

Effects of volatile constituents of a rosemary extract on allergic airway inflammation related to house dust mite allergen in mice

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Received March 10, 2005; Accepted April 30, 2005

Abstract. Perilla leaf extract is known to have anti-inflammatory properties. Recently, we have demonstrated that rosmarinic acid, a polyphenolic liquid component in perilla, inhibits the allergic airway inflammation induced by house dust mites (HDMs) *in vivo*. The purpose of this study was to evaluate the effects of intratracheal (i.t.) exposure to volatile constituents of a rosemary extract (VR), gaseous components in perilla, on a murine model of allergic asthma induced by HDM. C3H/HeN mice were treated 7 times weekly with i.t. exposure. The HDM allergen challenge elicited a pulmonary eosinophilic inflammation accompanied by an increase in the lung expression of interleukin (IL)-5, IL-13, and eotaxin. VR inhibited increases in the number of eosinophils, neutrophils, and mononuclear cells around the airways and those in the bronchoalveolar lavage fluid. VR exposure also significantly suppressed the expression of IL-13 enhanced by HDM allergen. These results suggest that i.t. exposure to VR can, at least partially, prevent allergic airway inflammation induced by HDM. The preventive effect is associated with inhibition of the enhanced local expression of IL-13.

Introduction

Allergic asthma is a chronic disease characterized by intermittent exacerbations of airway obstruction, hyper-responsiveness and inflammation. The prevalence of asthma

ranges between 5 and 25% in different studies and has doubled in the industrialized world over the past 20 years (1). Factors implicated in this increase include an altered indoor environment such as warmer housing, increased aeroallergen exposure, increased use of broad spectrum antibiotics with altered bacterial infection profiles, dietary changes, and air pollution (2,3).

Exposure to a number of aeroallergens has been shown to contribute to allergic asthma. Mite allergens have been involved in airway sensitization and allergy-related diseases. Like other inhaled allergens, house dust mite (HDM) sensitization can induce allergic airway inflammation in association with the enhanced lung expression of Th2 cytokines and allergen-specific immunoglobulin production *in vivo* (4).

Despite steady advances in asthma therapies, optimistic regulation of the disease remains to be fully established. Compared to the development of systemic anti-allergic agents, there are few therapeutic items targeting the intratracheal route other than an inhaled steroid and/or β_2 agonist. If other inhaled supplements mixed in indoor air can improve asthmatic manifestations, they would be candidates for alternative regimens. Phytochemicals have been widely used as an option without clinical evidence (5). Dried red perilla has been used as a folk medicine primarily in Japan and China. Perilla leaf extract has been reported to have anti-inflammatory (6), anti-allergic (7,8), and anti-oxidative properties (9). On the other hand, oxidative stress plays a role in the pathogenesis of allergic airway diseases (10). We have recently provided molecular evidence that oral administration of rosmarinic acid, a liquid component in perilla extract, inhibits inflammation induced by HDM allergen *in vivo* (11). We have designed a novel antioxidative system that can generate the volatile constituents in the gaseous phase from rosemary extract (12). We have also shown the anti-oxidative activities of the gaseous exposure to volatile constituents of a rosemary extract (VR) *in vitro* (12). These *in vivo* and *in vitro* studies elicit a hypothesis that treatment with pulmonary exposure to VR may inhibit allergic airway inflammation induced by HDM.

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Key words: rosemary, house dust mite, airway inflammation, cytokine

The aim of the present study was to elucidate the effects of VR on allergic airway inflammation induced by intratracheal instillation of HDM in mice. Furthermore, we elucidated its effects on the allergen-enhanced expression of cytokines and chemokines in the lung.

Materials and methods

Animals. Male C3H/HeN mice (7 weeks old) were obtained from Japan Clea Co. (Tokyo, Japan). Mice were used after body weight and infection were checked for 1 week. Animals were housed in a 12/12-h light/dark chamber room at a temperature between 23 and 25°C and 55 to 70% humidity, and given food and water *ad libitum*. This study adhered to the National Institute of Health guidelines for the use of experimental animals, and was approved by the National Institute for Environmental Studies Animal Care and Use Committee.

Preparation of VR. VR consisted of 1,8-cineole, (+)-camphor, (R)-(+)-limonene, borneol, and (-)- α -pinene (43:41:14:2.5:0.5, by weight percentage, respectively; Fig. 1) (12). A mixture of the above 5 compounds was obtained from Wako Pure Chemical Industry Ltd. (Osaka, Japan) and diluted with dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan), then used as a VR-challenge protocol.

