Benzaldehyde suppresses murine allergic asthma and rhinitis

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1. Introduction

Allergic asthma and rhinitis are caused by an IgE-mediated hypersensitivity reaction [1]. They worsen life quality and increase the economic burden [2]. The available medications, including antihistamines, mast cell stabilizers, and inhaled or systemic corticosteroids, have transient effects. Therefore, there is growing interest in research and development of more potent and long-acting therapeutic agents.

Benzaldehyde is an aromatic aldehyde commonly used in the cosmetic industry as a flavoring agent, fragrance, or denaturant [3]. In the United States and Europe, it is also used as a food additive. Previous research has suggested its effectiveness against some tumors [4], but only few studies have investigated its potential as an antiallergic drug. Moreover, Lacroix et al. have suggested that exposure to benzaldehyde-containing air decreases ovalbumin-induced allergic airway inflammation in mice with allergic asthma [5]. However, the effects of oral benzaldehyde have not been investigated.

To elucidate the antiallergic effects of oral benzaldehyde, we evaluated the number of nose-scratching events in 10 min, (ii) levels of total and ovalbumin-specific IgE in serum, (iii) differential counts of inflammatory cells in bronchoalveolar lavage (BAL) fluid, (iv) titers of some Th2 cytokines (IL-4, IL-5, IL-13) in BAL fluid, (v) histopathologic findings of lung and nasal tissues, and (vi) expressions of several pro-inflammatory (COX-2), antioxidation (extracellular SOD, HO-1), and hypoxia (HIF-1α, VEGF) in lung tissue were evaluated. The treated mice had significantly fewer nose-scratching events, less inflammatory cell infiltration in lung and nasal tissues, and lower HIF-1α and VEGF expressions in lung tissue than the allergic group. The number of eosinophils and neutrophils and Th2 cytokine titers in BAL fluid significantly decreased after the treatment (P < 0.05). These results imply that oral benzaldehyde exerts antiallergic effects in murine allergic asthma and rhinitis, possibly through inhibition of HIF-1α and VEGF.

2. Materials and methods

2.1. Animals

We used 20 female BALB/c mice (Orient Bio, Seongnam, Korea) aged 8–10 weeks and free from any murine-specific pathogens. They were raised in a well-controlled environment with a 12-h light–dark cycle and had unrestricted access to ovalbumin-free food and water. All mice were handled according to a protocol approved by the Animal Care and Use Committee of Inha University (INHA-140211-273).

2.2. Systemic sensitization and intranasal challenge

To induce allergic asthma and rhinitis, we adopted a published protocol with slight modifications [6–8]. Under pathogen-free conditions, 40-μg/kg ovalbumin (Sigma-Aldrich, St. Louis, MO) diluted in sterile...
saline and aluminum hydroxide gel (alum adjuvant, 40 mg/kg) was injected intraperitoneally on experimental days 1, 5, 14, and 21 for systemic sensitization. Thereafter, daily intranasal instillation of ovalbumin diluted in sterile saline (20 μL of 25-mg/mL ovalbumin per mouse) was performed until experimental day 35 (14 challenges in total).

The mice were divided into four equal groups (n = 5 mice/group). Negative control animals were exposed to normal saline (nonallergic group), while positive control animals were exposed to ovalbumin (allergic group). The remaining allergic animals received either 200- or 400-mg/kg benzaldehyde (Premier Botanicals Ltd, Independence, OR) by oral gavage feeding 30 min before the intranasal challenges.

2.4. Serum and BAL

events in 10 min was immediately recorded by two blind, independent observers. The number of nose-scratching events in 10 min was immediately recorded by two blind, independent observers.

2.3. Enumeration of nose-scratching events

Twenty-four hours after the last intranasal challenge, each mouse was intranasally exposed to ovalbumin. The number of nose-scratching events in 10 min was immediately recorded by two blind, independent observers.

2.4. Serum and BAL fluid collection

We used an aortic puncture technique to collect serum. BAL fluid was collected by normal saline lavage (~4 mL) through an intratracheal tube [7]. The fluid was filtered through wet gauze and centrifuged at 150 g for 10 min. The resultant pellet was suspended immediately in 4-mL saline. We determined the total cell numbers in duplicate with a hemocytometer. Then, a 100-μL aliquot was centrifuged (Cytospin 2 cytocentrifuge; Thermo Fisher Scientific, Pittsburgh, PA) and cell viability and total cell count were evaluated using the trypan blue exclusion test. Using centrifuged preparations stained with Diff-Quik (Baxter Scientific, Miami, FL), we determined differential cell counts in 500 cells per animal at 1000× magnification.

