

RESEARCH ARTICLE

Apigenin inhibits allergen-induced airway inflammation and switches immune response in a murine model of asthma

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Abstract

Many flavonoids were demonstrated to possess the antiallergic effect. Here we detected whether apigenin, a flavonoid, can attenuate allergen-induced airway inflammation and what is the possible mechanism in a murine model of asthma. Apigenin decreased the degree of the inflammatory cell infiltration, airway hyperresponsiveness, and total immunoglobulin E levels compared with the ovalbumin group. In addition, apigenin triggered the switching of the immune response to allergens toward a T-helper type 1 (Th1) profile. Our data clearly demonstrated that apigenin exhibits an anti-inflammatory activity in a murine asthma model, and can switch the immune response to allergens toward the Th1 profile.

Keywords: Apigenin; asthma; GATA-3; immune response; T-bet

Introduction

Allergen-induced airway inflammation in asthma is associated with elevated immunoglobulin E (IgE) in blood plasma and involves infiltration of eosinophils into the airway. These features result from an aberrant T-helper type 2 (Th2) immune response to allergen that leads to the recruitment of CD4⁺ Th2 lymphocytes in the airway. Dominant Th2 cells then generate more Th2 cytokine which cause B cells to synthesize IgE than normal.^(1,2) Th2 cell differentiation is driven by the transcription factor GATA-binding protein 3 (GATA-3), a member of the GATA family of zinc finger proteins which controls expression of Th2 cytokines,⁽³⁾ and is suppressed by T-box expressed in T cells (T-bet), a Th1-specific transcription factor, which is thought to induce interferon- γ production while inhibiting interleukin-4 (IL-4) production.^(4,5) In addition, recent reports have shown that infiltrated eosinophils in the

lung preferentially stimulate Th2 cell responses by presenting antigens.^(6,7) Therefore, it is proverbially considered that Th2 cells are dominant in asthmatic airway inflammation⁽¹⁾ and Th2 cytokines play an especially important role in the occurrence and development of asthma.⁽⁸⁾

Anti-inflammatory therapy is emphasized according to current treatment guidelines for asthma, and glucocorticoids are the most important and first-line therapeutic agents. Even though glucocorticoids are effective in preventing allergic inflammation, asthmatic patients who chronically inhaled glucocorticoids may develop serious side-effects, and those patients with severe forms of the disease or steroidal resistance get poor curative effect by inhale glucocorticoids.^(9–12) Furthermore, few asthmatic patients are able to obtain normal bronchial reactivity, even after years of steroid therapy. For these reasons, new or alternative anti-inflammatory agents are obviously needed to manage this disease.

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The flavonoids comprise a family of common phenolic plant pigments that have been identified as anticarcinogens and antioxidants.⁽¹³⁾ In the earlier studies, a variety of flavonoids exhibited profound immunoregulatory activity.^(14–16) Apigenin (Figure 1), a nonmutagenic and low toxicity flavone, is found in a variety of fruits and vegetables, including onions, parsley, and oranges, chamomile tea, wheat sprouts, and certain seasonings.⁽¹⁷⁾ Recently, some studies have convincingly demonstrated that apigenin possess anti-inflammatory, anticarcinogenic, and free radical-scavenging activities in a variety of *in vitro* systems.^(18–20) Yano et al.^(21,22) have demonstrated the regulation effect of apigenin on the immune system in ovalbumin (OVA)-sensitized mice, as evidenced by the decreased levels of total IgE. Sequentially, Yano⁽²³⁾ proved dietary apigenin attenuates exacerbation of atopic dermatitis (AD) symptoms through the reduction of serum IgE level expression. These data suggest that apigenin maybe can reduce total IgE level in asthma, a kind of inflammatory disorder like AD in term of mechanism, even suppress the infiltration of eosinophils and inhibit asthmatic symptoms. In order to prove this hypothesis, we investigated the effects of apigenin on airway inflammation and immune response in a murine model of asthma.

Materials and methods

Experimental animal

A total of 32 six-week-old female BALB/c mice, 18–22 g each, were purchased from Shanghai Laboratory Animals 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.

Antigen sensitization, challenge, and treatment

Mice were randomly divided into four groups of eight animals each: the control group, the OVA group, the low-dose apigenin group, and the high-dose apigenin

group. Mice in the last three groups were sensitized on days 0 and 14 by intraperitoneal (i.p.) 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. One week after the last sensitization, mice were exposed to aerosolized 1% OVA for 30 min each day three consecutive days. Mice in the two treatment-groups were consecutively i.p. injected with apigenin (Sigma, St. Louis, MO, USA) between days 15–23 with 2 mg/kg/day or 20 mg/kg/day, respectively. Apigenin, dissolved in 1% dimethylsulfoxide (DMSO) injection was administered in a solution with a total volume of 200 µL. Mice in the other groups received 1% DMSO without apigenin. For aerosolized challenges, mice were placed in a Plexiglas box (29 cm × 22 cm × 8 cm) and subjected to aerosolized OVA with an ultrasonic nebulizer (NE-U11B, Omuron, Tokyo, Japan). 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.

