

Ginsenoside Rh1 Attenuates Ovalbumin-induced Asthma by Regulating Th1/Th2 Cytokines Balance

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Running Title: Ginsenoside Rh1 benefits ovalbumin induced asthma

Abstract

Ginsenoside Rh1 (Rh1) has anti-inflammatory effects in asthma mice, but the underlying mechanism remains unclear. BALB/c mice were sensitized and challenged with ovalbumin (OVA) to construct asthma model. Mice received Rh1 or Tiotropium Bromide 0.5 h before OVA challenge. Airway morphology and airway remodeling were assessed by HE staining and Masson's trichrome staining, respectively. Th1/Th2 cytokines in serum or BALF were measured by ELISA kits. Rh1 significantly alleviated the lung resistance and airway resistance, and reduced the number of total inflammation cells, eosinophils, neutrophils, and lymphocytes in BALF of the asthmatic mice. The morphological changes and collagen deposition of airway were also reduced by Rh1 in asthmatic mice. The increase of Eotaxin, IL-4, IL-5, IL-13, and IL-33 and the decrease of IL-12 and IFN- γ in both BALF and serum of OVA exposed mice were reversed by Rh1. Rh1 attenuates OVA-induced asthma in the mice model by regulating Th1/Th2 cytokines balance.

Keywords: allergic asthma, Th1/Th2, Ginsenoside Rh1, inflammation, airway hyperresponsiveness

Introduction

Bronchial asthma (asthma) is a common chronic inflammatory airway disorder. Statistics shows that about 300 million people suffer from asthma worldwide, and this number is expected to reach 400 million by 2025 (Berair and Brightling, 2014). Asthma has high morbidity and mortality and imposes a huge economic burden on patients and society (Berair and Brightling, 2014). Currently available drugs for asthma can only control symptoms and prevent exacerbations, rather than cure the disease (Pepper et al., 2017). Therefore, it is meaningful to find a promising therapy method.

Variable airway obstruction and airway hyperresponsiveness (AHR) are the main characteristics of asthma (Boulet and Boulay, 2011, Gordon and R., 2008). AHR refers to the excessive responses of the airway to bronchoconstrictive stimuli including physical stimuli, chemical stimuli and biological stimuli (Lauzon and Martin, 2016). Although the pathogenesis of asthma is still unknown, studies have shown that the main biochemical and pathological changes in asthma are systemic and local (respiratory) immuno-inflammatory response disorders (Baptist and Busse, 2018). The most common disorder in asthma is non-specific airway inflammatory response (Mims, 2015), which is dominated by eosinophils, neutrophils, lymphocytes and their secreted inflammatory mediators as well as cytokine responses (Shi et al., 2011, Mims, 2015, Holgate, 2012). These inflammatory cells recruited in lung tissue will cause morphological changes. Besides, airway remodeling is also found in asthma (Yang et al., 2016). Some reports found that anti-IgE therapies attenuated airway remodeling (Ojiaku et al., 2017, Murphy et al., 2010), indicating that IgE may play an important role in asthma. Studies have also found that asthma was closely related to abnormal differentiation and function of Th1 and Th2 cells, as well as increased expression of secondary inflammatory cytokines and maladjustment of immunomodulatory mechanism (Shi et al., 2011, Liang et al., 2017). Th1 cells mainly secrete IL-2, IL-12, and IFN- γ which mediate cellular immunity, while Th2 cells secrete IL-4, IL-5, and IL-13 that mediate humoral immunity (Wei and Sheng Li, 2018). These cytokines can affect the level of IgE and immunoreaction (Nath et al.,

2007, Punnonen et al., 1997). Rebuilding Th1/Th2 cytokines balance was also reported as a possible treatment for asthma (Anatriello et al., 2019). Thus, research on the regulation of Th1/Th2 cytokines may help develop a promising asthma therapy.

Ginseng is a herb derived from the roots of several plants, mainly including Korean or Asian Ginseng (*Panax ginseng*), Siberian Ginseng (*Eleutherococcus senticosus*), and American Ginseng (*Panax quinquefolius*) (Kiefer and Pantuso, 2003). Ginseng is widely used as a traditional medicine due to its regulation of immune function, exercise performance and so on, from which dozens of effective monomer components have been isolated (Feng et al., 2015, Hou et al., 2014). Among these effective components, Ginsenoside Rb1, Rg3, compound K are reported to have an anti-asthmatic effect (Ren et al., 2019, Guo et al., 2017, Chen et al., 2015, Lee et al., 2016). Ginsenoside Rh1 also exerts obvious anti-inflammatory, antioxidant, anti-tumor, immunomodulatory effects, as well as a positive effect on the nervous system (Tam et al., 2018, Hou et al., 2014). In this study, we built an asthma model in mice by Ovalbumin (OVA) exposure, and then studied the effect and underlying mechanism of Rh1 in asthma model mice.

Materials and methods

Experimental animals and experimental protocols

Forty female BALB/c mice (specific pathogen-free, inbred) were bought from a commercial company (Guangzhou Sebiona biological technology Co. LTD, China, <http://www.sebiona.com>), which were 7 weeks old and weighed 20-30 g. Before the experiment, the mice were acclimatized for 7 days under the following conditions: 50 ± 10% relative humidity, 22 ± 1 °C, and a 12 h light/dark cycle, and they were given free access to rodent laboratory chow and tap water.

