

Full Paper

Salidroside Attenuates Allergic Airway Inflammation Through Negative Regulation of Nuclear Factor-Kappa B and p38 Mitogen-Activated Protein KinaseGuang Hai Yan¹ and Yun Ho Choi^{2,*}¹Department of Anatomy and Histology and Embryology, Yanbian University School of Basic Medical Sciences, 977 Gongyuan Road, Yanji City 133002, Jilin, China²Department of Anatomy, Medical School, Institute for Medical Sciences, Chonbuk National University, Jeonju, Jeonbuk 561-180, Republic of Korea

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Abstract. Salidroside is a biologically active ingredient of *Rhodiola rosea*, which has several interesting biological properties, including anti-oxidant and anti-inflammatory; however, its anti-allergic effects are poorly understood. The objective of this study is to determine whether salidroside attenuates the inflammatory response in an ovalbumin (OVA)-induced asthma model. OVA-sensitized/challenged mice show airway hyperresponsiveness (AHR) to inhaled methacholine and have an increased amount of T-helper2 type cytokines [interleukin (IL)-4, IL-5, and IL-13] and eosinophils in their bronchoalveolar lavage fluids and lung tissues. However, three successive intraperitoneal administrations of salidroside before the last OVA challenge result in significant inhibition of these asthmatic reactions. Moreover, OVA significantly increases the activation of nuclear factor-kappa B (NF- κ B) and p38 mitogen-activated protein kinase (MAPK) in lung tissues, whereas salidroside markedly suppresses NF- κ B translocation and reduces phosphorylation of p38 MAPK. Furthermore, salidroside attenuates the expression of intercellular adhesion molecule 1 and IL-6 through modulating the activities of p38 MAPK and NF- κ B in the BEAS-2B cells stimulated by proinflammatory cytokines. These findings indicate that salidroside protects against OVA-induced airway inflammation and AHR, at least in part via downregulation of NF- κ B and p38 MAPK activities. Our data support the utility of salidroside as a potential medicine for the treatment of asthma.

Keywords: salidroside, asthma, airway inflammation, nuclear factor-kappa B (NF- κ B), p38

Introduction

Asthma is a chronic inflammatory disease characterized by antigen-induced airway hyperresponsiveness (AHR), leukocytes accumulation into inflamed sites, airway mucosal hyperplasia, and airway wall remodeling (1). These effects are attributed to T-helper2 (Th2) cells, together with other inflammatory factors, including B cells, mast cells, eosinophils, cytokines, and chemokines. In particular, interleukin (IL)-4, IL-5, and IL-13, which are produced by Th2 cells, are all related to AHR and

inflammatory changes in the airway through the activation of eosinophils (2). As the influx and differentiation of Th2 cells are important factors in the induction and aggravation of asthma, increasing attention has been paid to investigations that target the activation of Th2 cells to prevent and treat asthma.

Nuclear factor-kappa B (NF- κ B), which is the ubiquitous eukaryotic transcription factor that regulates gene expression of proinflammatory cytokines and enzymes, has a key role in inflammatory and immune responses, including asthma (3). Its increased activation has been demonstrated in the lungs after allergen challenge and in airway epithelial cells and macrophages of asthmatic patients (4). Choi et al. also reported that pretreatment of NF- κ B p65 antisense results in a significant inhibition

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of established asthmatic reaction in a murine model (5). Taken together, the development of a new strategy to inhibit lung specific NF- κ B activity might constitute an interesting topic in the management of asthma.

Mitogen-activated protein kinases (MAPKs), which are composed of serine and threonine kinases, play critical roles in the activation of inflammatory cells. These kinases are divided into three major subgroups: extracellular signal-related kinase 1/2, p38, and c-Jun N-terminal kinase 1/2 (6). Among them, p38 MAPK is markedly activated in the lungs of asthmatic mice compared with normal mice. Subsequently, blockade of p38 MAPK has been shown to have an anti-inflammatory effect in allergic asthma, suggesting that the inhibition or regulation of p38 MAPK could be a potential therapy for asthma (7).

Salidroside, the 8-O- β -D-glucoside of tyrosol, is the main bioactive component of *Rhodiola rosea* (8). It has been reported to have various pharmacological properties including hepatoprotective, anti-cancer, antioxidative, and anti-inflammatory effects (9–11). However, no available study has evaluated the effect of salidroside treatment on ovalbumin-induced airway inflammation in a murine model of asthma. Thus, the aim of the present study is to investigate whether salidroside would exert a suppressive effect on pulmonary inflammation and AHR using an experimental asthma model.

Materials and Methods

Ethics statement

All experiments were approved by the Institutional Animal Care and Use Committee of Yanbian University School of Medical Sciences and were in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication 82–23, revised 1996) as well as ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines, produced by the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs). The permit number was SCXK(JI)2013-0012. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Animals and experimental protocols

Specific 7-week-old pathogen-free (SPF) inbred female BALB/c mice were purchased from House section of Yanbian University Health Science Center (Yanji, China). Mice were immunized intraperitoneally with 10 μ g of ovalbumin (OVA; chicken egg albumin from Sigma, St. Louis, MO, USA) plus 1.0 mg of aluminum hydroxide adjuvant (Imject[®] Alum; Pierce, Rockford,

