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Sulforaphane inhibits the Th2 immune response in ovalbumin-induced asthma

Jun Ho Park[1,#], Jong Won Kim[1,#], Chang-Min Lee[3,#], Yeong Dae Kim[2], Sung Woon Chung[2], In Duk Jung[3], Kyung Tae Noh[3], Jin Wook Park[3], Deok Rim Heo[3], Yong Kyoo Shin[4], Jong Keun Seo[5] & Yeong-Min Park[3,]*

[1]Department of Thoracic and Cardiovascular Surgery, Busan Medical Center, Busan 611-672, [2]Department of Thoracic and Cardiovascular Surgery, School of Medicine, Pusan National University, Busan 602-739, [3]Department of Microbiology and Immunology, Pusan National University School of Medicine, Yang-San 626-770, [4]Department of Pharmacology, Chungang University College of Medicine, Seoul 156-756, [5]Department of Dermatology, Busan Paik Hospital, College of Medicine, Inje University, Busan 614-735, Korea

Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)-butane), belonging to a family of natural compounds that are abundant in broccoli, has received significant therapeutic interest in recent years. However, the molecular basis of its effects remains to be elucidated. In this study, we attempt to determine whether sulforaphane regulates the inflammatory response in an ovalbumin (OVA)-induced murine asthma model. Mice were sensitized with OVA, treated with sulforaphane, and then challenged with OVA. Sulforaphane administration significantly alleviated the OVA-induced airway hyperresponsiveness to inhaled methacholine. Additionally, sulforaphane suppressed the increase in the levels of SOCS-3 and GATA-3 and IL-4 expression in the OVA-challenged mice. Collectively, our results demonstrate that sulforaphane regulates Th2 immune responses. This sutdy provides novel insights into the regulatory role of sulforaphane in allergen-induced Th2 inflammation and airway responses, which indicates its therapeutic potential for asthma and other allergic diseases. [BMB reports 2012; 45(5): 311-316]

INTRODUCTION

Asthma is a chronic inflammatory lung disease characterized by airway hyperresponsiveness (AHR) to allergens, airway edema, and increased mucus secretion (1, 2). Inflammation results from the infiltration of eosinophils, neutrophils, macrophages, and lymphocytes into the bronchial lumen and lung tissues (3, 4). Recruitment of these inflammatory cells from the blood to the site of inflammation is regarded as a critical event in the development and persistence of airway inflammation (5, 6). In particular, it has been reported that eosinophil infiltration into asthmatic lungs leads to degranulation and the release of eosinophil peroxidase (EPO), which results in airway epithelial damage and the development of AHR (7, 8). Studies have shown that eosinophils infiltrating the lung preferentially stimulate T-helper type 2 (Th2) cell responses by presenting antigens (9-11). Therefore, Th2 cells are dominant in the airways (12), and Th2 cytokines such as IL-4, IL-5, and IL-13 play a pivotal role in the pathophysiology of asthma (13-15).

Suppressor of cytokine signaling-3 (SOCS-3) is a member of the STAT-induced STAT inhibitor (SSI) family, which comprises cytokine-inducible negative regulators of cytokine signaling. *SOCS-3* expression is induced by various cytokines, including IL-6, IL-10, and interferon (IFN)-γ. The SOCS-3 protein can bind to and inhibit the activity of JAK2 kinase. Studies on the mouse counterpart of the *SOCS-3* gene suggest that it plays a role in the negative regulation of fetal liver hematopoiesis and placental development (16). Because they function as negative regulators of cytokine signaling, suppressor of cytokine signaling (SOCS) proteins are implicated in the pathogenesis of several inflammatory diseases. Cytokines secreted by Th2 cells are central to the development of allergic disease, and SOCS-3, which is mainly expressed by Th2 cells, regulates the onset and maintenance of Th2 cell-mediated allergic responses (17).

Sulforaphane has been demonstrated to play a role in various physiological responses in various *in vitro* systems, and its activities include anti-inflammatory anticarcinogenic activities and free radical scavenging (18). In a recent study, investigators identified sulforaphane as a potent inhibitor of nuclear transcription factor nuclear factor-κB (NF-κB), which performs pivotal functions in the regulation of cell growth, apoptosis, and cell cycle regulation (19). Moreover, sulforaphane actively inhibits I-κB kinase activity, I-κB degradation, DNA binding to NF-κB, and the activity of mitogen-activated protein kinases (MAPKs). Further, sulforaphane abrogates the pro-inflammatory and pro-allergic effects typically observed with cellular diesel exhaust particles (DEP) exposure (20). For these reasons, we proposed that sulforaphane regulates pro- and anti-inflammatory responses in many kind of diseases (18-20). In this

*Corresponding author. Tel: +82-51-510-8097; Fax: +82-55-382-8090;
E-mail: immunpym@pusan.ac.kr
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[#]These authors equally contributed.

