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Inhibitory Effects of the Methanol Extract of *Ganoderma lucidum* on Mosquito Allergy-Induced Itch-Associated Responses in Mice

Tsugunobu Andoh¹, Qun Zhang¹, Takumi Yamamoto¹, Manabu Tayama¹, Masao Hattori², Ken Tanaka³, and Yasushi Kuraishi^{1,*}

¹Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan

²Division of Metabolic Engineering, ³Division of Pharmacognosy, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0914, Japan

Received July 10, 2010; Accepted August 27, 2010

Abstract. Recently, we showed that a methanol extract of *Ganoderma lucidum* inhibits scratching, an itch-related response, induced by intradermal injections of some pruritogens in mice. The present study investigated whether *G. lucidum* extract would inhibit allergic itch. In mice sensitized with an extract of salivary gland of mosquito (ESGM), an intradermal injection of ESGM elicited scratching, which was suppressed by oral administration of *G. lucidum* extract (100 and 300 mg/kg). The scratching was inhibited by the H₁ histamine-receptor antagonist azelastine, but not by the peripherally acting H₁-antagonist terfenadine, at the oral dose of 30 mg/kg. In sensitized mice, ESGM increased the activity of cutaneous nerve, which was suppressed by *G. lucidum* extract (300 mg/kg). Although terfenadine (30 mg/kg) inhibited plasma extravasation induced by ESGM in the sensitized mice, *G. lucidum* extract (300 mg/kg) was without effect. These results suggest that *G. lucidum* extract relieves allergic itch through a peripheral action. The results support the idea that mast cells and H₁ histamine receptors are not the primary sites of the antipruritic action of *G. lucidum* extract.

Keywords: *Ganoderma lucidum*, itch and scratch, mosquito allergy, cutaneous nerve, plasma extravasation

Introduction

Itch, a skin sensation that provokes the desire to scratch, is a chief complaint among patients with pruritic diseases. Severe pruritus is an important issue related to quality of life. H₁ histamine-receptor antagonists are the drugs of first choice for the treatment of itch, but many pruritic diseases, except acute urticaria, respond poorly to H₁ histamine-receptor antagonists (1). Thus, the development of new drugs effective against antihistamine-resistant pruritus is expected.

The fruiting body of *Ganoderma lucidum* (Leyss. Ex. Fr.) KARST has been used as a crude drug in China, Japan, and Korea for the treatment of hypertension, chronic hepatitis, hyperglycemia, cancer, and chronic bronchitis

(2, 3). The extract of *G. lucidum* has been shown to inhibit the release of histamine from mast cells in vitro (4, 5), suggesting that *G. lucidum* extract has anti-allergic activity and is effective against mast cell-mediated itch. We have recently found that single oral administration of a methanol extract of *G. lucidum* inhibits scratching, an itch-associated response, induced by intradermal injections of serotonin and proteinase-activated receptor 2 (PAR₂) agonist in mice (6). In contrast, this extract does not suppress scratching induced by intradermal injections of histamine, compound 48/80, and substance P (6). Compound 48/80-induced scratching is at least partly mediated by histamine released from mast cells (7), and substance P-induced scratching is partly mediated by the release of several pruritogens from keratinocytes (8, 9). Therefore, mast cells and keratinocytes may not be important targets of the anti-pruritic action of *G. lucidum* extract. Itch induced by stimulation of PAR₂ and H₁ histamine receptors may be mediated by separate primary

*Corresponding author. kuraishi@pha.u-toyama.ac.jp
Published online in J-STAGE on October 8, 2010 (in advance)
doi: 10.1254/jphs.10180FP

afferents (10–12), and allergic itch may be mediated by primary afferents similar to those of PAR₂-associated itch (12). Thus, the findings that *G. lucidum* extract inhibits scratching induced by stimulation of PAR₂ receptor raise the possibility that this extract inhibits allergic itch.