2.5. Enzyme-linked immunosorbent assay (ELISA)

We evaluated the levels of total and ovalbumin-specific IgE in serum by ELISA as described previously [9]. Total IgE was measured and compared with a known concentration of mouse IgE standard (BD Biosciences, San Diego, CA). We used the optical density at 450 nm instead of calculating the concentration with a standard solution. The titers of IL-4, IL-5, and IL-13 were measured using individual ELISA kits (BioSource International, Camarillo, CA) according to the manufacturer’s instructions.

2.6. Histopathology

After fixation in a 10% formalin solution for 3 weeks, lung and nasal tissues were embedded in paraffin according to standard methods, and 4-μm-thick sections were stained with hematoxylin and eosin to detect cellular infiltration. The number of infiltrated cells around a single bronchiole and in 1 mm² of the lamina propria of the nasal mucosa was counted in 10 random high-power (400×) fields.

2.7. Western blot analysis

Lung tissue (left whole lung) from each animal was homogenized in lysis buffer using a Teflon homogenizer. We removed cellular debris of the homogenates by centrifuging at 1000 g for 10 min. Total protein contents were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL). SDS-polyacrylamide gel electrophoresis was used for separating aliquots of protein extracts (20–60 μg). We transferred them electrophoretically onto polyvinylidene difluoride membranes in a transfer buffer containing 25-mM Tris–HCl, 192-mM glycine, and 10% methanol. The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 and incubated with specific primary antibodies. They were then incubated with hors eradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce) and analyzed using Bio-1D software (SIM International Group, Newark, DE).

2.8. Statistical analysis

All statistical analyses were performed with SPSS version 19.0 software (IBM, Armonk, NY). We used the Kruskal–Wallis test and Mann–Whitney U-test for intergroup comparisons. P < 0.05 was considered significant.

3. Results

3.1. Nose-scratching events and serum IgE levels

In comparison with the nonallergic group, the allergic group showed a significantly increased number of nose-scratching events in 10 min after intranasal ovalbumin instillation. They also had significantly higher levels of total and ovalbumin-specific IgE in serum (P < 0.001). The treated mice showed significantly fewer nose-scratching events than the allergic animals (P < 0.01, Fig. 1), but they had no significant decrease in serum IgE levels (P > 0.05; Fig. 2).

3.2. Inflammatory cell counts in BAL fluid

The ovalbumin challenge significantly increased the counts of inflammatory cells such as eosinophils, neutrophils, and lymphocytes in BAL fluid compared with the saline challenge (P < 0.001). After benzaldehyde treatment, a significant decrease in the eosinophil and neutrophil counts was observed (P < 0.05; Fig. 3). The number of lymphocytes also decreased, although no significance difference was noted between the treated and the allergic groups.

3.3. Th2 cytokine titers in BAL fluid

The allergic group showed significantly increased IL-4, IL-5, and IL-13 levels in BAL fluid when compared with the nonallergic group.

![Fig. 1. Effect of oral benzaldehyde on murine nose-scratching behavior. The number of nose-scratching events in 10 min after the last intranasal challenge was compared among the nonallergic, allergic, and 200- and 400-mg/kg benzaldehyde groups. Data represent medians and ranges (n = five mice/group). ***P < 0.001 compared with the nonallergic group (Kruskal–Wallis test and Mann–Whitney U-test).](image-url)
After benzaldehyde treatment, the titers nearly normalized (no significant difference compared with the nonallergic group). Notably, the 400-mg/kg benzaldehyde group showed significantly lower IL-4 levels in BAL fluid than the 200-mg/kg benzaldehyde group ($P = 0.008$; Fig. 4).

3.4. Histopathologic findings of lung and nasal tissues

The allergic animals showed significantly more inflammatory cell infiltration around bronchioles and in the lamina propria of the nasal mucosa than the nonallergic group. After benzaldehyde treatment, the degree of cellular infiltration significantly decreased (Fig. 5). The number of infiltrated eosinophils around a single bronchiole and in 1 mm$^2$ of the lamina propria of the nasal mucosa also significantly decreased after the treatment ($P < 0.05$; Fig. 6).

3.5. Expressions of proteins related to various pathways

The allergic group showed increased expressions of Bcl-2, Bax, and caspase-3. Although the treated groups showed no significant differences in Bcl-2 and Bax expressions compared with the allergic group, caspase-3 expression significantly decreased in the 400-mg/kg benzaldehyde group (Fig. 7a).

Further, the allergic mice showed increased expressions of COX-2 and HO-1 and decreased expression of extracellular SOD compared with the nonallergic mice. After the treatment, significant suppression of COX-2 and HO-1 expressions was noted, but extracellular SOD expression was not significantly recovered compared with the allergic group (Fig. 7b).