IL, USA). A booster injection of 10 μ g of OVA plus 1.0 mg aluminum hydroxide adjuvant was given 10 days later. From day 17 to day 19, the immunized mice were challenged by exposure to an aerosol of 1% OVA in phosphate-buffered saline (PBS) for 20 min. Each group consisted of seven animals. The saline-sensitized and challenged mice were used as controls. Salidroside [25 and 50 mg/kg body weight (BW), Sigma], dissolved in saline, was administered by intraperitoneal injection to each animal at 24 h intervals on days 21–23, beginning 1 h before each provocation. A p38 MAPK inhibitor, SB239063 (0.75 mg/kg BW, Sigma), or a NF- κ B inhibitor, BAY 11-7085 (20 mg/kg BW, Sigma), dissolved in sterile dimethyl sulfoxide (DMSO)-PBS, was injected intraperitoneally to each animal twice on day 21 and 23. In the present study, a vehicle (0.1% v/v DMSO) had no significant effect on key characteristics of allergic asthma (AHR, inflammation, goblet cell metaplasia, and IgE) and important mediators (Th2 cytokines, eotaxin, and adhesion molecules) in an experimental murine model induced by exposure to OVA (data not shown).

Assessment of airway hyperresponsiveness

Airway responsiveness was measured 2 days after the last OVA challenge. Conscious unrestrained mice were placed in a barometric plethysmographic chamber (All Medicus, Seoul, Korea), and baseline readings were taken and averaged for 3 min. Aerosolized methacholine in increasing concentrations (from 2.5 to 50 mg/ml) was then nebulized through an inlet of the main chamber for 3 min, and readings were taken and averaged for 3 min after each nebulization. The bronchopulmonary resistances are expressed as enhanced pauses (Penh), according to the manufacturer's protocol. The results are expressed as the percentage increase in Penh over the baseline, following challenge performed with each concentration of methacholine, where the baseline Penh (after saline challenge) is expressed as 100%.

Collection of bronchoalveolar lavage (BAL) fluid and differential cell count

Immediately following assessment of airway responsiveness, mice were anesthetized and the tracheas were cannulated while gently massaging the thorax. The lungs were lavaged with 0.7 ml of PBS. The BAL fluid samples were collected and the number of total cells in a 0.05 ml aliquot was counted using a hemocytometer (Baxter Diagnostics, Deerfield, IL, USA). The remaining samples were centrifuged, and the supernatants were stored at -70°C until needed for the assay of total and OVA-specific IgE, TNF- α , IL-1 β , IL-4, IL-5, IL-13, eotaxin, intercellular adhesion molecule 1 (ICAM-1), and vascular

cell adhesion molecule 1 (VCAM-1) levels. The cell pellets were resuspended in PBS and cytospin preparations of the BAL cells were stained with Diff-Quik solution (International Reagents, Kobe). The cell differentials were then enumerated based on the cell morphology and staining profile. The counting was done by an observer kept unaware of the experimental treatments.

Cytokine measurement

Total and OVA-specific IgE, TNF- α , IL-1 β , IL-4, IL-5, IL-13, eotaxin, ICAM-1, and VCAM-1 levels in BAL fluids were determined using mouse enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The lower limits of detection for the cytokines were as follows (pg/ml): TNF- α , 5.1; IL-1 β , 2.0; IL-4, 3.3; IL-5, 5.0; IL-13, 1.5; eotaxin, 3.0; ICAM-1, 0.017; VCAM-1, 20.

Histological examination of murine lung tissue

Lungs were fixed with 10% formalin, and the tissues were embedded in paraffin. Fixed tissues were cut at 4 μ m, placed on glass slides, and deparaffinized. Sections were stained with hematoxylin-eosin and periodic acid-Schiff (PAS) for light microscopic examinations. For immunohistochemistry, sections were subjected to immunostaining using the antibody against NF- κ B p65 (Cell Signaling Technology, Danvers, MA, USA).

Western blot analysis

Freshly isolated lung tissues were homogenized in the presence of protease inhibitors and protein concentrations were determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA). A 30 μ g sample of protein from the lung homogenates was loaded per lane on a 12% SDS-PAGE gel. Electrophoresis was then performed. The proteins were then transferred to nitrocellulose membranes. Western blot analysis was performed using the polyclonal antibodies against TNF- α , IL-1 β , PARP, β -actin (Santa Cruz Biochemicals, Santa Cruz, CA, USA), IL-4, IL-5, IL-6, IL-13, eotaxin, ICAM-1, VCAM-1, phosphorylated (p)-p38, p38 (R&D Systems), chloride channel calcium activated protein 3 (CLCA3), or Muc5AC (Abcam, Cambridge, MA, USA). The binding of all the antibodies was detected using an ECL detection system (iNtRON Biotechnology, Seoul, Korea), according to the manufacturer's instructions.

Cytosolic and nuclear protein extractions for analysis of NF- κ B

Cytosolic or nuclear extractions from harvested lung tissues were performed as described previously (12). For western blot analysis, samples were processed by the

procedure mentioned above. The NF- κ B activation was assayed using the antibody against NF- κ B p65, inhibitory kappa B-alpha (I κ B- α , R&D Systems), or p-I κ B- α (Santa Cruz).

Electrophoretic mobility shift assay (EMSA)

To inhibit endogenous protease activity, 1 mM PMSF was added to lung nuclear extracts. An oligonucleotide containing the κ -chain binding site (κ B, 5'-CCGGTTA ACAGAGGGGGCTT-TCCGAG-3') was used as an EMSA probe. The two complimentary strands were annealed and labeled with [α - ^{32} P]dCTP. Labeled probe (10,000 cpm), 10 μ g of nuclear extract, and binding buffer (10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly(dI-dC), 1 mM dithiothreitol) were then incubated for 30 min at room temperature in a final volume of 20 μ l. Reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5 $\times$ Tris-borate buffer. DNA-protein interactions were specific for NF- κ B as demonstrated by competition EMSA using a 50-fold excess of unlabeled oligonucleotide.