Evaluation of airway responsiveness

Airway responsiveness to acetylcholine chloride (ACH) was measured in mice 24 h after the last challenge with an Anires animal lung function analysis system (SYNOL High-Tech, Beijing, China), as described previously.⁽²⁴⁾ Being anesthetized by i.p. injection of pentobarbital sodium (70 mg/kg) (Fluka Chemical Corp., Milwaukee, USA), mice were inserted into the trachea by a plastic tube, 2 mm internal diameter, via a tracheotomy for mechanical ventilation and the caudalis vein by a 27 gauge needle for drug administration. 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 stable airway pressure recording, ACH was administered by intravenous injection with a microinfusion pump at a rate of 36 mL/h in progressively increasing doses (0, 10, 30, 90, 270 µg/kg). After the administration of each dose, data was collected continuously from 5 sec to 1 min and maximum values of lung resistance were taken to express changes in murine airway hyperresponsiveness (AHR).

Analysis of BALF samples and serum

After evaluation of airway responsiveness, mice were bled by retro-orbital puncture using heparinized capillary tubes. Blood samples were centrifuged at 2000 g for 10 min at 4°C and serum was stored at –70°C until use. After extracting the blood sample, tracheas were inserted with a catheter by way of incisal opening sited in cervical part, and airway lumina were washed three times

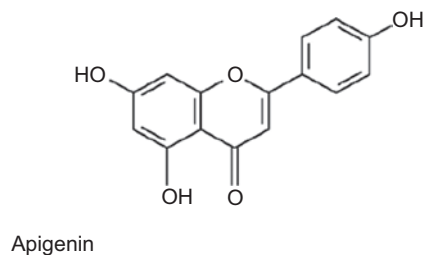


Figure 1. Chemical structure of apigenin.

with 0.4 mL, 0.3 mL, 0.3 mL sterile saline, respectively. Bronchoalveolar lavage fluid (BALF) was centrifuged at 1000 g for 10 min at 4°C and the number of total cell was counted with a hemacytometer. Smears of BALF cells were stained with Wright's staining for differential cell counting. The cells in the BALF samples were counted by two independent investigators in a single-blind study which analyzed at least 200 cells each of four different random locations using a microscope. Commercially available enzyme-linked immunosorbent assay kits were used to determine IL-4 and IFN- γ (Jingmei Biotech, Shanghai, China) levels in BALF and total serum IgE (BPP Biomedicals, Franklin, MA, USA). The limits of detection were 7 pg/mL for IFN- γ , 7 pg/mL for IL-4, and 1 ng/mL for total serum IgE.

Histological examination

After BALF was obtained, the left lung was removed, fixed in 10% neutral buffered formalin for 24 h, and then the specimens were dehydrated and embedded in paraffin in a standard manner. In order to carry out histological examination, 5 μ m sections of fixed embedded tissues were cut and stained with hematoxylin and eosin, according to routine laboratory procedure. Histological analyzes were performed by pathologists blinded to the treatment groups. For each mouse, five airway sections, distributed throughout the left lung, were analyzed use of a light microscope attached to an image-analysis system (Image-Pro Plus; Media Cybernetics, Minneapolis, MN, USA). The images were then cropped and corrected for brightness and contrast, but otherwise were not manipulated.

Western blot analysis

The right lung tissues from each group were added to lysis buffer and homogenized. After absolute schizolysis and centrifugation at 12,000 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. The protein from each mouse in the four groups were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated in blocking solution (5% dried milk in Tris-buffered saline) for 1 h at room temperature and then exposed to monoclonal antibody directed against GATA-3, T-bet (Cell Signaling Technology, Beverly, MA, USA) or anti- β -actin antibody (Jingmei Biotech, Shanghai, China) overnight at 4°C with gentle shaking. On the following day, the membrane was incubated with peroxidase-conjugated secondary antibody (1:1000) for 1 h at 37°C. 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

Experiments were repeated at least three times with consistent results. Data are expressed as the mean $\pm$ ASEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) and least significant difference (LSD) test with SPSS software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Apigenin decreases AHR in experimental asthma

To examine the effect of apigenin on AHR, we measured airway resistance in anaesthetized mice by invasive whole-body plethysmography. No significant difference was found in baseline airway resistance among the four groups. The airway resistance in the OVA and apigenin groups was obviously increased in a dose-dependent manner by administration of ACH, whereas only a slight increase could be detected in the control group. The airway resistance generated by administration of ACH at doses from 30 to 270 μ g/kg increased significantly in the OVA and apigenin groups compared with the control group. The OVA group had significantly greater airway resistance than the control group ($P < 0.01$; Figure 2). Treatment with apigenin led to a sharp decrease in airway resistance compared with the OVA group ($P < 0.05$; Figure 2) and the decrease was more obvious in the high-dose apigenin group.