Study protocol. Crude extract of HDM was purchased from LSL Co., Ltd. (Tokyo, Japan), and 1 μ g of HDM was dissolved in 100 μ l of phosphate-buffered saline (PBS) solution (Nissui Pharmaceutical, Tokyo, Japan) at pH 7.4 containing 0.05% Tween-80 (Nacalai Tesque, Kyoto, Japan). Each mouse was anaesthetized with 4% halothane, the solution was instilled via an intratracheal cannula (13), and the animals were randomized into four experimental groups. The vehicle group intratracheally received 100 μ l of PBS containing 0.05% Tween-80, 7 times weekly, and the HDM and HDM + VR groups intratracheally received 1 μ g of HDM extract in the same vehicle. HDM was injected intratracheally, 4 times bi-weekly. HDM + VR groups were exposed intratracheally to two doses of VR (9.6 μ g/body, 46 μ g/body) in 100 μ l of PBS, 7 times weekly. Both vehicle and HDM groups were exposed to equal volumes of PBS with the same protocol as

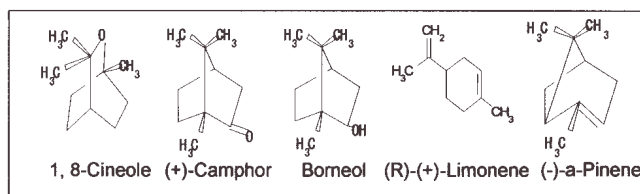


Figure 1. Structures of the volatile constituents of a rosemary extract (VR).

the HDM + VR groups. VR or vehicle was administered intratracheally 3 h before HDM instillation. All mice were sacrificed by deep anesthesia using diethylether 24 h after final HDM instillation.

Bronchoalveolar lavage (BAL). The trachea was cannulated after the collection of blood. Lungs were lavaged with 1.2 ml of sterile saline at 37°C, instilled bilaterally by syringe, and the lavaged fluid was harvested by gentle aspiration. This procedure was conducted twice more. Of the 3.6 ml that was instilled, an average volume of 90% was retrieved, and the amounts did not differ by treatment. Lavage fluid was combined and cooled to 4°C, then centrifuged at 300 g for 10 min; the total cell count was determined on a fresh fluid specimen using a hemocytometer. Differential cell counts were assessed on cytologic preparations. Slides were prepared using Autospin (Sakura Seiki Co., Tokyo, Japan) and stained with Diff-Quik (International reagents Co., Kobe, Japan), and a total of 500 cells were counted under oil-immersion microscopy ($n=8$ in each group). After BAL procedure, the lungs were removed, snap-frozen in liquid nitrogen, and stored at -80°C until they were analyzed for cytokines and chemokines.

Histological evaluation and morphometric analysis for the number of eosinophils, neutrophils, and mononuclear cells around the airways. In another experiment, the lungs were fixed by intratracheal instillation in 10% neutral phosphate-buffered formalin at a pressure of 20 cm H₂O after the collection of blood. After separation of the lobe, 2-mm blocks were taken for paraffin embedding. Sections (3- μ m thick) were stained with hematoxylin and eosin to evaluate the degree of infiltration by eosinophils, neutrophils, or mononuclear

Table I. Effects of volatile constituents in rosemary (VR) on the number of inflammatory cells in bronchoalveolar lavage (BAL) fluid.

Group	n	Eosinophils	Neutrophils	Mononuclear cells
		(x10 ⁴ in BAL fluid)		
Vehicle	8	0.16 $\pm$ 0.07	1.65 $\pm$ 0.48	24.3 $\pm$ 3.23
HDM	8	3.37 $\pm$ 0.91 ^b	3.20 $\pm$ 1.56	19.1 $\pm$ 2.36
HDM + VR (9.2 μ g/body)	8	1.74 $\pm$ 0.33 ^c	1.46 $\pm$ 0.25	19.9 $\pm$ 2.92
HDM + VR (46.0 μ g/body)	8	0.82 $\pm$ 0.22 ^d	0.86 $\pm$ 0.23	14.4 $\pm$ 2.61 ^a

BAL was performed 24 h after the final intratracheal instillation of house dust mite (HDM). Eosinophil, neutrophils, and mononuclear cell counts were assessed on cytological preparations stained with Diff-Quik. Values are the mean $\pm$ SE. n, number of animals. ^a $p<0.05$ versus vehicle, ^b $p<0.01$ versus vehicle, ^c $p<0.05$ versus HDM, ^d $p<0.01$ versus HDM.

