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Sulfuretin attenuates allergic airway inflammation in mice

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ABSTRACT

Sulfuretin is one of the main flavonoids produced by *Rhus verniciflua*, which is reported to inhibit the inflammatory response by suppressing the NF-κB pathway. Because NF-κB activation plays a pivotal role in the pathogenesis of allergic airway inflammation, we here examined the effect of sulfuretin on an ovalbumin-induced airway inflammation model in mice. We isolated sulfuretin from *R. verniciflua*. Sulfuretin was delivered intraperitoneally after the last ovalbumin challenge. Airway hyper-responsiveness, cytokines, mucin, and eosinophilic infiltration were analyzed in bronchoalveolar lavage fluid and lung tissue. A single administration of sulfuretin reduced airway inflammatory cell recruitment and peribronchiolar inflammation and suppressed the production of various cytokines in bronchoalveolar fluid. In addition, sulfuretin suppressed mucin production and prevented the development of airway hyper-responsiveness. The protective effect of sulfuretin was mediated by the inhibition of the NF-κB signaling pathway. Our results suggest that sulfuretin may have therapeutic potential for the treatment of allergic airway inflammation.

1. Introduction

Bronchial asthma is a chronic inflammatory disease characterized by airway obstruction in response to allergens, chronic eosinophilic airway inflammation, mucin hypersecretion, and non-specific airway hyper-responsiveness (AHR) [1]. Evidence reveals that these inflammatory responses are mediated by T-helper type 2 (Th2) cells, mast cells, B cells, and eosinophils [2,3]. Upon challenge with various allergens, these inflammatory cells infiltrate into the airway and produce Th2 cytokines, such as IL-4, IL-5, and IL-13 [2,3]. Therefore, targeted therapies have been directed toward preventing Th2 responses.

Nuclear factor-κB (NF-κB) plays a pivotal role for the production of Th2 cytokines and recruitment of inflammatory cells in the airways of murine asthma models [4,5]. Increased NF-κB activity has been identified in airway samples from asthma patients [6]. Mice that lack the p50 subunit of NF-κB are unable to mount airway eosinophilic inflammation and Th2 cytokines production [7]. Anti-inflammatory properties of corticosteroids are thought to be mediated by suppression of NF-κB [8]. We also recently demonstrated that adenoviral gene transfer of A20 prevents allergic airway inflammation through suppressing NF-κB activity [5]. Taken together, these studies support an importance of NF-κB activation in the development of asthma.

Sulfuretin is a major flavonoid isolated from the heartwood of *Rhus verniciflua*, which has been used to reduce oxidative stress [9], platelet aggregation [10], and mutagenesis [11]. Our recent study has shown that sulfuretin inhibits the inflammatory responses by suppressing the NF-κB pathway in type 1 diabetes models [12]. However, there is no report about the therapeutic efficacy of sulfuretin or *R. verniciflua* in the treatment of allergic airway inflammation. Due to the critical role of the NF-κB pathway in allergic airway inflammation, we isolated sulfuretin from *R. verniciflua* and examined the effectiveness of sulfuretin for reducing airway inflammatory reactions and improving asthma symptoms in an ovalbumin (OVA)-induced airway inflammation model.

2. Materials and methods

2.1. Animals and materials

Pathogen-free male BALB/c mice were obtained from Samtaco Inc. (Osan, Korea), housed in a laminar flow cabinet, and maintained on standard laboratory chow *ad libitum*. Mice were 7–8 weeks old at

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the start of each experiment. All experimental animals used in this study were maintained under the protocol approved by the Institutional Animal Care and Use Committee at Chonbuk National University. Sulfuretin was prepared and identified as previously described [12].

2.2. Immunization, challenge, and sulfuretin delivery in lung tissue

Mice were immunized intraperitoneally with 20 μg of OVA plus 2.25 mg aluminum hydroxide adjuvant on day 0 and OVA alone without alum on day 14. The immunized mice were exposed to aerosolized OVA on days 28 and 35. Aerosolization of OVA was performed using a chamber that was adapted for mice. Animals were exposed to OVA (1.5%) using an ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan; output 0.8 ml/min) for 20 min in a Plexiglas exposure chamber (24.5 × 40.5 × 15.0 cm). Control animals received the same immunization, but were exposed to aerosolized saline instead of OVA during airway challenge. A single intraperitoneal injection of sulfuretin (40 μg/kg in saline) was administered 2 h after the last OVA challenge.