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study, we attempted to characterize the effects of sulforaphane in a murine model of asthma. Our findings demonstrate for the first time that sulforaphane inhibits asthmatic syndrome and suppresses OVA-induced *SOCS-3* and *GATA-3* expression.

RESULTS

Sulforaphane reduces the population of inflammatory cells in bronchoalveolar lavage fluid and airway hyperresponsiveness

The total number of cells, including eosinophils, lymphocytes, and macrophages in bronchoalveolar lavage (BAL) fluid was significantly increased at 24 h after OVA inhalation compared with the number after saline inhalation (Fig. 1A). In particular, the increased number of eosinophils significantly decreased after sulforaphane administration, as detected by eosinophil peroxidase assay (Fig. 1B). Methacholine administration (2.5-50 mg/ml) significantly increased the percentage of Penh in OVA-sensitized and OVA-challenged mice compared with the controls. Penh is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. The dose-response curve of percentage Penh for OVA-sensitized and OVA-challenged mice treated with sulforaphane showed a right shift compared to that for untreated mice, and this shift was dose dependent (Fig. 1C). These results indicate that sulforaphane treatment reduces OVA-induced AHR (Fig. 1C).

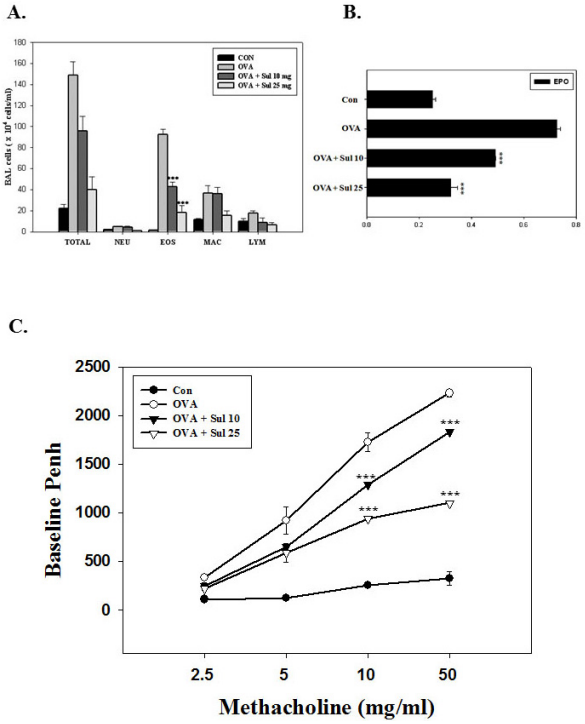

Fig. 1. Sulforaphane leads to a reduction in eosinophil number and airway hyperresponsiveness. (A) Airway responsiveness was measured at 24 h after the last challenge in mice that inhaled saline and were administered PBS (PBS), OVA-sensitized mice administered saline (OVA), and OVA-sensitized mice administered sulforaphane (OVA + 10 mg Sul or OVA + 25 mg Sul). The data represent the mean ± S.E.M. from five independent experiments. ***P < 0.001. (B) Eosinophil peroxidase (EPO) activity in BAL fluids of OVA-sensitized and -challenged mice. EPO is an indicator of the numbers of eosinophil levels. The results were from one representative experiment out of 5 performed. This experiment used five mice (n = 5). ***P < 0.001. (C) Mice were treated with PBS (CON), OVA plus 5 or 10 mg kg^{-1} d^{-1} (OVA + 10 mg Sul and OVA + 25 mg Sul, respectively), or OVA (OVA). The results are from one representative experiment out of five that were performed. This experiment used five mice (n = 5). ***P < 0.001 vs. OVA; NEU, neutrophils; EOS, eosinophils; LYM, lymphocytes; MAC, macrophages; Total, total cells.

