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Short communication

Involvement of leukotriene B_4 in dermatophyte-related itch in mice

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ABSTRACT

Background: Proteinase-activated receptor-2 (PAR_2) is involved in dermatophyte-induced scratching and leukotriene B_4 (LTB_4) release from keratinocytes. We investigated whether PAR_2-mediated LTB_4 production is involved in dermatophyte-induced scratching.
Methods: Dermatophyte extract was injected intradermally and scratching was observed in mice. LTB_4 was determined by enzyme immunoassay.
Results: Dermatophyte extract-induced scratching was inhibited by zileuton (5-lipoxygenase inhibitor), ONO-4057 (LTB_4 antagonist), FSLLRY-NH_2 (PAR_2 antagonist), and anti-PAR_2 antibody. Dermatophyte extract injection increased the cutaneous content of LTB_4, which was inhibited by zileuton and FSLLRY-NH_2.
Conclusion: These results suggest the involvement of LTB_4 in dermatophyte-associated itch. LTB_4 production might be due to PAR_2 stimulation in the skin.

Introduction

Dermatophytosis (or tinea) is caused by a fungal infection, typically by dermatophytes. The most common functional symptom of tinea pedis is pruritus, which is reported by about 60% of patients with moderate or severe tinea pedis [9]. Details of the underlying mechanisms of dermatophytosis-associated pruritus remain poorly understood. During the infection process, dermatophytes secrete various enzymes, including proteases [15]. Of these secreted enzymes, proteases, and especially endopeptidases (proteinases), cause itching in humans and itch-related behavior in mice when administered to the skin [8,18]. Proteinase-activated receptor 2 (PAR_2) has been implicated as having an important role in proteinase-mediated itching [18].

Trichophyton mentagrophytes, including *Arthroderma vanbreuseghemii* (ADV), is known to infect both animals and humans [10]. Intradermal injection of ADV extract into the rostral back of mice elicits an itch-related response (hind-paw scratching), which has been shown to be suppressed by both a serine protease inhibitor and a PAR_2 antagonist [7]. Taken together, these findings suggest the possibility that proteinases secreted by dermatophytes cause itching through the stimulation of PAR_2 receptors in the skin.

An injection of SLIGKV-NH_2 (tethered ligand peptide of human PAR_2 receptor) into the human skin provokes itch and pain [17]. Injections of pruritogenic and algogenic substances (such as histamine and capsaicin, respectively) into the murine cheek elicit hind-paw scratching and pain-related behavior (forelimb wiping) of the injection site, respectively [16], and an injection of SLIGRL-NH_2 (tethered ligand peptide of murine PAR_2 receptor) into the murine cheek provokes marked hind-paw scratching and little forelimb wiping, suggesting that stimulation of PAR_2 receptor in the skin mainly induces itching in mice [1]. Similarly, an injection of ADV extract into the murine cheek increased markedly hind-paw scratching and only slightly forelimb wiping, suggesting that ADV extract is pruritogenic with weak algogenic activity [7]. ADV extract-induced scratching is inhibited by a PAR_2 antagonist, which suggests that ADV extract-induced scratching is mediated by PAR_2 receptors in the skin [7].

PAR_2 receptors are expressed in epidermal keratinocytes [17] and nerve fibers in the skin [17]. Because dermatophytes infect the superficial skin and require keratin for growth [12], the epidermis may be a key site of pruritic dermatophyte infection. Epidermal keratinocytes produce itch mediators [5,6]. Of these, leukotriene (LT) B_4, a potent itch mediator [2], is produced by keratinocytes in response to pruritogenic stimuli [5,6] and is involved in dermatitis-associated pruritus [4]. The activation of PAR_2 receptors on keratinocytes has been shown to induce LTB_4 production [20]. Therefore, the purpose of this study was to determine whether

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LTB_4 produced by epidermal keratinocytes is involved in itching induced by ADV extract.

Materials and methods

Animals

Male Slc:ICR mice (5–8 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). They were housed in a room under controlled temperature (21–23 °C), humidity (45–65%), and light (7:00 AM to 7:00 PM) conditions. Food and water were available ad libitum. The study protocol was approved by the Committee for Animal Experiments at the University of Toyama.

