

Salidroside Mitigates Airway Inflammation in Asthmatic Mice via the AMPK/Akt/GSK3 β Signaling Pathway

Xue Luan^{a, b, c} Chunai Cui^{c, d} Jingzhi Jiang^{c, d} Chongyang Wang^{c, d} Li Li^{c, d}
Hongzi Li^{a, c} Chang Xu^{c, d} Liangchang Li^{c, d} Yongxue Chi^{a, c} Guanghai Yan^{c, d}

^aDepartment of Pediatrics, Affiliated Hospital of Yanbian University, Jilin, China; ^bDepartment of Pediatrics, First Hospital of Jilin University, Jilin, China; ^cJilin Key Laboratory for Immune and Targeting Research on Common Allergic Diseases, Yanbian University, Yanji, China; ^dDepartment of Anatomy, Histology and Embryology, Yanbian University Medical College, Jilin, China

Keywords

Salidroside · Acute asthma · Airway inflammation · Th1/Th2 imbalance · AMPK/Akt/GSK3 β

Abstract

Introduction: This study aimed to explore the effects and mechanisms of salidroside (SAL) in airway inflammation in asthmatic mice. **Methods:** Mice were sensitized with ovalbumin (OVA) to establish an asthma model. They were divided into the control group, OVA group, SAL low-dose group (SAL-L), SAL high-dose group (SAL-H), and dexamethasone (DXM) group. The airway reactivity of the mice was measured, and the total cells, neutrophils, eosinophils, and lymphocytes were counted, respectively. The levels of IL-4, IL-5, IL-13, and IFN- γ in bronchoalveolar lavage fluid (BALF) were detected by ELISA. Immunohistochemistry was used to detect the expression levels of p-AMPK, p-Akt, and p-GSK3 β . Western blot was used to detect cytokine levels in lung tissue and p-AMPK, p-Akt, and p-GSK3 β levels in LPS-induced 16HBE cells. **Results:** The airway hyperresponsiveness of asthmatic mice in the SAL-H group decreased ($p < 0.05$), and the total number of cells, neutrophils, eosinophils, and lymphocytes decreased significantly ($p < 0.05$). In addition, the

airways of mice showed airway inflammatory infiltration and goblet cell proliferation, and the corresponding cellular inflammatory factors IL-4, IL-5, and IL-13 were significantly decreased. However, the expression of IFN- γ in BALF and lung tissues was increased ($p < 0.05$). Moreover, after the mice were treated with SAL, the phosphorylation level of AMPK was significantly increased, which further reduced the phosphorylation levels of Akt and GSK3 β ($p < 0.05$). Both SAL and AMPK inhibitors exerted effects on LPS-induced 16HBE cells, consistent with in vivo results. **Conclusion:** SAL can inhibit bronchial hyperresponsiveness and reduce tracheal inflammation by increasing AMPK phosphorylation and inhibiting Akt and GSK3 β signaling pathways. © 2021 S. Karger AG, Basel

Introduction

Bronchial asthma is a chronic inflammatory disorder of the airways associated with airway hyperresponsiveness, which is characterized by recurrent episodes of wheezing, breathlessness, chest tightness, and coughing.

Xue Luan and Chunai Cui contributed equally. Edited by: A. Haczk, Sacramento, CA, USA.

It is estimated that by 2025, 400 million people worldwide will suffer from asthma [1, 2]. Due to increased use of inhaled glucocorticoid, the mortality rate of adults and children due to asthma has decreased over the past 25 years. However, the deaths caused by asthma have varied over the years [3]. Therefore, there is still an urgent need to develop more effective asthma prevention and treatment methods.

AMP-activated protein kinase (AMPK) can promote ATP production through catabolism or inactivate anabolic pathways that consume ATP. Therefore, AMPK accumulates energy during metabolic stress, thereby protecting cells under various pathological conditions [4]. Previously, the AMPK signaling pathway is considered to be a novel signaling pathway capable of regulating inflammation and immune responses [5, 6]. Studies have shown that the AMPK agonist AICAR reduces asthma-related inflammatory factors (such as IL-4, IL-5, and IL-13) and airway hyperresponsiveness [7]. In addition, in metformin-treated asthma mice, activation of the AMPK pathway partially relieved airway inflammation and reduced the intensity of oxidative stress responses [8]. AMPK is a key molecule associated with various antiasthmatic effects. As a downstream signaling molecule of the AMPK pathway, Akt plays an important role in cell survival, and its substrate is Ser/Thr protein kinase GSK3. Akt is capable of phosphorylating conserved amino terminal motifs in GSK3 α and GSK3 β . It is shown that the Akt/GSK3 β pathway is related to the anti-inflammatory effect in chronic asthma, inhibits airway hyperresponsiveness, and decreases airway resistance through phosphorylation signaling [9].

Salidroside (SAL) is the main active ingredient of the herbaceous shrub plant *Rhodiola* [10]. Studies have shown that SAL has anti-inflammatory, antitumor, and antimitochondrial oxidative stress effects [11–14]. In a diabetic mouse model, SAL caused dose-dependent increase in phosphorylation of AMPK/PI3K/Akt/GSK3 β in hepatocytes [15]. In addition, it is reported that SAL reduced atherosclerosis by inhibiting the PI3K/Akt pathway and enhancing AMPK phosphorylation [16]. Our previous research showed that SAL inhibited tracheal inflammation by downregulating NF- κ B and p38 MAPK [17]. Based on the above studies, we speculate that the anti-inflammatory activity of SAL may be attributed to the AMPK/Akt/GSK3 β signaling pathway. However, the role and mechanism of SAL in asthma has not been reported. In this study, we investigated the effect of SAL on the treatment of asthma in mice and the possible role of the AMPK/Akt/GSK3 β signaling pathway in the effect of SAL.

Materials and Methods

Animals

Fifty female BALB/c mice (aged 6–8 weeks; weighing 18 ± 5 g) were obtained from the Experimental Animal Center of Yanbian University (Jilin, China). All mice were housed in laboratory conditions free of specific pathogens and provided with adequate food and a comfortable environment. The study has been approved by the Ethics Committee of Yanbian University (Jilin, China).

Asthma Model

Mice were randomly divided into the control group, asthma model group (OVA group, Québec, Quebec), SAL (Yuanye, Shanghai, China) (low dose of 25 and high dose of 50 mg/kg, respectively) treatment groups, and dexamethasone (DXM) (2 mg/kg; Sigma, Phoenix, AZ, USA) treatment group, with 10 mice in each group. As shown in Figure 1, the mouse model of asthma was induced by OVA (Grade V; Sigma, Phoenix, AZ, USA) sensitization. In brief, mice were sensitized by intraperitoneal injection of 20 μ g OVA emulsified in 2 mg aluminum hydroxide gel (InvivoGen, San Diego, CA, USA) on days 0, 7, and 14. From day 21, mice received 5% OVA nebulization (NE-U12; Omron Corporation, Tokyo, Japan) in sterile saline for 0.5 h per day for 1 week. Before each OVA challenge, the SAL and DXM treatment groups were treated with intraperitoneal SAL and DXM, respectively. Mice in the control group were treated with a saline solution.

Airway Reactivity Measurement

As previously reported [18], airway responsiveness was analyzed using systematic and invasive plethysmography (Buxco Electronics, Troy, NY, USA). Changes in airway hyperresponsiveness were assessed by measuring pulmonary resistance (RL). In brief, the mice were weighed 24 h after the last challenge and intraperitoneally injected with 2% sodium pentobarbital (0.03 g/kg). The trachea was fully exposed. After incision of the trachea, a thin catheter with an inner diameter of 2 mm was inserted. After that, the catheter was connected to a ventilator for mechanical ventilation with a tidal volume of 0.2 mL and a frequency of 140 beats per min. After the ventilator was stabilized, PBS and Ach with a certain concentration (3.125, 6.25, 12.5, and 25 mg/mL, respectively) were administered to the mice through the ventilator using an ultrasonic nebulizer, and RL was recorded.

