experimental protocol.

Measurement of T-bet and GATA-3 protein by western blot analysis. The left lung tissues from each group were added to lysis buffer and homogenized. After absolute schizolysis and centrifugation at 12000 x g for 15 min at 4°C, the resulting supernatants were collected as the total protein samples. The protein concentrations were determined using the method of bicinchoninic acid. In total, 50 μg of total proteins isolated from the whole lung were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane for 2 h. The membrane was then incubated in blocking solution (5% dry milk in Tris-buffered saline) for 1 h at room temperature and subsequently exposed to 0.8 $\mu\text{g}/\text{ml}$ of the monoclonal antibodies anti-GATA-3, anti-T-bet or anti- β -actin (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C with gentle agitation. The following day, the membrane was incubated with peroxidase-conjugated goat antimouse IgG (1:1000) for 2 h at room temperature. Protein was detected by the ECL Plus (Amersham Biosciences, Piscataway, NJ, USA). Densitometric analysis was performed by ImageJ densitometry software (NIH) after scanning of the films. Target protein levels were normalized to β -actin protein levels.

Statistical analysis. Data were shown as the means \pm standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) and the post hoc least significant difference (LSD) test with SPSS software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Chrysin suppresses airway hyperreactivity in OVA sensitized mice. The airway resistance in anaesthetized mice was measured using invasive whole body plethysmography (Fig. 1). There were no significant differences in baseline airway resistance among the four groups. The airway resistance generated by the administration of ACh at doses from 30 to 270 $\mu\text{g}/\text{kg}$ increased significantly in the OVA-sensitized/challenged mice compared with the control mice. The OVA group had significantly greater airway resistance than the control group. Treatment with chrysin and budesonide resulted in a sharp decrease in airway resistance compared with the OVA group.

Chrysin attenuates allergic airway inflammation in mice. Aside from macrophages, few inflammatory cells were detected in control BALB/C mice. Sensitization and challenge with OVA resulted in a marked increase of total leukocyte and

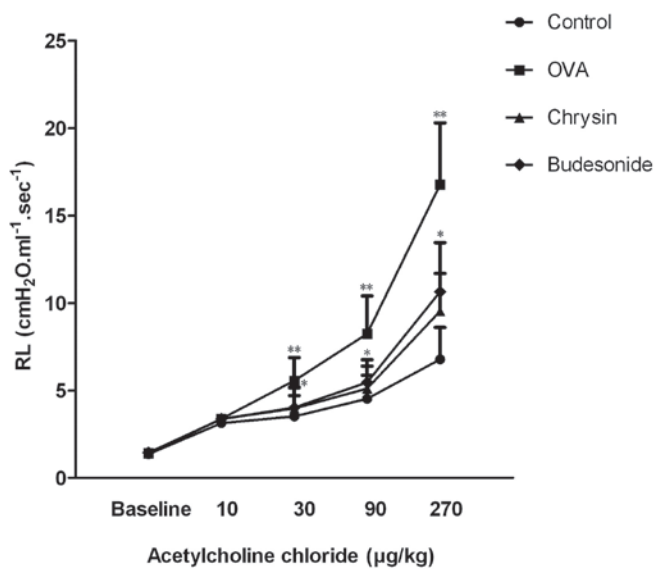


Figure 1. Treatment with chrysin decreased the development of AHR in a murine model of asthma. Data are the means \pm SEM (n=6 per group). **P<0.01 compared with the control group; *P<0.05 compared with the OVA group. AHR, airway hyperresponsiveness; OVA, ovalbumin.

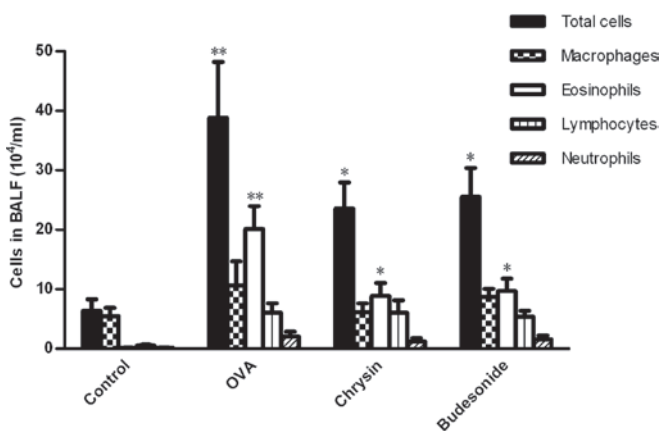


Figure 2. Treatment with chrysin inhibited the inflammatory cell accumulation in broncho-alveolar lavage fluid (BALF) in a murine model of asthma. Data are the means \pm SEM (n=6 per group). **P<0.01 compared with the control group; *P<0.05 compared with the OVA group. OVA, ovalbumin.