Materials

Zileuton (Sigma, St. Louis, MO, USA), indomethacin (Sigma), and 5-[2-(2-carboxyethyl)-3-[6-(4-methoxyphenyl)-5E-hexenyl] oxyphenoxy] valeric acid (ONO-4057; Ono Pharmaceutical Co., Ltd., Osaka, Japan) were dissolved in 0.5% carboxymethyl cellulose (Wako Pure Chemical Ind., Osaka, Japan). Diclofenac sodium (Research Biochemical Inc., Natick, MA, USA) was dissolved in tap water. Zileuton and ONO-4057 were administered orally 1 h before ADV extract injection, and indomethacin and diclofenac were administered orally 30 min before ADV extract injection. FSLLRY-NH_2 (selective PAR_2 antagonist peptide) was dissolved in saline and was injected intradermally together with ADV extract. Anti-PAR_2 monoclonal antibody (SAM-11, which has epitope of amino acid 37–50 of human PAR_2 containing the tethered ligand sequence and binds to human and murine PAR_2, according to the manufacturer's specifications; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and non-specific mouse IgG were dissolved in phosphate-buffered saline and injected intradermally 10 min before ADV extract injection. The doses of zileuton, ONO-4057, indomethacin, diclofenac, FSLLRY-NH_2, and antibody SAM-11 were selected from the published literature [5,6,18].

Dermatophytes and extract preparation

The dermatophyte ADV (ID #58445) was obtained from the National BioResource Project (http://www.nbrp.jp/). It was cultured in Sabouraud's dextrose liquid culture medium at 37 °C for more than 3 days. ADV extract was prepared as described previously [7].

Behavior experiments

The day before behavior experiments, the animals' hair was clipped over the rostral part of the back. For acclimation, mice were placed individually in an acrylic cage composed of four cells (13 cm × 9 cm × 35 cm) for at least 1 h prior to testing. Immediately after intradermal injection, mice were returned to the same cells, and their behaviors were videotaped for 1 h. No personnel were present in the observation room during this time. Playback of the video allowed for counting of injection site scratching by the hind paw. The series of movements in which the mice stretched either hind paw toward the injection site, leaned the head toward it, and rapidly scratched several times for about 1 s were considered as one bout of scratching [7].

LTB_4 enzyme immunoassay

The skin (8 mm in diameter) at the injection site was removed 5 min after injection, immediately weighed, shredded using scissors, and put into 0.2 mL of ice-cold ethanol containing 10 μM indomethacin and 10 μM zileuton. Lipid extraction and LTB_4 enzyme immunoassay were performed as previously described [4].

Data processing

Data are presented as means ± standard error of the mean (SEM). Data were analyzed with a Student's *t*-test, two-way repeated measures analysis of variance (ANOVA) and *post hoc* Tukey's test, or one-way ANOVA and *post hoc* Dunnett's test or Holm–Šidák test; $p < 0.05$ was considered significant. The statistical analyses were performed using Sigmaplot graphing and statistical software (version 11; Systat Software, Inc., Chicago, IL, USA).

Results

Scratching behavior induced by ADV extract

An intradermal injection of ADV extract (20 μg/site) into the rostral back elicited hind-paw scratching toward the injection site, inducing no other apparent behavioral responses. This effect peaked during the first 10-min period and almost completely subsided after 30 min (Fig. 1A). When scratching during the 1-h duration was compared between treatment groups, we found that ADV extract increased scratching bouts 2.2-fold compared to control (Fig. 1B).

Effects of inhibitors of 5-lipoxygenase, cyclooxygenase, and the LTB_4 receptor on ADV extract-induced scratching

Scratching induced by ADV extract (20 μg/site) was markedly inhibited by oral pretreatment with the 5-lipoxygenase inhibitor zileuton (10–100 mg/kg) and the LTB_4 receptor antagonist ONO-4057 (10–100 mg/kg). Nearly complete inhibition was observed after treatment with 30 and 100 mg/kg of zileuton or treatment