2.3. Bronchoalveolar lavage (BAL)

BAL was performed at 36 h after the last OVA challenge. Mice were anesthetized, and the trachea was cannulated while gently massaging the thorax. Lungs were lavaged with 0.7 ml PBS. The BAL fluid samples were collected and the number of cells per 100 μl aliquot was determined using a hemocytometer. The remaining sample was centrifuged, and the supernatant was stored at −70 °C until cytokine assays were performed. The pellet was resuspended in PBS, and a cytospin preparation of BAL cells was stained with Diff-Quik (International reagents Corp., Kobe, Japan). The different cell types were enumerated based on their morphology and staining profile.

2.4. Determination of AHR

AHR was assessed as a change in airway function after challenge with aerosolized methacholine via the airway. Mice were connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, Canada). Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each mouse was challenged with increasing concentrations of methacholine (2.5–50 mg/ml in saline) in an aerosol form. The data needed to calculate R_L was collected continuously following each methacholine challenge. Maximum R_L values were selected to express changes in airway function, which was represented as the percent change from baseline after saline aerosol treatment.

2.5. Cytokine assays

Tumor necrosis factor-α, IL-5, IL-13, and eotaxin levels in BAL were determined by ELISA (R&D Systems, Minneapolis, MN, USA). The lower limits of detection for the cytokines were as follows: TNF-α (>5.1 pg/ml), IL-5 (>5 pg/ml), IL-13 (>1.5 pg/ml), and eotaxin (>3 pg/ml).

2.6. EMSA

Nuclear extracts were prepared from the lung tissues. To inhibit endogenous protease activity, 1 mM PMSF was added. An oligonucleotide containing the κ-chain binding site (κB, 5′-CCGGTTAACAGA GGGGGCTTTCCGAG-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 μg 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 μl. Reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5 × Tris–borate buffer. DNA–protein interactions were specific for NF-κB as demonstrated by competition EMSA using a 50-fold excess of unlabeled oligonucleotide.

2.7. Western blot analysis

Lung tissues were homogenized with protease and phosphatase inhibitors and prepared in protein extraction solution (PRO-PREP, iNtRON, Sungnam, Korea). The homogenates, which contained 30 μg of protein, were separated by 10% SDS–PAGE and transferred to nitrocellulose sheets. The blot was probed with 1 μg/ml of primary antibodies for p50, p65, IκBα, proliferating cell nuclear antigen (PCNA), or β-actin (Santa Cruz Biochemicals, Santa Cruz, CA, USA). Alkaline phosphatase-conjugated anti-rabbit IgG (Santa Cruz Biochemicals) was used as a secondary antibody.

2.8. Histological studies and mucin analysis

Lungs were fixed with 10% formalin, and the tissues were embedded in paraffin. Fixed tissues were cut at 4 μm, placed on glass slides, and deparaffinized. Sections were stained with H&E for light microscopic examinations. For the detection of mucin, tissues were stained with Periodic Acid-Schiff. For quantification of mucin levels, BAL fluid was collected, and cells were removed by centrifugation. Lung mucin levels were measured using the mucin-binding lectin, jacalin (Calbiochem, San Diego, CA).

2.9. Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons were performed using one-way ANOVA, followed by the Fisher test. A value of $p < 0.05$ was accepted as an indication of statistical significance.

3. Results

3.1. Sulfuretin suppressed OVA-induced chemotaxis and airway inflammation

Airway inflammation was induced in BALB/c mice by intraperitoneal administration of OVA on days 0 and 14, followed by challenges with aerosolized OVA on days 28 and 35. As a negative control, mice were treated with saline. To determine the therapeutic effect of sulfuretin, mice received a single intraperitoneal injection of sulfuretin at a dose of 40 μg/kg 2 h after the last OVA challenge.

