

Original Article

Quercetin inhibits airway allergic inflammation through inhibiting the expression of NF- κ B activator 1

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Abstract: Objectives: Quercetin is a major bioflavonoid found in plants and used as antioxidant supplements on the market. Airway allergic inflammation is very common in asthma patients, which characterized by excessive eosinophils infiltration, IgE production and enhanced airway resistance due to Th-2 dominance. The present study was designed to investigate the anti-allergic potential of Quercetin as well as the underlying mechanism involved. Methods: An OVA-sensitized AHR animal model was established by inhalation of 1% OVA for consecutive 14 days after an initial injection of 10% OVA intraperitoneally. Then, the airway resistance was assayed by Buxco Resistance and Compliance (RC) system; cell enumeration was assayed by direct microscopic observation; IFN- γ , IL-4 and IL-17 secretion in BALF and IgE production in serum were assayed by ELISA. Real-time PCR and immunohistochemistry were used to test the expressions of NF- κ B activator 1 (Act1) which promotes allergic inflammation. Results: The results showed that Quercetin (50 mg/kg) decreased airway resistance, the number of total cells and eosinophils, the secretion of IL-4 and IL-17 and OVA-specific IgE induced by OVA. Moreover, Quercetin (50 mg/kg) also significantly decreased the expression of Act-1 induced by OVA. And the increase of Act-1 mRNA expression was obviously positively associated with the increased eosinophils infiltration ($P=0.0013$), secretion of IL-4 ($P=0.0006$) and IL-17 ($P=0.0034$) and production of IgE antibody ($P=0.0074$). Conclusions: Our data suggest that Quercetin effectively inhibits allergic inflammation including reductions of airway responsiveness, eosinophil infiltration, IL-4 and IL-17 secretion and IgE production by inhibiting the expression of Act1.

Keywords: Quercetin, allergic inflammation, airway resistance, Th2, Th17, NF- κ B activator 1

Introduction

Asthma is a disease of chronic airway allergic inflammation characterized by reversible airway obstruction, airway hyperresponsiveness (AHR), infiltration of eosinophils and T lymphocytes into the airway submucosa, mucus hypersecretion, and airway remodeling. Asthma is a complex noncommunicable disease associated with complex pathophysiology that carries a significant mortality rate and high morbidity all over the world, especially in children [1]. So far, it is well recognized that asthma is caused by Th2-driven inflammatory responses, which stimulate the releases of various cytokines such as interleukin (IL)-4, IL-5, IL-13 and enhance airway eosinophilia, IgE production and lung mucus release [2]. Excessive Th2 cells stimulate B cells to produce large quantities of antibodies and recruit eosinophils and neutro-

phils to sites of inflammation through the release of specific cytokines and chemokines including IL-4, IL-5, IL-13, IL-17, and IL-33 [3]. Another lineage of T cells that produce IL-17, has been recently identified in asthma patients and stimulates airway inflammation of allergic asthma [4]. Therefore, inhibition of Th2 and Th17-type cytokine production or stimulation of Th1-type cytokine production may be a useful therapeutic approach for some inflammatory diseases such as allergic asthma.

Quercetin is a major bioflavonoid found in plants such as tea, onions, apples, grains and grapes. A series of *in vitro* and *in vivo* studies suggested a potential therapeutic effect of Quercetin against inflammation which is the main characteristic of neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases [5-9]. Most

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recent studies have affirmed that Quercetin ameliorated the airway inflammation by reducing the production of inflammatory cytokines, relaxing tracheal rings, and reducing total number of cells in bronchoalveolar lavage fluid (BALF) and erythropoietin (EPO) in lungs [10, 11]. Moreover, Quercetin is reported to ameliorate various lung cancers and lung injuries in several *vitro* and *vivo* studies [12, 13]. However, more evidences are needed regarding whether Quercetin inhibits the process of chronic allergic inflammation in lungs.

