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RESEARCH ARTICLE



Suppression of leukotriene B₄ production is involved in the anti-pruritic action of *Grifola frondosa* in pollen allergy-induced ocular itching in mice

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ABSTRACT

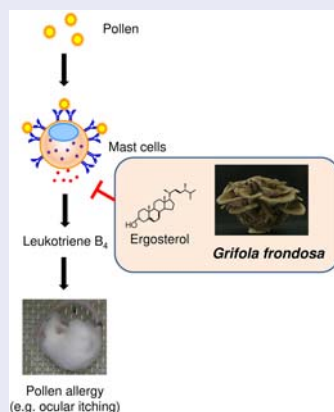
Grifola frondosa (*G. frondosa*) is an edible mushroom consumed as a health food in Asia that has recently been reported to have anti-allergic effects. In this study, we investigated the effects of *G. frondosa* on pollen allergy-induced ocular itching in mice. *G. frondosa* was boiled and freeze-dried (heat-treated *G. frondosa*). Oral administration of heat-treated *G. frondosa* (0.1 and 1 g/kg) suppressed pollen allergy-induced ocular itching and plasma extravasation but not histamine-induced plasma extravasation. Oral administration of ergosterol, an active ingredient in *G. frondosa* (5 and 50 mg/kg), or zileuton (100 mg/kg), which is an inhibitor of 5-lipoxygenase (the main enzyme required for leukotriene B₄ production), inhibited pollen allergy-induced ocular itching. In addition, ergosterol inhibited antigen-induced leukotriene B₄ production in mast cells. These findings suggest that *G. frondosa* and its active ingredient ergosterol prevent pollen allergy-induced itching through the inhibition of leukotriene B₄ production in mast cells.

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1. Introduction

Pollen allergy, also known as hay fever, is caused by pollen from plants, such as ragweed, grass, birch, and cedar, and has long been recognized as a major worldwide health concern (Canonica et al., 2007; Sakashita et al., 2010; Schatz, 2007). The number of patients suffering from pollen allergies has increased in recent years. In Japan, a recent study showed that over 35% of the population aged 20–49 years were sensitized to Japanese cedar pollen (Sakashita et al., 2010). Pollen-induced allergy is an IgE-mediated inflammatory condition with various symptoms, such as rhinorrhea in nasal mucosal membranes, sneezing, nasal congestion, ocular redness, tearing, and itching (Skoner, 2001). Moreover, these symptoms are associated with reduced quality of life and decreased work productivity (Petersen et al., 2008). The treatment of pollen allergies conventionally involves oral antihistamines and leukotriene receptor antagonists. More recently, sublingual desensitization therapy with allergen extracts has been increasingly administered. However, these pharmacotherapies and immunotherapies are associated with adverse effects, including drowsiness, dry mouth, chest congestion, upset stomach, and possibly anaphylaxis (Meltzer et al., 2017; Worm et al., 2013).

Grifola frondosa (*G. frondosa*) is a popular edible mushroom in Asia consumed as a health food and/or traditional medicine. Previous studies have shown that *G. frondosa* has pharmacological effects, such as anti-cancer (Deng et al., 2009; Masuda et al., 2017; Wesa et al., 2015), anti-diabetes (Aoki et al., 2018; Chen et al., 2019; Xiao et al., 2015), anti-inflammatory, and immunomodulatory activities (Lee et al., 2010; Tsao et al., 2013). In addition, it was recently reported that *G. frondosa* exhibits anti-allergic activity (Park et al., 2015). In our previous study, *G. frondosa* extract, its active component ergosterol, and its derivatives inhibited antigen-induced activation, including degranulation and histamine release, in the rat basophilic leukemia cell line RBL-2H3 (Kawai et al., 2018). Furthermore, oral administration of *G. frondosa* extract or ergosterol suppressed allergic reactions in ovalbumin-sensitized mice (Kawai et al., 2019). In this study, we investigated the effects of *G. frondosa* on pollen allergy-induced ocular itching in mice. In addition, we investigated the mechanisms underlying the anti-pruritic action of *G. frondosa*.

