



Hyperoside attenuates OVA-induced allergic airway inflammation by activating Nrf2



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ABSTRACT

Allergic airways disease (AAD) is one of the most common medical illnesses that is associated with an increased allergic airway inflammation. Hyperoside, an active compound isolated from *Rhododendron brachycarpum* G. Don, has been reported to have anti-inflammatory effect. The aim of this study was to analyze the protective effect of hyperoside on OVA-induced allergic airway inflammation in mice. In the present study, the mouse asthma model was induced by given OVA and hyperoside was administrated 1 h before OVA challenge. The levels of IL-4, IL-5, IL-13, and IgE were detected by ELISA. H&E staining was used to assess lung histopathological changes. The expression of NF- κ B p65, I κ B, HO-1, and Nf-E2 related factor 2 (Nrf2) were measured by western blot analysis. The results showed that hyperoside significantly reduced the inflammatory cells infiltration and the levels of IL-4, IL-5, IL-13, and IgE. Hyperoside significantly inhibited OVA-induced oxidative stress as demonstrated by decreased MDA, and increased GSH and SOD levels. Treatment of hyperoside also inhibited OVA-induced airway hyperresponsiveness (AHR). Furthermore, the results showed that treatment of hyperoside significantly inhibited LPS-induced NF- κ B activation. In addition, hyperoside was found to activate Nrf2/HO-1 signaling pathway. In conclusion, these results suggest that hyperoside ameliorates OVA-induced allergic airway inflammation by activating Nrf2 signaling pathway.

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1. Introduction

Allergic airways disease is a representative chronic disorder of the airways, which is characterized by inflammatory cells infiltration, airway hyper-responsive and airway inflammation [1]. Increased eosinophils were observed in patients of asthma and the production of Th2 cytokines IL-4, IL-5 and IL-13 were increased [2,3]. These cytokines play a critical role in the pathological process of asthma. Nf-E2 related factor 2 (Nrf2), an important transcription factor, has been reported to play critical role in asthma [4,5]. Studies showed that activating of Nrf2 could regulate the Th1/Th2 balance in the lung [6]. Furthermore, activating of Nrf2 could protect against OVA-induced asthma [7]. In recent years, the incidence and the perniciousness of asthma are increasing [8,9]. Thus, it is urgently needed to seek effective drugs to treat asthma.

Hyperoside, an active compound isolated from *Rhododendron brachycarpum* G. Don, has been reported to have anti-inflammatory and antioxidant effects [10]. Hyperoside has been reported to protect against cardiac ischemia reperfusion injury [11]. Also, hyperoside has been shown to inhibit carbon tetrachloride-liver injury in mice [12]. In

vitro, hyperoside was found to inhibit LPS-induced nitrite production in rat peritoneal macrophages [13]. Furthermore, hyperoside has been reported to inhibit LPS-induced TNF- α production in human endothelial cells [14]. Hyperoside also inhibits high glucose-induced vascular permeability, ROS production, and inflammatory response [10]. However, the protective effects of hyperoside against OVA-induced asthma remain unclear. In the present study, we analyzed the protective effect and mechanism of hyperoside on OVA-induced asthma in mice.

2. Materials and methods

2.1. Animals and reagents

BALB/c mice (18 to 20 g) were purchased from the Center of Experimental Animals of Wuhan University. The mice were housed in cages with the temperature was maintained at 24 ± 2 °C, and relative humidity was maintained at 40–80%. All the animal experiments were approved by the Institutional Animal Care and Use Committee of Wuhan University.

Hyperoside (purity > 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ovalbumin (OVA) and dimethyl sulfoxide (DMSO) were all purchased from Sigma (St. Louis, MO, USA). All the antibodies used in

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western blot were obtained from Cell Signaling Technology Inc. (Beverly, MA). The IL-4, IL-5 and IL-13 ELISA kits were provided Biolegend (California, USA). Other chemicals were according with reagent grade.

