International Immunopharmacology 22 (2014) 444–450

Contents lists available at ScienceDirect

International Immunopharmacology

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

# Benzaldehyde suppresses murine allergic asthma and rhinitis ☆

Tae Young Jang [a,1], Chang-Shin Park [b,1], Kyu-Sung Kim [a], Min-Jeong Heo [a], Young Hyo Kim [a,*]

[a] Department of Otorhinolaryngology, Head and Neck Surgery, Inha University College of Medicine, Incheon, Republic of Korea
[b] Department of Pharmacology, Hypoxia-Related Disease Research Center, Inha Research Institute for Medical Sciences, Inha University College of Medicine, Incheon, Republic of Korea

ARTICLE INFO

*Article history:*
Received 26 May 2014
Received in revised form 16 July 2014
Accepted 25 July 2014
Available online 5 August 2014

*Keywords:*
Allergic rhinitis
Asthma
Benzaldehyde
Hypoxia-inducible factor 1
Vascular endothelial growth factor

ABSTRACT

To evaluate the antiallergic effects of oral benzaldehyde in a murine model of allergic asthma and rhinitis, we divided 20 female BALB/c mice aged 8–10 weeks into nonallergic (intraperitoneally sensitized and intranasally challenged to normal saline), allergic (intraperitoneally sensitized and intranasally challenged to ovalbumin), and 200- and 400-mg/kg benzaldehyde (allergic but treated) groups. The number of nose-scratching events in 10 min, levels of total and ovalbumin-specific IgE in serum, differential counts of inflammatory cells in bronchoalveolar lavage (BAL) fluid, titers of Th2 cytokines (IL-4, IL-5, IL-13) in BAL fluid, histopathologic findings of lung and nasal tissues, and expressions of proteins involved in apoptosis (Bcl-2, Bax, caspase-3), inflammation (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.



## 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 (i) 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 and IL-13) in BAL fluid, (v) histopathologic findings of lung and nasal tissues, and (vi) expressions of several proteins involved in apoptosis (Bcl-2, Bax, caspase-3), inflammation (COX-2), antioxidation (extracellular SOD, HO-1), and hypoxia (Hif-1α, VEGF) in lung tissue using a murine model of allergic asthma and rhinitis.

## 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

☆ The study was summarized as an Oral Presentation at the 25th Congress of the European Rhinologic Society and 32nd International Symposium of Infection & Allergy of the Nose, Amsterdam, Netherlands, June 22–26, 2014.

* Corresponding author at: Department of Otorhinolaryngology, Head and Neck Surgery, Inha University College of Medicine, 27 Inhang-ro, Junggu, Incheon 400-711, Republic of Korea. Tel.: +82 32 890 2437; fax: +82 32 890 3580.
*E-mail address:* inhaorl@inha.ac.kr (Y.H. Kim).

[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.intimp.2014.07.029

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.

### 2.3. 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 $mm^2$ 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 horseradish 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 conducted 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

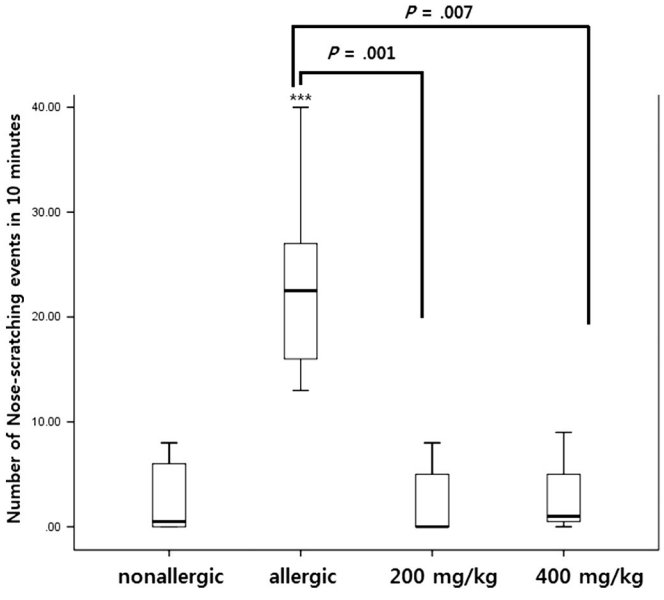


**Fig. 1.** Effect of oral benzaldehyde on murine nose-scratching behavior. The number of nose-scratching events in 10 min after the last intranasal challenge was compared among the nonallergic, allergic, and 200- and 400-mg/kg benzaldehyde groups. Data represent medians and ranges ($n$ = five mice/group). ***$P < 0.001$ compared with the nonallergic group (Kruskal–Wallis test and Mann–Whitney *U*-test).