2. Materials and methods

2.1. Animals

Male ICR mice (4–5 weeks old at the start of this study) were used in all experiments. All mice were purchased from Japan SLC (Shizuoka, Japan) and kept in a room under controlled temperature (ca. 22°C), humidity (ca. 55%), and light (from 7:00am to 7:00pm). Food and water were available *ad libitum*. The study protocol was approved by the Committee for Animal Experiments at the University of Toyama and conducted in accordance with the guidelines of the Japanese Pharmacological Society.

2.2. Materials

Fruit bodies of *G. frondosa* were cultivated by Hokuto Corporation (Nagano, Japan). The fruiting body of *G. frondosa* was cut into small pieces and boiled in an equal amount of

distilled water for 10 min. Heat-treated *G. frondosa* and the broth were freeze-dried and powdered (HTGF). Ergosterol, histamine dihydrochloride, and diphenhydramine hydrochloride (DPH) were purchased from Wako Pure Chemical Corporation (Osaka, Japan). Tranilast (TRL) and zileuton were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Cayman Chemical (Ann Arbor, MI, USA), respectively. Ragweed pollen (RP) from *Ambrosia trifida* (*A. trifida*) and dinitrophenyl-human serum albumin (DNP-HSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Sensitization and challenge of mice with RP

A mouse model of ocular allergy was used in accordance with a previously reported method (Andoh et al., 2012). RP from *A. trifida* was suspended in a gel containing 40 mg/mL aluminum hydroxide (Imject® Alum, Pierce, Rockford, IL, USA) and mixed for ≥ 30 min with a vortex mixer. Five-week-old male ICR mice were immunized with subcutaneous injections of RP (100 μ g) in adjuvant alum gel (4 mg) into their caudal back in a volume of 100 μ L twice a week for 3 weeks. After sensitization, RP-sensitized mice received an oral administration of HTGF resuspended in tap water or other drugs [TRL, DPH, chlorpheniramine maleate (CLP), and zileuton] in 0.5% carboxymethyl cellulose (CMC, Wako Pure Chemical Ind., Osaka, Japan). One hour after the oral administration of samples, mice were challenged by subconjunctival injection of RP allergen into the unilateral palpebra at a dose of 2 μ g/site in a volume of 2 μ L.

2.4. Behavioural observation

Behavioural observations were performed in accordance with a previously reported method (Andoh et al., 2012). Briefly, mice were placed in an acrylic cage consisting of four equal-sized cells (13 $\times$ 9 $\times$ 40 cm) for at least 1 h to allow for acclimation. They were returned to the same cells immediately after injection of the allergen or pruritogen, and their behaviour was videotaped for 2 h. All lab personnel were kept out of the observation room during this time. The number of times a mouse scratched its eye with its hind-paw was counted during video playback. A bout of scratching was defined as a mouse stretching its hind-paw on the treated side toward its eye, leaning its head toward the paw, rapidly scratching its eye several times for approximately 1 s, and lowering its hind-paw.

2.5. Histamine stimulation

Five-week-old male ICR mice were orally administered the samples as described in 2.3. One hour later, mice were stimulated by subconjunctival injection of histamine into the unilateral palpebra at a dose of 100 nmol/site in a volume of 2 μ L, as described previously (Zhang et al., 2010).

2.6. Plasma extravasation

Mice were given a 150- μ L intravenous injection of 1% Evans blue dissolved in physiological saline and subconjunctivally challenged with RP allergen 20 min later. Under deep

pentobarbital (80 mg/kg, intraperitoneal) anesthesia, mice were euthanized by cervical dislocation 20 min after challenge, and the ocular skin, including the conjunctiva, was immediately isolated. The bluish region of the sample was removed using an 8-mm diameter punch and incubated in 1 mL of dimethyl sulfoxide overnight. The concentration of dye extracted was determined spectrophotometrically at 620 nm.

2.7. Preparation and stimulation of bone marrow-derived mast cells (BMMCs)

The preparation and stimulation of BMMCs were performed in accordance with a previously reported method (Kawai et al., 2019). The sensitized BMMCs were then challenged with DNP-HSA (20 ng/mL) in the presence or absence of samples. Thirty minutes later, the supernatant was collected to measure leukotriene B₄ production.

2.8. Measurement of leukotriene B₄ (LTB₄) production

The supernatant (50 µL) was transferred to a 96-well enzyme-linked immunosorbent assay (ELISA) plate. The amount of LTB₄ was determined using ELISA kits in accordance with the manufacturer's instructions (Cayman Chemical, Co. Michigan, USA).