2.2. Experimental groups and establish of OVA-induced mouse asthma model

Mice were divided into six experimental groups ($n = 10$) as follows: Control group; hyperoside alone group (20 mg/kg); OVA group; OVA + hyperoside groups (5, 10, and 20 mg/kg); OVA + dexamethasone (DEX) (2 mg/kg) group. Mice were immunized on days 0 and 14 by intraperitoneal (i.p.) injection of 20 μ g OVA emulsified with 1 mg aluminum hydroxide in 200 μ l phosphate-buffered saline (PBS) (pH 7.4). On days 21, 22 and 23, 100 μ g of OVA in 20 μ l PBS was applied in mice by intranasal (i.n.) inhalations. Hyperoside was given at a dose of 5, 10 and 20 mg/kg body weight at day 21, 22 and 23. DEX was given at a dose of 2 mg/kg body weight at day 21, 22 and 23. For the control group, mice were exposed to same volume of PBS as immunization instead of OVA during airway challenge. The mice of hyperoside group were exposed to same volume of PBS as immunization instead of OVA during airway challenge and given at a dose of 20 mg/kg body weight at day 21, 22 and 23.

2.3. Collection of BALF and cell counting

The mice were anesthetized 24 h after the last dispose. The lungs were washed with 1.0 ml aliquots of cold PBS for 3 times to collect BALF. Then the BALF samples were centrifuged and the supernatants were collected. Cold PBS was used to resuspend the cell pellets. The numbers of eosinophils, neutrophils, macrophages and lymphocytes in BALF were stained by the Kwik-Diff staining.

2.4. Measurements of airway hyperresponsiveness(AHR)

Airway hyperresponsiveness (AHR) was detected using noninvasive whole-body plethysmography (Model PLY 3211; Buxco, Sharon, CT, USA) 24 h after the last OVA treatment.

2.5. Measurements of the levels of anti-OVA IgE in serum and cytokines in BALF

The mice were anesthetized 24 h after the last dispose. The blood samples were collected and settled at 4 °C for 2 h, followed by centrifugation at 1000 g for 10 min at 4 °C. The serum was stored at -80 °C. The levels of IL-4, IL-5 and IL-13 in BALF and IgE in serum were measured with specific mouse ELISA kits under the guidance of manufacturer's protocols (BioLegend, USA).

2.6. Lung histology

Lung tissues were fixed in 10% phosphate-buffer formalin and embedded in paraffin. The sections were cut into 5 μ m sections. Then the sections were stained with H&E staining to reveal the structural features.

2.7. MDA, SOD, and GSH assays

The mice were anesthetized 24 h after the last dispose. The lung samples were collected and homogenized. The levels of MDA, SOD, and GSH levels in lung homogenates were detected by using commercial kits purchased from the Beyotime Institute of Biotechnology (Shanghai, China) according to the instructions.

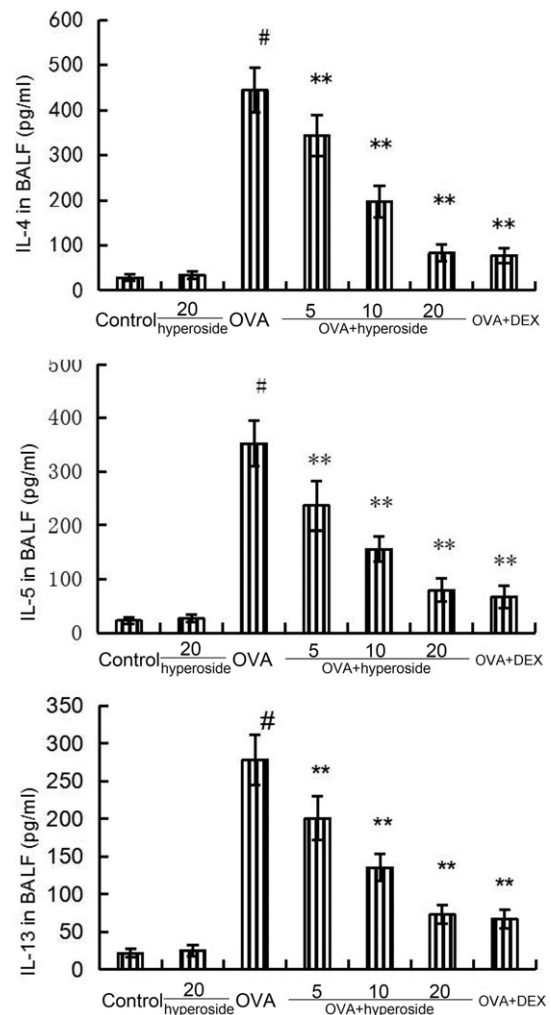


Fig. 1. Effects of hyperoside on OVA-induced IL-4, IL-5, and IL-13 productions in BALF were detected by ELISA. The values presented are the means $\pm$ SEM of three independent experiments. [#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA group.