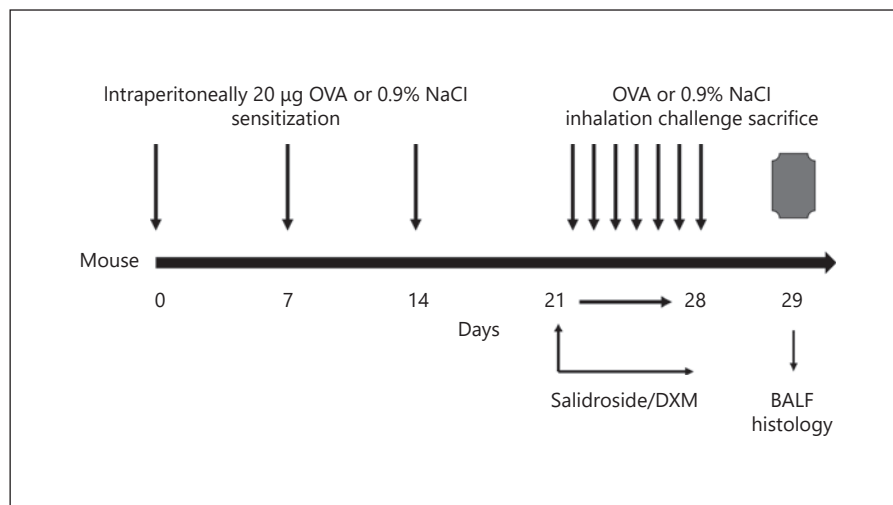
BALF Collection and Cell Counting

After airway hyperresponsiveness measurements, BALF was then collected by washing with 400 μ L of ice-cold PBS. The cells in BALF were collected by centrifugation at 1,000 rpm for 10 min at 4°C, and then the total number of inflammatory cells in BALF was counted. Based on morphological criteria, differential cell counts were performed by Wright staining. Meanwhile, the lavage supernatant was also collected.

ELISA

The concentrations of IL-4, IL-5, IL-13, and IFN- γ in BALF were measured according to the manufacturer's protocols using ELISA kits (R&D Systems, Minneapolis, MN, USA). The microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) was used to detect the optical density and calculate the cytokine concentration.

Fig. 1. Schematic diagram of OVA-induced asthma model. Mice were sensitized with intraperitoneal injections of 20 µg of OVA emulsified in aluminum hydroxide in a total volume of 0.2 mL on days 0, 7, and 14, followed by 7 consecutive challenges by exposure to OVA aerosol for 30 min every day. Prior to daily OVA nebulization challenge, SAL (25 mg/kg, 50 mg/kg) or DXM (2 mg/kg) was administered in the SAL-L, SAL-H, or DXM groups, respectively. The control mice were treated with saline for the sensitization and challenge. Mice were sacrificed 24 h after the final challenge. BALF samples were collected, and the lungs were dissected for analysis. OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; DXM, dexamethasone; SAL, salidroside.



Histopathology

Lung tissue was collected after airway hyperresponsiveness measurements fixed with 4% paraformaldehyde for 0.5 h and then embedded in paraffin. The tissue was cut into 5-µm sections at room temperature and stained with hematoxylin and eosin (H&E) to observe the trachea inflammatory response. Airway goblet cell proliferation and mucus secretion were evaluated by periodic acid-Schiff (PAS) staining. Under $\times 200$ magnification, 3 slices were randomly selected from each mouse, and 5–8 fields of view with round bronchial cross section and a diameter of about 100–200 µm were randomly taken from each slice. The inflammation score was calculated by evaluating the infiltration of inflammatory cells around the bronchi. The semiquantitative scoring system (0–4) was used. The percentage of PAS staining positive cells in airway epithelial cells was calculated. Immunohistochemistry was performed using p-AMPK, p-Akt, and p-GSK3β antibodies (Cell Signaling Technology, Danvers, MA, USA) and observed under an optical microscope (Olympus BX50; Olympus, Tokyo, Japan).

Cell Culture

Human bronchial epithelial cell line 16HBE cells (Rockville, MD, USA) were pretreated with SAL (5 mM) and compound C (5 mM; Selleck, Houston, TX, USA) for 6 h. Thereafter, the cells were stimulated with LPS and then cultured for 24 h.

Immunofluorescence

Immunofluorescence was performed in 16HBE cells and lung tissue sections. In brief, endogenous peroxidase was inactivated in 3% H_2O_2 . After blocking nonspecific antibodies, the samples were incubated with anti-p-AMPK (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Afterward, Rhodamine-conjugated AffiniPure goat anti-rabbit IgG was then used to detect p-AMPK. The nuclei were stained with DAPI. Cells were observed under an Olympus BX61 fluorescence microscope (Olympus, Tokyo, Japan).

Western Blot

Freshly isolated lung tissue was homogenized in the presence of a protease inhibitor, and the protein concentration was mea-

sured using Bradford reagent (Bio-Rad, Hercules, CA, USA). 16HBE cells were lysed with RIPA (Solarbio, Beijing, China) containing PMSF and phosphatase inhibitor to isolate total proteins. The proteins were subjected to SDS-PAGE gel electrophoresis (20 µg protein per lane) and then transferred to a nitrocellulose membrane for Western blot detection. The antibodies, including β-actin, IL-4, IL-5, IL-13, IFN-γ, GAPDH, AMPK, p-AMPK, Akt, p-Akt, GSK3β, and p-GSK3β, were purchased from Santa Cruz Biochemicals, Dallas, TX, USA. Detection was performed using the ECL detection system (iNtRON Biotechnology, Seoul, South Korea). Optical density scanning was performed with Gel Doc XR (Bio-Rad, Inc) to calculate the relative protein expression level.

Statistical Analysis

Results were analyzed using statistical software SPSS 21.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as mean $\pm$ SD. Comparisons among multiple groups were performed using one-way analysis of variance followed by the LSD method. A *p* value of <0.05 indicates that the difference is statistically significant.

Results

SAL Reduces Airway Hyperresponsiveness in Asthmatic Mice

To assess the effect of SAL on the airway hyperresponsiveness, RL was detected. No significant difference in baseline airway resistance was observed in the 5 groups. When Ach was administered at a dose from 3.125 to 25 mg/mL, the RL value of the OVA group and the SAL-L group was significantly higher than that of the control group ($p < 0.05$). However, compared with the control group, the RL of the SAL-H group and the DXM group was significantly reduced ($p < 0.05$) (Fig. 2). These results

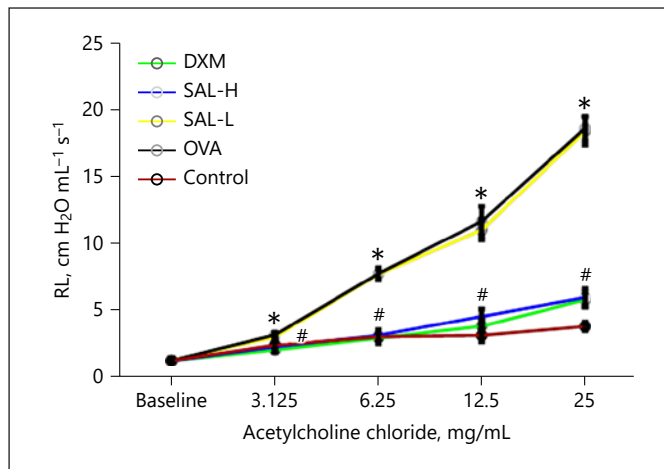


Fig. 2. SAL reduced airway hyperactivity in asthmatic mice. Data are presented as mean $\pm$ SD ($n = 10$ per group). * $p < 0.05$ compared with the control group. # $p < 0.05$ compared with the OVA group. SAL, salidroside; OVA, ovalbumin; DXM, dexamethasone.