To examine the effect of sulfuretin on chemotaxis, that is, recruitment of inflammatory cells into the airway, total and differential cell counts were performed in BAL fluid. In the saline-treated mice, OVA challenge resulted in a marked increase of eosinophils and a slight increase of lymphocytes when compared to control mice (Fig. 1A). However, treatment with sulfuretin significantly attenuated the OVA challenge-induced increases ($p < 0.01$). The observed reduction in chemotaxis into the airway was well-correlated with the histological changes of lung parenchyma. Lungs from OVA-challenged mice treated with saline showed widespread perivascular and peribronchiolar inflammatory cell infiltrates (Fig. 1B). The majority of the infiltrated inflammatory cells were eosinophils. However, treatment with sulfuretin resulted in a significant reduction of inflammatory cell infiltration. These results indicate that treatment with sulfuretin efficiently

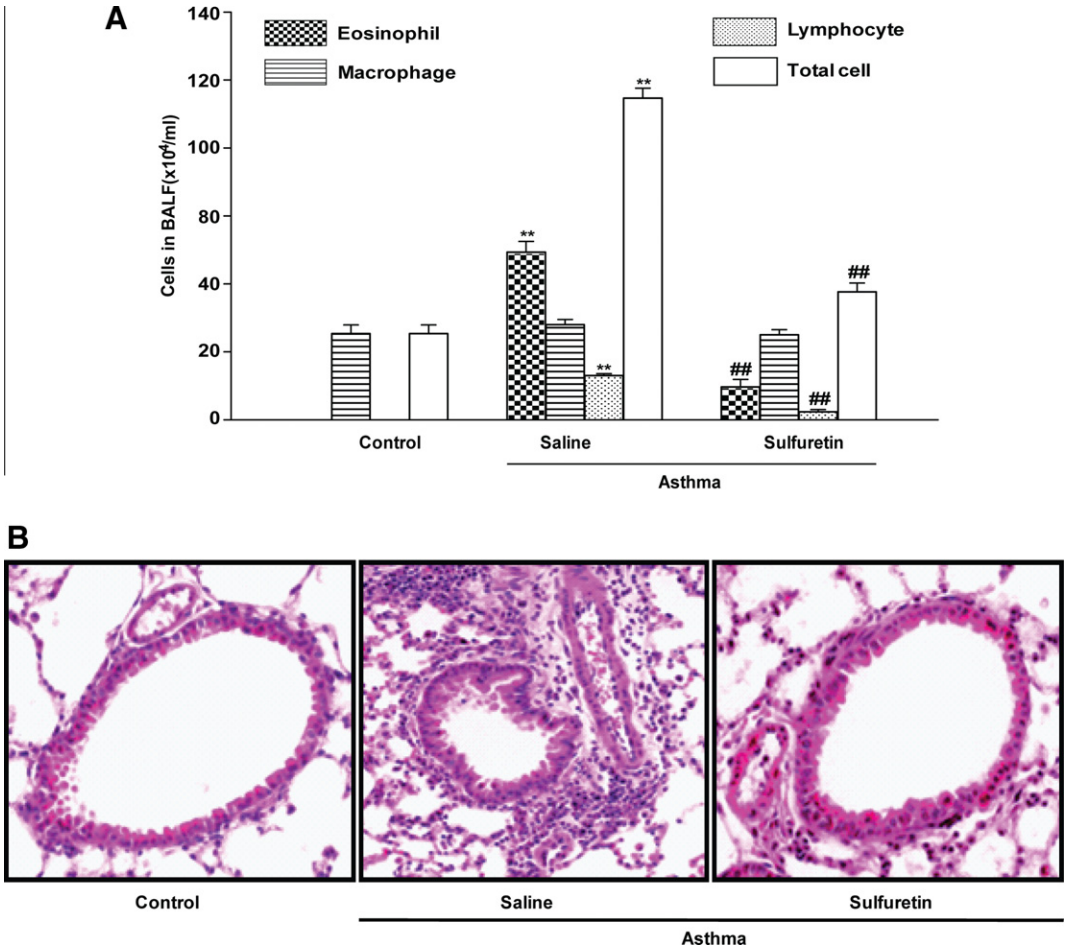

Fig. 1. Differential cell counts in BAL fluid and histological evaluation of lung inflammation following OVA challenge and treatment with sulfuretin. (A) Mice were injected intraperitoneally with sulfuretin 2 h after the last OVA challenge. The effect of sulfuretin on the OVA challenge-induced differential cell counts in BAL fluid was analyzed 36 h after the last OVA challenge. Results from three independent experiments with 5–7 mice/group are given as mean ± SEM. $^{**}p < 0.01$ vs. untreated control; $^{\#\#}p < 0.01$ vs. saline-treated group. (B) Paraffin-embedded lung sections were prepared 36 h after the last OVA challenge and stained with H&E (100×). Data are representative of three independent experiments.

inhibits the infiltration of inflammatory cells and attenuates allergic airway inflammation.

3.2. Sulfuretin decreased NF-κB activation

Based on the knowledge that NF-κB plays a key role in allergic inflammation of the lung by inducing the transcription of various pro-inflammatory mediators [13], we hypothesized that sulfuretin would attenuate airway inflammatory reactions by suppressing NF-κB activation. To address this issue, we first studied the nuclear translocation and DNA binding activity of NF-κB in lung tissues after OVA challenge. There