Cell culture

The human bronchial epithelial cell line BEAS-2B cells were purchased from American Type Culture Collection (Rockville, MD, USA). The cells were grown and maintained as previously described (13). We pretreated these cells for 6 h with salidroside (5 μ M), SB239063 (10 μ M), or BAY 11-7085 (5 μ M). For TNF- α /IL-4 stimulation, the medium was replaced, and the cells were cultured in medium containing 50 ng/ml each cytokine as mentioned for up to 24 h (14). None of the agents used significantly affected cell morphology or viability under these conditions (data not shown). Subsequently, cells were lysed and total proteins were extracted as described above.

Densitometric analysis and statistical analyses

All immunoreactive and phosphorylation signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad, Hercules, CA, USA). Data are expressed as the mean $\pm$ S.E.M. Statistical evaluation of the data was performed using ANOVA, followed by Dunnett's *post-hoc* test, employing Prism 5 software (GraphPad Software, San Diego, CA, USA). Results with $P < 0.05$ were considered statistically significant.

Results

Salidroside inhibits OVA-induced AHR, chemotaxis, and inflammatory reactions in experimental asthma

One functional consequence of the inflammatory

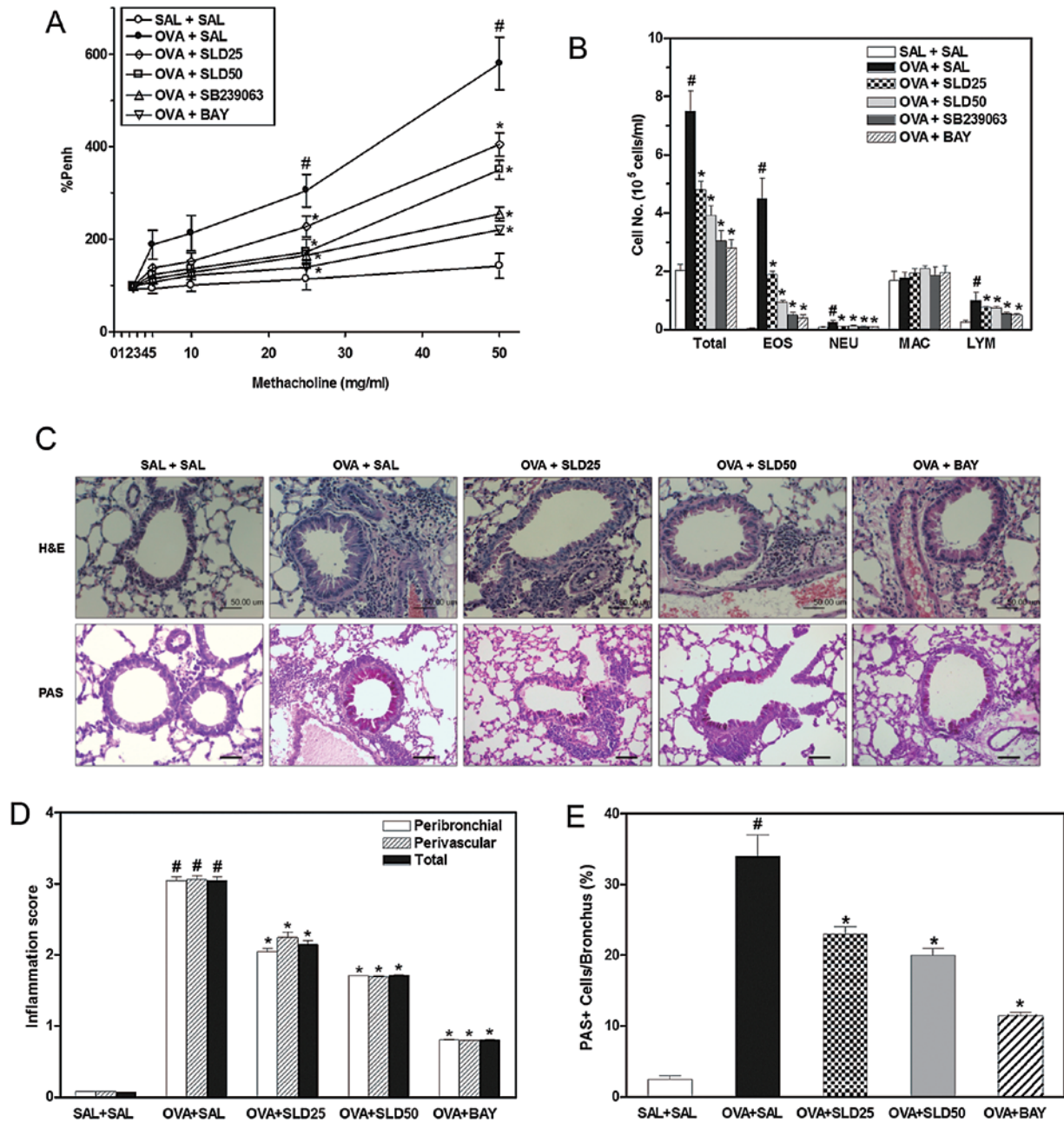


Fig. 1. Airway hyperresponsiveness, differential cell counts in BAL fluids, and histological evaluation of lung inflammation following OVA sensitization and treatment with solidoside. A) All animals were nebulized with various concentrations of methacholine as a bronchoconstrictor. Data are shown as the percentage increase in Penh over the baseline, where the baseline Penh of the saline-treated control group is expressed as 100%. B) The effect of solidoside, SB239063, or BAY 11-7085 on OVA-induced differential cell counts in BAL fluid were analyzed. EOS, eosinophil; NEU, neutrophil; MAC, macrophage; LYM, lymphocyte. C) Paraffin-embedded lung sections were stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS). Magnification 200 $\times$. Bars indicate 50 μ m. Data are representative of three independent experiments. D) Inflammation scores. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. E) Quantitation of airway mucus expression. Sampling was performed 48 h after the last OVA challenge in mice. Results are given as the mean $\pm$ S.E.M. # P < 0.05 vs. SAL + SAL, * P < 0.05 vs. OVA + SAL. Saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), solidoside 25 mg/kg (OVA + SLD25), solidoside 50 mg/kg (OVA + SLD50), SB239063 (OVA + SB239063), and BAY 11-7085 (OVA + BAY).

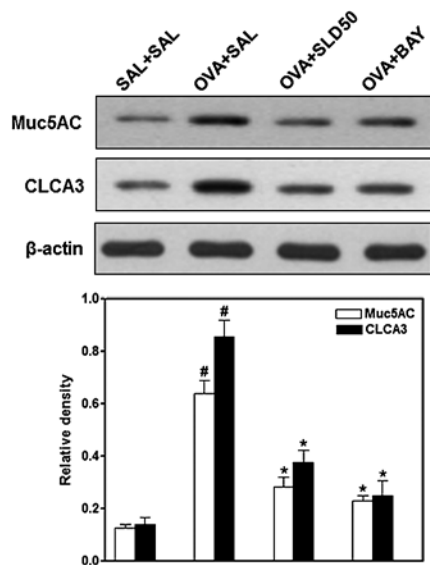


Fig. 2. Effect of salidroside on the protein expression of Muc5AC and CLCA3 in OVA-inhaled mice. Protein expressions of Muc5AC and CLCA3 in lung tissues were evaluated 48 h after the last OVA challenge. Density ratio vs. β -actin was measured using a densitometer. Results are given as the mean $\pm$ S.E.M. [#] $P < 0.05$ vs. SAL + SAL, ^{*} $P < 0.05$ vs. OVA + SAL. Saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), salidroside 50 mg/kg (OVA + SLD50), and BAY 11-7085 (OVA + BAY).