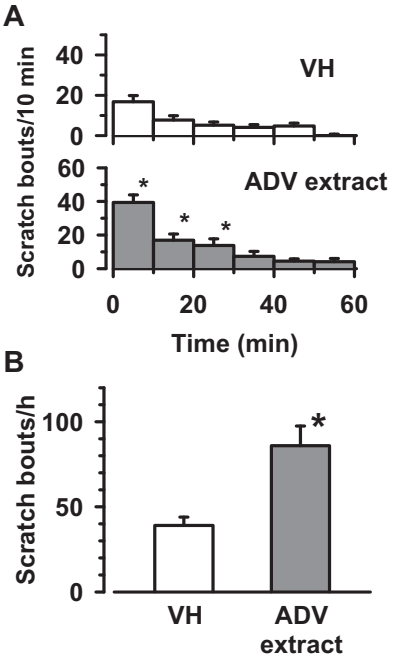

Fig. 1. Scratching response to an intradermal injection of dermatophyte extract into the rostral back in mice. Hind-paw scratching of the injection site was counted for 60 min after an intradermal injection of an extract of the dermatophyte *Arthroderma vanbreuseghemii* (ADV) or vehicle (VH). (A) Time-course of scratching after VH and ADV extract (20 μg/site) injections. $^*p < 0.05$ vs. VH (Tukey's test). (B) Total number of scratching bouts for 1 h. $^*p < 0.05$ (Student's *t*-test). The values represent the mean ± SEM ($n = 16$).

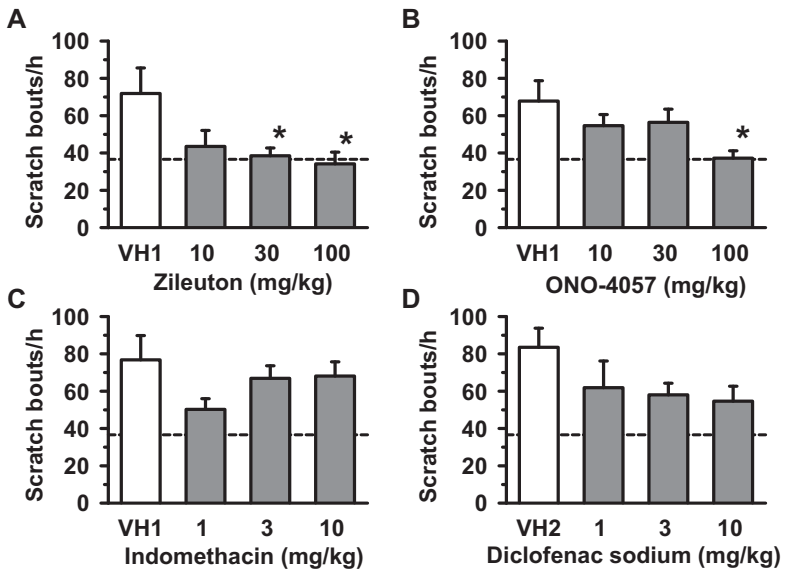

Fig. 2. Effects of inhibition of eicosanoid systems on dermatophyte extract-induced scratching in mice. Hind-paw scratching of the injection site was counted for 60 min after an intradermal injection of *Arthroderma vanbreuseghemii* (ADV) extract (20 μg/site). (A) Zileuton or vehicle (VH1, 0.5% carboxymethyl cellulose) was administered orally 60 min before ADV extract injection. $n = 8$ in each group. (B) ONO-4057 or VH1was administered orally 60 min before ADV extract injection. $n = 16$ each group. (C) Indomethacin or VH1 was administered orally 30 min before ADV extract injection. $n = 8$ in each group. (D) Diclofenac sodium or VH2 (tap water) was administered orally 30 min before ADV extract injection. $n = 8$ in each group. Dashed lines represent the average value of scratch bouts in mice given intradermal injection of saline. The values represent the mean ± SEM. $^{*}p < 0.05$ vs. VH1 or VH2 (Dunnett's test). These experiments were performed twice and similar results were obtained.

with 100 mg/kg of ONO-4057 (Fig. 2A and B). Oral pretreatment with the cyclooxygenase inhibitors indomethacin (1–10 mg/kg) or diclofenac sodium (1–10 mg/kg) had a tendency to decrease the ADV extract-induced scratching, but the effects were not statistically significant (Fig. 2C and D).