There are no apparent responses to mosquito bites and an injection of an extract of salivary glands of mosquito (ESGM) in naive mice. However, after repeated exposure, mice show marked scratching after mosquito bites and ESGM injection (13). Although the non-sedative H₁ histamine-receptor antagonist terfenadine suppresses plasma extravasation, it does not inhibit scratching responses to mosquito bites and ESGM injection in sensitized mice (13, 14). The allergic itch response has been suggested to be mediated by 5-lipoxygenase metabolites (15). In the present experiments, we investigated the efficacy of *G. lucidum* extract against allergic itch by using a mouse model of mosquito allergy.

Materials and Methods

Animals

Five-week-old male ICR mice (Japan SLC, Ltd., Shizuoka) were used. They were housed under controlled temperature ($23 \pm 1^\circ\text{C}$), humidity ($60 \pm 5\%$), and light (lights on from 0800 to 2000). Food and water were freely available. Procedures for animal experiments were approved by the Committee for Animal Experiments at the University of Toyama and were conducted in accordance with the guidelines of The Japanese Pharmacological Society.

Materials

Azelastine hydrochloride (Sigma, St. Louis, MO, USA) and terfenadine (Sigma) were suspended in 0.5% carboxymethyl cellulose and administered orally 30 min before allergen challenge. The doses of azelastine hydrochloride and terfenadine were selected from the previous reports on itch-related response and plasma extravasation, respectively (13, 14).

Preparation of methanol extract of *G. lucidum*

The dried powder (1 kg) of *G. lucidum* [harvested in China; lot No. LUD-703(GHI); Koshiro Co., Ltd., Kyoto] was extracted three times with methanol for 3 h, and the methanol extract was then dried with a yield of 17.8%, as described previously (6); more than 30 g of extract is kept at the Department of Applied Pharmacology. The extract was suspended in 5% gum arabic before use and was administered orally 1 h before ESGM challenge.

Chromatographic fingerprint

A dried extract of *G. lucidum* was dissolved in methanol at a concentration of 10 mg/ml and filtered through a 0.2- μm Millipore filter. One microliter of this solution was used for LC-MS analysis with a LC-IT-TOF mass spectrometer, equipped with an electrospray ionization interface (Shimadzu, Kyoto). The electrospray ionization parameters were as follows: source voltage, -3.5 kV ; capillary temperature, 200°C ; and nebulizer gas, 1.5 l/min. The mass spectrometer was operated in negative ion mode scanning from m/z 100 to 2000. A symmetry C₁₈ column (2.1-mm inside diameter $\times$ 150 mm; Waters, Milford, MA, USA) was used with a column temperature of 40°C . The mobile phase comprised a binary eluent of 0.1% aqueous formic acid (A) and CH₃CN (B) using a liner gradient program of 30%–32% B, 0–40 min; 32%–40% B, 40–60 min; 40% B, 60–65 min; 40%–82% B, 65–70 min; and 82%–100% B, 75–80 min. The flow rate was 0.2 ml/min. Annotation of an unknown compound was preferably assigned to a compound that had been previously reported on *G. lucidum* by comparing the high-resolution mass spectral data with those in the literature (16). The chromatographic fingerprint is shown in Fig. 1.

Sensitization and challenge

The thoraxes, including the salivary gland, were isolated from female mosquitoes (*Aedes albopictus*), homogenized in distilled water, and centrifuged at $9,000 \times g$ for 30 min as described (13). The extract was lyophilized and kept at -80°C until use. ESGM was dissolved in

Fig. 1. Chromatograms of methanol extract of *G. lucidum*. For experimental procedures, see Materials and Methods. 1, ganoderic acid C2; 2, lucidenic acid N; 3, ganoderic acid B; 4, ganoderic acid K; 5, ganoderic acid H; 6, ganoderenic acid D; 7, ganoderic acid F; 8, ganolucidic acid D; 9, 12-acetoxyganoderic acid F.

saline before use and was injected intradermally into the caudal or rostral back for sensitization or challenge, respectively, twice a week for 4 weeks; the dose and volume of injection per site were 10 μ g and 50 μ l, respectively.