process that underlies asthma is an AHR. AHR was determined by Penh and was substantially increased in OVA-challenged mice in response to methacholine (Mch) inhalation as compared with control mice. Salidroside, SB239063, or BAY 11-7085 dramatically prevented AHR to inhaled Mch, as shown in Fig. 1A, suggesting that immune-mediated pathology *in vivo* was modified. Next, to examine the effect of salidroside on chemotaxis, that is, recruitment of inflammatory cells into airway, inflammatory cells were counted in BAL fluids. We analyzed the cellular composition of the BAL fluids of mice 48 h after the last OVA challenge. In the saline-treated mice, OVA challenge resulted in a marked increase of eosinophils and slight increases of neutrophils and lymphocytes when compared with control mice (Fig. 1B). However, pretreatment of salidroside, SB239063, or BAY 11-7085 significantly attenuated OVA-induced recruitment of eosinophils ($P < 0.05$). The observed reduction in chemotaxis into the airway correlated with the histological changes of lung parenchyma. Lungs from OVA-challenged mice showed widespread perivascular and peribronchiolar inflammatory cell infiltrates (Fig. 1: C and D). Moreover, the percentage of mucus-producing goblet cells (indicated by PAS staining) in OVA-inhaled mice was

substantially greater than that in control mice (Fig. 1: C and E). However, administration of salidroside led to a significant reduction of inflammatory cell infiltration and goblet cell hyperplasia. To further assess the goblet cell response in asthmatic mice, the levels of Muc5AC and CLCA3, which are the markers of goblet cell metaplasia, were measured by western blot analysis. The protein expression of Muc5AC and CLCA3 was substantially increased in OVA-inhaled mice compared with saline-inhaled mice. In line with histological findings, salidroside and BAY 11-7085 markedly reduced the expression of these proteins in OVA-challenged mice (Fig. 2). On the other hand, pretreatment with salidroside (25 and 50 mg/kg BW) alone in the control mice did not induce airway inflammation or lung damage (data not shown). Taken together, the present results indicate that salidroside efficiently attenuates allergic airway inflammation and mucus hypersecretion in OVA-induced asthmatic mice.

Salidroside attenuates the release of total and OVA-specific IgE into BAL fluids of OVA-inhaled mice

Total and OVA-specific IgE levels were determined by ELISA in each experimental group. IgE levels in BAL fluids were dramatically elevated in OVA-challenged mice, compared with control mice. However, the administration of salidroside or BAY 11-7085 to OVA-inhaled mice led to a significant reduction in the total and OVA-specific IgE levels (Fig. 3A).

