Biochemical and Biophysical Research Communications 400 (2010) 83–88

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Sulfuretin attenuates allergic airway inflammation in mice

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ARTICLE INFO

Article history:
Received 31 July 2010
Available online 7 August 2010

Keywords:
Sulfuretin
Asthma
NF-κB
Airway inflammation
Th2 cytokine

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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doi:10.1016/j.bbrc.2010.08.014

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