Involvement of Leukotriene B\(_4\) Released from Keratinocytes in Itch-associated Response to Intradermal Interleukin-31 in Mice

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A recent study suggests that interleukin-31 (IL-31) exerts its effect via indirect mechanisms rather than through direct stimulation of cutaneous nerves. However, the underlying peripheral mechanisms of IL-31-induced itch in the skin remain unclear. Therefore, the present study investigated the peripheral mechanisms underlying IL-31-induced itch in mice. IL-31-induced itch-related response was inhibited by anti-allergic drugs (tranilast and azelastine), but not by an \(H_1\) histamine receptor antagonist (terfenadine). Furthermore, a 5-lipoxygenase inhibitor (zileuton), but not a cyclooxygenase inhibitor (indomethacin), and a leukotriene B\(_4\) \((\text{LTB}_4)\) receptor antagonist (CMHVA) attenuated the action of IL-31. IL-31 receptor-immunoreactivity was observed in the epidermis and primary sensory neurones. IL-31 receptor mRNA was expressed in mouse keratinocytes and dorsal root ganglia neurones. IL-31 increased the production of \(\text{LTB}_4\) in mouse keratinocytes. These results suggest that IL-31 elicits itch not only through direct action on primary sensory neurones, but also by inducing \(\text{LTB}_4\) production in keratinocytes.

Key words: itch; leukotriene B\(_4\); interleukin-31; keratinocytes; scratching; dorsal root ganglia.

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MATERIALS AND METHODS

Animals

Male ICR mice aged 4–11 weeks were used in this study. The mice were purchased from Japan SLC (Shizuoka, Japan). For one part of the experiment, neonatal mice were used for the isolation of cutaneous keratinocytes. Neonatal mice were obtained from late pregnant mice purchased from Japan SLC (Shizuoka). They were housed in a room with controlled temperature (21–23°C), humidity (45–65%), and light (from 07.00h to 19.00h). Food and water were freely available. All experimental procedures involving animals were approved by the Committee for Animal Experiments at the University of Toyama and conducted in accordance with the guidelines of the Japanese Pharmacological Society.

Drugs

Recombinant mouse IL-31 was prepared in Chugai Pharmaceutical Co., Ltd (Shizuoka, Japan). Briefly, IL-31 was purified from the supernatant of mouse IL-31-transformed Chinese hamster ovary cells using hydroxyapatite column (Bio-Rad Laboratories, Inc., Hercules, CA, USA), union-exchange column (GE healthcare, Chicago, IL, USA), and gel filtration column (GE healthcare). IL-31 was dis-
solved in phosphate buffered-saline (PBS) and administered intradermally. Naltrexone hydrochloride (Sigma-Aldrich, St Louis, MO, USA) (18–20) was dissolved in physiological saline and administered subcutaneously 15 min before IL-31 injection. Tefradine (16, 18, 20) and tranilast (Sigma-Aldrich) (21) were dissolved in 0.5% sodium carboxymethyl cellulose and administered orally 30 and 60 min before IL-31 injection, respectively. Azelastine hydrochloride (Sigma-Aldrich) (16, 18, 22) was dissolved in tap water and administered orally 30 min before IL-31 injection. Indomethacin (Sigma-Aldrich), zileuton (Ono Pharmaceutical Co. Ltd, Osaka, Japan) (15, 18, 23), and CMHVA (5-[2-(2-carboxyethyl)-3-[6-(4-methoxyphenyl)-SE-hexenyl]oxyphenoxy] valeric acid) (Ono Pharmaceutical Co. Ltd) (15, 16, 18, 24, 25) were dissolved in 0.5% sodium carboxymethyl cellulose and administered orally 30, 60, and 60 min before IL-31 injection, respectively.

**Behavioural tests**

The mouse hair was clipped over the rostral part of the back the day before the experiment. For acclimation, the mice were housed individually in the observation cage (an acrylic cage composed of 4 cells) for at least 1 h. Immediately after the intradermal injection, the mice were put back into the same cells, and their behaviour was videotaped for 1 h by personnel kept out of the observation room. Scratching by the hind paws at the injection site was observed during video playback (26). The mice stretched either the left or the right hind paw, leaned their head towards the paws, rapidly scratched themselves, and brought their toenails to their mouth. A series of these movements was counted as one bout of scratching (15).