Salidroside reduces the levels of cytokines involved in the pathophysiology of asthma in OVA-challenged mice

Allergic asthmatic inflammation is known to be caused by the secretion of a series of proinflammatory (TNF- α and IL-1 β) and Th2 cytokines (IL-4, IL-5, and IL-13) (15). To assess the effect of salidroside on pulmonary inflammation in asthmatic mice, the levels of these cytokines in lung tissues as well as BAL fluids were measured. First, ELISA showed that the levels of TNF- α , IL-1 β , IL-4, IL-5, and IL-13 in BAL fluids were significantly increased in OVA-challenged mice compared with the levels in control mice (Fig. 3B). The increased levels of these cytokines were significantly decreased by the administration of salidroside or BAY 11-7085. Consistent with these results, western blot analysis revealed that protein expressions of TNF- α , IL-1 β , IL-4, IL-5, and IL-13 in lung tissues were significantly up-regulated in allergic mice compared with those in control mice (Fig. 3: C and D). The elevated levels of these cytokines after OVA challenge were significantly reduced by salidroside or BAY 11-7085. Moreover, given that chemokines and leukocyte-endothelial adhesion molecules are important in the recruitment and

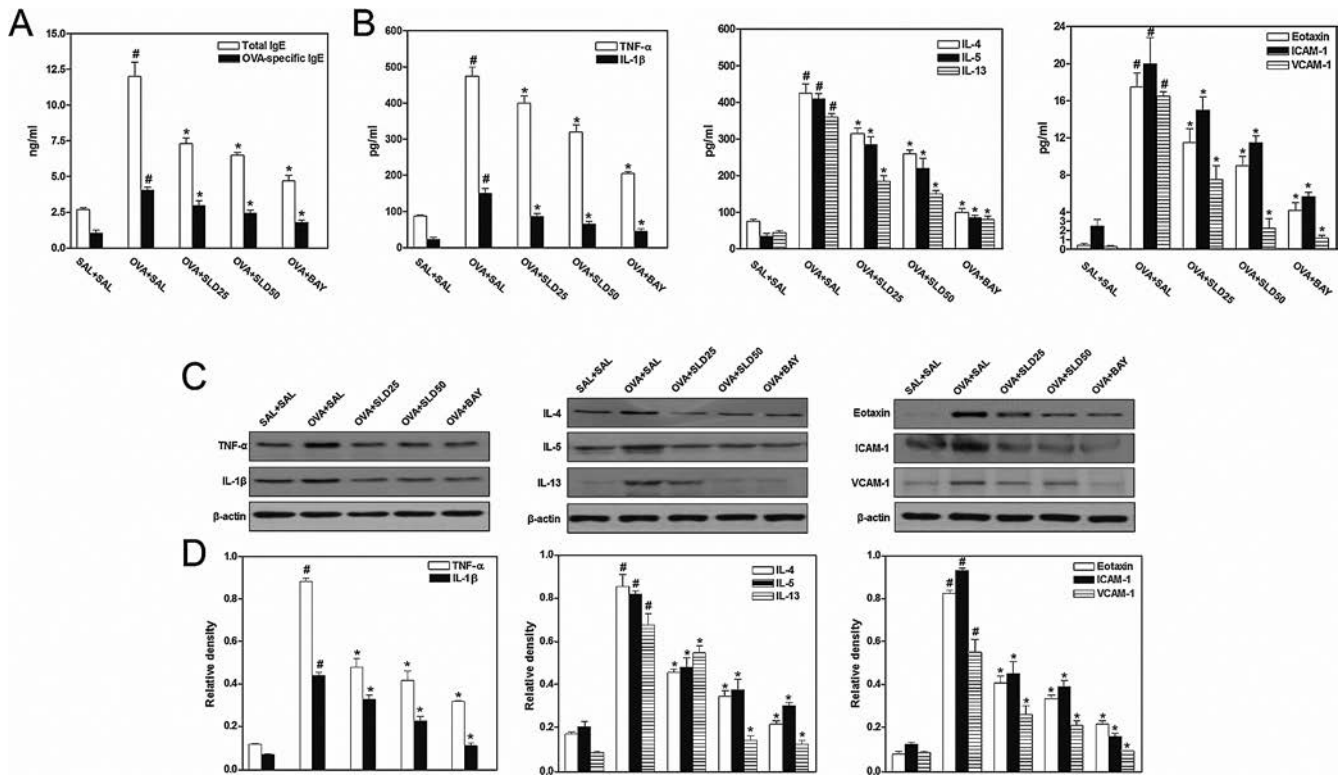


Fig. 3. Assessment of IgE, pro-inflammatory and Th2 cytokines, and adhesion molecules in BAL fluids and lung tissues of OVA-sensitized mice treated with solidoside. A, B) The levels of IgE and various cytokines were quantified by ELISAs. C, D) Protein expressions of these cytokines in lung tissues were also measured by western blotting. Density ratio vs. β -actin was measured using a densitometer. A representative immunoblot of three independent experiments is shown. Sampling was performed 48 h after the last OVA challenge in mice. Results are given as the mean $\pm$ S.E.M. [#] $P < 0.05$ vs. SAL + SAL, ^{*} $P < 0.05$ vs. OVA + SAL. Saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), solidoside 25 mg/kg (OVA + SLD25), solidoside 50 mg/kg (OVA + SLD50), and BAY 11-7085 (OVA + BAY).

migration of leukocytes to the sites of inflammation (16), the levels of eotaxin, ICAM-1, and VCAM-1 were measured. Changes in the levels of these cytokines were similar to those seen for the aforementioned cytokines, indicating that the OVA challenge-induced increase in cytokine levels can be reversed by solidoside or BAY 11-7085.