Materials and methods

Reagents

Quercetin dissolved in dimethylsulfoxide (DMSO) were from Sigma, USA. Interleukin IFN- γ , IL-4 and IL-17 (rat) and anti-rat ovalbumin (OVA) IgE antibodies, ELISA kits were purchased from Boster Company, Wuhan, China.

Animals and treatments

Sprague Dawley (SD) rats, each weighing 200 ± 20 g, were obtained from the experimental animal center, first affiliated hospital of Zhengzhou University and maintained in a pathogen-free environment. All procedures were reviewed and approved by the Animal Care and Use Committee of Zhengzhou University. Animals were randomly divided into four groups with 6 animals in each group: control group, OVA group, control + Quercetin group and OVA + Quercetin group. The OVA group challenged with 1% OVA for 30 min for consecutive 14 days after an initial injection of 10% OVA + 10% Al(OH)₃ intraperitoneally, and the Quercetin group pretreated with Quercetin (0, 10, 25, 50 mg/kg) 1 h before OVA challenge [14].

Airway resistance assay

Airway resistance was assessed *in vivo* in anesthetized rats by measuring changes in lung resistance in response to increasing doses of inhaled methacholine (Mch). The animals were intubated with a 6 mm tracheal cannula. The other end of the catheter was connected to Buxco Resistance and Compliance (RC) system (Buxco, USA). So the intrapulmonary pressure and airflow signal could be recorded synchronically. Baseline values were recorded from data obtained after challenge with aerosolized

Phosphate buffered saline (PBS). Airway resistance (AR) then could be recorded as increased percentage from baseline (%).

Cell enumeration of BALF

Immediately following assessment of AR, rats were sacrificed with an i.p injection of sodium pentobarbitone (100 mg/kg). The trachea was cannulated and BALF was obtained by washing the airways three times with 1 mL of PBS through a tracheal cannula. Coefficient of recovery was about 80%. Then, the BALF was centrifuged and the cell pellet was suspended in 200 μ L of PBS and counted using a hemocytometer. The cell suspensions were then centrifuged onto glass slides using a cytospin centrifuge at 1000 g for 5 min at room temperature. Cyto-centrifuged cells were air dried and stained with Wright staining which allows differential counting of various cells. At least 400 cells per sample were counted by direct microscopic observation.

ELISA assay of BALF

After centrifugation of BALF, the supernatant was used for determination of cytokine levels. Interleukin IFN- γ , IL-4 and IL-17 production in BALF and anti-OVA IgE in serum were measured by ELISA according to the manufacturer's instructions. Briefly, Total 100 μ L supernatants were added into 96-well plate and incubated for 1 h, followed by 100 μ L enzyme-linked antibodies incubation for 0.5 h at 37°C. After washed for 3 times with washing buffer, the chromogenic reagent was added and incubated for 0.5 h, followed with 2 M H₂SO₄ termination reaction. The 450 nm absorbance was determined by microplate reader. Each sample was repeated three times.

Real-time PCR assay

RNA was isolated from rat lung tissues using Trizol reagent (Invitrogen, China) according to the instructions of the manufacturer. Each sample was reverse transcribed into cDNA and analyzed by quantitative real-time PCR with SYBR Green I. The primers (Invitrogen, China) for Act-1 were 5'AGTCAGCGAGCCTGCGTC3' and 5'CCATCTCCTGGCTACCGC3' (197 bp). Briefly, 2 μ L (out of 20 μ L) of the reverse-transcribed reaction mixture was added to a 20 μ L PCR mixture for 35 cycles. Each cycle included 94°C