Apigenin attenuates airway inflammation

Apart from macrophages, very few inflammatory cells were detected in control BALB/C mice. A significant increase in total cell number was exhibited in OVA-sensitized and challenged animals, when compared to the control mice. This increase resulted from a marked eosinophilia and a significant enhancement in the number of macrophages. The effect of apigenin on allergen-induced inflammatory cell infiltration was assessed in animals treated with two different dose of apigenin during the last 9 days before put to death. As exhibited in Figure 3, apigenin suppressed allergen-induced inflammatory cell infiltration, and the high-dose apigenin showed the greater suppression than the low-dose apigenin. The anti-inflammatory effect of apigenin was further demonstrated in following histological examination of hematoxylin-eosin stained sections. A marked affluence of inflammatory cells into

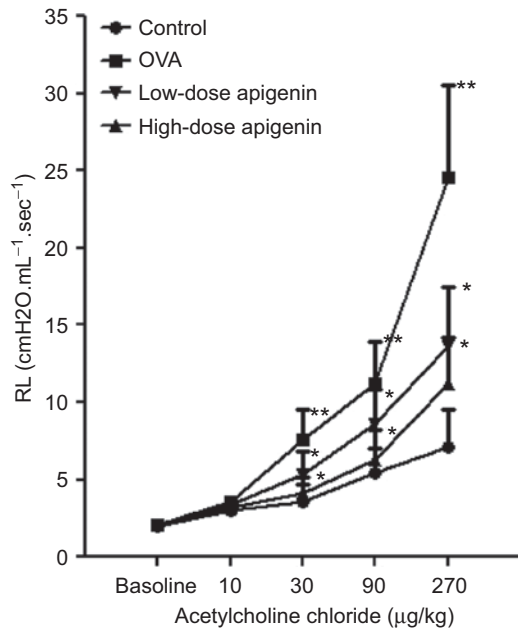


Figure 2. Effects of apigenin on the development of airway hyper-responsiveness (AHR) in a murine model of asthma. Apigenin (▲, ▼) decreased AHR compared with the ovalbumin (OVA)-sensitized and -challenged mice (■). (●), control. Data are the mean SEM ($n=8$ per group). * $P<0.05$ compared with the OVA group; ** $P<0.01$ compared with the control group.

the airway and around the blood vessels was observed in OVA-sensitized/challenged mice, but not in the saline treated control mice. Mice treated with apigenin showed a marked diminution in inflammatory cell infiltration within the peribronchiolar and perivascular regions (Figure 4A–D).

Apigenin alters levels of Th1/Th2 cytokines in BALF and total IgE

Levels of the Th2 cytokine, IL-4, in BALF was significantly increased, but the concentration of IFN- γ was decreased in OVA-sensitized and challenged mice compared with the control group ($P<0.05$; Table 1). Administration of apigenin reduced the levels of these Th2 cytokine in BALF compared with the OVA group ($P<0.05$; Table 1). By contrast, it raised the average concentration of IFN- γ ($P<0.05$; Table 1) in BAL fluid. Total serum IgE levels were significantly enhanced in the OVA group compared with the control group ($P<0.05$; Table 1). Treatment with apigenin reduced total serum IgE levels to near baseline compared with the OVA group ($P<0.05$; Table 1).

Apigenin alters the ratio of T-bet and GATA-3 in lung

The relative amount of target protein was quantified as the density of the target protein versus β -actin. After