was an increase in the levels of the p50 and p65 subunits of NF-κB in the nuclei from lung tissues of OVA-challenged mice (Fig. 2A), as well as an increase in the binding activity of lung nuclear extracts to a NF-κB consensus sequence (Fig. 2B), as compared to control mice. Immunostaining of p50 and p65 subunits in lung tissues also confirmed the nuclear translocation of p50 and p65 subunits (Fig. 2C). However, nuclear extracts prepared from sulfuretin-treated mice showed suppressed nuclear translocation and NF-κB DNA binding. We also examined the alteration of IκBα levels in the cytosol fraction. Lung tissues from OVA-challenged mice showed a decreased level of IκBα protein in the cytosol, when compared to a similar fraction of control lung, but the increased IκBα degradation, as a result of the OVA challenge, was markedly suppressed by sulfuretin treatment (Fig. 2A).

3.3. Sulfuretin reduced the levels of cytokines involved in the pathophysiology of airway inflammation in BAL fluid

The levels of secreted cytokines in BAL fluid were assessed. OVA challenge resulted in a 14.8-fold increase in TNF-α. Treatment with sulfuretin significantly diminished OVA challenge-mediated TNF-α production (Fig. 3A). OVA challenge resulted in 11.4- and 4.1-fold increases in the levels of IL-5 and IL-13 in BAL fluid, respectively (Fig. 3B and C). However, sulfuretin significantly diminished the OVA challenge-mediated increase in IL-5 and IL-13 by 63.9% and 62.6%, respectively. In addition, chemotactic cytokine for the recruitment of eosinophils (eotaxin) was assayed. Eotaxin level in the BAL fluid of mice treated with sulfuretin was significantly lower than that in the saline group (Fig. 3D). This result was consistent with the reduced number of eosinophils in the BAL fluid of sulfuretin-injected mice (Fig. 1).

3.4. Sulfuretin attenuated excessive production of mucin in the airway

Bronchiolar mucin accumulation, an additional hallmark most commonly associated with the chronic airway inflammatory response, can contribute to airway obstruction, dyspnea, and cough in asthma patients [14]. We, therefore, evaluated whether sulfuretin affects mucin production from bronchial goblet cells. Minimal intrabronchial mucin was observed in control mice,