2.8. Western blot analysis

Total protein from lung tissues were extracted by protein extraction kits that included phosphatase inhibitors and a protease inhibitor. Briefly, lung tissues were added to cell lysis buffer and homogenized on ice

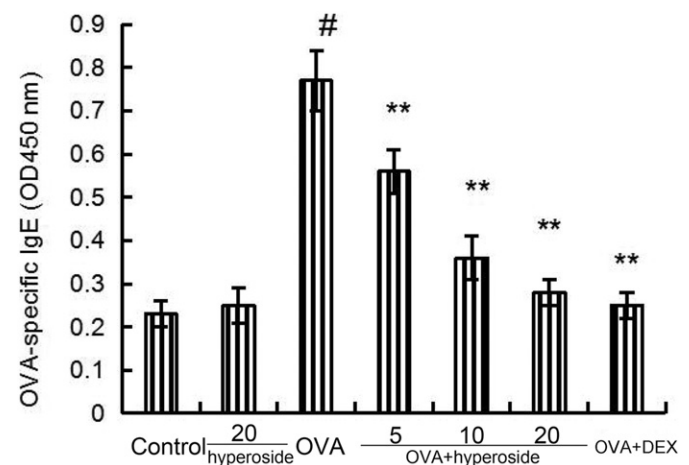


Fig. 2. Effects of hyperoside on OVA-induced IgE in serum were detected by ELISA. The values presented are the means $\pm$ SEM of three independent experiments. [#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA group.

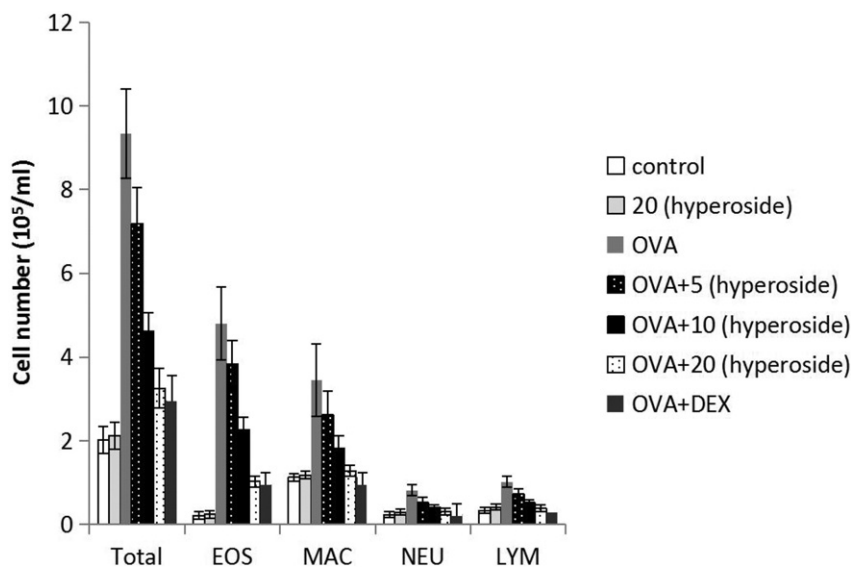


Fig. 3. Effects of hyperoside on OVA-induced inflammatory cell count in the BALF. The values presented are the means $\pm$ SEM of three independent experiments. [#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA group.

with phosphatase inhibitors and a protease inhibitor. The homogenate was centrifuged at 14,000 g for 10 min at 4 °C and the supernatant was aliquoted and then stored at -80°C after removing a small aliquot for protein quantification. Nuclear and cytoplasmic proteins from lung tissues were extracted using detecting kit (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. A BCA protein assay kit was used to determine the protein concentrations. The proteins (30 μg) were separated by 10% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked in 5% BSA for 1 h. Then the membranes were probed overnight with primary antibodies. After washing, the membranes were incubated with HRP-labeled secondary antibodies. Finally, the membranes were detected by the enhanced chemiluminescence detection reagents.