Ethics Statement

This research was approved by the Committee of Experimental Animals of Hangzhou Hospital of Traditional Chinese Medicine (approval number: HZ2019091322). All animal experiments were performed in the Hangzhou Hospital of Traditional Chinese Medicine in compliance with the guidelines of China Council on Animal Care and Use. Measurements were taken to minimize pain and discomfort

of the animals during the whole experiment. The mice were sacrificed after the experiment.

Sensitization, challenge, and treatment of mice

BALB/c mice were evenly divided into 4 groups: Control group, OVA group, OVA+Tio group, and OVA+Rh1 group. Mice were subjected to OVA sensitization and challenges and received treatment with Tiotropium Bromide (Tio, H20140933, Boehringer Ingelheim Pharma GmbH & Co. KG, <https://www.boehringer-ingelheim.cn>) or Rh1 (purity>97%, cat. no. A0240, Chengdu Must Bio-Technology Co., Ltd., China, <http://www.cdmust.com/index.aspx>). Rh1 was extracted from the root of Panax ginseng. Briefly, animals were immunized by intravenous injection of a solution containing 20 µg of OVA (A003049-001, Shanghai Sangon Biotech Co., Ltd, China, <https://www.sangon.com>) and 2 mg of aluminumhydroxide (20001060, Sinopharm Chemical Reagent Co. Ltd, China, <https://www.reagent.com.cn>) on the 1st and 7th days. Seven days after the last sensitization, mice were exposed to the ultrasonic atomized 2% OVA solution for 30 min. The exposure was given three times a week for 6 weeks.

The sensitized mice in the OVA+Tio group were administered 0.1 mmol/L Tio nasal drops 0.5 h before being challenged. As pointed out in Li's research, 100 mg/kg Ginsenoside Rh2, a similar homologous compound to Rh1, attenuates allergic airway inflammation in a murine model of asthma(Li et al., 2015), and therefore, the sensitized mice in the OVA + Rh1 group were orally given 100 mg/kg Rh1 (Rh1 was dissolved in 0.1% dimethyl sulfoxide) before being challenged. The ten mice in the Control group were sensitized and challenged with saline.

Assessment of airway hyperresponsiveness in asthmatic mice

According to Meng's study(Meng et al., 2018), AHR tests were performed by the forced oscillation technique. Airway responsiveness of mice was determined by increasing the doses of inhaled methacholine (Mch). Briefly, mice were anesthetized by intraperitoneally injection of 50 mg/kg pentobarbital sodium 24 h after the final OVA challenge. Then a catheter was inserted into mouse trachea and mechanically ventilated by the FlexiVent (scireq, Canada). First, the baseline was measured. Then

the atomized Mch solution was used to test the lung function of asthmatic mice. The concentration of Mch increased gradually in the order of 3.125, 6.25, 12.5, 25, 50, and 100 mg/mL. Each concentration was tested for 5 min, followed by a 3-min rest. Data were collected per minute. At last, the lung resistance (LR, cm H₂O/ml/s) and airway resistance (AR, cm H₂O/ml/s) were plotted as functions of Mch at different doses.

Sample preparation

Twenty-four hours after the final allergen challenge, broncho alveolar lavage fluid (BALF), serum and lung tissue were collected. Mice were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium. BALF collection was performed as described in a previous report (Sethi and Naura, 2018). Briefly, the thoracic cavity of mice was opened and the upper part of the trachea was cannulated. The lung of mice was washed twice with 1 mL of cold phosphate-buffered saline (PBS, pH 7.4) (P1022, Solarbio). The collected BALF was centrifuged (3000×g, 10 min) at 4 °C. Cell pellet was re-suspended by PBS for cell counting, and the supernatant was stored at -80 °C. Blood was collected from the eyeballs of mice and centrifuged (3000 g, 10 min, 4 °C) to separate serum as described previously (Luo et al., 2014). The mice were euthanized by intraperitoneal injection of 150 mg/kg pentobarbital sodium, and the lungs were harvested immediately. Euthanasia was confirmed successful when the mice met the criteria below: arrest of pulse and breathing, loss of corneal reflex, and disappearance of respiratory sounds and heartbeat sounds upon examination with a stethoscope. Briefly, the mice's chest was opened, and the lung was separated and washed by distilled water.

Detection of total inflammatory cells and differential cells (neutrophils, eosinophils, and lymphocytes) in the BALF of asthmatic mice

Total inflammatory cells and differential cells (neutrophils, eosinophils, and lymphocytes) in the BALF of asthmatic mice were counted according to Sethi's study (Sethi et al., 2019), with slight modification. Briefly, the cell pellet re-suspended solution was diluted to an appropriate concentration. Then the total inflammatory cells were counted by a Blood cell counter (AMQAX1000, Invitrogen, America). The numbers of differential cells (neutrophils, eosinophils, and lymphocytes) in BALF

were measured by the Diff-Quick stain kit (G1541, Solarbio). Briefly, the BALF cell pellet re-suspended solution was smeared on a slide. After being dried naturally in the air, the slide was fixed with Diff-Quick Fixative for 20 s. Then the slide was successively stained with Diff-Quik I for 10 s and with Diff-Quik II for 20 s. During each staining, the slide was shaken vertically 2-3 times. At last, the slide was observed under the microscope and counted by the Blood cell counter, followed by washing with water. The purple cells were lymphocytes, the cells with gray-blue cytoplasm and purple nucleus were monocytes, and those appearing pink to clear with many large red to orange granules in the cytoplasm and blue nucleus were Eosinophils.