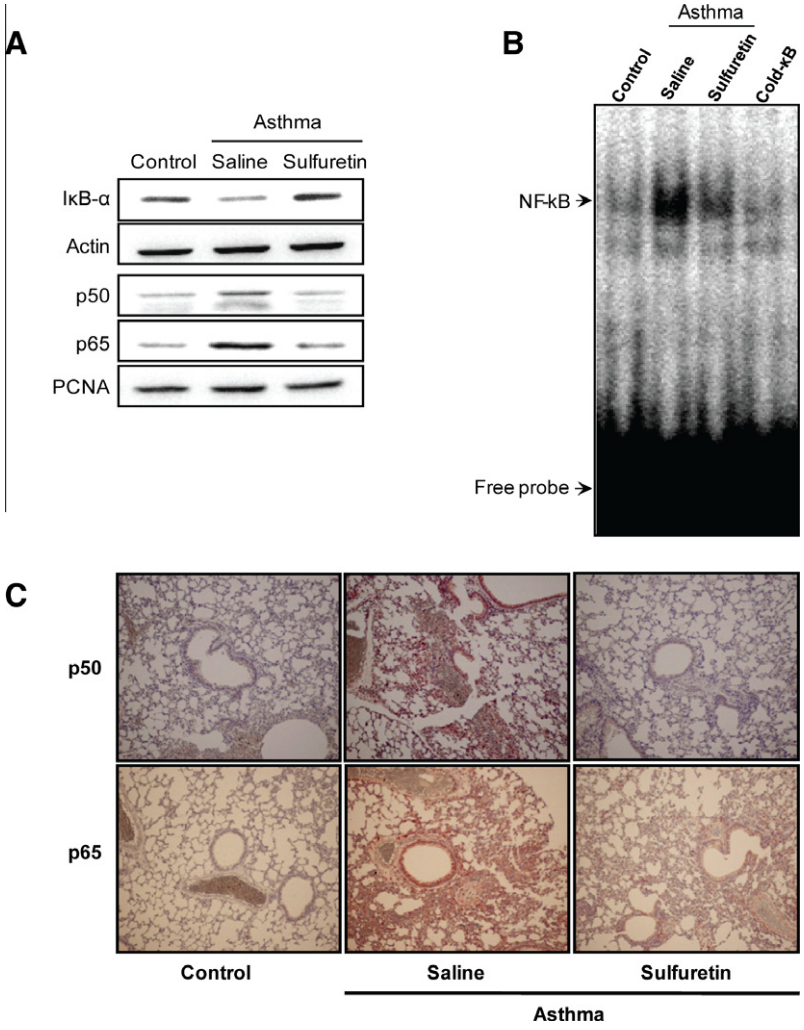

Fig. 2. Effect of sulfuretin on OVA challenge-induced NF-κB activation. Mice were injected intraperitoneally with sulfuretin 3 h after the last OVA challenge. Lung homogenates were prepared 2 h after the last OVA challenge. The translocation of p65 and p50 to the nucleus, and IκBα degradation in cytoplasm (A) and NF-κB DNA binding activity (B) were assessed by Western blotting and EMSA, respectively. Actin and PCNA were used as loading controls for cytosolic and nuclear proteins, respectively. (C) Nuclear translocation of the p65 and p50 subunits of NF-κB was examined by immunohistochemistry staining. Similar results were obtained from three independent experiments.

whereas in OVA-challenged mice, there was excessive mucin content that caused partial or complete obstruction of airways. The number of goblet cells increased in OVA-challenged mice (Fig. S1A). In contrast, mucin production was effectively attenuated to near normal levels by sulfuretin treatment. The measured mucin content correlated well with the histology data (Fig. S1B).

3.5. Sulfuretin prevented the development of AHR

One functional consequence of the inflammatory process that underlies asthma is a hyper-responsiveness to methacholine, a bronchoconstrictor. We, therefore, examined whether sulfuretin also influenced this endpoint. As shown in Fig. 4, OVA-challenged mice developed AHR in response to inhaled methacholine. However, sulfuretin reduced AHR in OVA-immunized mice by approximately 52% ($p < 0.01$) and restored AHR to levels similar to that of the control.

4. Discussion

The present study was designed to investigate whether negative regulation of NF-κB by sulfuretin would affect OVA challenge-induced inflammatory responses in the airways. To this end, sulfuretin was delivered intraperitoneally into the OVA-challenged mice. The results show that sulfuretin suppressed OVA challenge-induced NF-κB activation. This event led to further reduction of the pathological inflammatory responses in the airway tissues.