2.9. Statistical analysis

Data are presented as the mean ± standard error. The statistical significance of differences in means among groups was analyzed using a one-way analysis of variance (ANOVA), followed by a post-hoc Holm–Šidák test. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of HTGF on RP allergy-induced ocular scratching and plasma extravasation in mice

Subcutaneous injections of RP and alum twice a week for 3 weeks increased the serum concentration of total IgE in ICR mice (data not shown). In mice sensitized by RP, challenge with RP allergens in the eyelid markedly increased hind-paw scratching of the treated eye and plasma extravasation in the eyelid (Figure 1a,b). However, oral pretreatment with HTGF significantly and dose-dependently inhibited ocular scratching and plasma extravasation induced by RP allergens at a dose of 0.1 and 1 g/kg. Oral pretreatment with TRL also significantly inhibited these symptoms at a dose of 300 mg/kg (Figure 1a,b).

3.2. Effects of HTGF on histamine-induced plasma extravasation in mice

In ICR mice, challenge with histamine in the eyelid increased plasma extravasation (Figure 2). In addition, oral pretreatment with DPH and CLP decreased plasma extravasation in these mice (Figure 2). Although there was a tendency toward decreased plasma extravasation in the eyelids of mice, oral pretreatment with HTGF did not significantly decrease plasma extravasation.

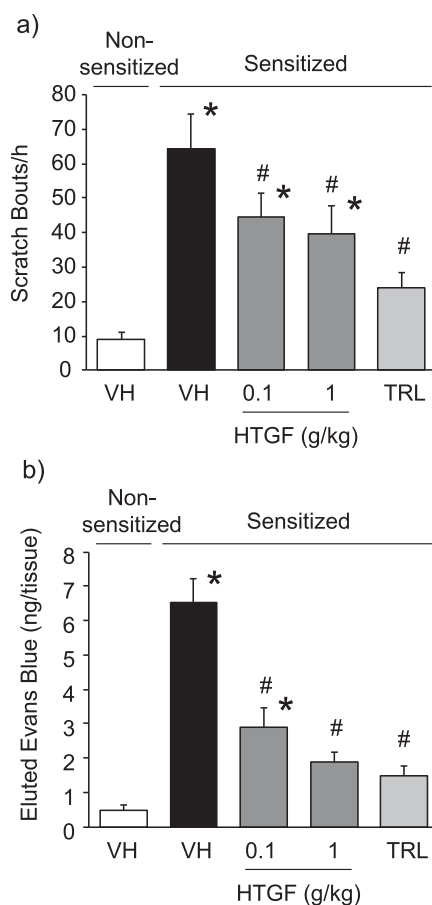


Figure 1. Effects of heat-treated *G. frondosa* (HTGF) on ragweed pollen (RP) allergen-induced ocular allergy in sensitized mice. (a) Effects of HTGF on RP allergen-induced ocular scratching. (b) Effects of HTGF on RP allergen-induced plasma extravasation. Mice immunized with RP and alum (Sensitized) or alum only (Non-sensitized) were orally administered vehicle (VH), HTGF (0.1 and 1 g/kg), and tranilast (TRL, 300 mg/kg) 1 h before injection of the PR allergen. Values are presented as the mean and SEM ((a), $n = 13-14$; (b), $n = 8$). Significance: * $p < 0.05$ vs. non-sensitized mice with VH. # $p < 0.05$ vs. sensitized mice with VH.

3.3. Effects of ergosterol on RP allergy-induced ocular scratching

Subconjunctival injection of RP significantly increased ocular scratching in RP-sensitized mice compared with non-sensitized mice (Figure 3). Similar to the 5-lipoxygenase inhibitor zileuton, peroral preadministration of ergosterol significantly inhibited RP allergy-induced scratching compared with the vehicle (Figure 3).