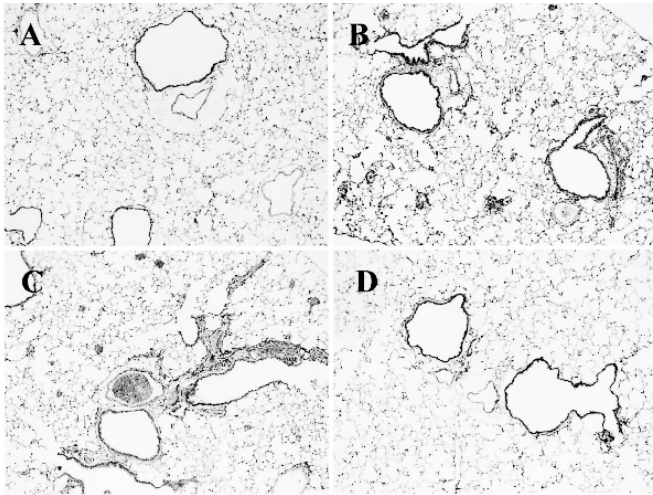


Figure 2. Low-power photomicrographs of lung sections stained with hematoxylin and eosin 24 h after the final intratracheal instillation of house dust mite (HDM). Lung sections were obtained from mice instilled with (A) vehicle, (B) HDM, (C) HDM + VR (9.2 µg/body), and (D) HDM + VR (46 µg/body). Original magnification, $\times 100$.

cells around all airways by two technicians (K.I. and H.T.) in a blinded fashion. The length of the basement membrane of the airways was measured by videomicrometer (Olympus, Tokyo, Japan) in each sample slide. The number of eosinophils, neutrophils, and mononuclear cells around the airways was counted with a micrometer under oil immersion ($n=7$ to 9 in each group). Results were expressed as the number of inflammatory cells per millimeter of basement membrane, as described previously (13).

Quantitation of cytokine and chemokine protein levels in lung tissue supernatants. Frozen lungs were homogenized with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (Sigma, St. Louis, MO), 0.1 mM phenylmethanesulfonyl fluoride (Nacalai Tesque), 1 µM pepstatin A (Peptide Institute, Osaka, Japan), and 2 µM leupeptin (Peptide Institute), as described previously (14). Homogenates were then centrifuged at 105,000 g for 1 h and supernatants were stored at -80°C . Enzyme-linked immunosorbent assays (ELISA) for interleukin

(IL)-5, IL-13, eotaxin, MIP-1 α , and thymus and activation-regulated chemokine (TARC) in the lung tissue supernatants were conducted using matching antibody pairs according to the manufacturer's instructions. The second antibodies were conjugated to horseradish peroxidase. Subtractive readings of 550 nm from the readings at 450 nm were converted to pg/ml using values obtained from standard curves generated with the limits of detection of 5 pg/ml, 1.5 pg/ml, 3 pg/ml, 2 pg/ml, and 5 pg/ml for IL-5, IL-13, eotaxin, MIP-1 α , and TARC, respectively ($n=8$ in each group).

Statistical analysis. Data were reported as mean $\pm$ SE. Differences among groups were analyzed by ANOVA followed by Fisher's protected least-significant difference test (Stat view version 4.0; Abacus Concepts, Inc, Berkeley, CA), as described previously (13). Significance was assigned to p -values <0.05 .

Results

Effects of VR on cell counts in BAL fluid. To estimate the effects of VR on airway inflammation, we first investigated the cellular profiles of BAL fluid (Table I). The number of eosinophils in BAL fluid was significantly greater in the HDM group than in the vehicle group ($p<0.01$). In the presence of allergen challenge, VR significantly inhibited the number of eosinophils in a concentration-dependent manner ($p<0.05$ for 9.2 µg/body and $p<0.01$ for 46 µg/body versus HDM alone). HDM challenge increased the number of neutrophils compared with vehicle challenge, although the difference did not reach statistical significance. VR also suppressed the increased number of neutrophils induced by HDM, but the differences did not achieve statistical significance.