Observation of airway morphology in asthmatic mice

Fresh lung tissue was fixed with 4% formaldehyde, paraffin-embedded, and cut into 5 μ m sections. Then the sections were stained with Hematoxylin&Eosin (H&E) Staining Kit (G1120, Solarbio) in accordance with Fang's study(Fang and Ping). Briefly, the paraffin was removed from the sections using xylene (X112051, Aladdin). After this, the sections were successively processed with high concentration alcohol, low concentration alcohol, and distilled water, followed by 5 min of staining with Hematoxylin Dye. After being washed by running water, the sections were differentiated by the Differentiation Fluid for 30 seconds. Then the sections were soaked in tap water for 15 min and in Eosin Dye for 2 min. Subsequently, the sections were washed by running water, re-dehydrated with pure alcohol, and transparentized by xylene. At last, the transparent sections were dripped with Canada gum, covered with cover glass and sealed until the gum dried slightly, and then the mice lung tissue samples were imaged at 100x or 200x under the microscope.

Measurement of IgE and cytokines in BALF and serum from asthmatic mice

The total IgE, OVA-specific IgE, Th1 cytokines (IL-12, IFN- γ), and Th2 cytokines (Eotaxin, IL-4, IL-5, IL-13, IL-33) in serum or BALF were measured by ELISA kits. The total IgE in serum was quantified by an anti-mouse IgE ELISA kit (ab157718, Abcam, England). OVA-specific IgE was measured by the OVA-specific IgE ELISA kit (439807, BioLegend, Inc., USA, <https://www.biolegend.com>). The cytokines were correspondingly measured by an IL-12 ELISA kit (SEKM-0013,

Solarbio), an IFN- γ ELISA kit (SEKM-0031, Solarbio), Eotaxin (SEKM-0073, Solarbio), an IL-4 ELISA kit (SEKM-0005, Solarbio), an IL-5 ELISA kit (SEKM-0006, Solarbio), an IL-13 ELISA kit (SEKM-0014, Solarbio), and an IL-33 ELISA kit (SEKM-0028, Solarbio).

ELISA was performed following the procedure provided by the manufacturer. Briefly, an appropriate number of antibody coated well strips were removed. For measurement of OVA-specific IgE, the 96-well plate was pre-coated with OVA, and all reagents were equilibrated to room temperature (30 min). Then all the reagents, samples, and standards were prepared as instructed. The standards or samples were added to each well and incubated at room temperature for 30 min. After aspirating and washing each well 4 times, the prepared horseradish peroxidase (HRP) labeled secondary detector antibody was added into the wells. After incubation at room temperature for 30 min, each well was subjected to another 4 rounds of aspiration and washing. Chromogen Substrate Solution was added into each well to stop the interaction and the color development at 450 nm was recorded by a microplate reader (Multiskan Sky, Thermo Fisher Scientific (China) Inc., China, <https://www.thermofisher.com/cn/zh/home.html>) immediately.

Quantification of airway remodeling in asthmatic mice

Airway remodeling was assessed as reported in a previous study (Cui et al., 2019). The lung tissue was stained with Masson's trichrome (G1340, Solarbio). Briefly, paraffin sections were dewaxed and dehydrated, and then washed with tap water and distilled water in turn. The nucleus was dyed with Weigert Hematoxylin for 5-10 min, followed by differentiation in acidic ethanol differentiation solution for 10 s and washing with distilled water. Then the sections were processed with Masson Blue Liquer 5-15 s and dyed with Fuchsin Acid Solution for 5-10 min. After washing the sections with Weak Acid Solution for a while, 1% Phosphomolybdic Acid Solution was used to treat the sections for 1-2 min. Without washing, the sections were dyed directly with Aniline Blue Dye for 1-2 min. At last, the sections were washed for a moment with Weak Acid Solution, followed by 95% ethanol and anhydrous ethanol dehydration, xylene transparentization, and neutral gum sealing. Then the sections

were observed under the microscope at 100 x or 200 x. The blue or light green area should be collagen fiber while the red area should be the muscle or the cytoplasm.

The proportion of the area stained with Masson's trichrome was calculated to assess the extent of subepithelial fibrosis. The Masson's trichrome positive area was quantified by using the previously determined color threshold for Masson's trichrome. Then the area between the airway basal membrane and the airway adventitia was outlined and quantified. Subepithelial collagen deposition was expressed as collagen (Masson's trichrome positive) area relative to the total measured area.

Statistical Analysis

Each experiment was repeated at least 3 times. All statistics was analyzed with SPSS 18.0 (Chicago, USA). One-way ANOVA followed by Tukey's multiple comparisons test was used to compare the difference between groups. Results are expressed as mean $\pm$ standard deviation (SD). Statistical significance was identified as $P < 0.05$.

Results

Rh1 alleviated AHR in OVA-induced asthma model mice

BALB/c mice were induced by OVA to build an asthma model and then treated with Rh1. Then forced oscillation technique was used to analyze lung mechanics in mice. Fig. 1A exhibited the structure of Rh1, whose chemical formula was $C_{36}H_{62}O_9$. Fig. 1B showed the modeling and administration processes that we used to successfully construct a mouse asthma model. The data in Fig. 1C and Fig. 1D showed that the LR and AR generated by Mch (from 0 to 100 mg/ml) administration increased as the concentration of Mch increased, respectively. According to Fig. 1C, the LR of mice in the OVA group was significantly greater than that in the Control group under treatments with 25 mg/ml, 50 mg/ml and 100 mg/ml Mch ($P < 0.01$, $P < 0.001$, $P < 0.001$). The LR in the OVA+Tio group and the OVA+Rh1 group was significantly lower than that in the OVA group under 25 mg/ml Mch treatment, ($P < 0.01$), and the difference was more obvious when the dose of Mch was 50 mg/ml or 100 mg/ml ($P < 0.001$).