3.4. Ergosterol inhibits leukotriene B_4 production in antigen-stimulated BMMCs

To investigate the effects of ergosterol, an active component in *G. frondosa*, on antigen-induced leukotriene production in mast cells, we measured 5-lipoxygenase and leukotriene A_4 hydrolase mRNA expression (data not shown) and LTB $_4$ production in

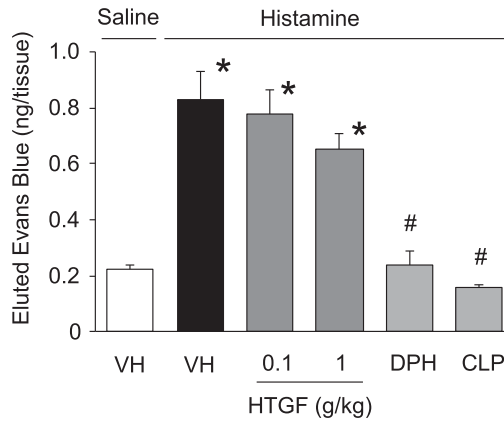


Figure 2. Effects of heat-treated *G. frondosa* (HTGF) on histamine-induced plasma extravasation. Mice were orally administered vehicle (VH), HTGF (0.1 and 1 g/kg), diphenhydramine hydrochloride (DPH, 10 mg/kg), and chlorpheniramine maleate (CLP, 30 mg/kg) 1 h before subconjunctival injection of histamine (100 nmol/site) or physiological saline. Values are presented as the mean and SEM ($n = 6$). Significance: * $p < 0.05$ vs. saline + VH, # $p < 0.001$ vs. histamine + VH.

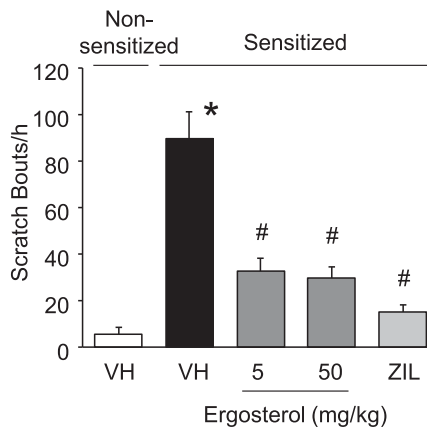


Figure 3. Effects of ergosterol on ragweed pollen (RP) allergen-induced ocular allergy in sensitized mice. Mice immunized with RP and alum (Sensitized) or alum only (Non-sensitized) were orally administered vehicle (VH), ergosterol (5 and 50 mg/kg), and zileuton (ZIL, 100 mg/kg) 1 h before injection of the PR allergen. Values are presented as the mean and SEM ($n = 4$). Significance: * $p < 0.05$ vs. non-sensitized mice with VH. # $p < 0.05$ vs. sensitized mice with VH.

antigen-stimulated BMMCs. LTB_4 production was significantly suppressed by ergosterol at concentrations of 10, 20, and 50 $\mu\text{g/mL}$ in a dose-dependent manner (Figure 4).

4. Discussion

In this study, the effects of HTGF and its active ingredient ergosterol on pollen allergy-induced ocular itching and the underlying mechanisms were investigated using mice and mast cells. Specifically, we used HTGF for *in vivo* experiments and ergosterol for *in vivo*

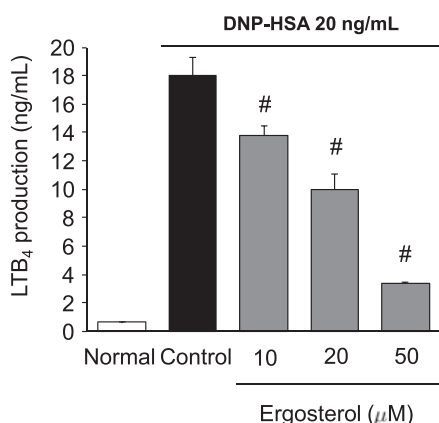


Figure 4. Effect of ergosterol on LTB₄ production in antigen-stimulated BMMCs. IgE-sensitized BMMCs pretreated with ergosterol for 20 min were stimulated with DNP-HSA (20 ng/mL) for 30 min, and the production of LTB₄ was measured. Each bar represents the mean $\pm$ SEM ($n = 4$). Significance: # $p < 0.05$ vs Control.

and *in vitro* experiments. Although HTGF concentrations cannot be converted accurately because dry *G. frondosa* contains 0.5% ergosterol, doses of 0.1 and 1 g/kg HTGF correspond to doses of 0.5 and 5 mg/kg ergosterol, respectively. Therefore, the doses used in our *in vivo* experiments are considered relevant. Although the kinetics of ergosterol in humans and mice have not been elucidated, we used concentrations of 10–50 μ M in the *in vitro* experiments. The total blood volume in mice accounts for 7.3% of their total body weight (Wu et al., 1981). For a mouse weighing 40 g, doses of 0.5 and 5 mg/kg ergosterol (MW: 396.65) correspond to concentrations of 0.017 and 0.17 mM ergosterol, respectively. Thus, the doses used in our *in vitro* experiments are also considered relevant.