eosinophil counts in BALF. The number of total inflammatory cells and eosinophils were significantly decreased in BALF following treatment with chrysin (Fig. 2). The anti-inflammatory effect of chrysin was demonstrated following histological examination of the H&E- and PAS-stained sections. A significant change of inflammatory cells in the airway and around the blood vessels was observed in OVA-sensitized/challenged mice, but not in the saline-treated control mice. The majority of the infiltrated inflammatory cells were eosinophils. Chrysin significantly attenuated eosinophil-rich inflammatory leukocyte infiltration compared with OVA-challenged mice (Fig. 3).

Chrysin inhibits airway goblet cell hyperplasia and mucus production in a murine model of asthma. Marked goblet

Table I. Cytokine levels in BALF and serum total IgE were measured by ELISA.

Group	IL-4 (pg/ml)	IFN- γ (pg/ml)	IgE (ng/ml)
Control	19.02 \pm 6.65	28.72 \pm 5.28	53.8 \pm 14.21
OVA	119.15 \pm 32.43 ^a	14.62 \pm 3.25 ^a	226.0 \pm 26.83 ^a
Chrysin	55.23 \pm 9.38 ^b	24.85 \pm 4.32 ^b	98.0 \pm 22.61 ^b
Budesonide	53.70 \pm 10.25 ^b	23.35 \pm 5.14 ^b	105.7 \pm 19.15 ^b

Treatment with chrysin enhances the level of IFN- γ and reduces the levels of the Th2-type cytokine IL-4 in BALF and total serum IgE in a murine model of asthma. Data are the means \pm SEM (n=6 per group). ^aP<0.05 compared with the control group, ^bP<0.05 compared with the OVA group. BALF, bronchoalveolar lavage fluid; IgE, immunoglobulin E; OVA, ovalbumin.

cell hyperplasia and mucus hypersecretion in the bronchi were observed in the OVA-challenged mice, but not in the control mice. Chrysin significantly attenuated OVA-induced overproduction of mucus and goblet cell hyperplasia in mice (Fig. 4a-d). PAS staining scores were calculated for each experimental group, as described in Materials and methods. Mucus secretion was markedly increased in mice in the OVA group with enhanced staining scores (Fig. 4e). There was a significant reduction in the proportion of goblet cells in the airway of mice administered chrysin.

Chrysin alters the levels of Th1/Th2 cytokines in BALF and total IgE in serum. BALF was collected 24 h after the last OVA challenge, and the concentration of IFN- γ and IL-4 were assessed using ELISA. The level of the Th2 cytokine IL-4 in BALF was significantly increased, but the concentration of IFN- γ was decreased in the OVA group compared with the control group (P<0.05, Table I). Total serum IgE levels were significantly elevated in the OVA group compared with the control group. Treatment with chrysin reduced total serum IgE levels compared with the OVA group (P<0.05; Table I).

Chrysin alters the ratio of T-bet and GATA-3 in the lung. The relative amount of target protein was quantified as the density of the target protein versus β -actin. Following normalization of the data according to the expression of β -actin protein, the relative density of T-bet and GATA-3 are presented as fold numbers (Fig. 5A). The ratio of T-bet and GATA-3 was significantly decreased in the OVA group compared with the control group (P<0.01; Fig. 5B). Treatment with chrysin markedly increased the ratio of T-bet and GATA-3 compared with OVA-sensitized/challenged mice (P<0.05; Fig. 5B).