Several lines of evidence suggest a central role of NF-κB in the pathogenesis of asthma. NF-κB induces multiple pro-inflammatory genes that can contribute to allergic asthma [4,5]. Therefore, several therapeutic attempts targeting the NF-κB signaling pathway have been made to treat allergic airway inflammation. Specifically, p65 antisense oligonucleotide [4], p65-targeting small interfering ribonucleic acid [15], NF-κB decoy oligonucleotide [16], and proteasome inhibitors affecting NF-κB signaling pathway [17] have demonstrated to be effective in experimental asthma models. Our previous study showed that the inactivation of NF-κB by A20, which results in a decrease in the inflammatory airway response, also follows this strategy [5]. In this study, we isolated sulfuretin from *R. verniciflua* and demonstrated that sulfuretin effectively attenuates allergic airway inflammation and asthmatic symptoms. Our data further suggest that the therapeutic effect of sulfuretin results from the inhibition of the NF-κB signaling pathway. Sulfuretin inhibited OVA challenge-induced nuclear

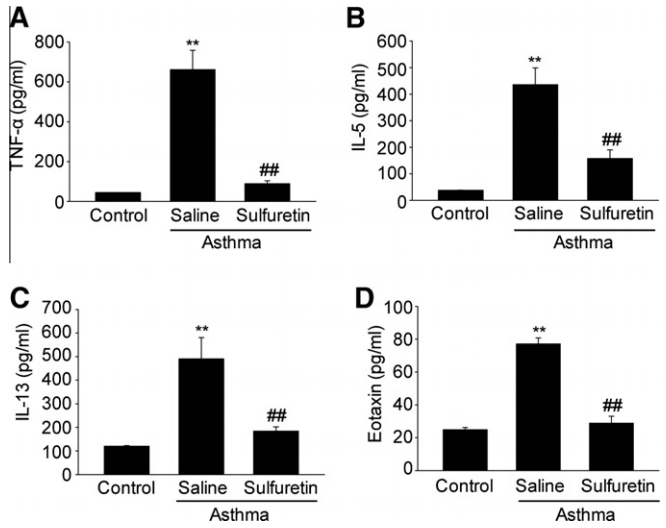

Fig. 3. Assessment of pro-inflammatory cytokines in the BAL fluid of OVA-sensitized mice treated with sulfuretin. Various cytokines in BAL fluid were measured by specific ELISAs. The level of TNF-α was determined 3 h after the last OVA challenge (A) and the levels of IL-5 (B), IL-13 (C), and eotaxin (D) were determined 24 h after the last OVA challenge. Results of three independent experiments with three mice/group are expressed as mean ± SEM. $^{**}p < 0.01$ vs. untreated control; $^{\#\#}p < 0.01$ vs. saline-treated group.

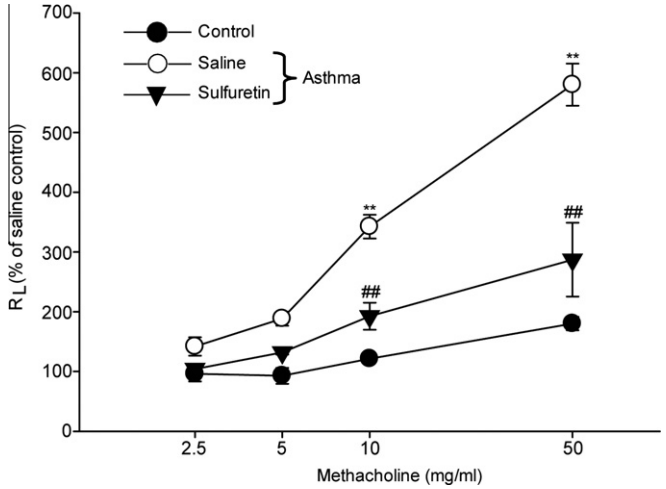

Fig. 4. AHR responses in OVA-sensitized mice treated with sulfuretin. All animals were sensitized to OVA as described in Fig. 2 and nebulized with various concentrations of methacholine (2.5, 5, 10, 50 mg/ml) as a bronchoconstrictor. Data are shown as airway resistance (cm H_2O per s per ml) and percent reduction, where the resistance of the vehicle-treated control group would be 100%. Each value represents the mean ± SEM of three independent experiments. $^{**}p < 0.01$ vs. control; $^{\#\#}p < 0.01$ vs. saline-treated group.