Effects of VR on inflammatory cells around the airways. We next examined whether VR could inhibit airway inflammation induced by HDM in histological examination (Fig. 2). Intratracheal instillation with HDM led to the recruitment of eosinophils and neutrophils around the airways (Fig. 2B), whereas that with vehicle did not (Fig. 2A). The magnitude of peribronchovascular inflammation in the HDM + VR (9.2 µg/body) group (Fig. 2C) was not significantly different from that in the HDM group. Intratracheal exposure

Table II. Effects of VR on the number of inflammatory cells in lung tissue.

Group	n	Eosinophils	Neutrophils	Mononuclear cells
		(n/mm basement membrane)		
Vehicle	7	0.20 $\pm$ 0.08	0.80 $\pm$ 0.22	0.47 $\pm$ 0.16
HDM	8	5.07 $\pm$ 1.10 ^a	2.36 $\pm$ 0.37 ^a	2.47 $\pm$ 0.53 ^a
HDM + VR (9.2 µg/body)	8	5.88 $\pm$ 1.38	2.18 $\pm$ 0.29	2.45 $\pm$ 0.31
HDM + VR (46 µg/body)	9	3.34 $\pm$ 0.50	1.25 $\pm$ 0.24	1.74 $\pm$ 0.23

Four groups of mice were intratracheally inoculated with vehicle, HDM, or HDM + VR (9.6 and 46 µg/body) for 6 weeks. Lungs were removed and fixed 24 h after the last intratracheal administration. Sections were stained with Diff-Quik for the measurement of inflammatory cells around the airway. Results are expressed as the number of cells per length of basement membrane of airways. Results are expressed as number of cells per length of basement membrane of airways. Values are mean $\pm$ SE. n, number of animals. ^a $p<0.05$ versus vehicle.

Table III. Protein levels of cytokines and chemokines in lung tissue supernatants.

Group	n	IL-5	IL-13	Eotaxin	MIP-1 α	TARC
		(pg/total lung tissue supernatants)				
Vehicle	8	13.2 $\pm$ 3.4	5.5 $\pm$ 3.6	90.8 $\pm$ 16.0	0.2 $\pm$ 0.2	74.2 $\pm$ 21.6
HDM	8	99.7 $\pm$ 15.4 ^a	267.0 $\pm$ 46.8 ^a	660.7 $\pm$ 106.5 ^a	13.2 $\pm$ 8.6	100.2 $\pm$ 14.3
HDM + VR (46 μ g/body)	8	81.1 $\pm$ 17.9	134.5 $\pm$ 27.6 ^b	432.6 $\pm$ 73.2	6.2 $\pm$ 2.8	66.5 $\pm$ 12.4

Lungs from mice were obtained 24 h after the last administration of vehicle or HDM. Cytokine and chemokine levels in the lung tissue supernatants were measured by enzyme-linked immunosorbent assays. n, number of animals. Results are mean $\pm$ SE. ^ap<0.01 versus vehicle group. ^bp<0.01 versus HDM.

to VR (46 μ g/body) inhibited the allergic inflammation induced by HDM as indicated by a reduction in recruitment of inflammatory cells (Fig. 2C). To quantitate the infiltration of inflammatory cells around the airways, we estimated the magnitude of histopathological changes in lung specimens stained with Diff-Quik (Table II). Instillation of HDM significantly increased the number of infiltrated eosinophils, neutrophils, and mononuclear cells compared with vehicle exposure (p<0.05). The 46 μ g/body of VR suppressed the increased number of cells, although the difference did not reach statistical significance.

Effect of VR on cytokine and chemokine levels in lung tissue. To elucidate the effects of VR on the lung expression of cytokines and chemokines, we quantitated protein levels of IL-5, IL-13, eotaxin, MIP-1 α , and TARC in lung tissue supernatants 24 h after the last intratracheal instillation (Table III). The HDM group showed increases in IL-5, IL-13, and eotaxin levels compared with the vehicle group (p<0.01). The levels were lower in the HDM + VR (46 μ g/body) group than the HDM group (p<0.01 for IL-13, and N.S. for IL-5 and eotaxin). HDM challenge increased levels of MIP-1 α and TARC without significance, and the levels were lower in the HDM + VR (46 μ g/body) group than the HDM group, although the difference did not reach statistical significance.

Discussion

In the present study, HDM challenge caused allergic airway inflammation in mice, which could be partially inhibited by pretreatment with VR. The inhibitory effects of VR are associated with a decrease in the enhanced local protein expression of IL-13.