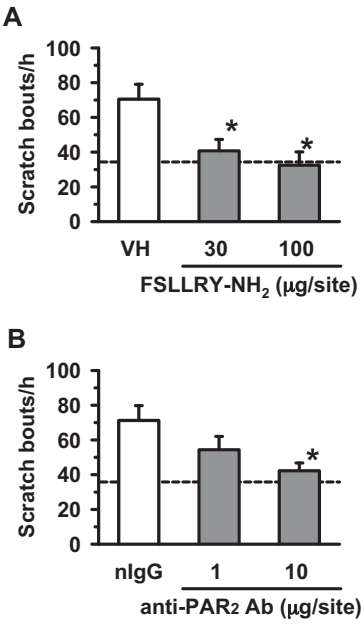

Fig. 3. Effects of PAR_2 antagonist and anti-PAR_2 antibody (Ab) on dermatophyte extract-induced scratching in mice. Hind-paw scratching of the injection site was counted for 60 min after an intradermal injection of *Arthroderma vanbreuseghemii* (ADV) extract (20 μg/site). (A) ADV extract was injected intradermally with or without the PAR_2 antagonist FSLLRY-NH_2 (30 and 100 μg/site). (B) Anti-PAR_2 monoclonal Ab (1 and 10 μg/site) and non-specific IgG (nIgG, 10 μg/site) were injected intradermally 10 min before ADV extract injection. The dashed line represents the average value of scratch bouts in mice given an intradermal injection of saline. The values represent the mean ± SEM ($n = 8$). $^{*}p < 0.05$ vs. nIgG (Dunnett's test). These experiments were performed twice and similar results were obtained.

Effects of PAR_2 antagonist and anti-PAR_2 antibody on ADV extract-induced scratching

Scratching induced by ADV extract (20 μg/site) was markedly inhibited by intradermal co-injection with FSLLRY-NH_2 (30 and 100 μg/site) (Fig. 3A). In addition, ADV extract-induced scratching was dose-dependently inhibited by intradermal pretreatment with a PAR_2 neutralizing antibody (1 and 10 μg/site) dose-dependently inhibited scratching induced by ADV extract (20 μg/site), as compared with a non-specific IgG (Fig. 3B).

LTB_4 production

Cutaneous LTB_4 content was significantly increased 5 min after intradermal injection of ADV extract (20 μg/site) (Fig. 4). This increase was significantly and almost completely prevented by oral pretreatment with zileuton (100 mg/kg) and intradermal co-injection with FSLLRY-NH_2 (100 μg/site) (Fig. 4).

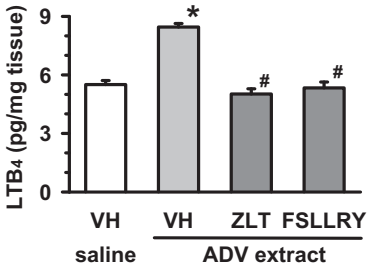

Fig. 4. Increased production of leukotriene (LT) B_4 after dermatophyte extract injection in the mouse skin. *Arthroderma vanbreuseghemii* (ADV) extract (20 μg/site) and saline were injected intradermally, and 5 min later LTB_4 content was measured in the treated skin. Zileuton (ZLT, 100 mg/kg; $n = 9$) and the vehicle (VH; $n = 8$ in each group) were administered orally 1 h before ADV extract injection. FSLLRY-NH_2 (FSLLRY, 100 μg/site; $n = 8$) was injected intradermally together with ADV extract. The values represent the mean ± SEM. $^{*}p < 0.05$ vs. VH + saline, $^{\#}p < 0.05$ vs. VH + ADV extract (Holm–Šidák test). These experiments were performed twice and similar results were obtained.

Discussion

An intradermal injection of ADV extract (20 μg/site) elicited hind-paw scratching, confirming our previous report, which showed that intradermal ADV extract (1–20 μg/site) causes a dose-dependent increase in hind-paw scratching [7].