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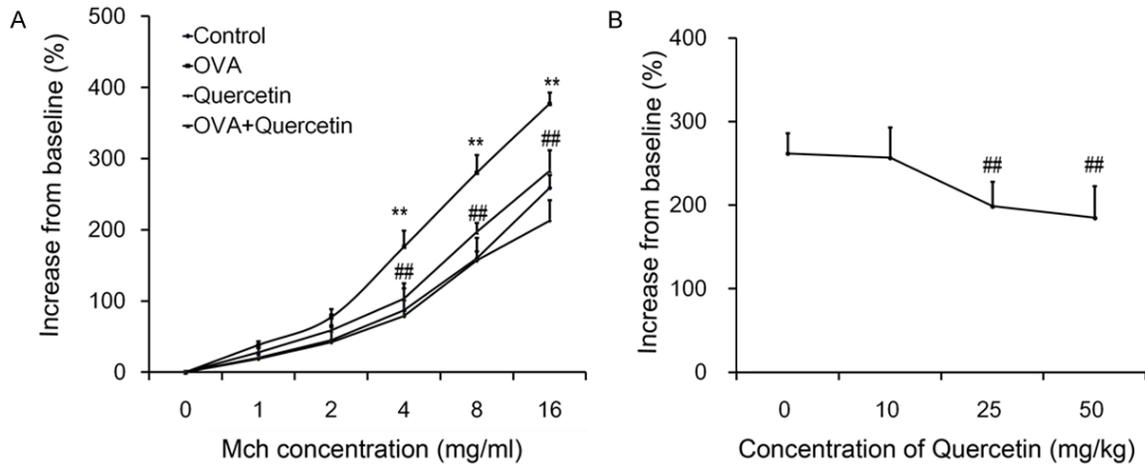


Figure 1. Quercetin inhibited AR in an OVA-induced AHR model (n=4). A: AR assay induced by different concentrations of Mch. OVA resulted in increased AR to Mch (at the concentrations of 8 and 16 mg/mL) and Quercetin abolished the increased AR. $**P < 0.01$ versus Control; $###P < 0.01$ versus OVA group. B: Different concentrations of Quercetin inhibited AR in a concentration dependent manner in an OVA-induced AHR model when the animals were challenged by 8 mg/ml of Mch. $###P < 0.01$ versus OVA group (at the concentration of 0). Measurements of lung mechanics were analyzed by two-factor ANOVA.

for 20 seconds, 60°C for 30 seconds and 72°C for 30 seconds after an initial 94°C for 5 min. Raw data were normalized to β -actin (5'TGACGTGGACATCCGCAAAG3' and 5'CTGGAAGGTGGACAGCGAGG3') [14].

Immunohistochemistry

After BALF was collected, the left lung was inflated with a 1:1 (v/v) mixture of optimal cutting temperature compound and 4% paraformaldehyde in PBS and then longitudinal slices were cut with a scalpel blade to allow for cross-sectioning of a maximum of airways. These slices were then placed in tissue cassettes and fixed for an additional 24 h in 4% paraformaldehyde in PBS, before processing into paraffin. 5- μ m sections were cut from each paraffin block. Endogenous peroxidase was inhibited by soaking tissue sections in 3% H_2O_2 . After rinsing in PBS, sections were incubated with goat serum to block nonspecific binding of antibodies, then sections were incubated overnight at 4°C with polyclonal rabbit anti rat Act-1 antibody (Santa Cruz Biotechnology, 1:200 dilution). After washing in PBS, the sections were incubated with biotinylated goat anti rabbit IgG for 1 h, and washed again. sABC was incubated with the sections for 60 min at room temperature. After washing in PBS, the signal was detected with 3,3'-diaminobenzidine. Omission of primary or secondary antisera was included

as a method of providing a control for each biopsy.

Statistical analysis

Calculations were performed with SPSS software. All values were expressed as Mean \pm SE. Measurements of lung mechanics were analyzed by two-factor ANOVA. Other data were compared by use of the Student's *t*-test. The relationships between variables were explored by simple correlation and multiple regression analyses. *P* value of less than 0.05 was considered significant.