The allergic group showed increased HIF-1$\alpha$ and VEGF expressions compared with the nonallergic group; the expressions were significantly suppressed by the benzaldehyde treatment (Fig. 7c).

4. Discussion

In our study, benzaldehyde-treated allergic mice showed significantly fewer nose-scratching episodes than untreated allergic mice. To the best of our knowledge, this is the first study reporting the antiallergic effects of oral benzaldehyde in murine allergic asthma and rhinitis. Patients with allergic rhinitis suffer from impaired life quality due to allergic symptoms such as runny nose and sneezing. Therefore, benzaldehyde treatment could significantly improve life quality of such patients.

In several studies of the antiallergic effects of therapeutic antibodies, we found that protocols in which treatment was started in the ovalbumin injection period led to decreased serum IgE levels [6], but the levels did not significantly decrease when treatment was started after sensitization [10]. In the present study, we started the treatment after complete sensitization to ovalbumin, so oral benzaldehyde should have had minimal or no effect on the serum titers of IgE. The significant post-treatment reduction in serum IgE levels despite systemic
sensitization implies that benzaldehyde may have antiallergic effects through mechanisms independent of mast cell degranulation and IgE. This result is clinically meaningful because patients with allergic asthma and rhinitis are systemically sensitized to provocative allergens.

Benzaldehyde treatment significantly reduced the counts of eosinophils, neutrophils, and lymphocytes in BAL fluid. Similarly, Lacroix et al. suggested that inhaled benzaldehyde decreases eosinophil and neutrophil counts in BAL fluid [5]. As eosinophils, neutrophils, and lymphocytes play a pivotal role in the initiation and maintenance of allergic inflammation, a decrease in the cell counts implies a beneficial antiallergic effect.

Th2 cytokines contribute to the initiation and maintenance of allergic responses [11], and are involved in airway eosinophilia and hyperresponsiveness [12]. More specifically, IL-4 expression is related to differentiation of Th2 lymphocytes, synthesis of IgE, upregulation of IgE receptors, and hypersecretion of mucus [13–15]. IL-5 plays a role in eosinophilic inflammation and its infiltration of the airway [16]. IL-13 is related to differentiation of B lymphocytes and isotype switching of antibody to IgE [17]. Oral benzaldehyde significantly reduced Th2 cytokine titers in BAL fluid, suggesting that it may exert its antiallergic effect by blocking Th2 cytokine activity. The histopathologic findings support these results.

We found the upregulation of several proteins associated with apoptosis in the allergic mice, which is in agreement with previous findings. Bcl-2 is upregulated in BAL fluid of patients with severe asthma [18] and nasal mucosa of patients with allergic rhinitis [19]. In a rat model of experimentally induced allergic rhinitis, Suo et al. found that Bcl-2 and Bax are significantly upregulated in glandular cells, epithelial cells, and eosinophils [20]. After challenge with house dust mite extracts, caspase-3 is upregulated in human bronchial and nasal epithelial cells [21]. However, benzaldehyde treatment didn’t cause a significant change in the overall expression of these proteins in this study. Therefore, we speculate that the antiallergic effects of benzaldehyde are independent of the antiapoptotic pathway.

COX-2 expression was significantly increased in the allergic mice but significantly decreased after the benzaldehyde treatment. Inhibition of COX-2 exerts an antiallergic effect through the downregulation of prostaglandin D2 [22]. The expression of extracellular SOD significantly declined in the allergic mice. There are few studies on the role of extracellular SOD in allergic airway inflammation. In epithelial mucosa of patients with eosinophilic sinusitis, SOD activity is significantly suppressed [23]. Extracellular SOD inhibits maturation of dendritic cells, controls activation and proliferation of T lymphocytes, and regulates differentiation of Th2 and Th17 cells [24]. Therefore, extracellular SOD appears to ameliorate ovalbumin-induced allergic airway inflammation.

Fig. 4. Effects of oral benzaldehyde on Th2 cytokine titers in BAL fluid. (a) IL-4, (b) IL-5, and (c) IL-13 titers in BAL fluid were compared among the nonallergic, allergic, and 200- and 400-mg/kg benzaldehyde groups. Data represent medians and ranges (n = five mice/group). **P < 0.01, ***P < 0.001 versus the nonallergic group (Kruskal–Wallis test and Mann–Whitney U-test).

Fig. 5. Histopathologic findings of lung and nasal tissues. (a–d) Lung and (e–h) nasal tissue sections of the nonallergic, allergic, and 200- and 400-mg/kg benzaldehyde groups were stained with hematoxylin and eosin and compared under 400× magnification.
The antiallergic effects of benzaldehyde may be independent of extracellular SOD and antioxidative mechanisms.