Oral administration of HTGF and ergosterol inhibited pollen allergy-induced ocular itching in mice. The mouse model of immediate pollen allergy-induced ocular itching used in this study critically depends on mast cells (Andoh et al., 2012). Recent reports have shown that *G. frondosa* extracts and ergosterol inhibit the antigen-induced histamine release of mast cells, and the oral administration of *G. frondosa* extracts or ergosterol inhibits antigen-induced allergic responses by suppressing histamine release in mast cells in mice (Kawai et al., 2018; Kawai et al., 2019). Histamine is partly involved in pollen allergy-induced ocular itching (Andoh et al., 2012). However, oral HTGF did not inhibit histamine-induced vascular permeability, suggesting that HTGF does not have H₁ histamine receptor antagonistic activity.

LTB₄ is biosynthesized from arachidonic acid via leukotriene A₄ hydrolase and 5-lipoxygenase and is an itch mediator (Andoh et al., 2012; Andoh & Kuraishi, 1998). In mice sensitized with RP, both histamine and LTB₄ are involved in pollen allergy-induced ocular itching (Andoh et al., 2012). LTB₄ directly activates primary afferents (Andoh & Kuraishi, 2005) and elicits itching (Andoh & Kuraishi, 1998). Mast cells are reported to produce LTB₄ (Miyahara et al., 2009; Yamashita et al., 2000). Specifically, mast cells express H₄ histamine receptors (Lippert et al., 2004), and the activation of these receptors leads to LTB₄ production (Takeshita et al., 2003). Therefore, it is

suggested that histamine released from mast cells not only causes itching but also acts in an autocrine/paracrine manner to produce LTB₄, resulting in enhanced itching. Taken together, LTB₄ is important for the induction of allergic itching. In this study, ergosterol inhibited pollen allergy-induced itching in mice and LTB₄ production in antigen-stimulated BMMCs (Figures 3 and 4). Thus, these results suggest that the inhibition of LTB₄ production is involved in the anti-pruritic activity of ergosterol (and HTGF).

The mechanisms by which ergosterol inhibits allergic reaction-induced LTB₄ production remain unknown. IgE-mediated mast cell activation induces LTB₄ production (Miyahara et al., 2009; Yamashita et al., 2000). As a possible mechanism, our recent study demonstrated that ergosterol inhibits the antigen-induced aggregation of high-affinity IgE receptors on mast cells (Kawai et al., 2018). Therefore, the inhibition of high-affinity IgE receptor aggregation by ergosterol may be involved in the regulation of LTB₄ production.

In addition to ergosterol, vitamin D has been reported as an active ingredient in *G. frondosa* with anti-allergic effects (Liu et al., 2017; Cho et al., 2019). Oral administration of vitamin D in humans did not affect the serum concentration of LTB₄ compared with the placebo (Gendelman et al., 2015). However, vitamin D inhibits mast cell proliferation and calcium ionophore-induced histamine release in mast cells (Toyota et al., 1996). Therefore, vitamin D may also be involved in the anti-pruritic and anti-inflammatory effects of HTGF.

5. Conclusion

Our findings suggest that *G. frondosa* and its active ingredient ergosterol prevent pollen allergy-induced itching by suppressing histamine release and lipid mediators (e.g. LTB₄) in mast cells. The number of patients experiencing pollen allergies is increasing worldwide. The currently available anti-allergic drugs used to treat these allergies are associated with adverse side effects. In contrast, *G. frondosa* is a safe food with no known side effects. The amount of *G. frondosa* used in this study can be converted into an amount that can be safely ingested daily by humans. Taken together, the results strongly suggest that *G. frondosa* is a valuable medicinal food that may be used as an alternative medicine for the treatment of pollen allergies.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability

Experimental data used to support the findings of this study are available from the corresponding author upon request.

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