Results

Quercetin inhibited AR in an OVA-induced AHR model

After the animals were challenged by different concentrations of Mch (0, 1, 4, 8 and 16 mg/ml), the AR of animals increased in a dose-dependent manner. The AR of OVA-treated animals, which were challenged by 4, 8 and 16 mg/mL of Mch increased significantly compared with those of the control animals. AR of OVA-treated animals significantly decreased after treatment with Quercetin (50 mg/kg) ($**P < 0.01$, **Figure 1A**). In OVA-treated animals, different concentrations of Quercetin inhibited AR in a concentration dependent manner when the animals were challenged by 8 mg/ml of Mch ($###P < 0.01$, **Figure 1B**).

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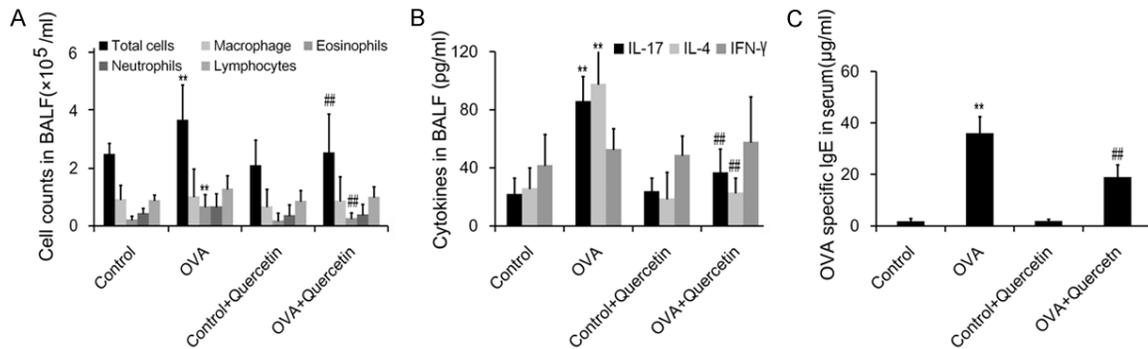


Figure 2. Quercetin inhibited eosinophils infiltration, IL-4, IL-17 and IgE production in an OVA-induced AHR model (n=6, ** $P < 0.01$, versus Control; ## $P < 0.01$, versus OVA group). A: Total and differential cells counts in BALF; B: Production of IL-4, IL-17 and IFN- γ in BALF; C: Secretion of OVA-specific IgE antibody in Serum. The data were compared by use of the Student's t -test.

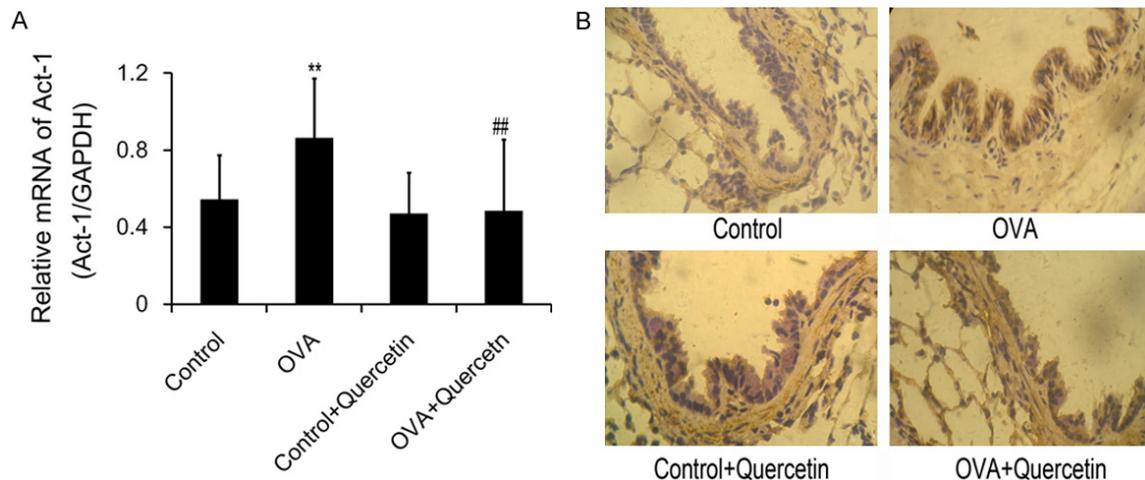


Figure 3. Quercetin inhibited Act-1 expression induced by OVA stress (n=4, ** $P < 0.01$ versus Control; ## $P < 0.01$ versus OVA group). A: Act-1 expression in lungs was determined by real time PCR. The data were compared by use of the Student's t -test. B: Act1 expression in lungs was determined by immunohistochemistry ($\times 400$).