As show in Fig. 1D, the AR of mice in the OVA group was significantly greater

than that of Control group mice under 12.5 mg/ml Mch treatment ($P<0.05$), and the difference was more obvious when the dose of Mch was 25 mg/ml, 50 mg/ml or 100 mg/ml ($P<0.01$, $P<0.001$, $P<0.05$). Data in Fig. 1D suggested that the AR of mice in the OVA+Tio group was significantly lower than that of OVA group mice under 12.5 mg/ml Mch treatment ($P<0.05$), and the difference was more obvious when the dose of Mch was 25 mg/ml, 50 mg/ml or 100 mg/ml ($P<0.01$, $P<0.001$, $P<0.001$). Besides, the AR of mice in the OVA+Rh1 group was significantly lower than that of OVA group mice under 25 mg/ml Mch treatment ($P<0.05$), and the difference was more obvious when the dose of Mch was 50 mg/ml or 100 mg/ml ($P<0.01$, $P<0.001$).

It could be concluded from the above results that both pretreatment with Tio and oral feeding of Rh1 could reverse the increases in LR and AR in OVA-induced asthma mice.

Rh1 alleviated OVA-induced airway inflammatory cell recruitment in asthma model mice

Total BALF cells and differential BALF cells (eosinophils, neutrophils, and lymphocytes) were counted to evaluate the effect of Rh1 on airway inflammation. As illustrated by Fig. 1E, the number of total inflammatory cells in the OVA group was significantly higher ($P<0.001$) than that in the Control group, while the numbers of total inflammatory cells in the OVA+Tio group and the OVA+Rh1 group were both significantly lower ($P<0.001$) than that in the OVA group. As shown in Fig. 1F-H, the numbers of eosinophils, neutrophils, and lymphocytes in the OVA group were significantly larger ($P<0.001$) than those in the Control group, while the numbers of the three cells in the OVA+Tio group and the OVA+Rh1 group were both significantly smaller ($P<0.001$) than that in the OVA group. All these results mean that Rh1 alleviated OVA-induced airway inflammatory cell recruitment.

Rh1 alleviated airway morphological changes and serum IgE level increase in OVA-induced asthma model mice

We performed H&E staining of airway tissue to investigate the changes in airway morphology. The results (Fig. 2A) showed that mice challenged with OVA in the OVA group had a smaller airway compared with those in the Control group,

suggesting that OVA exposure enhanced lung tissue inflammation. By contrast, the airway size of mice in the OVA+Tio group and the OVA+Rh1 group was greater than that of OVA group mice (Fig. 2B-C). This indicates that the inflammation in the OVA+Tio group and the OVA+Rh1 group was reduced by Tio and Rh1, respectively.

The levels of total IgE and OVA-specific IgE in serum were measured by ELISA kits. We found that the levels of total IgE (Fig. 2B) and OVA-specific IgE (Fig. 2C) in serum of OVA group mice were significantly augmented compared with those in the Control group ($P<0.001$). However, the levels of total IgE and OVA-specific IgE in serum of mice in the OVA+Tio group and the OVA+Rh1 group were significantly decreased compared with those in the OVA group ($P<0.001$). This means that both Tio and Rh1 reduced the levels of total IgE and OVA-specific IgE in serum of the OVA-induced asthma mice.

Rh1 alleviated airway remodeling in OVA-induced asthma model mice

Airway remodeling in asthma was observed by Masson's trichrome staining. The area of peribronchial trichrome staining (Masson's trichrome positive) in the OVA group was larger than that in the Control group (Fig. 3), while the OVA-induced increase in the Masson's trichrome positive area was reversed by Rh1 and Tio. Quantitative analysis also confirmed these results (Fig. 3). The airway of asthmatic mice in both the OVA+Tio group and the OVA+Rh1 group showed significantly less collagen deposition compared with OVA group mice ($P<0.001$, $P<0.01$, Fig. 3). Collectively, the above findings indicate that Rh1 attenuates airway remodeling in the mouse asthma model.

Rh1 regulated the concentration of Th1/Th2 cytokines in BALF and serum of OVA-induced asthma model mice

Compared with the Control group, significantly higher concentrations of Eotaxin (Fig. 4A), IL-4 (Fig. 4B), IL-5 (Fig. 4C), IL-13 (Fig. 4D) and IL-33 (Fig. 4E) yet significantly lower concentrations of IL-12 (Fig. 4F) and IFN- γ (Fig. 4G) were detected in BALF from asthmatic mice in the OVA group ($P<0.001$). Fig. 4 also illustrated that in BALF from asthmatic mice in the OVA+Tio group or the OVA+Rh1 group, the levels of IL-4, IL-5, IL-13, IL-33, and Eotaxin were significantly decreased

yet those of IL-12 and IFN- γ were significantly increased, as compared with the OVA group ($P<0.001$). These results mean that Rh1 reversed the increase in Eotaxin, IL-4, IL-5, IL-13, and IL-33 levels and the decrease in IL-12 and IFN- γ levels in the BALF of OVA exposed mice.