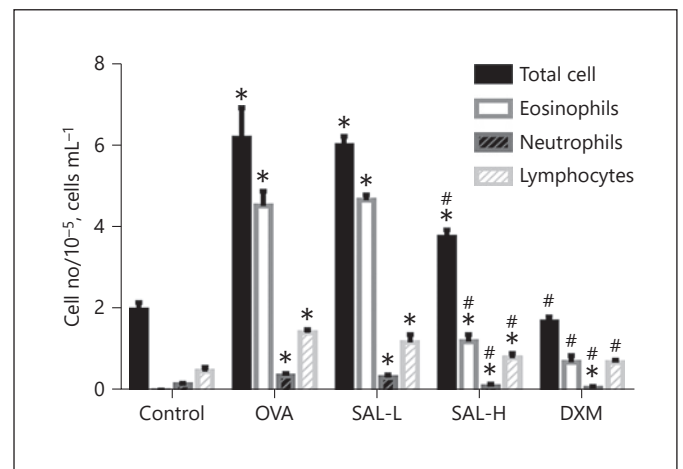


Fig. 3. Effects of SAL on total and differential cellular component count in BALF in asthmatic mice. Data are presented as mean $\pm$ SD ($n = 10$ per group, $10^5/\text{mL}$). * $p < 0.05$ compared with the control group. # $p < 0.05$ compared with the OVA group. SAL, salidroside; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; DXM, dexamethasone.

indicate that SAL reduces airway hyperresponsiveness in asthmatic mice in a dose-dependent manner.

SAL Reduces the Infiltration of Inflammatory Cells in the Lung Tissue of Asthmatic Mice

Inflammatory cell infiltration is one of the main features of airway inflammation. To investigate the effect of SAL on inflammatory cell infiltration in asthmatic mice, we counted the number of cells in BALF of each group. The data showed that the infiltration of lymphocytes, eosinophils, and neutrophils in BALF of the OVA and SAL-L groups was significantly higher than that of the control group ($p < 0.05$). However, the SAL-H or DXM groups significantly reduced the number of these cells ($p < 0.05$) (Fig. 3).

Further histopathological analysis of the lungs (H&E and PAS staining) showed that mice in the OVA and SAL-L groups showed obvious thickening of airway wall, airway smooth muscles, and submucosa, mucosal epithelial hyperplasia, and increased mucus secretion (Fig. 4a). In addition, compared with the control group, PAS staining showed an obvious increase in goblet hyperplasia in the OVA and SAL-L groups (Fig. 4b). In contrast, inflammation score and goblet cell proliferation were less in the SAL-H and DXM groups. Compared with the control group, the OVA group had significantly higher inflammation score ($p < 0.05$, Fig. 4c), suggesting that there are a great number of infiltrating inflamma-

tory cells around the airway. The SAL groups clearly showed lower inflammatory scores than the OVA group in a dose-dependent manner ($p < 0.05$). As shown in Figure 4d, the percentage of PAS-positive cells in the OVA group was significantly higher than that of the control group ($p < 0.05$), but the SAL-H group could obviously reduce the percentage of PAS-positive cells in the airway epithelium in a dose-dependent manner ($p < 0.05$). These results indicate that SAL relieves airway inflammation and inhibits infiltration of inflammatory cells in asthmatic mice.

SAL Decreases IL-4, IL-5, and IL-13 and Increases IFN- γ in BALF of Asthmatic Mice

We analyzed the levels of IL-4, IL-5, IL-13, and IFN- γ in BALF and lung tissues by ELISA and Western blot, respectively. ELISA results showed that the levels of IL-4, IL-5, and IL-13 in BALF in the OVA and SAL-L groups were significantly increased, while the levels of IFN- γ were significantly lower than those in the control group ($p < 0.05$). However, in the SAL-H and DXM groups, levels of IL-4, IL-5, and IL-13 were significantly reduced, while IFN- γ levels were significantly increased than those in the OVA group ($p < 0.05$) (Fig. 5a). Consistent with these results, Western blot showed that IL-4, IL-5, and IL-13 protein levels in the OVA and SAL-L groups were significantly increased, while IFN- γ protein levels were significantly lower than those in the control group ($p < 0.05$).