Quercetin inhibited eosinophils infiltration, IL-4, IL-17 and IgE production in an OVA-induced AHR model

The lungs of animals treated with OVA showed a significant increase in the number of total cellular scores and eosinophils. Quercetin (50 mg/kg) decreased the total cells and eosinophils in BALF induced by OVA stress (**Figure 2A**). The secretions of IL-4 and IL-17 in the lungs of OVA-challenged animals increased compared to those in the control group (**Figure 2B**). Quercetin (50 mg/kg) administration resulted in significant reduction in IL-4 and IL-17 (**Figure 2B**). OVA-specific IgE in serum could be observed in all OVA-treated animals by using a qualitative ELISA Kit. Quercetin (50 mg/kg) decreased OVA-specific IgE (**Figure 2C**).

Quercetin inhibited Act-1 expression induced by OVA stress

By using real-time PCR, we observed that OVA stimulated the expression of Act-1 and Quercetin (50 mg/kg) significantly decreased its expression in lungs induced by OVA ($P < 0.01$, **Figure 3A**). The results of immunohistochemistry also showed that OVA promoted the expression of Act-1, which mainly distributed in the epithelial cells. Quercetin (50 mg/kg) significantly decreased the expression of Act-1 in lungs induced by OVA (**Figure 3B**).

Correlation between Act-1 expression and airway inflammation

Correlated analysis results showed the increase of Act-1 mRNA expression was obviously posi-

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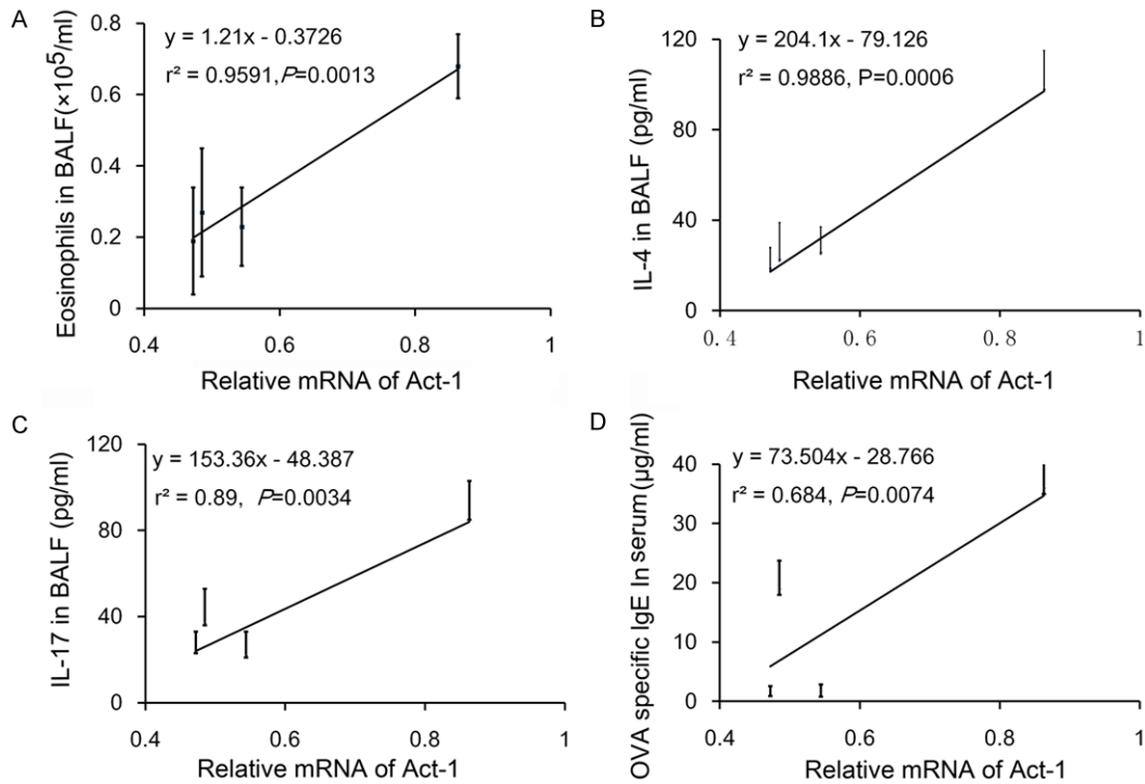