Eotaxin (Fig. 4H), IL-4 (Fig. 4I), IL-5 (Fig. 4J), IL-13 (Fig. 4K), and IL-33 (Fig. 4L) presented significantly higher levels in the serum of OVA group mice than in that of Control group mice ($P<0.001$). However, the levels of these cytokines in the OVA+Tio group and the OVA+Rh1 group were significantly lower than those in the OVA group ($P<0.001$). Oppositely, IL-12 (Fig. 4M) and IFN- γ (Fig. 4N) levels in the serum of OVA group mice were significantly lower than those in the Control group ($P<0.001$). On the contrary, the serum of mice in the OVA+Tio group and the OVA+Rh1 group exhibited significantly increased levels of IL-12 (Fig. 4M) and IFN- γ (Fig. 4N), compared with the OVA group ($P<0.001$).

In summary, the increase of Eotaxin, IL-4, IL-5, IL-13, and IL-33, and the decrease of IL-12 and IFN- γ in both BALF and serum of OVA exposed mice were significantly reversed by Tio and Rh1 ($P<0.001$), which means that Rh1 can also regulate the concentration of Th1/Th2 cytokines like Tio.

Discussion

In this study, Rh1 significantly alleviated the AHR in the OVA exposed asthmatic mice. At the same time, the concentrations of total inflammation cells and differential inflammation cells, which included eosinophils, neutrophils, and lymphocytes, were also down-regulated by Rh1. The changes in airway remodeling and collagen deposition of asthma mice were reversed by Rh1. Both IgE and OVA-specific IgE in BALF of the OVA-induced asthma model mice were significantly down-regulated by Rh1. The increase of Eotaxin, IL-4, IL-5, IL-13, and IL-33, and the decrease of IL-12 and IFN- γ in both BALF and serum of OVA exposed asthmatic mice were reversed by Rh1.

AHR is a common characteristic of asthma (Boulet and Boulay, 2011). Ginsenoside Rh2 was reported to inhibit AHR (Li et al.). In our research, homogenous materials Rh2 and Rh1 also alleviated the AHR challenged by Mch, indicating that

Rh1 may be a promising therapeutic drug for asthma. Besides, Rh1 was reported to affect immune cells and regulate the immune system (Tam et al., 2018). Here, we also found that Rh1 significantly reduced the numbers of total inflammatory cells, eosinophils, neutrophils, and lymphocytes in BALF. This means that Rh1 attenuated inflammation. In addition, Rh1 may have an anti-asthmatic effect, for it alleviated AHR and inhibited inflammation cell recruitment. Considering that asthma is also characterized by airway narrowing in morphology (Nishimoto et al., 2017), reducing morphological changes should be an anti-asthmatic therapy. IgE is reported to have a central role in the pathogenesis of allergic asthma (Djukanovic et al., 2016, Geha et al., 2003), and IgE targeted biological therapies for severe asthma have been developed and approved (Oishi and Matsunaga, 2018, Patel and Sur, 2017). Thus, regulating the level of IgE may be key to the anti-asthmatic effect of Rh1. To test Rh1's anti-asthmatic effect, we studied changes in morphology and IgE level in the asthmatic mice. We found that Rh1 inhibited morphological changes in OVA-induced asthmatic mice, which is consistent with its inhibitory effect on inflammation cell recruitment. We also found that Rh1 reversed the increase of IgE and OVA-specific IgE in OVA-induced asthma. These results confirmed Rh1's anti-asthmatic effect. Previous studies reported that anti-IgE therapies might influence airway remodeling by attenuating its progress or reversing long-term changes (Ojiaku et al., 2017, Murphy et al., 2010). In this study, Rh1 could reduce collagen deposition and inhibit airway remodeling in the OVA-induced asthma model mice, which is in accordance with the result that Rh1 decreased the level of IgE. Thus, IgE level is a key factor involved in the attenuating effect of Rh1 on asthma. Airway remodeling is caused by chronic inflammation for a repetitive cycle of injury and repair in the airway wall (Yang et al., 2016). Subepithelial fibrosis is a feature of airway structural changes (Berair and Brightling, 2014, Bourdin et al., 2012). Hence, the anti-asthmatic effect of Rh1 was further proved by its inhibition on airway remodeling and collagen deposition.

Th1 and Th2 cells, as well as secondary inflammatory cytokines, are related to asthma (Shi et al., 2011, Liang et al., 2017). Interfering with Th1/Th2 cytokines can

attenuate the asthma-induced inflammation and airway remodeling (Anatriello et al., 2019). Besides, IL-5 targeted biological therapies for severe asthma have been developed and approved (Oishi and Matsunaga, 2018). We found that Rh1 increased the levels of Th1 cytokines (IFN- γ , IL-12) and decreased those of Th2 (Eotaxin, IL-4, IL-5, IL-13, IL-33) cytokines in BALF and serum of asthmatic mice. Previous studies also revealed that Rh1 regulated CD4⁺ T cell activation and Th1/Th2 balance (Feng et al., 2015, Wang et al., 2000, Zhang Chun-jing et al.), which thus corresponds the regulating effect of Rh1 on Th1/Th2 cytokines to its anti-asthmatic effect. However, how Rh1 attenuates asthma has not been studied, and the roles of Th1/Th2 cytokines in the attenuating effect of Rh1 on asthma remain unclear. In the present study, Rh1 inhibited the increases in the concentrations of Eotaxin, IL-4, IL-5, IL-13, and IL-33, and reversed the decrease of IL-12 and IFN- γ in both BALF and serum of OVA-induced mice. These results indicated that Rh1 regulated Th1/Th2 balance in asthma mouse.