Solidoside suppresses the nuclear translocation of NF- κ B and phosphorylation of I κ B- α in lung tissues of allergic mice

In view of the present data that AHR and eosinophilic inflammation in asthmatic mice is blocked by BAY 11-7085, a specific NF- κ B inhibitor, as well as the knowledge that NF- κ B plays a key role in allergic inflammation of the lung by inducing the transcription of various proinflammatory mediators (3), we hypothesized that solidoside would attenuate airway inflammatory reactions by suppressing NF- κ B activation. To address this issue, we first studied the nuclear translocation and

DNA binding activity of the NF- κ B p65 subunit in lung tissues after OVA challenge. There was a decrease and increase of NF- κ B p65 levels in the cytosols and nuclei from OVA-challenged lungs, respectively (Fig. 4A). Moreover, EMSA showed an increase in the binding activity of lung nuclear extracts to NF- κ B consensus sequence in OVA-exposed mice (Fig. 4B) compared with control mice. Specificity of the DNA-protein interactions for NF- κ B was demonstrated by competition assays using a 50-fold excess of unlabeled oligonucleotide (Fig. 4B, lane 6). Furthermore, immunostaining of p65 subunit in lung tissues confirmed the nuclear translocation of p65 subunit (Fig. 4C). In contrast, cytosolic and nuclear extracts from solidoside or SB239063-treated mice showed the suppression of nuclear translocation and NF- κ B binding. Next, the effects of solidoside on OVA-induced phosphorylation and degradation of I κ B- α were evaluated to clarify the molecular mechanisms by which solidoside inhibits NF- κ B activity. Solidoside significantly reduced the OVA-induced

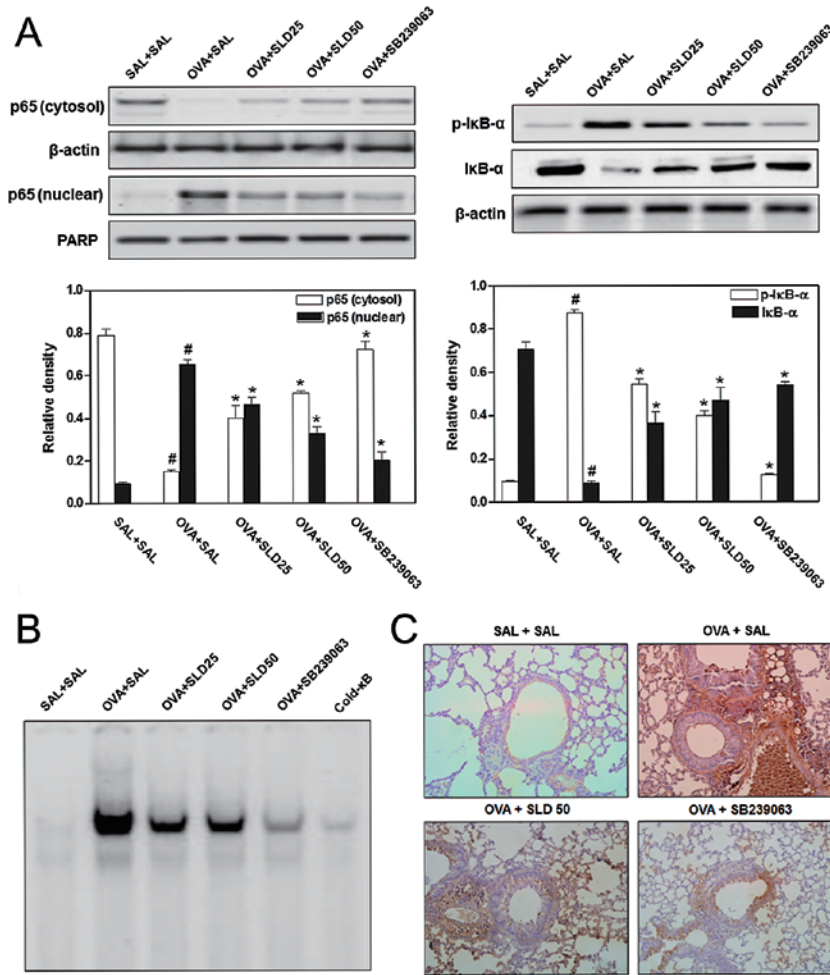


Fig. 4. Effect of salidroside on OVA-induced NF-κB activation. The translocation of p65 to the nucleus as well as IκB-α phosphorylation and degradation in cytoplasm (A) and NF-κB DNA binding activity (B) were assessed by western blotting and EMSA, respectively. Density ratio vs. β-actin was measured using a densitometer. C) Nuclear translocation of the NF-κB p65 subunit was examined by immunohistochemistry staining. Representative images are shown from three independent experiments. Results are given as the mean ± S.E.M. #P < 0.05 vs. SAL + SAL, *P < 0.05 vs. OVA + SAL. Saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), salidroside 25 mg/kg (OVA + SLD25), salidroside 50 mg/kg (OVA + SLD50), and SB239063 (OVA + SB239063).

phosphorylation and degradation of IκB-α in the cytosol from lung tissues, as did SB239063. Taken together, these findings indicate that salidroside represses NF-κB transcriptional activity possibly by stabilizing IκB-α and impairing the nuclear transport of p65 subunit in lung tissues from OVA-challenged mice.

Salidroside blocks OVA-induced phosphorylation of p38 MAPK in asthmatic mice

To investigate whether the inhibition of asthmatic response by salidroside is mediated through p38 MAPK, phosphorylation of p38 MAPK was examined in OVA-challenged mice pretreated with salidroside or SB239063, a p38 MAPK inhibitor. As shown in Fig. 5, salidroside or SB239063 effectively inhibited OVA-induced phosphorylation of p38 MAPK in allergic mice. On the other hand, the expression of p38 was unaffected by OVA, salidroside, or SB239063.