We have shown that ADV extract-induced scratching is inhibited by both the PAR_2 antagonist FSLLRY-NH_2 and the serine protease inhibitor nafamostat [7]. In the present study, we reconfirmed the inhibitory activity of FSLLRY-NH_2 and observed that a PAR_2-neutralizing antibody also inhibited ADV extract-induced scratching. The PAR_2 receptor is activated via the proteolytic cleavage of its N-terminal sequence by serine proteases. ADV extract cleaves a synthetic N-terminal peptide of PAR_2 similar to both trypsin and tryptase [7]. Therefore, serine protease(s) present in the ADV extract may elicit scratching through PAR_2 activation, although pruritogenic serine proteases have not yet been shown to be a component of ADV extract. In the skin, PAR_2 has been shown to be expressed mainly in nerve fibers [17] and epidermal keratinocytes [17]. Since ADV extract is equally effective at inducing scratching behavior in both mast cell-deficient mice and normal littermates [7], mast cells may not be essential for the pruritogenic action of ADV extract. Thus, an intradermal injection of ADV extract may primarily act on nerve fibers and epidermal keratinocytes to induce scratching.

ADV extract-induced scratching was inhibited by the LTB_4 receptor antagonist ONO-4057, suggesting that dermatophyte-associated itching involves LTB_4, a potent pruritogen in mice [2]. A key enzyme for LTB_4 production is 5-lipoxygenase [14]. Thus, the observation that the 5-lipoxygenase inhibitor zileuton suppressed scratching and LTB_4 production induced by the ADV extract supports the idea that LTB_4 is involved in dermatophyte-associated itching. Since ADV extract-induced scratching was fast in onset and short in duration, component and/or resident cells in the skin may be responsible. 5-Lipoxygenase is present in epidermal keratinocytes [4], and cultured keratinocytes produce LTB_4 upon stimulation with PAR_2 agonist [20]. Thus, it is plausible that PAR_2-mediated LTB_4 produced by epidermal keratinocytes is involved in ADV extract-induced scratching. Mast cells also produce LTB_4 following activation [13]. However, mast-cell deficiency does not reduce scratching induced by ADV extract or PAR_2 agonist [7], suggesting that mast cells do not play a key role in ADV extract-induced scratching. We do not rule out the possibility that LTB_4 produced by infiltrating leukocytes is involved under dermatitis conditions.

There are two LTB_4 receptor subtypes, BLT1 and BLT2, which have high and low binding affinities for LTB_4, respectively [19]. Since ONO-4057 has a similar affinity for BLT1 and BLT2 receptors [19], the results of the present study did not detect the relative contributions of BLT1 and BLT2 receptors in ADV extract-induced scratching. In the dorsal root ganglia and skin in normal mice, BLT1 receptor mRNA is expressed, but BLT2 receptor mRNA cannot be detected [3]. LTB_4 administration increases Ca^{2+} influx in cultured dorsal root ganglion neurons [3]. Most (73%) of the BLT1 immunoreactive neurons are small or medium in size and TRPV1 channel immunoreactivity (a marker of unmyelinated C-fiber neurons) has been shown to be present in BLT1-positive neurons [3]. Therefore, it is suggested that BLT1 receptors are responsible for an initiation phase of ADV extract-induced scratching. On the other hand, LTB_4 promotes the chemotaxis of leukocytes that express BLT1 and BLT2 receptors [19]. Therefore, it is possible that BLT2 receptors are involved in a late phase of ADV extract-induced scratching [11].

In this study, the cyclooxygenase inhibitors indomethacin and diclofenac did not significantly affect the ADV extract-induced scratching. Indomethacin does not inhibit scratching induced by PAR_2 agonist, substance P, and sphingosylphosphorylcholine in mice [5,6,20], although it inhibits prostaglandin E_2 production induced by PAR_2 agonist and substance P in cultured keratinocytes [5,20]. Intradermal injection of prostaglandin E_2 does not elicit scratching in mice [2]. With these findings taken into account, the present results suggest that although its production is increased by ADV extract, prostaglandin E_2 does not play a key role in ADV extract-induced scratching.

In summary, data shown here suggest that dermatophyte-contained serine protease(s) act on PAR_2 in the epidermis to produce LTB_4, which causes itching. This may be a causal mechanism of dermatophytosis-associated pruritus.

Conflict of interest

The authors state no conflict of interest.

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