The immune response is controlled by CD4-positive (CD4⁺) T cells that are activated by antigen presenting cells (APC) and differentiate into distinct subsets of effector Th cells, including Th1, Th2, Th17, and iTreg (Zhu et al., 2010). Previous report also showed that Rh1 modulates CD4⁺ T cells activation and Th1/Th2 balance (Feng et al., 2015). Thus, Rh1 may influence APC and then activate differentiation of CD4-positive (CD4⁺) T cells into Th subsets. However, how Rh1 exert its effects on Th1/Th2 cytokines balance in asthma is unclear, which requires further investigations.

This study did not probe into the molecular mechanism underlying the imbalance of Th1/Th2 cytokines, and therefore, the relative RNA level should be focused on in future study. Besides, Rh1's anti-asthmatic effect should be studied in the human body.

In conclusion, Rh1 has an anti-asthmatic effect by inhibiting AHR, reduces airway inflammation cell recruitment and IgE level, attenuates airway morphological changes and inhibits airway remodeling, and the possible underlying mechanism lies in the regulation of Th1/Th2 cytokines balance. Our results suggest that Rh1 might be

a promising therapeutic approach for asthma. However, its anti-asthmatic effect still needs to be further investigated in future research.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

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Drafting the article or critically revising it for important intellectual content: All authors

Final approval of the version to be published: All authors

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: All authors

Availability of Data and Materials

The analysed data sets generated during the study are available from the corresponding author on reasonable request.

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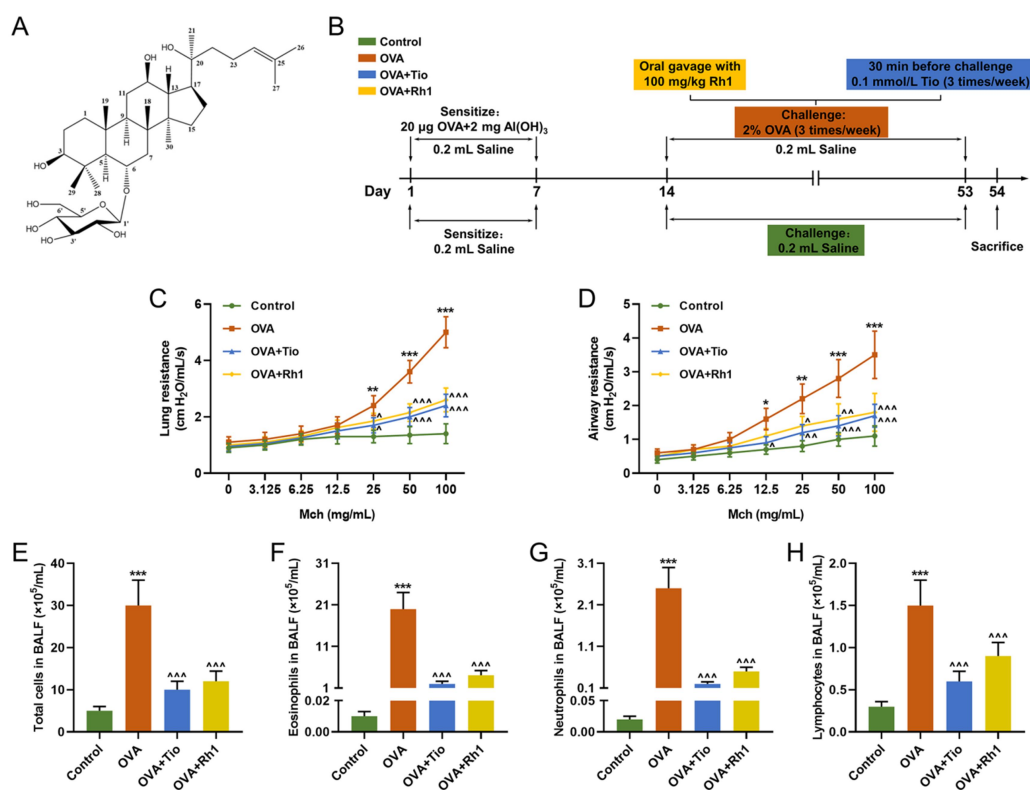


Figure 1 Ginsenoside Rh1 alleviates airway hyperresponsiveness and inflammation cell recruitment in ovalbumin (OVA)-induced asthma model mice. (A) Structure of Ginsenoside Rh1. (B) Experimental protocol for establishment of OVA-induced asthma mice model. (C, D) Airway resistance and lung resistance of mice that inhaled 0-100 mg/ml methacholine (Mch) were measured by forced oscillation method. (E) The number of total inflammatory cells in the broncho alveolar lavage fluid (BALF) of mice was measured by Blood Cell Counter. (F-H) The numbers of eosinophils, neutrophils, and lymphocytes in the BALF of mice were measured by a Quick-Diff Stain kit. Data are shown as mean $\pm$ SD. N=10 mice /group. * P <0.05, ** P <0.01, *** P <0.001, OVA group vs. Control group. ^ P <0.05, ^^ P <0.01, ^^ P <0.001, OVA+Tio group or OVA+Rh1 group vs. OVA group.