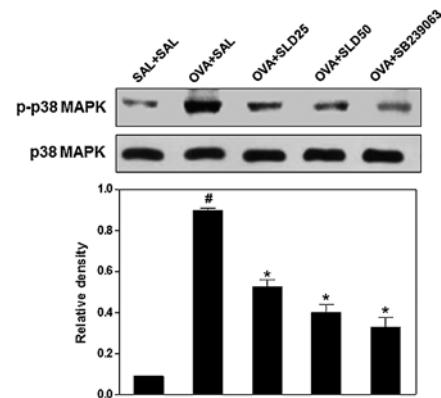


Fig. 5. Effect of salidroside on OVA-induced p38 MAPK activation. Protein expressions of phosphorylated (p)-p38 and p38 in lung tissues were evaluated 48 h after the last OVA challenge. Density ratio vs. β-actin was measured using a densitometer. Results are given as the mean ± S.E.M. #P < 0.05 vs. SAL + SAL, *P < 0.05 vs. OVA + SAL. Saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), salidroside 25 mg/kg (OVA + SLD25), salidroside 50 mg/kg (OVA + SLD50), and SB239063 (OVA + SB239063).

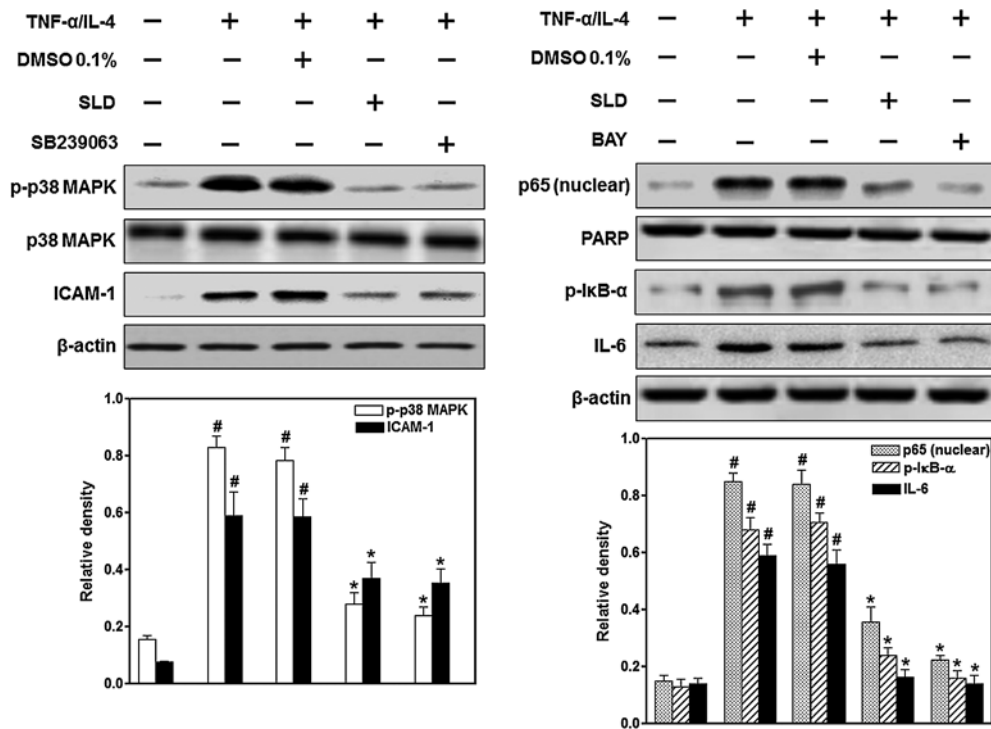


Fig. 6. Effect of solidoside on expression of p38 MAPK and other signaling intermediates in TNF- α /IL-4-treated BEAS-2B cells. Western blotting of the levels of phosphorylated (p-) p38 MAPK, ICAM-1, and other signaling intermediates in TNF- α /IL-4-treated BEAS-2B cells. BEAS-2B cells were pretreated with solidoside (SLD, 5 μ M), SB239063, or BAY 11-7085 for 6 h prior to challenge with TNF- α /IL-4. Representative images with respective densitometry data are shown. Density ratio vs. β -actin was measured using a densitometer. Results are given as the mean $\pm$ S.E.M. # P < 0.05 vs. the untreated group, * P < 0.05 vs. the group treated by TNF- α /IL-4.

Solidoside abrogates TNF- α /IL-4-induced expression of ICAM-1 and IL-6 in BEAS-2B cells

To further confirm that solidoside directly regulates the activity of p38 MAPK and NF- κ B in vitro, Western blot analyses of the expression of p-p38 MAPK, p38 MAPK, ICAM-1, nuclear p65 subunit, p-I κ B- α , and IL-6 in BEAS-2B cells were performed. According to our results, the levels of p-p38 MAPK, ICAM-1, nuclear p65 subunit, p-I κ B- α , and IL-6 were remarkably increased at 24 h in BEAS-2B cells treated by TNF- α /IL-4 (Fig. 6). The elevated levels of these proteins were significantly decreased by solidoside.