2.9. Statistical analysis

All data were expressed as mean $\pm$ SEM. To test the significance in multiple group comparisons, one-way ANOVA the Tukey-Kramer method were used. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Hyperoside reduces OVA-induced Th2 cytokine levels in BALF

ELISA was applied in this study to investigate the effects of hyperoside on OVA-induced Th2 cytokine production. The levels of IL-4, IL-5 and IL-13 in BALF of hyperoside group were equal to the control group. Compared with control group, levels of IL-4, IL-5 and IL-13 in BALF increased significantly in OVA group. However, the increases were significantly suppressed by treatment of hyperoside or DEX (Fig. 1).

3.2. Hyperoside decreases OVA-specific serum IgE level

The effects of hyperoside on OVA-induced IgE level was detected by ELISA. As shown in Fig. 2, OVA-specific IgE Level was observed to have an apparent increase in OVA-challenged mice compared with that in control group. In addition, administration of hyperoside or DEX significantly decreased the serum IgE level induced by OVA. The level of serum IgE in hyperoside group did not increase when compared to the control group.

3.3. Effects of hyperoside on inflammatory cells in BALF

The numbers of total cell, eosinophils, neutrophil, and macrophages in BALF were examined 24 h after the last OVA challenge. The results showed that the numbers of total cell, eosinophils, neutrophil, and macrophages in BALF increased significantly when compared to the control group. However, the numbers of total cell, eosinophils, neutrophil, and macrophages in OVA + hyperoside groups showed significant decreases compared with the control group (Fig. 3). The numbers of total cell, eosinophils, neutrophil, and macrophages in hyperoside group did not increase when compared to the control group (Fig. 3).

3.4. Hyperoside inhibits AHR in OVA-induced mouse asthma

The effects of hyperoside on OVA-induced AHR were detected by measuring Penh in response to increased doses of methacholine. The results showed that Penh value was obviously improved in response to methacholine inhalation in OVA-challenged group compared with the control group mice. However, a significant reduction of Penh value was observed in mice treated with hyperoside or DEX. The Penh value

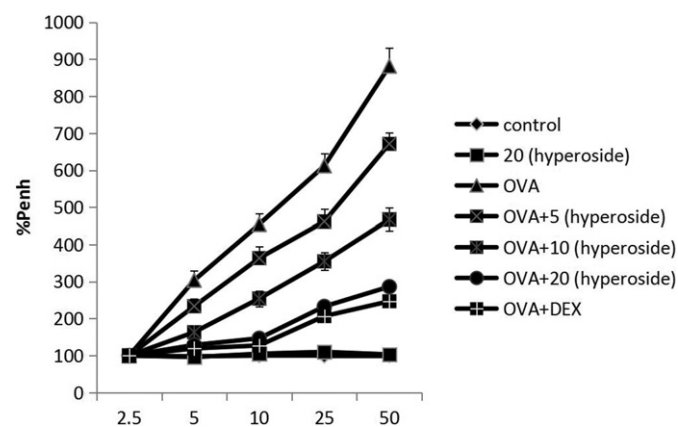


Fig. 4. Hyperoside alleviated OVA-induced airway hyperresponsiveness. The effects of hyperoside on AHR were measured using noninvasive whole-body plethysmography (Buxco, Sharon, CT). The values presented are the means $\pm$ SEM of three independent experiments. [#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA group.

of hyperoside group did not increase when compared to the control group (Fig. 4).

3.5. Hyperoside inhibits histopathological changes in OVA-induced mouse asthma

H&E staining was applied in this study to investigate the effects of hyperoside on lung histological changes. The results showed that OVA-challenged mice showed marked inflammatory cell infiltration in the peribronchial and perivascular areas and airway epithelial thickening. However, treatment of hyperoside or DEX significantly attenuated OVA-induced pathological changes (Fig. 5).

3.6. Effects of hyperoside on oxidative stress in lung tissues

The effects of hyperoside on MDA, SOD, and GSH levels were detected in this study. As shown in Fig. 6, OVA increased the level of MDA and decreased the levels of SOD and GSH significantly. However, administration of hyperoside or DEX significantly decreased MDA level induced by OVA. Administration of hyperoside or DEX significantly increased SOD and GSH levels induced by OVA.

3.7. Hyperoside inhibits NF- κ B signaling pathway activation

Western blot analysis was applied in this study to investigate the effects of hyperoside on OVA-induced NF- κ B activation. The results showed that the levels of phosphorylation of NF- κ B p65 and I κ B α were significantly increased in OVA-challenged group mice. However, the levels of phosphorylation of NF- κ B p65 and I κ B α were significantly inhibited by treatment of hyperoside (Fig. 7).