Behavioral observation

The animals were put into an acrylic cage composed of four equal-sized cells (13 $\times$ 9 $\times$ 40 cm) for at least 1 h for acclimatization. Immediately after the intradermal injection of ESGM, they were put back into the same cells and their behavior was videotaped for 1 h with personnel kept out of the observation room. The frequency of scratching around the injected site by the hind paws was counted by video playback. A series of the following movements was counted as one bout of scratching (17): A mouse stretched either of its hind paws toward the injection site, leaned its head toward it, rapidly scratched several times for about 1 s, and lowered the hind paw.

Recording of the activity of cutaneous nerve

Procedures for recording the activity of cutaneous nerve were roughly similar to those previously described (14, 18). Briefly, under pentobarbital (80 mg/kg) anesthesia, the skin of the rostral back was turned inside out, and the activity of the exposed cutaneous nerve innervating the rostral back was recorded extracellularly using bipolar electrodes of silver wire (Unique Medical Co., Ltd., Tokyo) and an AC amplifier (MEG-2100; Nihon Kohden, Tokyo) with a band-pass filter (high-cut filter, 3 kHz; low-cut filter, 150 Hz). ESGM was injected intradermally into the receptive field.

Plasma extravasation

Plasma extravasation was determined as described (19). Briefly, 1% Evans blue dissolved in saline was injected into the tail vein in a volume of 0.15 ml and intradermal challenge with ESGM was performed 20 min later. The skin was isolated 20 min after challenge under deep pentobarbital anesthesia, and the bluish area of the skin was punched out (1.7-cm diameter). The skin sample was incubated in 2 ml of dimethyl sulfoxide overnight, and the concentration of dye extracted was determined spectrophotometrically at 620 nm.

Data processing

Data are presented as means and S.E.M. Statistical significance was analyzed using Dunnett's or Bonferroni multiple comparisons; $P < 0.05$ was considered significant.

Results

Effects of *G. lucidum* extract on scratching

An intradermal injection of ESGM in the rostral back did not elicit scratching in naive mice (Fig. 2A). ESGM was then injected into the caudal back of the same mice three times twice a week, and the fifth injection into the rostral back also did not elicit scratching, with the number of scratch bouts similar to that observed in naive mice injected with saline (Fig. 2A). In contrast, the eighth injection of ESGM caused significant increase in scratching (Fig. 2A). The responses peaked during the initial 10-min period and almost subsided by 60 min (Fig. 2B).

Fig. 2. Effects of *G. lucidum* extract and H₁ histamine-receptor antagonists on scratching induced by challenge with mosquito allergen. Scratch bouts toward the rostral back were counted for 60 min after challenge. A) Extract of salivary gland of mosquito (10 μ g/site) was injected intradermally into the rostral (1st, 5th, and 8th injections) or caudal back (2nd, 3rd, 4th, 6th, and 7th injections) of the mice. Saline (SL) was injected into the naive mice. * $P < 0.05$ vs. SL. B) Time course of scratching after the 8th injection of extract of salivary gland of mosquito (10 μ g/site). C) *G. lucidum* extract and vehicle (VH) were administered orally 1 h before challenge (the 8th injection of extract of salivary gland of mosquito). * $P < 0.05$ vs. VH (Dunnett's multiple comparisons). D) Azelastine hydrochloride (AZL) and terfenadine (TRF) were administered orally at a dose of 30 mg/kg 30 min before challenge. * $P < 0.05$ vs. VH (Dunnett's multiple comparisons). Values represent means and S.E.M. for 8 (A, B, and D) and 16 animals (C).

ESGM-induced scratching in the sensitized mice was inhibited by pretreatment with *G. lucidum* extract at oral doses of 100 and 300 mg/kg; the effect was significant at a dose of 300 mg/kg (Fig. 2C). The inhibition was marked at the first 10-min period; the number of scratch bouts was 76.4 ± 11.3 and 38.9 ± 7.2 ($n = 8$ each) in the vehicle and *G. lucidum* extract groups, respectively. The scratching was significantly inhibited by azelastine hydrochloride, but not terfenadine, at an oral dose of 30 mg/kg (Fig. 2D).