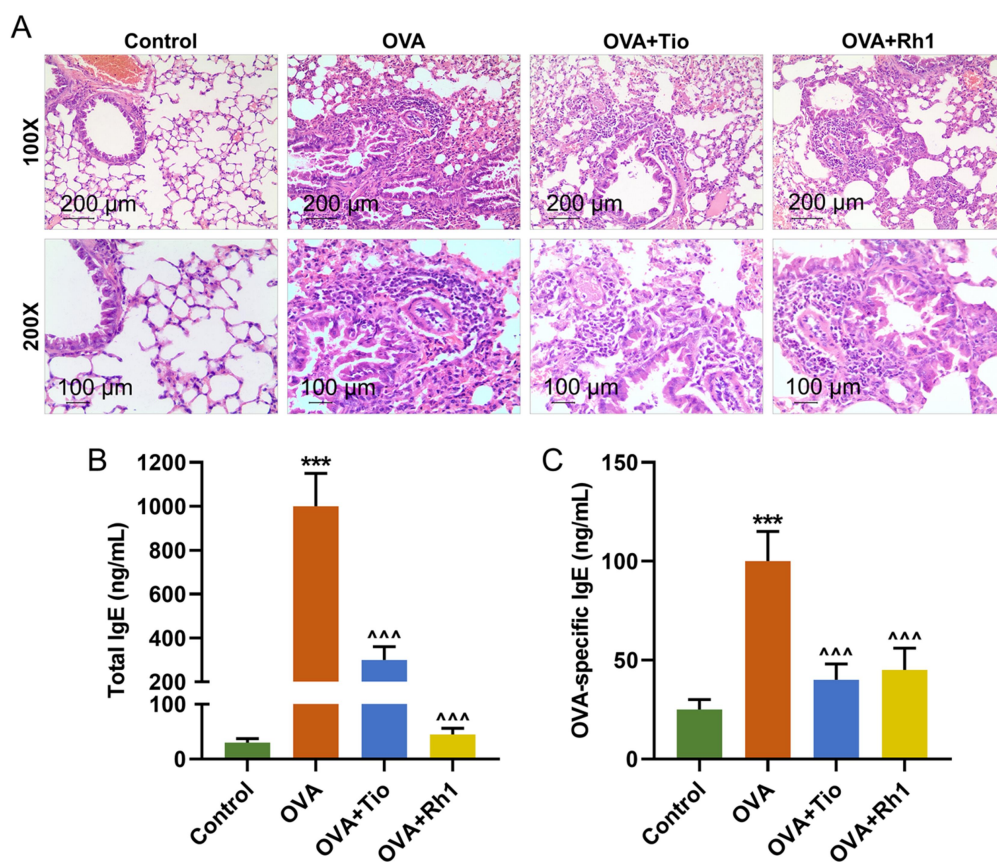


Figure 2. Ginsenoside Rh1 alleviates airway morphological changes and IgE level increase in ovalbumin (OVA)-induced asthma model mice. (A) Hematoxylin&Eosin stained lung tissue of mice was observed under a microscope (100×, 200×). (B) The level of total IgE in serum of mice was measured by ELISA kits. (C) The level of OVA-specific IgE in serum of mice was measured by ELISA kits. Data are shown as mean $\pm$ SD. N=10 mice /group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, OVA group vs. Control group. ^ $P<0.05$, ^^ $P<0.01$, ^^^ $P<0.001$, OVA+Tio group or OVA+Rh1 group vs. OVA group.

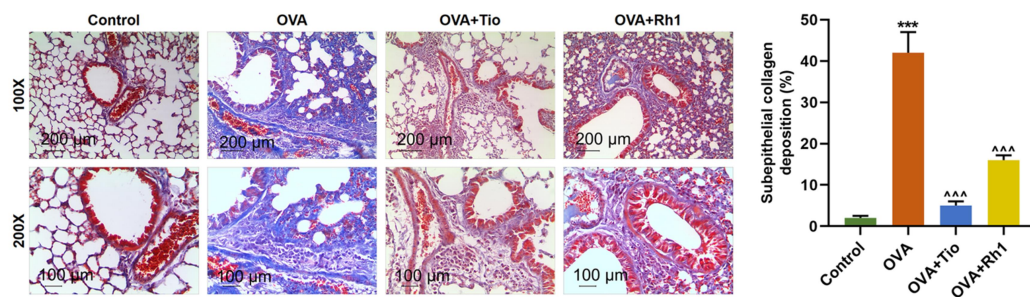


Figure 3. Ginsenoside Rh1 alleviates ovalbumin (OVA)-induced airway remodeling and collagen deposition in OVA-induced asthma model mice. (Left part) Masson's trichrome stained lung tissue of mice was observed with a microscope (100 $\times$, 200 $\times$). (Right part) Subepithelial collagen deposition(%) in mice airway was assessed by Masson's trichrome staining. Data are shown as mean $\pm$ SD. N=10 mice /group. * P <0.05, ** P <0.01, *** P <0.001, OVA group vs. Control group. ^ P <0.05, ^^ P <0.01, ^^ P <0.001, OVA+Tio group or OVA+Rh1 group vs. OVA group.