3.8. Effects of hyperoside on Nrf2 signaling pathway

The effects of hyperoside on Nrf2 signaling pathway were detected by western blot analysis. As shown in Fig. 8, the expression of Nrf2 and HO-1 was decreased by OVA. However, treatment of hyperoside up-regulated the expression of Nrf2 and HO-1 in a dose-dependent manner (Fig. 8).

4. Discussion

In recent years, extractives from natural herbaceous plants are gaining increasing attention for their prominent biological activities [15]. For many chronic diseases, herbal drugs have been reported to have an effective treatment [16]. Hyperoside is an active compound isolated from *Rhododendron brachycarpum* G. Don. In the present study, our results showed that hyperoside protected against OVA-induced asthma by inhibiting the development of AHR, pulmonary allergic inflammation, and IgE production.

It is well known that inflammatory cells and multiple mediators were included in allergic airway inflammation process and AHR development [17]. Once challenged by allergen, the levels of Th2 type cytokines were increased and induced the Th2 dominant immune response [18,19]. IL-4, IL-5, and IL-13 are major Th2 cytokines that played critical roles in the development of asthma [20,21]. Studies showed that Th2 cytokines could induce the expression of IgE [22]. In the model of OVA-induced mice asthma, the productions of Th2 cytokines were significantly increased [23]. While, a large body of studies showed that inhibition of Th2 cytokines could attenuate OVA-induced asthma [24]. To investigate the protective effects of hyperoside, the effects of hyperoside on OVA-induced Th2 cytokines were detected. Our results showed hyperoside significantly suppressed OVA-induced IL-4, IL-5, and IL-13 production. In addition, OVA-induced IgE and AHR were also attenuated by treatment of hyperoside. These results suggested that hyperoside had protective effects against OVA-induced