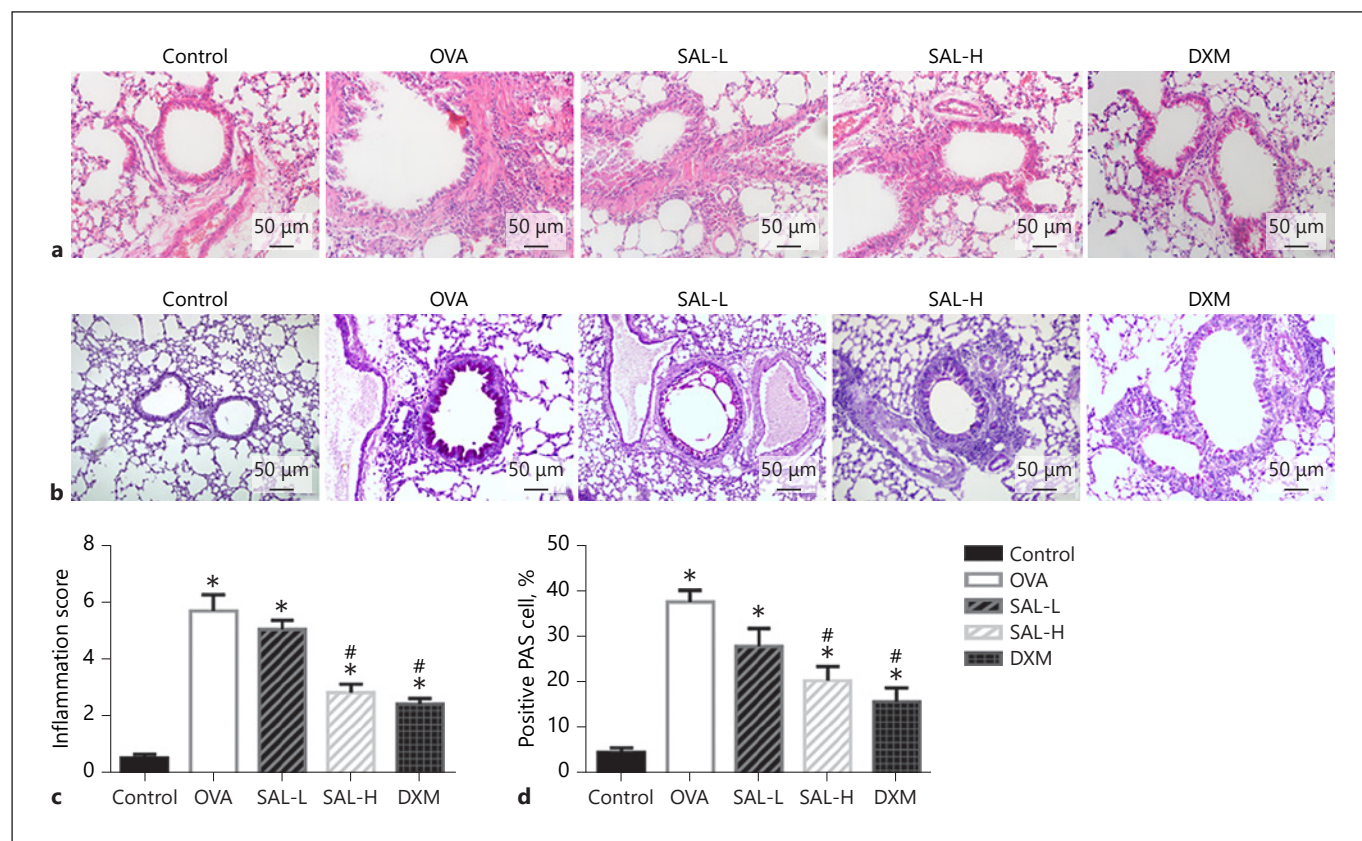


Fig. 4. Effect of SAL on airway inflammation and goblet cell hyperplasia in asthmatic mice. **a** Lung sections were stained with H&E to observe the inflammatory cells. **b** PAS staining was used to measure mucus production around the airways ($\times 200$ magnification; scale bar, 50 μm). **c, d** The column chart of H&E and PAS represents mean $\pm$ SD ($n = 3$). * $p < 0.05$ versus the control group. # $p < 0.05$ versus the OVA group. SAL, salidroside; OVA, ovalbumin; DXM, dexamethasone.

However, IL-4, IL-5, and IL-13 protein levels were significantly reduced, while IFN- γ was increased in the SAL-H and DXM groups than those in the OVA group ($p < 0.05$) (Fig. 5b, c). These results indicate that high-dose SAL inhibits IL-4, IL-5, and IL-13 levels, whereas increases IFN- γ level.

SAL Regulates Inflammatory Response in Asthmatic Mice via the AMPK/Akt/GSK3 β Signaling Pathway

To determine the effects of SAL on phosphorylation of AMPK, Akt, and GSK3 β in lung tissue, immunohistochemical staining was performed (Fig. 6a). Compared with the control group, p-AMPK decreased, while p-Akt and p-GSK3 β increased in the airway of the OVA group. In the SAL-H and DXM groups, p-AMPK increased, while p-Akt and p-GSK3 β decreased compared to the OVA group. The Western blot results of lung tissue

showed that the total protein of AMPK, Akt, and GSK3 β in each group was not significantly changed (Fig. 6b). However, p-AMPK levels in the OVA and SAL-L groups were significantly lower than those in the control group ($p < 0.05$), and p-AMPK expression levels in the SAL-H and DXM groups were significantly increased than those in the OVA group ($p < 0.05$) (Fig. 6c). In addition, in the OVA and SAL-L groups, p-Akt and p-GSK3 β were significantly upregulated ($p < 0.05$), while in the SAL-H and DXM groups, p-Akt and p-GSK3 β were significantly downregulated ($p < 0.05$) (Fig. 6b, c). The immunofluorescence results are consistent with the above results (Fig. 6d). These results indicate that SAL can alleviate airway inflammation in asthmatic mice by promoting AMPK phosphorylation, which in turn reduces Akt and p-GSK3 β phosphorylation.

SAL Regulates Inflammatory Response in LPS-Induced 16HBE Cells via the AMPK/Akt/GSK3 β Signaling Pathway

To further demonstrate that SAL directly regulates AMPK, Akt, and GSK3 β in vitro, we treated 16HBE cells with LPS. The level of p-AMPK was decreased, whereas the levels of p-Akt and p-GSK3 β were increased after LPS intervention in comparison with the control group ($p < 0.05$) (Fig. 7a, 7b). After SAL treatment, the level of p-AMPK was significantly increased,

and the levels of p-Akt and p-GSK3 β were significantly reduced ($p < 0.05$). Interestingly, SAL resulted in an increase in the level of p-AMPK and a decrease in levels of p-Akt and p-GSK3 β compared to the effects after LPS intervention ($p < 0.05$). Moreover, compound C decreased the expression of p-AMPK (Fig. 7a, 7b). The expression of p-AMPK in LPS-treated 16HBE cells was observed by immunofluorescence (Fig. 7c). P-AMPK had mild expression in 16HBE cells. Downregulation of p-AMPK in LPS and/or compound C-treated 16HBE