Previous *in vitro* and *in vivo* studies have shown that perilla leaf extract inhibits histamine release, antigen-specific IgE production, and passive cutaneous anaphylaxis reaction (7,8). However, the ingredients in perilla leaf responsible for its anti-allergic reactions remain unclear. We focused on rosmarinic acid, a liquid component in perilla leaf extract, and demonstrated that oral administration of rosmarinic acid inhibits allergic inflammation induced by HDM *in vivo* (11). The inhibitory effects have been concomitant with the suppression of increases in cytokines, such as IL-4 and IL-5, and chemokines such as eotaxin and macrophage chemo-attractant protein-1, and allergen-specific antibody (11). The study raises the possibility that gaseous components in perilla

can also inhibit allergic airway inflammation *in vivo*. In the present study, the number of BAL eosinophils was significantly smaller in the HDM + VR group than the HDM group, which indicates that VR can ameliorate allergic airway inflammation related to HDM challenge. On the other hand, the magnitude of inflammation around airways in the lung specimens was shown to be less in HDM + VR groups than the HDM group, although it did not achieve statistical significance (Table II). This discrepancy in the effects of VR may be explained by its exposure route. Intratracheal exposure to VR might result in more prominent effects on alveolar spaces than those on interstitial spaces. Our previous study has shown that oral administration of rosmarinic acid improved allergic airway inflammation in both BAL and histopathological findings (11). This suggests that rosmarinic acid administered through a systemic route could reduce inflammation in the lung parenchyma and alveolar spaces. The present results and our previous observations (11) suggest that intratracheal exposure to VR should be effective in inhibiting infiltration of the inflammatory cells within the alveolar spaces.

A headspace analysis has shown that rosemary extract includes 5 typical volatile compounds, i. e. 1,8-cineole, (+)-camphor, (R)-(+)-limonene, borneol, and (-)- α -pinene (43:41:14:2.5:0.5 weight percentage, respectively) (12). Also, the *in vitro* study has shown that 1,8-cineole is most attributable to the antioxidative effects of VR among the compounds (12). Thus, future research should identify the compounds responsible for the anti-inflammatory effects in the present model in order to synthesize more potent anti-allergic supplements.

IL-13 is induced by the airway challenge with HDM (15), and has been shown to be a critical mediator in the effector phase of allergic airway inflammation (16,17). Specifically, IL-13 plays an essential role in the pathogenesis of airway hyperresponsiveness, pulmonary mucous cell hyperplasia (18), and airway remodeling (19). In the present study, VR significantly inhibited the enhanced lung expression of IL-13 by HDM. Thus, VR inhibited the HDM-induced airway inflammation, at least in part, through the suppression of IL-13. Alternatively, the marked suppression of cytokine expression in lung tissue supernatants may indicate that inhaled VR could have an effect on the lung parenchyma via alveolar spaces. However, VR could not inhibit goblet cell proliferation induced by HDM in the designated period of our study (data not shown). A prolonged exposure to VR may ameliorate chronic features of asthma.

In addition to other allergic airway inflammation models, HDM-induced airway inflammation is reportedly concomitant with the enhanced lung expression of Th2 cytokines such as IL-13 and IL-5, and chemokines such as eotaxin *in vivo* (15). On the other hand, the neutralization of MIP-1 α significantly reduced the recruitment of eosinophil during the allergic airway response. TARC induces chemotaxis for Th2 type CD4⁺ T lymphocytes (20) and plays an important role in the pathogenesis of allergic airway inflammation and hyper-responsiveness (21). In the present study, VR exposure suppressed the lung expression of all cytokines and chemokines without significance. Therefore, the mechanisms by which VR ameliorated the HDM-induced airway inflammation can be explained, in part, through the inhibition of these cytokines and chemokines. Indeed, the total levels of IL-5, eotaxin, MIP-1 α , and TARC in the lung tissue supernatants were significantly greater in the HDM group than the vehicle group (986.9 $\pm$ 78.9 versus 178.3 $\pm$ 39.9; $p < 0.01$), in which they were significantly inhibited by VR (46 μ g/body) treatment (586.5 $\pm$ 97.2; $p < 0.05$).

In conclusion, intratracheal exposure to VR at least partly inhibits allergic airway inflammation induced by HDM, and the inhibition is concomitant with the suppression of lung expression of IL-13. These results indicate that VR exposed through an intratracheal route may be a candidate for supportive therapy of allergic airway diseases such as asthma.

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