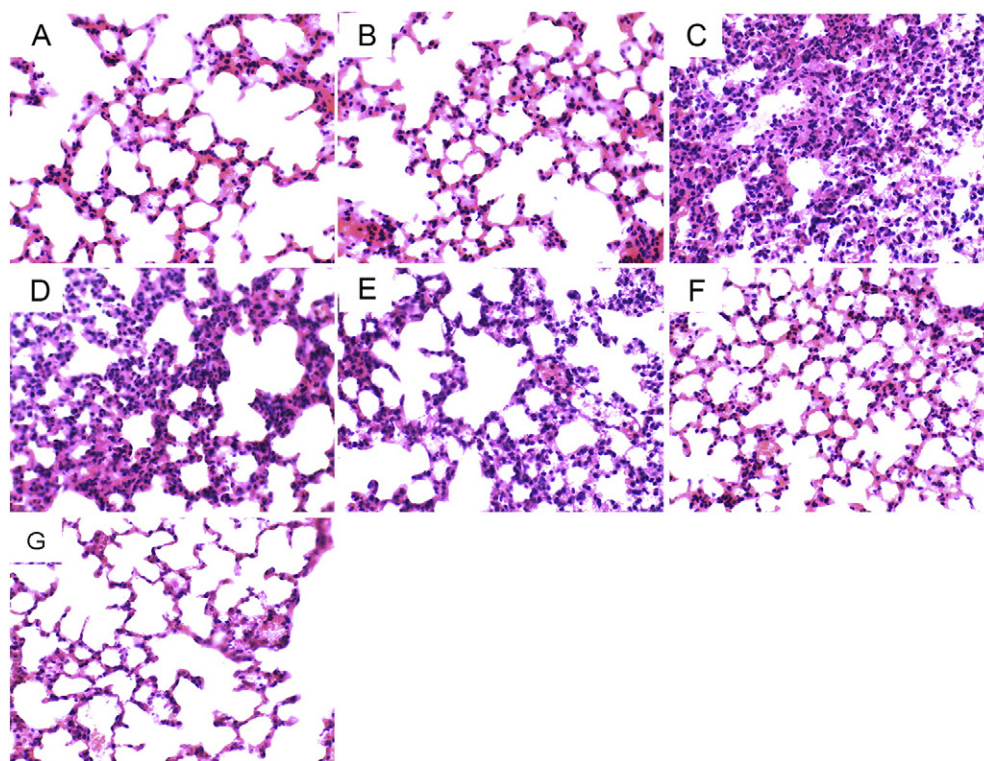


Fig. 5. Effects of hyperoside on histopathological changes in lung tissues in OVA-induced asthma. Lungs tissues from each experimental group were processed for histological evaluation. Representative histological changes of lung obtained from mice of different groups. A: Control group, B: hyperoside (20 mg/kg) group, C: OVA group, D: OVA + hyperoside (5 mg/kg) group, E: OVA + hyperoside (10 mg/kg) group, F: OVA + hyperoside (20 mg/kg) group, G: OVA + DEX group (Hematoxylin and eosin staining, magnification 200 $\times$).

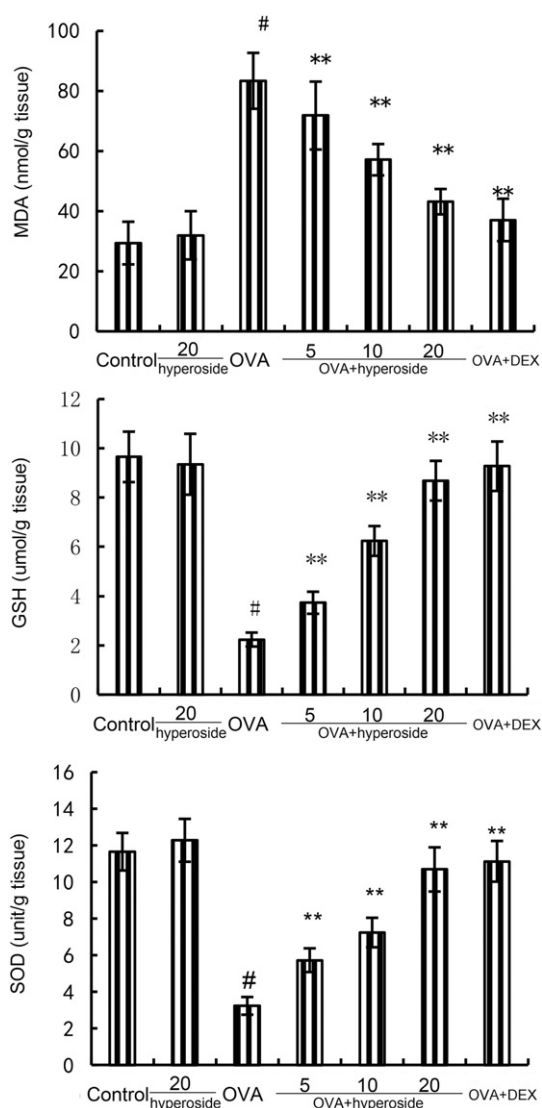


Fig. 6. Effects of hyperoside on oxidative stress in lung tissues. The effects of hyperoside on MDA, SOD, and GSH levels were detected in this study. The values presented are the means $\pm$ SEM of three independent experiments. [#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA group.

asthma. Oxidative stress is an important feature in the pathogenesis of asthma. The infiltration of neutrophils is main sources of ROS. ROS could lead to DNA damage and lipid peroxidation. GSH, an important indicator of oxidative stress, plays critical roles in the maintenance of epithelial integrity. In this study, hyperoside significantly inhibited OVA-induced oxidative stress as demonstrated by decreased MDA, and increased GSH and SOD levels. These results suggested that hyperoside protected against OVA-induced asthma by inflammatory and oxidative responses.