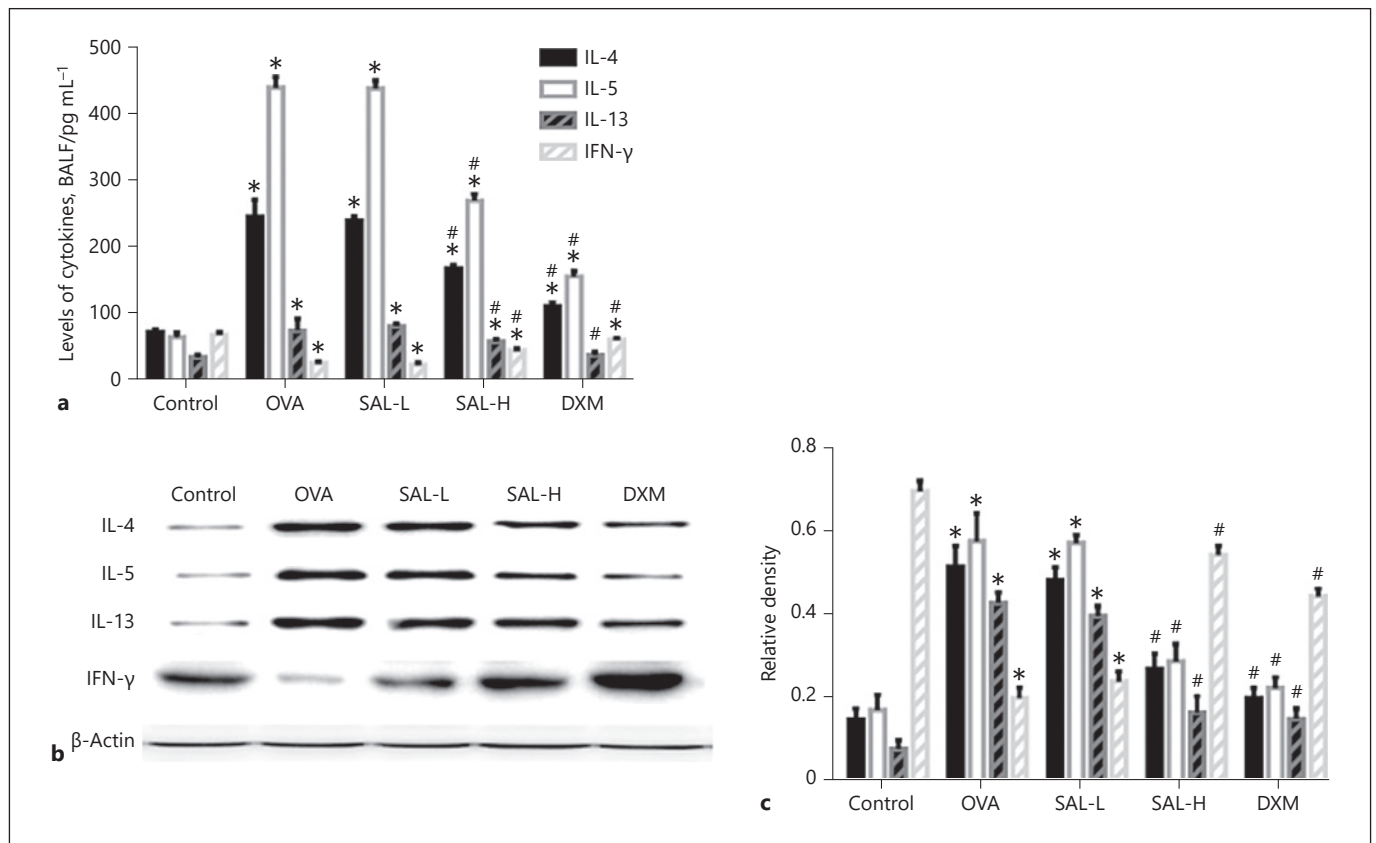


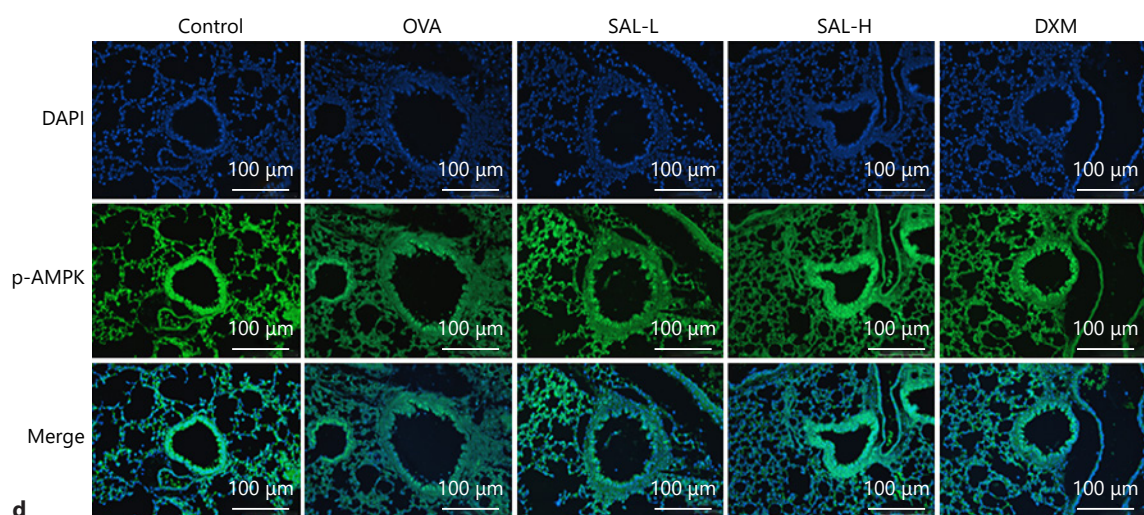
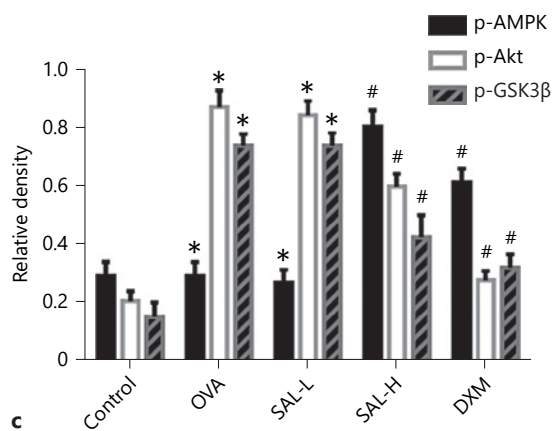
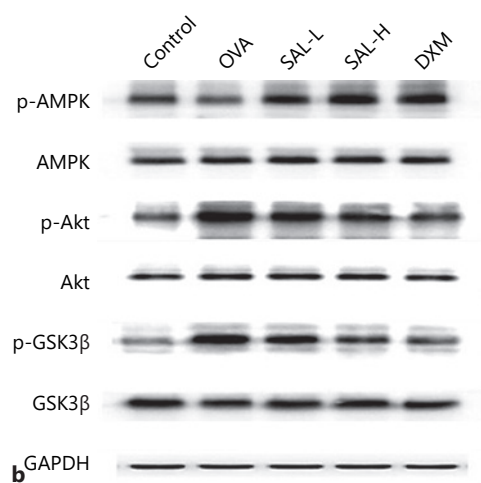
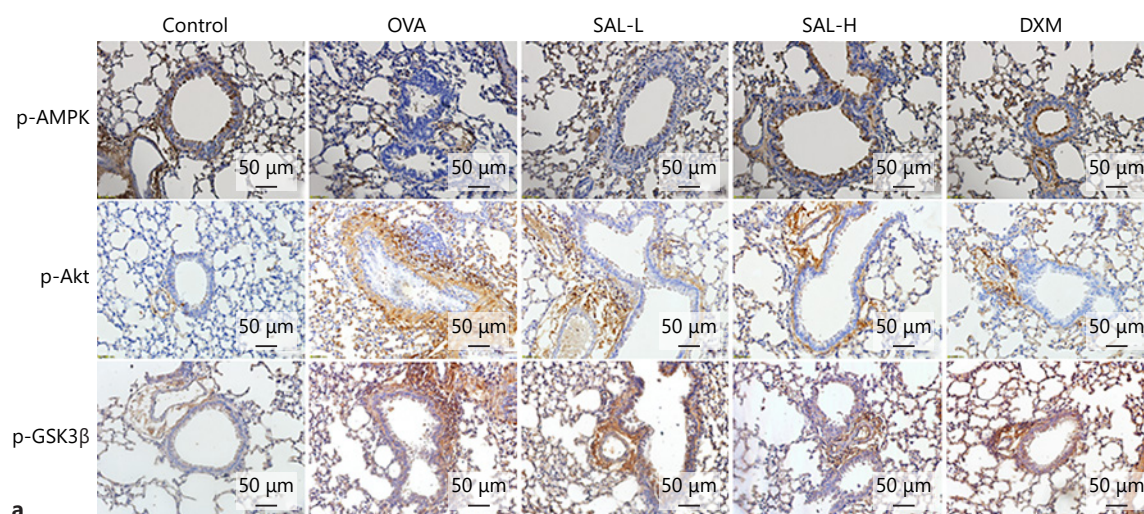
Fig. 5. Effects of SAL on levels of IL-4, IL-5, IL-13, and IFN- γ in BALF and lung tissues of asthmatic mice. **a** Cytokine levels were tested by ELISA. Data are presented as mean $\pm$ SD ($n = 10$ per group, pg/L). **b, c** Protein levels of the cytokines in the lung after 48 h were measured by Western blotting. Density ratio against

β -actin was measured using a densitometer. * $p < 0.05$ compared with the control group. # $p < 0.05$ compared with the OVA group. SAL, salidroside; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; DXM, dexamethasone.

Fig. 6. Effect of SAL on the expression of AMPK, Akt, and GSK3 β phosphorylation in lung tissues of asthmatic mice. **a** Immunohistochemical staining was performed to measure the expression of p-AMPK, p-Akt, and p-GSK3 β ($\times 200$; scale bar, 5 μ m). **b, c** The expression of AMPK, Akt, and GSK3 β phosphorylation was analyzed by Western blotting. Density ratio against GAPDH was mea-

sured using a densitometer. Data are presented as mean $\pm$ SD ($n = 3$ per group). * $p < 0.05$ compared with the control group. # $p < 0.05$ compared with the OVA group. **d** The expression of p-AMPK in lung tissue was observed by immunofluorescence. SAL, salidroside; OVA, ovalbumin; DXM, dexamethasone.

(For figure see next page.)