Discussion

In the current study, the effects of chrysin on allergy-induced airway inflammation in a murine model of allergic asthma were evaluated. The findings have shown that chrysin attenuated OVA-induced airway inflammation and mucus secretion in the lung tissues of allergic mice. Chrysin administration enhanced IFN- γ and decreased IL-4 levels in BALF and total

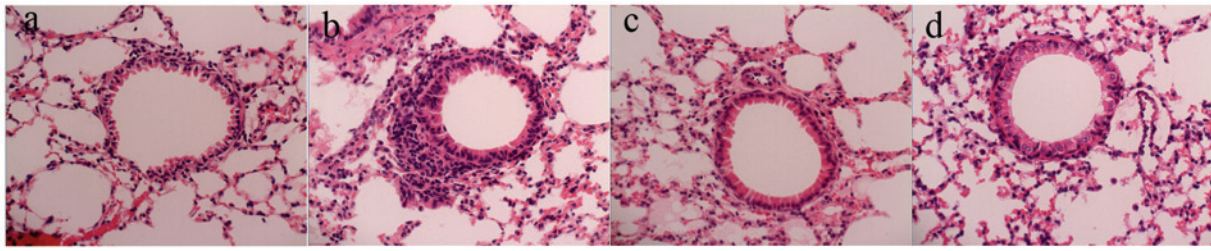


Figure 3. Treatment with chrysin attenuates airway inflammation in a murine model of asthma. Hematoxylin and eosin staining (original magnification, x100) of: (a) Control; (b) OVA; (c) chrysin; and (d) budesonide groups. OVA, ovalbumin.

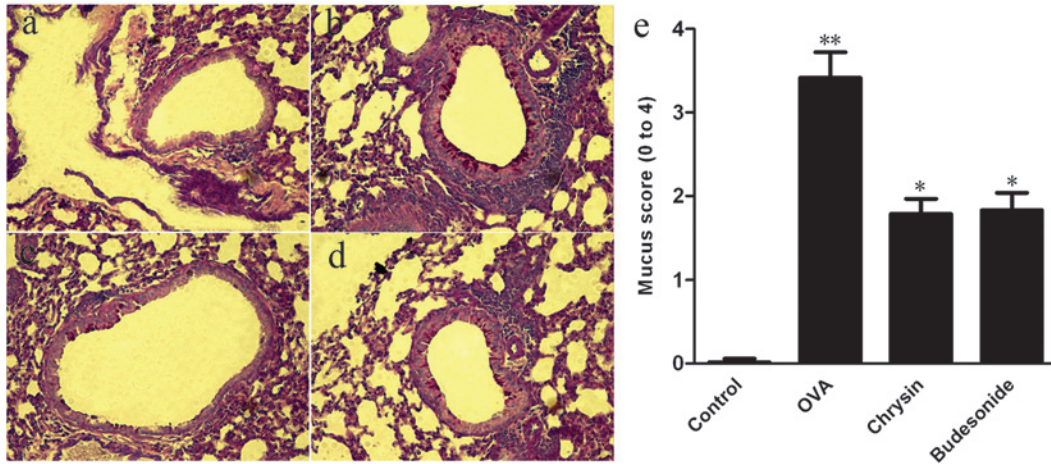


Figure 4. Treatment with paeonol inhibits antigen-induced mucus production in a murine model of asthma. Periodic acid Schiff (PAS) staining (original magnification, x200) of: (a) Control; (b) OVA; (c) chrysin; and (d) budesonide groups. (e) Quantitative analyses of mucus production in lung sections were performed as described in Materials and methods. Data are the means ± SEM (n=6 per group). **P<0.01 compared with the control group; *P<0.05 compared with the OVA group. OVA, ovalbumin.