translocation of p65 and p50 subunits, as well as cytosolic IκBα degradation, as demonstrated by EMSA and Western blotting. These findings are in agreement with a previous study from an animal asthma model showing that mice lacking the p50 or c-Rel subunits of NF-κB develop less airway inflammation upon antigen challenge [7]. We have extended the favorable effects of sulfuretin against inflammatory insults in a type 1 diabetes animal model. We have found that sulfuretin protects pancreatic β-cells from cytokine or streptozotocin toxicity through suppressing the NF-κB pathway [12]. Taken together, our studies underscore the importance of NF-κB in inflammatory diseases and the effectiveness of sulfuretin in controlling inflammatory diseases.

The overall effect of sulfuretin on inflammation is mediated primarily by down-regulating the synthesis of a number of cytokines. Sulfuretin effectively down-regulates eosinophil recruitment, as evidenced by the decreased level of eotaxin and reduced eosinophilia in BAL fluid at later stages after OVA challenge. In addition, histological evaluation reveals that sulfuretin is also able to reduce OVA challenge-induced peribronchiolar inflammation (eosinophilic infiltration). Given that eosinophilic infiltration of the airway is a characteristic feature of allergic airway inflammation, we speculate that sulfuretin alleviates airway inflammation by decreasing the severity of eosinophilia. IL-5 has been shown to provide an essential signal for the expansion and mobilization of eosinophils from the bone marrow into the lung after allergen exposure [18]. IL-13 is known to induce expression of eotaxin in airway epithelial cells [19]. In addition, IL-13 and eotaxin contain NF-κB binding sites in the promoter regions of their genes [20,21]. In our experiments, IL-5, IL-13, and eotaxin levels in the BAL fluid were lower in mice receiving sulfuretin than in saline-treated mice. Therefore, it is possible that sulfuretin interferes with the recruitment of eosinophils by reducing Th2 cytokines and eotaxin levels.

We also demonstrated that sulfuretin effectively inhibited OVA challenge-induced AHR to methacholine and abrogated the increased mucin accumulation. A possible explanation for this finding may be that sulfuretin reduces the cytokine levels. AHR could be caused by a direct effect of TNF-α on airway smooth muscle. Studies have demonstrated that incubation of tracheal tissues with TNF-α increases the contractile responses to methacholine or other bronchoconstrictors [22,23]. An alternative mechanism by which sulfuretin could modulate the AHR response is through the down-regulation of Th2 responses. In mice and human, antigen-induced AHR results from Th2-dependent allergic responses, and Th2-associated cytokines are involved in the induction of AHR and mucin production [24]. Therefore, down-regulation of TNF-α and Th2 cytokines by sulfuretin may result in reduced AHR and suppressed mucin secretion in the airway.

Treatment with sulfuretin abrogates OVA challenge-induced inflammatory parameters, such as increases in the number of eosinophils and cytokine levels in BAL fluid, a marked infiltration of inflammatory cells into the lung tissues, mucin hypersecretion, and the development of AHR. Although we argue that the suppression of NF-κB is a principal therapeutic mechanism of sulfuretin, this study does not exclude the possibility that unidentified mechanisms of sulfuretin may contribute to its beneficial effects. At present, inhaled corticosteroid is recognized as the most efficient therapy for asthma because it is highly effective in controlling asthma symptoms and preventing acute exacerbations. Nevertheless, there is still a subgroup of patients who have severe asthma that is resistant to corticosteroid therapy; this subtype of bronchial asthma is termed difficult-to-control or refractory asthma [25]. Local application of sulfuretin might be used as a potential adjuvant for the treatment of allergic airway inflammation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.08.014.

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