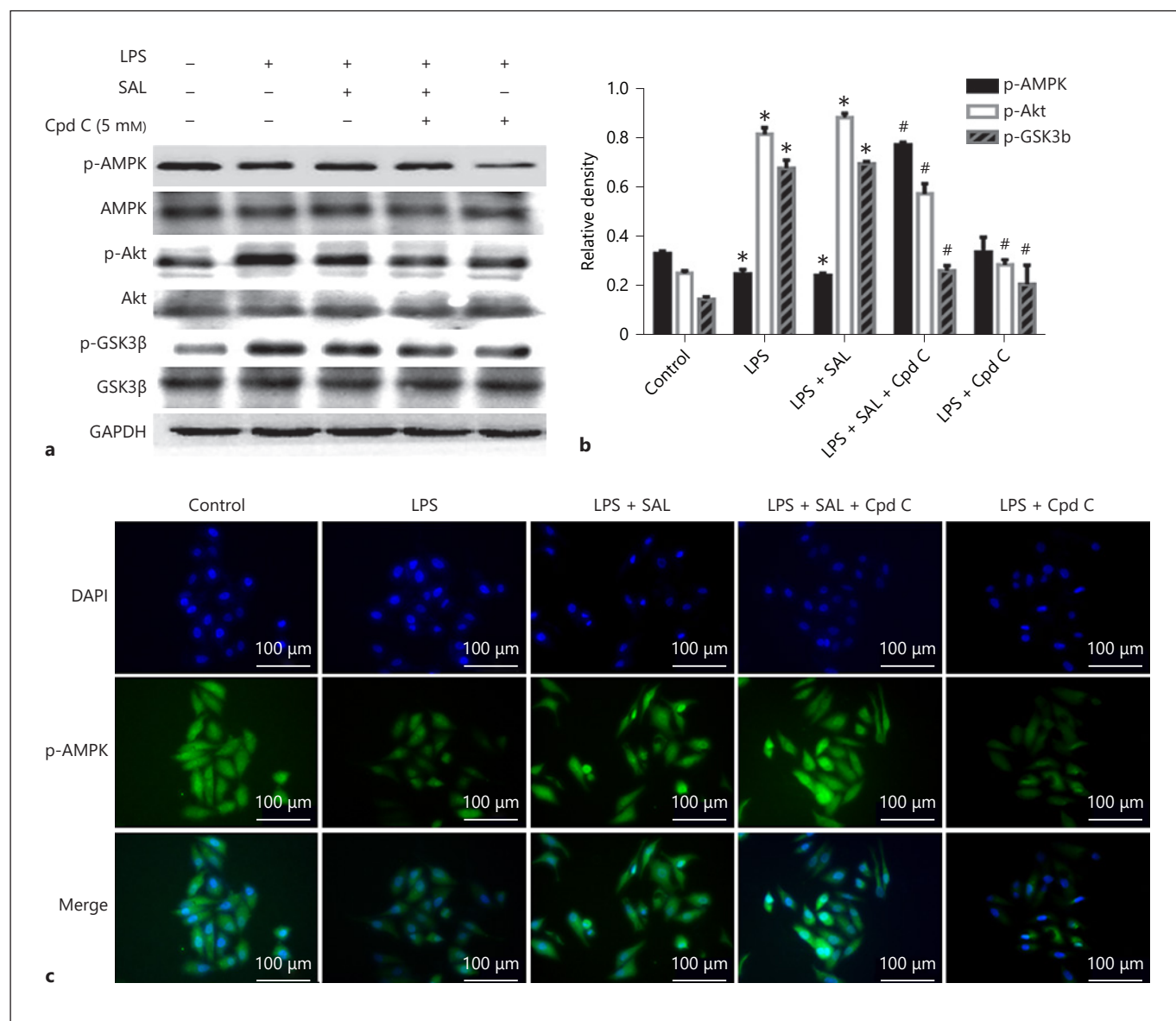


Fig. 7. Effect of SAL on the expression of AMPK, Akt, and GSK3 β phosphorylation in LPS-treated 16HBE cells. 16HBE cells were pretreated with SAL (5 mM) and compound C (5 mM) for 6 h prior to challenge with LPS. **a, b** Western blotting of the levels of phosphorylated (p-) AMPK, (p-) Akt, and (p-) GSK3 β in 16HBE cells. Representative images and densitometry data are shown.

Data are presented as mean $\pm$ SD ($n = 3$ per group). * $p < 0.05$ compared with the untreated group. # $p < 0.05$ compared with the group treated by LPS. **c** The expression of p-AMPK in LPS-treated 16HBE cells was observed by immunofluorescence. SAL, salidroside.

cells was observed, and upregulation of p-AMPK was observed when SAL was added. Therefore, the anti-inflammatory effect of SAL on asthmatic mice may be achieved by targeting AMPK and then regulating the Akt/GSK3 β signaling pathway.

Discussion/Conclusion

Asthma is a chronic respiratory disease that is featured by bronchoconstriction. Over time, it eventually causes airway damage [19, 20]. Corticosteroids play an important role in preventing and treating airway inflammation in asthma, but in the long term, it significantly increases

the risk of diabetes, which in turn leads to poor glycemic control, glaucoma, cataracts, bruising or purple, adrenal insufficiency, and osteoporosis [21]. These risks indicate the urgency of exploring other effective treatments or interventions. It is well known that airway hyperresponsiveness is directly related to excessive mucus secretion that causes airway obstruction [22] and is one of the most basic pathological features of bronchial asthma. In this study, we found that high-dose SAL significantly reduced airway hyperresponsiveness in asthmatic mice. During chronic asthma, a significant feature of antigen-induced bronchitis is an increased number of inflammatory cells in the respiratory tract and subepithelial space of the lung, including eosinophils, mast cells, lymphocytes, and neutrophils [23]. Therefore, we explored the effects of SAL on the infiltration and accumulation of inflammatory cells in BALF after repeated OVA challenges. The results showed that SAL significantly reduced the number of lymphocytes, eosinophils, and neutrophils in BALF. HE staining and PAS staining showed that SAL significantly relieved airway wall thickening, airway smooth muscle thickening, inflammatory cell infiltration, and other pathological features in lung tissues of asthmatic mice and also reduced goblet cell proliferation. These results indicate that SAL has a strong anti-inflammatory effect on bronchial asthma.

Previous study has shown that SAL had an antiasthmatic effect, which is related to regulating the Th1/Th2 balance [24]. Th1/Th2 balance is one of the basic ways to regulate the body's immune response. Under normal conditions, Th1/Th2 is balanced. However, genetic variation and environmental factors can break this balance. Th2 bias is a key factor in the formation and development of asthma. Inflammatory factors secreted by Th2, including IL-4, IL-5, and IL-13, are key molecules that cause allergic asthma. These cytokines are often overexpressed in the bronchial mucosa of asthma patients [25–28]. Neutrophils, but not eosinophils, in asthma patients show overexpression of IFN- γ in their sputum cells, which may indicate ongoing innate immune activation [29]. This study showed that SAL significantly reduced IL-4, IL-5, and IL-13 levels in asthmatic mice and increased IFN- γ levels in lung tissue and BALF. This alleviates the effects of the Th1/Th2 imbalance, which in turn eases the progression of asthma.