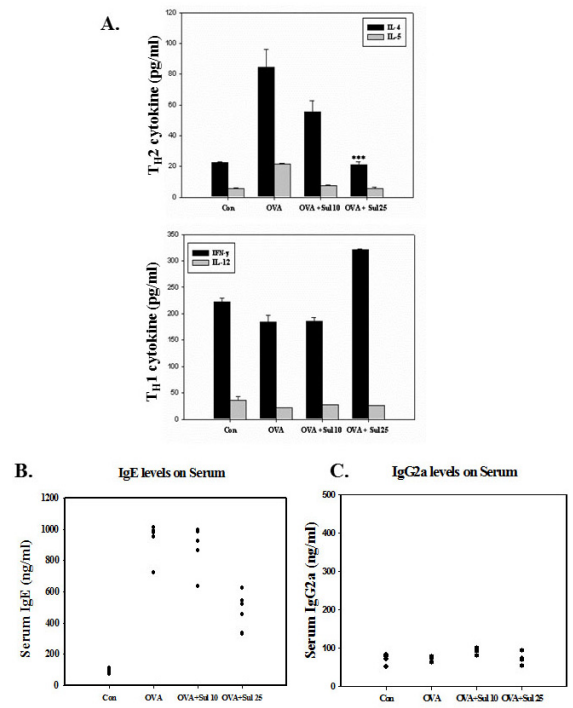

Fig. 2. Effect of sulforaphane treatment on Th2 cytokine and OVA-specific IgE levels. OVA-sensitized mice were treated as described in Materials and Methods. (A) BAL fluid was obtained at 4 h after the last airway challenge, and the IL-4, IL-5, IL-12, and IFN-γ cytokine levels in the BAL fluid. The data shown represent the mean ± SEM from six independent experiments ***P < 0.001 vs. OVA. Further, blood was collected by cardiac puncture. (B and C) IgE (B) and IgG2a (C) levels were measured at 4 h after the last challenge in mice that inhaled saline and were administered PBS (PBS), OVA-sensitized mice administered saline (OVA), and OVA-sensitized mice administered sulforaphane (OVA + 10 mg Sul or OVA + 25 mg Sul). ELISA was used for analysis in three independent experiments.

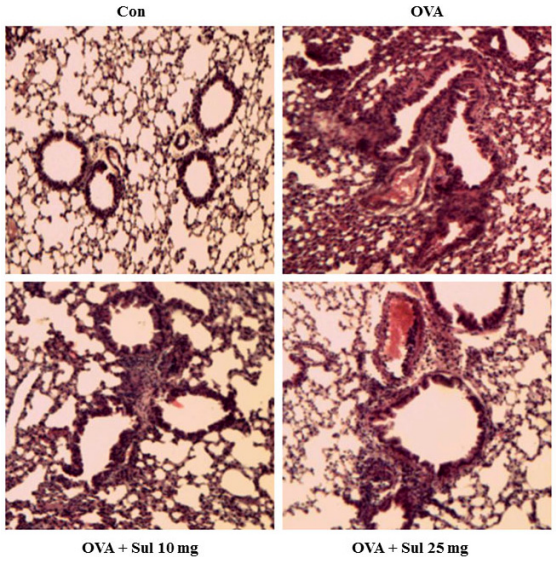

Fig. 3 Sulforaphane inhibits lung inflammation and inflammatory cell infiltration. Mice were sensitized and challenged as described in Materials and Methods. Sections were obtained from the lungs of mice that received the control treatment (CON), OVA + sulforaphane at 10 mg kg^{-1} d^{-1} (OVA + 10 mg Sul), OVA + sulforaphane at 25 mg kg^{-1} d^{-1} (OVA + 25 mg Sul), and OVA (OVA). The lungs were removed at 2 days after the last airway challenge. Sections were stained with hematoxylin and eosin (×200).

Sulforaphane reduces the levels of Th2 cytokines and OVA-specific IgE levels of OVA-sensitized and OVA-challenged mice

BAL fluid was obtained 4 h after the last airway challenge. The levels of the Th2 cytokines IL-4 and IL-5 in the BAL fluid were significantly increased in the OVA-challenged mice than in the control mice. Sulforaphane administration inhibited secreted IL-4 and IL-5 levels. Although the levels of IL-4 and IL-5 were higher in the OVA-sensitized and OVA-challenged mice than in saline-sensitized and saline-challenged mice, the levels of the Th1 cytokines IFN-γ and IL-12 were not different in the two mouse groups. These results indicate that sulforaphane functions as an attenuator of Th2 cytokines rather than Th1 cytokines in airways challenged with OVA (Fig. 2A). We also investigated the expression of OVA-specific IgE, which is associated with the Th2 response in airway inflammation. As predicted, IgE expression was remarkably increased because of OVA challenge (Fig. 2B). However, sulforaphane significantly decreased the level of OVA-specific IgE. Further, the level of IgG2a was not affected much by sulforaphane (Fig. 2C). These data indicate that sulforaphane modulates IgE levels associated with the Th2 response in OVA-induced asthma models (Fig. 2B).