Effect of *G. lucidum* extract on the firing in cutaneous nerve branch

In mice administered ESGM injections seven times, an intradermal injection of ESGM, but not saline, into the receptive field significantly increased the firing of the cutaneous nerve (Fig. 3). The increased firing was significantly suppressed by pretreatment with *G. lucidum* extract at an oral dose of 300 mg/kg (Fig. 3).

Fig. 3. Effect of *G. lucidum* extract on the activity of cutaneous nerve induced by challenge with mosquito allergen. Extract of salivary gland of mosquito (ESGM) was injected intradermally into the rostral back of the mouse given ESGM injections seven times. *G. lucidum* extract (300 mg/kg) and vehicle (VH) were administered orally 1 h before the ESGM injection. Nerve activity was recorded extracellularly using bipolar electrodes. Upper panel shows the typical traces of cutaneous nerve firing for 60 s before (Pre-ESGM) and 1 min after (Post-ESGM) the injection of ESGM. Lower panel shows the effect of *G. lucidum* extract. Data are presented as a percentage of the firing rate for 10 min after ESGM or saline injection, as compared with firing rate 5 min before injection. Values represent the means and S.E.M. for 3–4 animals. * $P < 0.05$ vs. saline, # $P < 0.05$ vs. VH plus ESGM challenge (Bonferroni multiple comparisons).

Effect of *G. lucidum* extract on plasma extravasation

An intradermal injection of ESGM caused marked plasma extravasation in sensitized mice (Fig. 4). The plasma extravasation was not affected by *G. lucidum* extract at an oral dose of 300 mg/kg (Fig. 4A). In contrast, it was almost abolished by terfenadine at an oral dose of 30 mg/kg (Fig. 4B).

Discussion

G. lucidum extract (300 mg/kg) inhibited itch-associated behavior induced by mosquito allergy, suggesting its efficacy against allergic itch. Mosquito allergy markedly increased the firing of cutaneous nerve, which was also inhibited by *G. lucidum* extract at the same dose, thus suggesting that anti-pruritic effect is mediated mainly by a peripheral action. Triterpenes and unsaturated fatty acids, constituents of *G. lucidum*, were reported to sup-

Fig. 4. Effects of *G. lucidum* extract and terfenadine (TRF) on plasma extravasation induced by challenge with mosquito allergen. Administration of extract of salivary gland of mosquito (ESGM) and *G. lucidum* extract was performed as described in the Fig. 3 legend. TRF (30 mg/kg) was administered orally 30 min before allergen challenge. Evans blue solution was injected intravenously 20 min before challenge and the skin of the challenge site was isolated 20 min after the challenge. VH, vehicle. Values represent the means and S.E.M. for six animals. * $P < 0.05$ vs. saline and # $P < 0.05$ vs. allergy control (Bonferroni multiple comparisons).

press the release of histamine from mast cells induced by compound 48/80 in vitro (4, 5). However, high concentrations (more than 1 mM) of ganoderic acids are needed to substantially inhibit the release of histamine (4). In the present experiments, *G. lucidum* extract did not suppress mosquito allergy-induced increase in vascular permeability. In addition, *G. lucidum* extract does not inhibit scratching induced by compound 48/80 and histamine (6). Therefore, the anti-pruritic effect of *G. lucidum* extract may not be due to anti-allergic action, that is, the inhibition of histamine release from mast cells. This idea is supported by the results that the H₁ histamine-receptor antagonist terfenadine did not suppress itch-associated behavior induced by mosquito allergy at a dose that almost completely inhibits plasma extravasation. Azelastine, another H₁-receptor antagonist, suppressed the itch-associated behavior, which may be partly due to the direct inhibitory action on the primary sensory neurons (14).