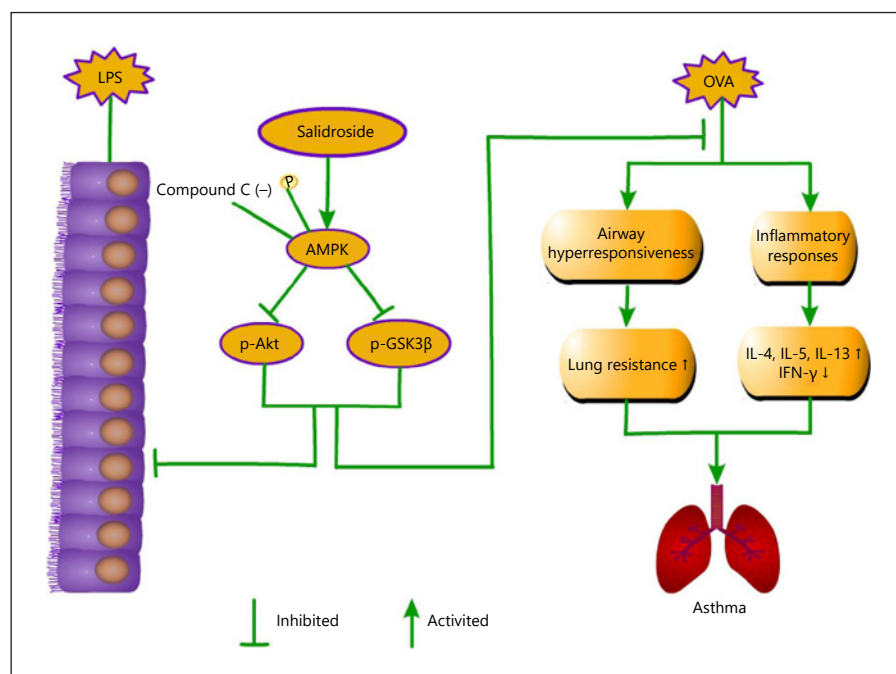
AMPK is a serine/threonine protein kinase that is widely expressed and highly conserved in eukaryotes. It is the main regulator of cellular energy metabolism homeostasis and redox response [30]. AMPK is activated after phosphorylation of N-terminal Thr172, but AMPK has completely different mechanisms in different diseases

[31, 32]. Studies have shown that LKB1 and AMPK α 1 deficiency increases T-effector protein synthesis and promotes IFN- γ production [33]. Here, we found that SAL promoted the expression of IFN- γ in the lung tissue of asthmatic mice, which confirms the positive regulatory effect of AMPK on Th1 cytokines. In addition, it is shown that the treatment of obese asthmatic mice with AMPK agonist metformin reduces the accumulation of eosinophils in the lungs, restores the number of cells in BALF, and increases the phosphorylation of AMPK in lung tissue [34]. Similarly, we found that SAL showed anti-inflammatory effects on lung tissue of asthmatic mice and LPS-induced 16HBE cells by activating AMPK and enhancing its phosphorylation.

The protein kinase Akt plays an important role in glucose metabolism and protein synthesis and has antiapoptosis effects [35]. GSK3 β is a downstream signaling molecule of Akt. It is well known that Akt activation results in GSK3 β phosphorylation [36]. Glycyrrhizin reduces activation of the PI3K/Akt/GSK3 β signaling pathway, thereby inhibiting related cytokines and reducing airway inflammatory diseases [37]. Our study found that SAL inhibits airway inflammation in asthmatic mice by reducing the phosphorylation of Akt and GSK3 β . As an upstream signaling molecule of GSK3 β , AMPK can effectively block the phosphorylation of GSK3 β in RAW264.7 cells [38]. In this study, we found that compound C, an AMPK inhibitor, reduced the levels of p-Akt and p-GSK3 β . Therefore, we believe that SAL further regulates the Akt/GSK3 β signaling pathway via AMPK, thereby inhibiting airway hyperresponsiveness and reducing tracheal inflammation in asthmatic mice. This may provide a strong molecular basis for SAL to use as a new target for the prevention or treatment of allergic asthma.

In conclusion, as summarized in Figure 8, we report here that SAL could reduce airway hyperresponsiveness and inflammation, including reducing the number of inflammatory cells in total cells, neutrophils, eosinophils, and lymphocytes in BALF, inhibiting airway inflammation and mucus secretion, and promoting the expression level of Th1 cytokines and inhibiting the expression level of Th2 cytokines. SAL could promote the phosphorylation of AMPK and inhibit the phosphorylation of Akt and GSK3 β in the lung tissue of asthmatic mouse models and in the 16HBE cells, indicating that the anti-inflammatory effect of SAL on the airway may be through upregulating the expression of AMPK and downregulating the expression of Akt and GSK3 β . This conclusion may provide a strong molecular basis for SAL to be used as a new drug to prevent or treat allergic asthma.

Fig. 8. Schematic diagram of the potential mechanism of SAL in inhibiting airway inflammation in OVA-induced asthmatic mice and 16HBE cells. SAL significantly activates AMPK. The activated AMPK promotes the phosphorylation of Akt and GSK3 β and inhibits the OVA-induced increase in airway resistance, the increase in Th1 cytokine expression, and the decrease in Th2 cytokine expression in asthmatic mice, thereby reducing airway hyperresponsiveness and inflammation. In addition, SAL also reduces the inflammatory response of 16HBE airway epithelial cells induced by LPS through this pathway. SAL, salidroside; OVA, ovalbumin.



Statement of Ethics

The study has been approved by the Ethics Committee of Yanbian University (Jilin, China).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study was funded by the National Natural Science Foundation of China (Nos.: 81860729, 81970018) Jilin Provincial Basic Research Project of Central Guided Local Science and Technology Development Fund (No.: 202002020JC).

Author Contributions

Xue Luan, Chunai Cui, Jingzhi Jiang, Chongyang Wang, Li Li, Hongzi Li, Chang Xu, and Liangchang Li collected and analyzed the data. Xue Luan and Chunai Cui wrote the manuscript. Yongxue Chi and Guanghai Yan designed the study and revised the manuscript. Guanghai Yan collected the funds. All authors read and approved the final manuscript.

Data Availability Statement

All data generated or analysed during this study are included in this article.

References

- 1 Pawankar R, Canonica GW, Holgate ST, Lockett RF. Allergic diseases and asthma: a major global health concern. *Curr Opin Allergy Clin Immunol*. 2012;12(1):39–41.
- 2 Papi A, Brightling C, Pedersen SE, Reddel HK. Asthma. *Lancet*. 2018;391(10122):783–800.
- 3 Abram M, Wegmann M, Fokuhl V, Sonar S, Luger EO, Kerzel S, et al. Nerve growth factor and neurotrophin-3 mediate survival of pulmonary plasma cells during the allergic airway inflammation. *J Immunol*. 2009;182(8):4705–12.
- 4 Escobar DA, Botero-Quintero AM, Kautza BC, Luciano J, Loughran P, Darwiche S, et al. Adenosine monophosphate-activated protein kinase activation protects against sepsis-induced organ injury and inflammation. *J Surg Res*. 2015;194(1):262–72.
- 5 Nath N, Giri S, Prasad R, Salem ML, Singh AK, Singh I. 5-aminoimidazole-4-carboxamide ribonucleoside: a novel immunomodulator with therapeutic efficacy in experimental autoimmune encephalomyelitis. *J Immunol*. 2005;175(1):566–74.
- 6 Peairs A, Radjavi A, Davis S, Li L, Ahmed A, Giri S, et al. Activation of AMPK inhibits inflammation in MRL/lpr mouse mesangial cells. *Clin Exp Immunol*. 2009;156(3):542–51.
- 7 Park SJ, Lee KS, Kim SR, Chae HJ, Yoo WH, Kim DI, et al. AMPK activation reduces vascular permeability and airway inflammation by regulating HIF/VEGFA pathway in a murine model of toluene diisocyanate-induced asthma. *Inflamm Res*. 2012;61(10):1069–83.