Figure 4. The relationship between Act-1 expression and eosinophils (A), secretion of IL-4 (B) and IL-17 (C) and production of IgE (D). The relationships between variables were explored by simple correlation and multiple regression analyses.

tively associated with the increased eosinophils infiltration (**Figure 4A**, $P = 0.0013$), secretion of IL-4 (**Figure 4B**, $P = 0.0006$) and IL-17 (**Figure 4C**, $P = 0.0034$) and production of IgE antibody (**Figure 4D**, $P = 0.0074$).

Discussion

Asthma is a chronic airway inflammatory disease characterized by intense leukocyte and eosinophilic infiltration accompanied by mucus hypersecretion and airway hyperresponsiveness. The exact mechanisms of allergic inflammation in asthma remain unclear but are considered to be dependent on the sustained infiltration and activation of numerous inflammatory cells including eosinophils, neutrophils, lymphocytes, and macrophages, followed by synthesis and release of a variety of pro-inflammatory mediators and cytokines [15, 16]. Eosinophil infiltration is a typical features of allergic asthma. Several mediators secreted from eosinophils can amplify portions of the inflammatory cascade and increase AHR, inflammatory cell accumulation, and mucus

secretion [17]. In our study, we found that Quercetin inhibited eosinophil infiltration significantly induced by OVA, indicating Quercetin is very effective to eliminate eosinophil accumulation.

Initiation of allergic response occurs with allergen presentation by antigen-presenting cells to CD4^+ T cells. Antigen-activated CD4^+ T cells orchestrate several characteristic features of asthma, including the secretion of Th2-type cytokines such as IL-4, IL-5, and IL-13 and IL-17, which are responsible for IgE production by B cells and eosinophil activation and recruitment [18, 19]. In addition, Th17 cells are implicated in the pathogenesis of antigen-induced airway inflammation [20]. Th17 cells secrete the cytokine IL-17 which mediate recruitment of neutrophils and induce the release of pro-inflammatory mediators. Our findings indicated that Quercetin significantly decreased Th2-type and Th17-type cytokine production. The immunoglobulin IgE may be an important target in treatments for allergic asthma and is closely associated with Th2 and Th17-type responses. Admi-

nistration of Quercetin markedly attenuated the increased OVA-specific IgE levels in serum.

Act-1 is a key component in IL-17A signaling and induces Th2 differentiation [21]. Swaidani's research showed that epithelium-derived Act-1 has the essential role in allergic pulmonary inflammation through the distinct impact of the IL-17R-Act1 and IL-25R-Act1 axes and acts as a potential therapeutic target for allergic pulmonary inflammation [22, 23]. In our present study, we observed Quercetin decreased the expression of Act-1 induced by OVA stress, which is associated with the increased infiltration of eosinophil, secretion of IL-4 and IL-17 and production of IgE antibody.

In conclusion, these findings support the evidence that Quercetin inhibited allergic inflammation in an asthmatic mouse model. Quercetin may have potential as an effective antiasthmatic agent through decrease of allergic inflammation.

Disclosure of conflict of interest

None.

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