HO-1, a specific regulator of endogenous carbon monoxide, has a protective effect in allergic airway inflammation. It inhibits mast cell degranulation and synthesis of several Th2 cytokines. HO-1 thus protects against airway inflammation and hyper-responsiveness and hyper-secretion of mucus [25]. In our study, HO-1 expression significantly increased after induction of allergic asthma and rhinitis, but was significantly suppressed after the benzaldehyde treatment. Interestingly, suppression of HO-1 expression was concentration dependent. According to Yu et al., ovalbumin-sensitized guinea pigs show high expression of HO-1, which is suppressed after treatment with dexamethasone [26]. Elhini et al. also suggested that HO-1 is upregulated in patients with persistent allergic rhinitis [27]. Therefore, HO-1 is a potential therapeutic target for allergic airway inflammation [25,28,29]. HO-1 suppression may contribute to the antiallergic effects of benzaldehyde.

HIF is a heterodimer composed of 2 subunits (α and β). Expression of the β subunit is constitutive, for HIF expression is mainly regulated through its α subunit [30]. Under hypoxia, HIF upregulates several target genes including pro-inflammatory cytokines.

**Fig. 6.** Effects of oral benzaldehyde on inflammatory cell infiltration in lung and nasal tissues. The number of infiltrated cells (a) around a single bronchiole and (b) in 1 mm² of the lamina propria of the nasal mucosa was compared among the nonallergic, allergic, and 200- and 400-mg/kg benzaldehyde groups. Data represent medians and ranges (n = five mice/group). *P < 0.05, **P < 0.01 versus the nonallergic group (Kruskal-Wallis test and Mann-Whitney U-test).

**Fig. 7.** Effects of oral benzaldehyde on expressions of proteins involved in apoptosis, inflammation, antioxidation, and hypoxia. Expressions of (a) Bcl-2, Bax, and caspase-3; (b) COX-2, extracellular SOD, and HO-1; and (c) HIF-1α and VEGF in lung tissue were quantified by Western blot analysis and SDS-PAGE and compared among the nonallergic, allergic, and 200- and 400-mg/kg benzaldehyde groups. A: nonallergic group, B: allergic group, C: 200 mg/kg treatment group, D: 400 mg/kg treatment group. Data represent means ± SD (n = five mice/group). *P < 0.05, **P < 0.01 versus the nonallergic group; *P < 0.05, **P < 0.01 versus the allergic group.
Benzaldehyde is categorized as a food additive in the United States and also a flavoring substance in European countries [3]. A commercially available US perfume contains up to 0.5% of benzaldehyde [3]. It is also naturally present in ordinary foods such as alcoholic beverages, dairy products, tea, coffee, cocoa, and fruit, whose concentration ranges from 0.01 to 8.9 ppm [3]. In other words, humans are exposed to low-dose benzaldehyde by subcutaneous or oral routes. In several human clinical studies, systemic allergic reaction, phototoxicity, photosensitization, and reproductive or developmental toxicity to benzaldehyde were absent or rare [3]. In rats, benzaldehyde demonstrated no carcinogenicity. On the contrary, it showed carcinostatic or antitumor effects [3]. Further study to evaluate the safety and efficacy of oral or intranasal benzaldehyde in allergic asthma or rhinitis is required. The 200- and 400-mg/kg benzaldehyde groups showed few differences in this study. To achieve the maximal antiallergic effects with minimal side effects, a lower drug concentration is desirable. We are now performing similar experiments with much lower concentrations of oral benzaldehyde (20–50 mg/kg).

In conclusion, oral benzaldehyde exerts antiallergic effects in murine allergic asthma and rhinitis, possibly through inhibition of HIF-1α and VEGF expressions.

Author contributions

Tae Young Jang wrote the manuscript. Chang-Shin Park performed the animal experiments and collected the data. Kyu-Sung Kim interpreted the results and proofread the manuscript. Min-Jeong Heo conducted the animal experiments, collected the data, and performed the statistical analysis. Young Hyo Kim designed the study, performed the statistical analysis, and prepared the figures.

Conflict of interest

There is no conflict of interest related to this study.

Acknowledgments

Tae Young Jang received a grant (NRF-2013 M1A3A3 A20242309) from the Space Core Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science, ICT and Future Planning (MSIP), South Korea. Chang-Shin Park received a grant (Medical Research Center [MRC] No. 2014009392) from the NRF funded by the MSIP. Young Hyo Kim received support from the Basic Science Research Program through the NRF funded by the Ministry of Education, Science, and Technology (NRF-2013R1A1A1063682). Tae Young Jang, Chang-Shin Park, Kyu-Sung Kim, and Young Hyo Kim received Inha University Research Grants. Min-Jeong Heo has no relationships to declare.

References