- 8 Park CS, Bang BR, Kwon HS, Moon KA, Kim TB, Lee KY, et al. Metformin reduces airway inflammation and remodeling via activation of AMP-activated protein kinase. *Biochem Pharmacol*. 2012;84(12):1660–70.
- 9 Yadav UC, Naura AS, Aguilera-Aguirre L, Boldogh I, Boulares HA, Calhoun WJ, et al. Aldose reductase inhibition prevents allergic airway remodeling through PI3K/AKT/GSK3 β pathway in mice. *PLoS One*. 2013;8(2):e57442.
- 10 Chiang HM, Chen HC, Wu CS, Wu PY, Wen KC. Rhodiola plants: chemistry and biological activity. *J Food Drug Anal*. 2015 Sep;23(3):359–69.
- 11 Qin Y, Liu HJ, Li M, Zhai DH, Tang YH, Yang L, et al. Salidroside improves the hypoxic tumor microenvironment and reverses the drug resistance of platinum drugs via HIF-1 α signaling pathway. *EBioMedicine*. 2018;38:25–36.
- 12 Sun MY, Ma DS, Zhao S, Wang L, Ma CY, Bai Y. Salidroside mitigates hypoxia/reoxygenation injury by alleviating endoplasmic reticulum stress induced apoptosis in H9c2 cardiomyocytes. *Mol Med Rep*. 2018;18(4):3760–8.
- 13 Zhang Y, Zhao Q. Salidroside attenuates interleukin-1 β -induced inflammation in human osteoarthritis chondrocytes. *J Cell Biochem*. 2018;120(2):1203.
- 14 Chen X, Fang C. Effect of salidroside on bone marrow haematopoiesis in a mouse model of myelosuppressed anaemia. *J Radiat Res*. 2019;60(2):197–203.
- 15 Zheng T, Yang X, Wu D, Xing S, Bian F, Li W, et al. Salidroside ameliorates insulin resistance through activation of a mitochondria-associated AMPK/PI3K/Akt/GSK3 β pathway. *Br J Pharmacol*. 2015;172(13):3284–301.
- 16 Xing SS, Yang XY, Zheng T, Li WJ, Wu D, Chi JY, et al. Salidroside improves endothelial function and alleviates atherosclerosis by activating a mitochondria-related AMPK/PI3K/Akt/eNOS pathway. *Vascul Pharmacol*. 2015;72:141–52.
- 17 Yan GH, Choi YH. Salidroside attenuates allergic airway inflammation through negative regulation of nuclear factor-kappa B and p38 mitogen-activated protein kinase. *J Pharmacol Sci*. 2014;126(2):126–35.
- 18 Yao J, Jiang M, Zhang Y, Liu X, Du Q, Feng G. Chrysin alleviates allergic inflammation and airway remodeling in a murine model of chronic asthma. *Int Immunopharmacol*. 2016;32:24–31.
- 19 Dougherty RH, Fahy JV. Acute exacerbations of asthma: epidemiology, biology and the exacerbation-prone phenotype. *Clin Exp Allergy*. 2009 Feb;39(2):193–202.
- 20 Chauhan BF, Ducharme FM. Anti-leukotriene agents compared to inhaled corticosteroids in the management of recurrent and/or chronic asthma in adults and children. *Cochrane Database Syst Rev*. 2012;5(5):CD002314–CD14.
- 21 Kelada O, Marignol L. Erythropoietin-stimulating agents and clinical outcomes in metastatic breast cancer patients with chemotherapy-induced anemia: a closed debate? *Tumour Biol*. 2014;35(6):5095–100.
- 22 Morcillo EJ, Cortijo J. Mucus and MUC in asthma. *Curr Opin Pulm Med*. 2006 Jan;12(1):1–6.
- 23 Yadav UC, Ramana KV, Aguilera-Aguirre L, Boldogh I, Boulares HA, Srivastava SK. Inhibition of aldose reductase prevents experimental allergic airway inflammation in mice. *PLoS One*. 2009;4(8):e6535.
- 24 Wang J, Jin RG, Xiao L, Wang QJ, Yan TH. Anti-asthma effects of synthetic salidroside through regulation of Th1/Th2 balance. *Chin J Nat Med*. 2014;12(7):500–4.
- 25 Bentley AM, Meng Q, Robinson DS, Hamid Q, Kay AB, Durham SR. Increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics. *Am J Respir Cell Mol Biol*. 1993;8(1):35–42.
- 26 Robinson DS, Ying S, Bentley AM, Meng Q, North J, Durham SR, et al. Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. *J Allergy Clin Immunol*. 1993;92(3):397–403.
- 27 Boniface S, Koscher V, Mamessier E, El Biaze M, Dupuy P, Lorec AM, et al. Assessment of T lymphocyte cytokine production in induced sputum from asthmatics: a flow cytometry study. *Clin Exp Allergy*. 2003 Sep;33(9):1238–43.
- 28 Cho SH, Stanciu LA, Holgate ST, Johnston SL. Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma. *Am J Respir Crit Care Med*. 2005;171(3):224–30.
- 29 da Silva J, Hilzendeger C, Moermans C, Schleich F, Henket M, Kebabdzic T, et al. Raised interferon- β , type 3 interferon and interferon-stimulated genes: evidence of innate immune activation in neutrophilic asthma. *Clin Exp Allergy*. 2017;47(3):313–23.
- 30 Novikova DS, Garabadzhiu AV, Melino G, Barlev NA, Tribulovich VG. AMP-activated protein kinase: structure, function, and role in pathological processes. *Biochemistry*. 2015 Feb;80(2):127–44.
- 31 Grahame Hardie D. AMP-activated protein kinase: a key regulator of energy balance with many roles in human disease. *J Intern Med*. 2014 Dec;276(6):543–59.
- 32 Kim J, Yang G, Kim Y, Kim J, Ha J. AMPK activators: mechanisms of action and physiological activities. *Exp Mol Med*. 2016 Apr 1;48:e224.
- 33 MacIver NJ, Blagih J, Saucillo DC, Tonelli L, Griss T, Rathmell JC, et al. The liver kinase B1 is a central regulator of T cell development, activation, and metabolism. *J Immunol*. 2011;187(8):4187–98.
- 34 Calixto MC, Lintomen L, André DM, Leiria LO, Ferreira D, Lellis-Santos C, et al. Metformin attenuates the exacerbation of the allergic eosinophilic inflammation in high fat-diet-induced obesity in mice. *PLoS One*. 2013;8(10):e76786.
- 35 Wang X, Liu Y, Yang Z, Zhang Z, Zhou W, Ye Z, et al. Glucose metabolism-related protein 1 (GMRP1) regulates pancreatic beta cell proliferation and apoptosis via activation of Akt signalling pathway in rats and mice. *Diabetologia*. 2011 04/01;54:852–63.
- 36 Hazeki K, Nigorikawa K, Hazeki O. Role of phosphoinositide 3-kinase in innate immunity. *Biol Pharm Bull*. 2007 Sep;30(9):1617–23.
- 37 Kao TC, Shyu MH, Yen GC. Glycyrrhizic acid and 18 β -glycyrrhetic acid inhibit inflammation via PI3K/Akt/GSK3 β signaling and glucocorticoid receptor activation. *J Agric Food Chem*. 2010;58(15):8623–9.
- 38 Lv H, Liu Q, Wen Z, Feng H, Deng X, Ci X. Xanthohumol ameliorates lipopolysaccharide (LPS)-induced acute lung injury via induction of AMPK/GSK3 β -Nrf2 signal axis. *Redox Biol*. 2017 Aug;12:311–24.