Scratching induced by PAR₂-activating peptide is significantly inhibited by *G. lucidum* extract at a dose of 1,000 mg/kg, and there is a tendency toward a reduction at doses of 300 and 100 mg/kg (6). Itch induced by mosquito allergy and stimulation of PAR₂ receptor in the skin may be signaled by the same group of primary afferents (12). In our preliminary experiments, mosquito allergy increases the release of serine protease activity in the skin and mosquito allergy-induced scratching was inhibited by blockade of PAR₂ receptors (unpublished observation). Therefore, it is possible that the suppression of mosquito allergy-induced itch by *G. lucidum* extract is mediated by the inhibition of the action of serine protease(s) on PAR₂ receptors. In the near future, we will examine the effects of *G. lucidum* extract on the binding of PAR₂ ligand to PAR₂ receptors and on the activity of serine protease.

PAR₂ receptors are present in epidermal keratinocytes and primary sensory neurons (20–22). Epidermal keratinocytes release several itch mediators (9, 23–25). Intradermal substance P acts on NK₁ tachykinin receptors on keratinocytes to release itch mediators and itch enhancers (8, 9). Since *G. lucidum* extract does not inhibit substance P-induced scratching (6), the inhibitory action on allergic itch may not be due to the inhibition of release of itch mediators from keratinocytes and blockade of NK₁ tachykinin receptors.

G. lucidum extract does not inhibit scratching induced by intradermal injections of histamine, serotonin, and compound 48/80 (6). Therefore, it may not exert broad inhibitory action, including local anesthetic action, on the primary afferents. As mentioned above, *G. lucidum* extract suppressed scratching induced by mosquito allergy and stimulation of PAR₂ receptor without effect on

histamine-induced scratching (present study and ref. 6). Similarly, acute topical application of tacrolimus inhibits scratching induced by mosquito allergy and PAR₂-receptor stimulation without effect on histamine-induced scratching (26). TRPV1-expressing primary afferents play an important role in itch/scratch induced by allergy and PAR₂-receptor stimulation and limited roles in histamine-induced itch/scratch (10, 11, 19). PAR₂ receptors are expressed in TRPV1-positive sensory neurons, and PAR₂ receptor stimulation decreases the threshold of TRPV1 activation (20). Taken together, these findings suggest that inhibition of allergic itch by *G. lucidum* is due to the blockade of PAR₂ receptors and/or neuron type-specific inhibitory action on TRPV1-positive primary afferents.

Azelastine inhibits scratching induced by histamine, substance P, compound 48/80, and mosquito allergy (14, 27, 28). It exerts inhibitory influence on many kinds of cells including sensory neurons (14), epidermal keratinocytes (27), and mast cells (29). It markedly inhibits high K⁺-induced increase in intracellular Ca²⁺ concentration in cultured mouse sensory neurons (14). In contrast, *G. lucidum* extract does not inhibit scratching induced by histamine, substance P, and compound 48/80, although it inhibits mosquito allergy-induced scratching (6). It is suggested that the antipruritic mechanisms of *G. lucidum* extract is different from those of azelastine. It may not exert broad inhibitory influence (e.g., blockade of calcium channels) on primary sensory neurons and epidermal keratinocytes.

Scratching induced by mosquito allergy is suppressed by a 5-lipoxygenase inhibitor and a 5-lipoxygenase activating peptide inhibitor, suggesting the involvement of 5-lipoxygenase metabolites in itch of mosquito allergy (15). Mosquito allergy-induced scratching is not inhibited by a leukotriene B₄ antagonist and cysteinyl leukotriene antagonists (15), but it is suppressed by an antagonist to lipoxin A₄, a 5-lipoxygenase metabolite (30). Although an intradermal injection of lipoxin A₄ does not elicit scratching in naive mice, it induces scratching in sensitized mice and also mice given adoptive transfer of CD4⁺ cells from the skin of sensitized ones (30). These findings taken together suggest that lipoxin A₄ acts on CD4⁺ cells to release some itch mediator(s). Thus, we will examine the effects of *G. lucidum* extract on the pruritogenic actions of lipoxin A₄ and itch mediator(s) released from CD4⁺ cells in the near future.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (22790063) and by Grants for Health Science from the Health, Labour, and Welfare Ministry, Japan (22141101).

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