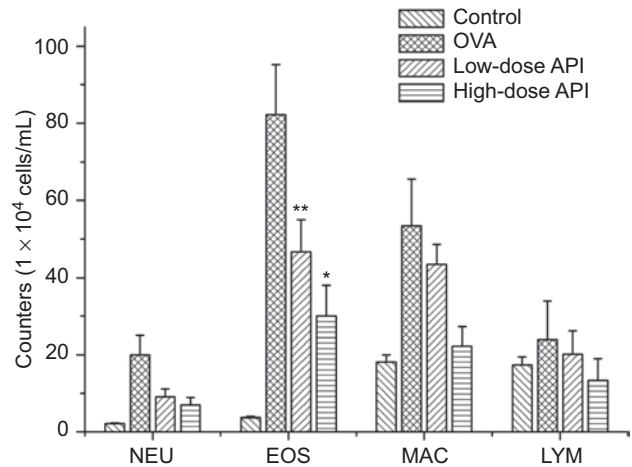


Figure 3. Effects of apigenin on inflammatory cell accumulation in bronchoalveolar lavage fluid (BALF) in a murine model of asthma. Apigenin decreased accumulation of macrophages, eosinophils, lymphocytes and neutrophils specifically, compared with the OVA group. Data are the mean SEM ($n=8$ per group). * $P<0.05$ compared with the OVA group; ** $P<0.01$ compared with the control group.

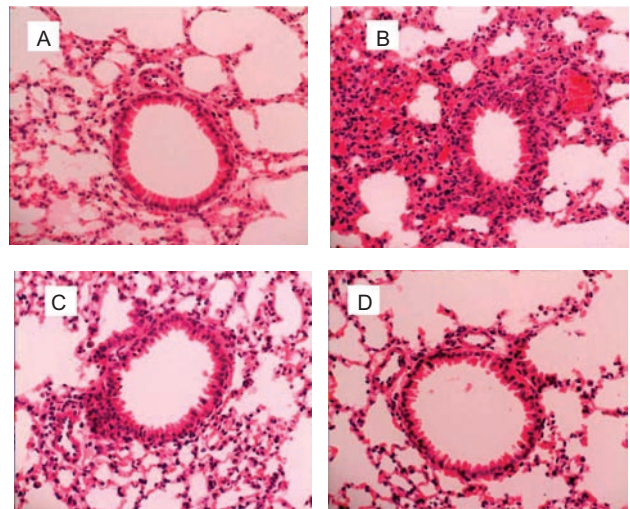


Figure 4. Treatment with apigenin attenuates airway inflammation in a murine model of asthma. (A–D) hematoxylin and eosin staining (original magnification $\times 200$) of lung tissue from (A) control, (B) ovalbumin (OVA)-sensitized and -challenged, and (C) low-dose apigenin-treated mice, (D) high-dose apigenin-treated mice.

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 (Figure 5). There was a significant difference in the ratio of T-bet and GATA-3 in asthmatic mice compared with normal control subjects ($P<0.05$; Figure 5). Treatment with apigenin increased the ratio of T-bet and GATA-3 compared with asthmatic mice, and the alteration was more obvious in the high-dose group than in the low-dose group.

Table 1. Serum total IgE, cytokines levels in BALF were measured by ELISA.

Groups	Number	IL-4 (pg/mL)	IFN- γ (pg/mL)	IgE (ng/mL)
Control	8	21.75 $\pm$ 4.29	27.75 $\pm$ 4.48	18.02 $\pm$ 4.97
OVA	8	127.68 $\pm$ 28.39*	19.50 $\pm$ 3.13*	192.81 $\pm$ 17.64*
Low-dose apigenin	8	49.87 $\pm$ 3.27**	24.91 $\pm$ 5.06**	76.33 $\pm$ 27.15**
High-dose apigenin	8	34.82 $\pm$ 4.23**	23.90 $\pm$ 6.86**	40.92 $\pm$ 12.35**

The results are the mean SEM ($n=8$).

BALF, Bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; IFN- γ , interferon- γ ; IgE, immunoglobulin E; IL-4, interleukin-4; OVA, ovalbumin.

* $P < 0.05$ compared with the control group; ** $P < 0.05$ compared with the OVA group.

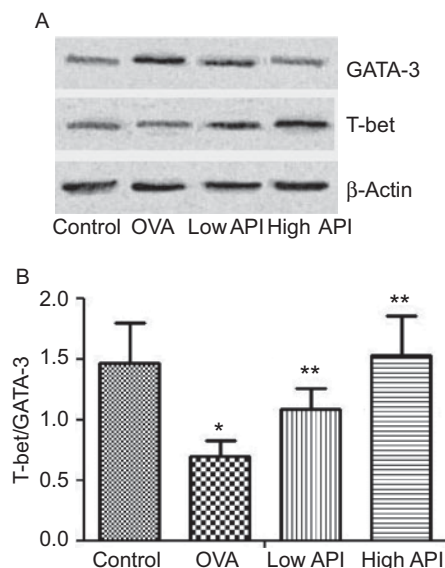


Figure 5. Treatment with apigenin increased the ratio of T-bet and GATA-3 in lung in a murine model of asthma. Apigenin increased the relative density of T-bet and decreased the relative density of GATA-3, and increased the ratio of T-bet and GATA-3 compared with the OVA group. Data are the mean SEM ($n=8$ per group). * $P < 0.05$ compared with the control group; ** $P < 0.01$ compared with the OVA group.