Discussion

Allergic airway inflammation, characterized by increased infiltration of leukocytes, such as eosinophils, and considerable secretion of mucus into the airways, is a major factor in the pathogenesis of asthma. Especially, the eosinophil has long been recognized as the principal effector cell and plays pathogenic roles in asthma by its release of cytotoxic granule proteins (17). Our present findings show that solidoside prevents eosinophilic

infiltration into the airways, as evidenced by a significant drop in total cell counts and eosinophil counts in BAL fluid. Likewise, tissue eosinophilia is also inhibited, as revealed by a marked reduction of inflammatory cell infiltration in histological examination. Eosinophilic transmigration into the airways is a multistep process that is orchestrated by not only Th2 cytokines such as IL-4, IL-5, and IL-13 but also proinflammatory cytokines including TNF- α , IL-1 β , and IL-6, and coordinated by chemotactic cytokine (eotaxin) in combination with adhesion molecules, including ICAM-1 and VCAM-1 (18, 19). IL-4 is required for B cell maturation and IgE synthesis and participates in the initiation of Th2 inflammatory responses. IL-5 is pivotal for growth, differentiation, recruitment, and survival of eosinophils. IL-13 can potentially induce mucus hypersecretion, eotaxin expression, airway inflammation, and AHR (20, 21). TNF- α , IL-1 β , and IL-6 also exert similar responses, which include upregulation of eosinophil chemoattractants and adhesion molecules, recruitment of eosinophils, increase of cytokine release, and enhancement of AHR (15). According to the current study, solidoside attenuates the increased release of Th2 and proinflammatory cyto-

kines, eotaxin, and adhesion molecules into the airway of OVA-challenged mice. From these findings, we speculate that salidroside can prevent eosinophilic airway inflammation by diminishing secretion of aforementioned cytokines into lungs.

AHR is a hallmark clinical symptom of asthma, which is defined as the abnormal increase in airflow limitation in response to a provoking stimulus. Although there are less data surrounding the precise mechanisms whereby airway inflammation enhances AHR, it is convincing that various mediators released during allergic inflammation play a critical role in AHR development (22). For example, it has been established that IL-5 plays a crucial role in AHR by mobilizing and activating eosinophils, leading to the release of proinflammatory products such as major basic protein and cysteinyl-leukotrienes, which are closely associated with AHR (23). Similarly, IL-4 and IL-13 have been shown to induce AHR in murine asthma models in which cysteinyl-leukotrienes might be causative agents of AHR (24). Moreover, AHR could be brought about by a direct effect of TNF- α on airway smooth muscle (25). Furthermore, IgE-mediated mast cell activation may contribute to AHR by producing a wide array of inflammatory mediators and cytokines (26), which extends the potential importance of our unpublished results that salidroside inhibits IgE-induced mast cell degranulation (data not shown) in the suggested mechanisms of AHR. As such, the observed reduction of AHR by salidroside may be related to decrease in Th2 cytokine production, tissue eosinophilia, TNF- α levels, and mast cell degranulation by salidroside.

The transcription factor NF- κ B regulates a wide variety of target genes that encode multiple inflammatory cytokines, such as TNF- α , IL-1 β , IL-6, IL-4, IL-5, IL-13, ICAM-1, and VCAM-1, all of which are closely implicated in the pathogenesis of asthma, as mentioned above (3). Our results indicate that salidroside exerts anti-NF- κ B actions in lung tissues of OVA-challenged mice. Moreover, suppression of NF- κ B activity by BAY 11-7085, a specific inhibitor of NF- κ B, has been shown to induce not only a reduction in the levels of the aforementioned cytokines, but also amelioration in eosinophilic airway inflammation and AHR in our model, which is in line with the previous report (27). Taken together, it is proposed that the mechanism underlying the anti-asthmatic effects of salidroside may be attributed to inhibition of NF- κ B transcriptional activity and to subsequent reduction of proinflammatory chemical mediators.

As described previously, p38 MAPK has been considered to play a cardinal role in allergic asthma (7). Supporting this contention, our study has shown that the augmentation in asthmatic symptoms as well as the

phosphorylation of p38 MAPK after OVA inhalation is significantly reduced after the administration of SB239063, a selective p38 MAPK inhibitor. Moreover, SB239063 prominently represses NF- κ B activity in OVA-challenged lungs, suggesting that p38 MAPK acts upstream of the NF- κ B signaling pathway in allergic airway disease. Likewise, salidroside dramatically decreases the activity of p38 MAPK and NF- κ B in lung tissues of OVA-challenged mice. Accordingly, these observations encourage the view that treatment of allergic mice with salidroside leads to the suppression of p38 MAPK and the subsequent disruption of NF- κ B activity, reversing pathophysiologic features of asthma.

Subsequently, to further assess whether salidroside directly modulates the activities of p38 MAPK and NF- κ B in allergic response, an *in vitro* experiment was performed using BEAS-2B cells. According to our data, TNF- α /IL-4 stimulation facilitates the nuclear accumulation of p65 as well as the phosphorylation of p38 MAPK and I κ B- α and increases the levels of ICAM-1 and IL-6 in BEAS-2B cells. However, SB239063 represses the expression of ICAM-1 by attenuating the phosphorylation of p38 MAPK in these cells incubated by TNF- α /IL-4. Moreover, BAY 11-7085 has been revealed to inhibit IL-6 production, which is mediated by the suppression of the NF- κ B signaling pathway. Likewise, our *in vitro* system has demonstrated that salidroside reduces the amounts of ICAM-1 and IL-6 by directly blocking p38 MAPK phosphorylation and NF- κ B transcriptional activity, respectively.

In conclusion, our data indicate that salidroside could ameliorate asthmatic inflammation and AHR by downregulating proinflammatory and Th2 cytokines via inhibiting the p38 MAPK-NF- κ B module. These findings suggest that salidroside is a potential anti-inflammatory agent in asthma treatment.

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Conflicts of Interest

The authors indicated no potential conflicts of interest.

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