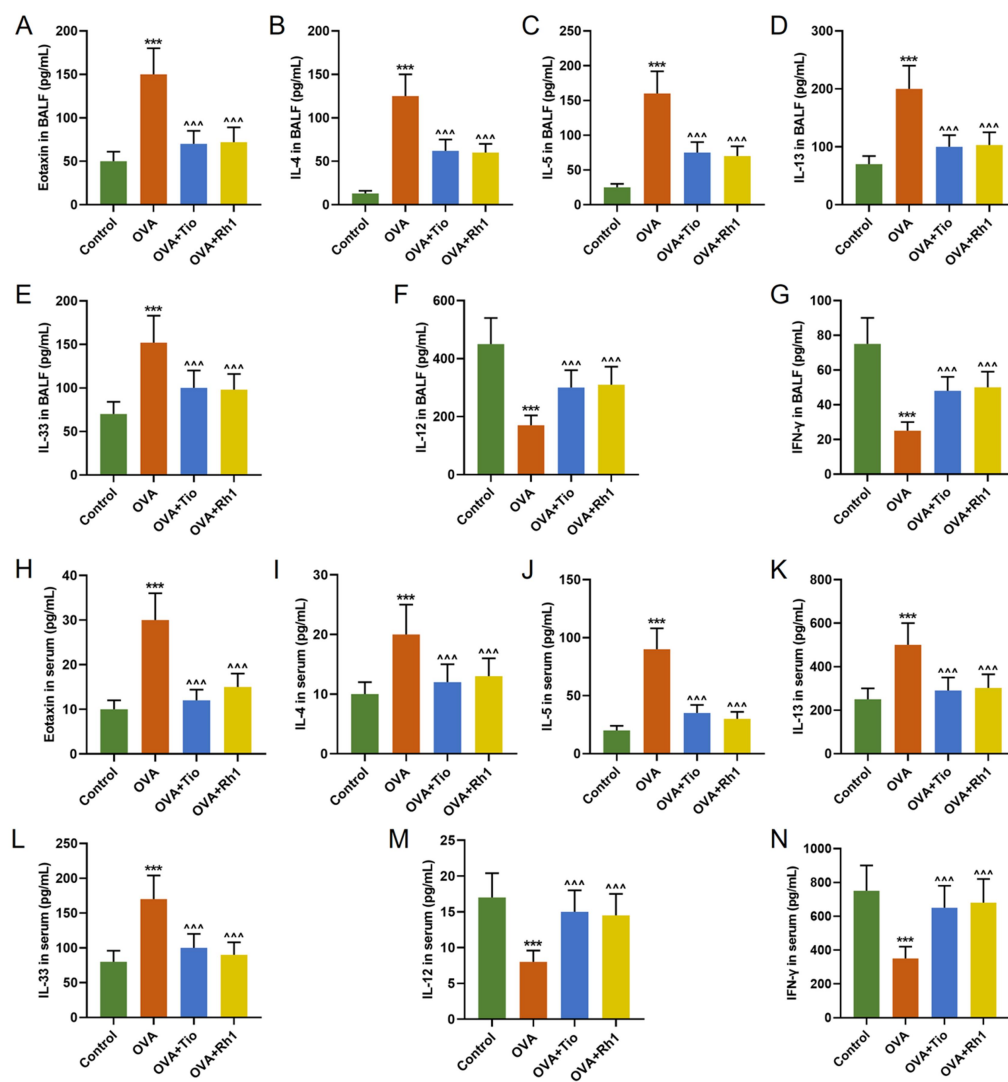
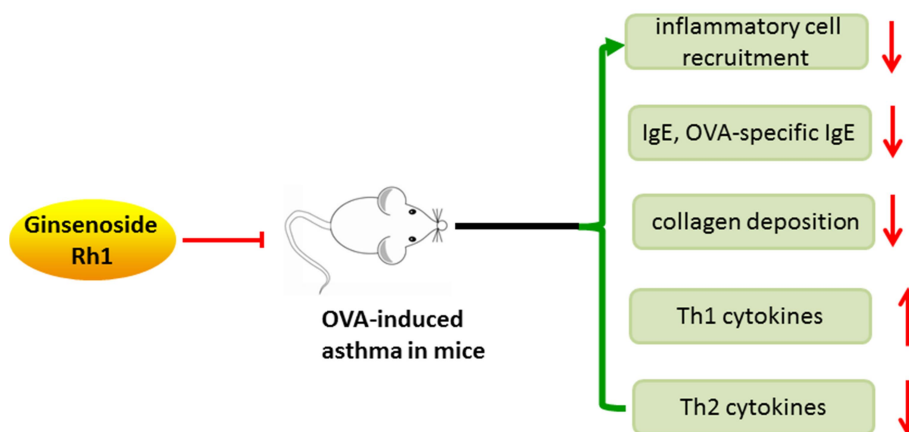


Figure 4. Ginsenoside Rh1 regulates the concentration of Th1/Th2 cytokines in broncho alveolar lavage fluid (BALF) and serum of ovalbumin-induced asthma model mice. (A) Eotaxin level in BALF from mice was measured by the ELISA kit. (B) IL-4 level in BALF from mice was measured by the ELISA kit. (C) IL-5 level in BALF from mice was measured by the ELISA kit. (D) IL-13 level in BALF from mice was measured by the ELISA kit. (E) IL-33 level in BALF from mice was measured by the ELISA kit. (F) IL-12 level in BALF from mice was measured by the ELISA kit. (G) IFN-γ level in BALF from mice was measured by the ELISA kit. (H) Eotaxin level in serum of mice was measured by the ELISA kit. (I) IL-4 level in serum of mice was measured by the ELISA kit. (J) IL-5 level in serum of mice was measured by the

ELISA kit. (K) IL-13 level in serum of mice was measured by the ELISA kit. (L) IL-33 level in serum of mice was measured by the ELISA kit. (M) IL-12 level in serum of mice was measured by the ELISA kit. (N) IFN- γ level in serum of mice was measured by the ELISA kit. Data are shown as mean $\pm$ SD. N=10 mice /group. * P <0.05, ** P <0.01, *** P <0.001, OVA group vs. Control group. ^ P <0.05, ^^ P <0.01, ^^^ P <0.001, OVA+Tio group or OVA+Rh1 group vs. OVA group.

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Graphical abstract caption.

Ginsenoside Rh1 attenuates ovalbumin-induced asthma by regulating Th1/Th2 cytokines balance, indicating a promising therapeutic approach for asthma.