Discussion

In the present study, we have assessed the effects of apigenin on allergen-induced airway inflammation and immune response in acute experimental asthma. Our results showed that apigenin can decrease airway inflammation induced by allergens and switches the immune response to allergen from a Th2 to a Th1 profile. The switching of the immune response occurred when apigenin was administered from the end day of the last sensitization to the end of the challenge.

It has been reported that apigenin take part in the anti-inflammatory and immunoregulatory activity *in vitro* and *in vivo*.^(25,26) In the present study, we found that injection of apigenin markedly reduced total serum IgE, suppressed airway inflammatory cells infiltration induced by allergens, resulting in decreased number of eosinophils and total inflammatory cells in BALF, and the high-dose apigenin exhibited the more conspicuous effect in

anti-inflammation. Lung histology validated an apigenin ablation of airway inflammation in the lung. These findings suggested that apigenin is an anti-inflammatory or immunoregulatory herbal extract that appears beneficial for the treatment of allergic asthma.

It is widely accepted that T cells play an important role in the injurious lung immune responses associated with asthma. CD4⁺ Th cells can be divided into Th1 and Th2 groups functionally based on the various kinds of cytokine that they produce. The different patterns of T cell differentiation generate the different inflammatory effectors, and those inflammatory effectors are correlated with the extent and type of damage observed in airway. Under normal physiologic conditions, the ratio of Th1 to Th2 cells maintains a suitable level. Once the balance between Th1 and Th2 is broken, diseases will occur. The two major Th-specific transcription factors, T-bet and GATA-3, regulating the expression of cytokine genes are characteristics of Th1 or Th2, and play crucial roles in Th cell differentiation. It has been reported that change in the T-bet/GATA-3 ratio reflect change in the Th1/Th2 balance. Therefore, T-bet/GATA-3 ratio may be used to evaluate the immune balance of the Th1/Th2 responses in asthma. In addition, the increased IL-4 production is correlated with excessive Th2 cell responses, and IFN- γ is correlated with Th1 cell responses. In our studies, the ratio of T-bet to GATA-3 decreased in the asthma group compared with the control group, and reverted partly in the apigenin treatment groups. At an equal pace, IL-4 production was depressed, and IFN- γ level increased in treatment groups. And the degree of these change in above were more prominent in the high-dose apigenin group than those in the low-dose group. The data suggests that apigenin administered from the end day of the last day of sensitization to the end of challenge can switch the immune response to allergens in BALB/c mouse, the high-dose apigenin are more effective.

The pathophysiology of AHR is complex because many factors contribute to its development. Allergen-induced airway inflammation is an important one of these factors, and Th2 cytokines, especially IL-4, are critical in allergic inflammation and development of AHR.⁽²⁷⁾ In the present study, the airway resistance in the apigenin groups was decreased by administration of ACH compared with the

OVA group and the decrease in the high-dose group was larger than that in the low-dose group. This alteration was in accordance with the change of the number of eosinophils and total inflammatory cells and the expression of IL-4 observed in BALF. When these changes are combined, the apigenin-induced inhibition of hyperresponsiveness may correlate with the reduction in airway inflammation.

The pathogenesis of asthma is not entirely clear and apigenin may also have additional effects on the other immune cells. Th17 cells are considered to take an important role are implicated in the pathogenesis of a number of inflammatory diseases.^(28–30) It has been demonstrated that Th17 cells are increased in the lung and draining lymph nodes of antigen-sensitized CC10 IL-23 mice upon antigen inhalation.⁽³¹⁾ Moreover, Laura McKinley found that Th17 cell-mediated airway inflammation is steroid-resistant.⁽³²⁾ In addition, adoptive transfer of antigen-specific CD4⁺CD25⁺ T-regulatory cells was found to suppress allergic inflammation and hyperreactivity via a mechanism dependent upon IL-10.⁽³³⁾ Importantly, when delivered after the onset of disease, T-regulatory cells were able to downregulate established inflammation and prevent airway remodeling.⁽³⁴⁾ Whether apigenin can influence the effects or differentiation of Th17 cells and T-regulatory cells remain unknown.

In conclusion, our data clearly demonstrates that apigenin inhibits allergen-induced airway inflammation, AHR, and can switch the immune response to allergens toward a Th1 profile. Overall, apigenin is promising to be a beneficial medication for the treatment of asthma by ameliorating allergic responses.

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Declaration of interest

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