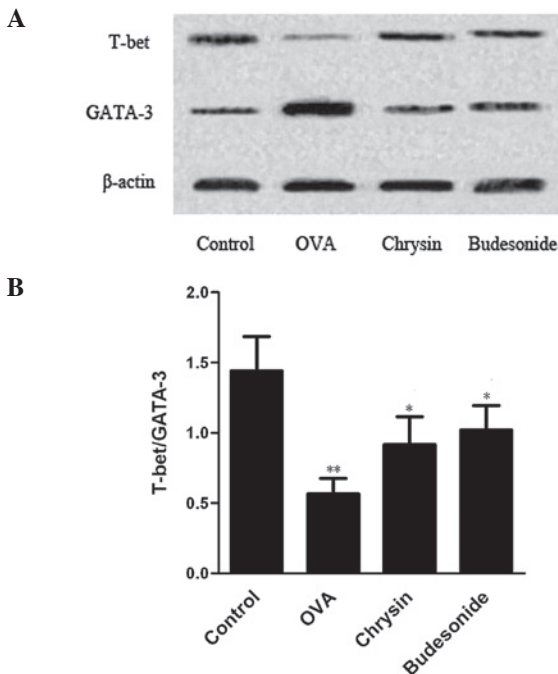


Figure 5. Treatment with chrysin increased the ratio of T-bet and GATA-3 in the lung in a murine model of asthma. Treatment with chrysin (A) increased the relative density of T-bet and decreased the relative density of GATA-3, and (B) increased the ratio of T-bet and GATA-3 compared with the OVA group. Data are the means ± SEM (n=6 per group). **P<0.01 compared with the control group; *P<0.05 compared with the OVA group. OVA, ovalbumin.

IgE in serum. In addition, treatment with chrysin regulates the level of T-bet and GATA-3 levels in lungs. Furthermore, we showed that chrysin markedly suppressed AHR in a murine model of asthma.

A previous study has demonstrated the regulation effect of chrysin on the immune system in mice, as evidenced by the decreased levels of total IgE and Th2 cytokines (17). In asthma, allergen-induced Th2 cells in the lungs lead to eosinophilic airway inflammation, increased secretion of mucus and recurring bronchospasm (18). In this study, we observed that treatment with chrysin markedly reduced not only the eosinophil count, but also the Th2 cytokine IL-4 level in BALF. Lung histology also validated the fact that chrysin suppressed the eosinophilic airway inflammation and mucus-producing goblet cells in the airway. These results suggest that chrysin is an anti-inflammatory or anti-allergic herbal extract that appears to be beneficial for the treatment of allergic asthma.

It is widely accepted that Th1/Th2 imbalance plays a significant role in asthma. CD4⁺ Th cells can be divided into Th1 and Th2 groups defined by functional differences and cytokine profiles. Induction of the transcription factors T-bet and GATA-3 play crucial roles in Th cell differentiation into Th2 and Th1 cells (19-20). T-bet, a member of the T-box family of transcription factors, was a master determinant of Th1 lineage, and T-bet-deficient mice exhibited a profound

lack of Th1 immune responses (21). GATA-3 is a transcription factor that is specifically expressed in Th2 cells and plays a critical role in the differentiation of Th2 cells from uncommitted CD4⁺ lymphocytes. It has been reported that IFN- γ and IL-4 levels in the lymphocytes of asthmatic patients were correlated with the ratio of T-bet/GATA-3, which may be used as an objective indicator of immune imbalance in patients with allergic asthma (21). Therefore, resetting the T-bet/GATA-3 ratio imbalance may have an important therapeutic role for asthma (22-24). In this study, the ratio of T-bet to GATA-3 was markedly decreased accompanied by an increase of the Th2 cytokine IL-4 and inhibition of synthesis of the Th1 cytokine IFN- γ in the asthma group compared with the control group, and partly reverted in the chrysin treatment group.

The pathophysiology of AHR is complex and a number of factors contribute to its development. Chronic airway inflammation is an important factor that results in AHR. Th2 cytokines, particularly IL-4, which regulate inflammatory cell recruitment to the lung, are critical in allergic inflammation and the development of AHR (25-26). Administration of the Th1 cytokine IFN- γ effectively attenuates established allergy-induced airway inflammation and AHR in mice (27). In addition, eosinophils are crucial in the development of AHR, since AHR is absent in mice whose eosinophils are completely ablated (28). Our findings demonstrated, for the first time, that treatment with chrysin significantly inhibited AHR in a model of allergic asthma. The inhibition of AHR may be attributed to restoring the Th1/Th2 imbalance and the reduction of eosinophilic airway inflammation in allergic mice.

In conclusion, our data have shown that treatment with chrysin reduces airway inflammation and AHR due to the alteration of Th1/Th2 polarization via the suppression of GATA-3 and the increase of T-bet expression in OVA-induced allergic mice. Therefore, the use of chrysin is a promising approach towards a novel asthma therapy.

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