Sulforaphane ameliorates the pathological changes in OVA-induced asthma

Histological analysis of OVA-exposed mice revealed the typical pathologic features of asthma: compared with the control mice, numerous inflammatory cells, including eosinophils, had infiltrated the lungs around the bronchioles (Fig. 3). The number of infiltrated inflammatory cells was remarkably lowered in the lung tissues of mice treated with sulforaphane. Severe inflammation was observed in the lung tissue at 24 h after OVA inhalation compared with the condition after saline inhalation. The above-mentioned pathophysiological phenomena were dramatically inhibited by sulforaphane administration, and the results suggest that sulforaphane inhibits OVA-induced inflammation in the lungs, including the influx of eosinophils and inflammatory cells.

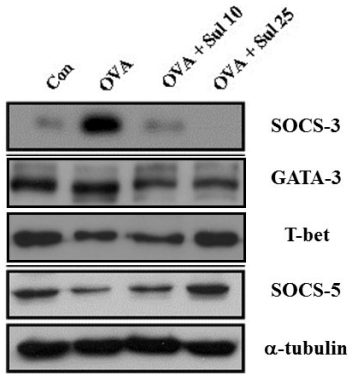

Fig. 4. Effect of sulforaphane on SOCS-3 and GATA-3 expression in the lung tissues of OVA-sensitized and OVA-challenged mice. Sampling was performed at 24 h after the last challenge in mice that inhaled saline and were administered saline (CON), mice that inhaled OVA and were administered saline (OVA), mice that inhaled OVA and were administered 10 mg kg^{-1} d^{-1} sulforaphane (OVA + 10 mg Sul), and mice that inhaled OVA and were administered 25 mg kg^{-1} d^{-1} sulforaphane (OVA + 25 mg Sul). The data represent the mean ± S.E.M. from six independent experiments.

Sulforaphane decreases SOCS-3 and GATA-3 expression in the lung tissues of OVA-sensitized and OVA-challenged mice

Western blot analysis revealed that SOCS-3 and GATA-3 expression in lung tissues was significantly increased 24 h after OVA inhalation compared with the expression after saline inhalation (Fig. 4). This increase in protein expression was however decreased when sulforaphane was administered. Conversely, sulforaphane treatment induced an increase in SOCS-5 and T-bet expression, which is associated with the Th1 response.

DISCUSSION

This study is the first to provide experimental evidence demonstrating that sulforaphane inhibits OVA-induced airway inflammation in a murine model of asthma. Sulforaphane markedly inhibited asthmatic reactions such as leukocytic recruitment into the airway and lung inflammation. Further, the results indicated that sulforaphane regulates the Th1/Th2 balance, which is mediated by SOCS-3 and GATA-3.

It has been well characterized that in chronic asthma, Th2 lymphocytes infiltrate the lungs and produce inflammatory cytokines, including IL-4, IL-5, and IL-13 (21). These cytokines may induce the expression of inflammatory molecules in both the endothelial cells of the bronchial circulation and the epithelial cells in the airway, and they may also promote the recruitment of lymphocytes and eosinophils (22). One of these cytokines, IL-4, induces isotype switching from IgG to IgE production in B cells (23). Animal studies have shown that the immunological processes involved in airway inflammation in asthma are characterized by the proliferation and activation of Th2 $CD4^+$ T-cells. Ultimately, the above-mentioned cytokines lead to degranulation of effector/proinflammatory cells with the release of mediators and oxidants, which then lead to the injury and inflammation observed in asthma. OVA-induced asthma has been recognized as a disease that results from chronic airway inflammation characteristically associated with the infiltration of lymphocytes, eosinophils, and neutrophils into the bronchial lumen (24).

We found that sulforaphane reduced SOCS-3 and GATA-3 expression and increased SOCS-5 and T-bet expression in OVA-sensitized and OVA-challenged mice (Fig. 4). This suggests that sulforaphane treatment is a novel and selective way to simultaneously suppress GATA-3 and SOCS-3 and increase T-bet and SOCS-5 expression in asthmatic reactions in vivo. These genes are strongly associated with Th1 and Th2 cytokine regulation. We also examined Th1/Th2 cytokine production in BAL fluid cells and consistently found that sulforaphane suppressed the increase in the levels of IL-4, a Th2 cytokine, in OVA-sensitized and OVA-challenged mice but increased IFN-γ production. Our data also demonstrate that sulforaphane suppressed the increase in the levels of GATA3 mRNA in OVA-challenged mice. Taken together, the results suggest that SOCS-3 and GATA-3 might not only be candidate genes for asthma but also regulators of the Th1/Th2 balance.