In various pathophysiological conditions, NF- κ B has been considered to play a pivotal role in inflammatory phenotypic changes through regulation of inflammatory genes expression [25]. In inactive cells, NF- κ B p65 is in the cytoplasm and sequestered by I κ B inhibitory proteins. Once stimulating by LPS or OVA, NF- κ B p65 translocates into the nucleus to regulate inflammatory cytokines gene transcription [26]. Recent studies showed that allergic inflammation was adjusted by NF- κ B activation [27]. To investigate the anti-inflammatory mechanism of hyperoside, the effects of hyperoside on NF- κ B signaling pathway were detected. The results of this study showed that OVA-induced NF- κ B activation was significantly inhibited by treatment of hyperoside. Previous studies showed that hyperoside inhibited high glucose-induced vascular inflammatory response by inhibiting NF- κ B activation [10]. And our results were consistent with previous study. Nrf2 is a transcription factor implicated in the transactivation of gene coding detoxifying enzymes [28]. Activation of Nrf2 could up-regulate the expression of HO-1, the main anti-oxidant molecule. Nrf2/HO-1 signaling pathway has been known to play critical roles in the regulation of oxidant stress [29]. Recently, Nrf2 has been reported to be involved in inflammatory response. Activation of Nrf2 could inhibit LPS-induced NF- κ B activation [30]. In addition, studies demonstrated that activation of Nrf2 had the ability to inhibited OVA-induced airway inflammation [31]. To further investigate the protective mechanism of hyperoside, the effects of hyperoside on Nrf2/HO-1 signaling pathway were measured. The results showed that hyperoside attenuated OVA-induced allergic airway inflammation by activating Nrf2 signaling pathway.

In conclusion, the results of this study reveal the protective effect of hyperoside is associated with attenuation of allergic airway inflammation and AHR. Hyperoside attenuates OVA-induced allergic airway inflammation by activating Nrf2 signaling pathway. Further researches will be carried out to assess the therapeutic effect of hyperoside into a clinical practice.

Conflict of interest

The authors have no conflict of interest to declare.

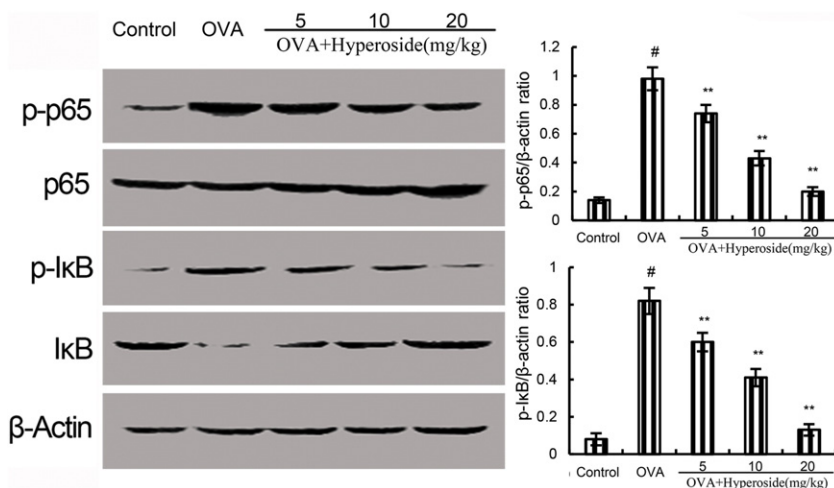


Fig. 7. Effects of hyperoside on OVA-induced NF- κ B activation. The expression of NF- κ B p65 and I κ B α were detected by western blotting. The values presented are the means $\pm$ SEM of three independent experiments. [#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA group.

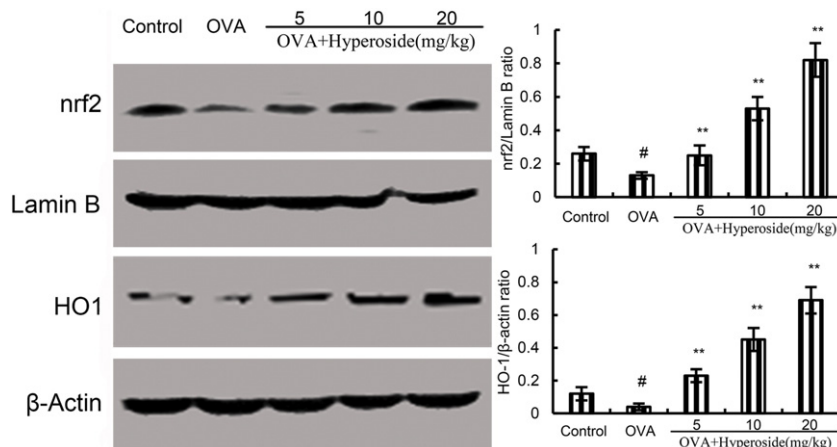


Fig. 8. Effects of hyperoside on Nrf2 signaling pathway. The expression of Nrf2 and HO-1 were detected by western blotting. The values presented are the means $\pm$ SEM of three independent experiments. [#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA group.

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