In conclusion, our results strongly suggest that sulforaphane reduces allergic airway inflammation by inducing an alteration in the Th1/Th2 balance via suppression of SOCS-3 and GATA-3.

MATERIALS AND METHODS

Animals and experimental protocol

Female BALB/c mice aged 6-8 weeks and free of murine-specific pathogens were obtained from Charles River Laboratories (Yokohama, Japan). All experimental animals used in this study were maintained under a protocol approved by the Institutional Animal Care and Use Committee of the Pusan National University Medical School. The mice were immunized intraperitoneally (i.p.) with 20 μg of OVA (Sigma-Aldrich, St. Louis, MO, USA) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL, USA) on days 1 and 15. The mice were then challenged with OVA (inhalation of 5% OVA) for 30 min each day from days 21 to 23. BAL fluid was collected at 24 h after the last challenge. For lavage extraction, mice (n = 6 per group) were killed using ether overdose. The chest cavity was exposed for expansion, after which the trachea was carefully intubated and a catheter was secured with ligatures. Prewarmed saline solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and maintained at 4^oC. A fraction of each pool was then centrifuged, and the supernatants were stored at -70^oC until use.

Sulforaphane administration

The mice were injected i.p. with 200 μl of either 10 or 25 mg/kg sulforaphane (Sigma) each day from days 17 to 20.

Total cell counting

The total number of cells was counted with a hemocytometer. Smears of BAL cells prepared with Cytospin II (Shandon, Runcorn, UK) were stained with Diff-Quik solution (Dade Diagnostics of P.R. Inc., Aguada, Puerto Rico) for differential cell counting. Two independent, blinded investigators counted the cells using a microscope. Approximately 200 cells were counted in each of four random fields.

Histopathological analysis

At 48 h after the last challenge, the lungs of the mice were removed after sacrifice. Before removal, the lungs and trachea were filled intratracheally with a fixative (4% paraformaldehyde) after a ligature was placed around the trachea. Lung tissues were fixed with 10% (v/v) paraformaldehyde. The specimens were dehydrated and embedded in paraffin. For histological examination, 4 μm sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Nussloch, Germany), placed on glass slides, deparaffinized, and sequentially stained with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI, USA).

Assay of Th1/Th2 cytokines and OVA-specific IgE levels

The levels of IL-4 and IL-5 were quantified in the supernatant of BAL fluid using enzyme immunoassays performed according to the manufacturer's protocol (IL-4 and IL-5; R&D Systems, Inc., Minneapolis, MN, USA). The serum IgE levels were measured using an ELISA kit according to the manufacturer's protocol (R&D Systems).

Western blot analysis

Lung tissues were homogenized, washed with PBS, and incubated in lysis buffer containing a protease inhibitor cocktail (Sigma) to obtain lung protein extracts. The samples were loaded on 10% SDS-PAGE gels and separated at 120 V for 90 min, after which they were electronically transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked with 5% nonfat milk in washing buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1% Tween 20) and incubated with the indicated antibodies in buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1% Tween 20; 1% nonfat milk) for 1 h at room

temperature. The membranes were subsequently washed and incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Determination of airway responsiveness to methacholine

Airway responsiveness was measured 24 h after the last challenge with the mice in an unrestrained conscious state, as described previously (25). The mice were placed in a barometric plethysmographic chamber (All Medicus Co., Seoul, Korea), and baseline readings were taken for 3 min and averaged. Aerosolized methacholine in increasing concentrations (2.5-50 mg/ml) was nebulized through an inlet of the main chamber for 3 min. Readings were taken for 3 min after each nebulization and averaged. Enhanced pause (Penh), calculated as (expiratory time/relaxation time $-$ 1) $\times$ (peak expiratory flow/peak inspiratory flow) according to the manufacturer's protocol. Here, Penh was used as a measure of airway responsiveness to methacholine. The results are expressed as the percentage increase in Penh following challenge with each concentration of methacholine, where the baseline Penh (after saline challenge) is expressed as 100%. Penh values were averaged over 3 min after each nebulization and evaluated.

Densitometric analysis and statistics

Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the mean $\pm$ S.E.M. Analysis of variance (ANOVA) was used to compare the experimental group values to the control values, while comparisons between multiple groups were performed using Tukey's multiple comparison test. A P value of less than 0.05 was considered statistically significant.

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