**Immunohistochemistry**

Under anaesthesia with pentobarbital (80 mg/kg, intraperitoneal), the mice were transcardially perfused with PBS and then 4% paraformaldehyde (PFA). The rostral back skin was isolated and post-fixed with 4% PFA at 4°C overnight. The skin was immersed in 30% sucrose solution for 4°C for at least 2 days. The tissue was embedded in Tissue-Tek® optimum cutting temperature (OCT) compound (Sakura Finetec Co., Ltd, Tokyo) and stored at −80°C until use. The frozen sections were sectioned at 20 μm with a cryostat (Leica, Wetzlar, Germany). After being washed with PBS 3 times, the sections were treated with 0.3% Triton X-100 in PBS and then with 0.25% foetal bovine serum to block non-specific immunoglobulin binding. The sections were incubated with goat anti-IL-31 receptor A (IL-31RA) antibody (AF2107; 1:500, R&D Systems, Inc., Minneapolis, MN, USA) and rabbit anti-protein gene product 9.5 antibody (RA95101; 1:2000, Ultraclone Ltd, Isle of Wight, UK) at 4°C overnight. After washing, the preparations were incubated with Alexa Fluor 594-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-goat IgG antibodies (Life Technologies, Carlsbad, CA, USA) for 1 h at room temperature. The fluorescence signals were observed using a confocal laser-scanning microscope (Bio-Rad Laboratories Inc., Hercules, USA).

**Primary cultures of murine keratinocytes and dorsal ganglion neurones**

The skin from neonatal mice was removed and treated with 0.05% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) dissolved in serum-free MCDB 153 medium (Sigma-Aldrich) containing 0.67% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Dojindo, Kumamoto, Japan), 0.12% sodium bicarbonate (Wako Pure Chemical Industries Ltd, Osaka, Japan), 0.01% penicillin G (Meiji Seika Pharma Co., Ltd, Tokyo, Japan), and 0.006% kanamycin (Wako Pure Chemical Industries Ltd) at 37°C overnight. The epidermal sheets were peeled gently from the underlying dermis. The keratinocytes were dissociated by gently shaking the serum-free MCDB 153 medium containing the epidermal sheet. After washing with keratinocyte growth medium (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland), the keratinocytes were cultured in collagen-coated 24-well or 6-cm-diameter plates.

The bilateral DRGs at C1–T13 levels were removed from the 6-week-old mice. DRG was treated with 0.25% collagenase (Wako Pure Chemical Industries Ltd) in serum-free Dulbecco’s Modified Eagle’s medium (DMEM) containing antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C for 1 h and was shaken gently for dissociation. The dissociated cells were treated with 0.25% trypsin-EDTA at 37°C for 15 min and then the cells were washed with DMEM containing antibiotics. The cells suspended in DMEM containing antibiotics, 10% foetal bovine serum, and 50 μM cytosine β-D-arabinofuranoside (Sigma-Aldrich), and cultured in poly-D-lysine-coated 6-cm-diameter plates.

**Reverse transcription and PCR**

The total RNA from the primary cultures of murine keratinocytes and DRG neurones was extracted by using GeneElute Mammalian Total RNA miniprep kit (Sigma-Aldrich). The total RNA (1 μg) was reverse transcribed into the cDNA using oligo (dT)16 primer and Reverscript III® (Wako Pure Chemical Industries Ltd). PCR was performed as described previously (16). The primer sequences used were as follows: IL-31RA (sense) 5'-gtgcgtgctgtagtgaagac-3'; IL-31RA (antisense) 5'-taaacgctgctgtagtgaagac-3'; OSMRβ (sense) 5'-aaggtttctcgcgtgtagtgaagac-3'; OSMRβ (antisense) 5'-aaggtttctcgcgtgtagtgaagac-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense) 5'-ccaggtcatcagtaac-3'; GAPDH (antisense) 5'-taccttcctgagtcgtt-3'. The reaction product was separated on 2% agarose gel and stained with ethidium bromide.

**Measurement of LTB4**

To measure LTB4 production in keratinocytes, the culture medium (200 μl) was collected (removed) from the primary cultures of murine keratinocytes 10 min after IL-31 administration and assayed for LTB4 with an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA). Zileuton and the vehicle (dimethyl sulphoxide, final concentration 0.001%) were treated 60 min before IL-31 application. Anti-IL-31RA antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and non-specific IgG (Sigma-Aldrich) were treated 30 min before IL-31 application. The remaining keratinocytes were treated with 1% Triton X-100 and used for protein determination by using a protein assay kit (Bio-Rad Laboratories, Inc.). The amount of LTB4 was normalized to the amount of protein.

**Data processing**

Data are presented as means ± standard error of the mean (SEM). The statistical significance was analysed using the Student’s t-test (2 groups), one-way analysis of variance (ANOVA), or 2-way repeated measures-ANOVA followed by a post hoc Holm–Šidak test (3 or more groups). A p-value < 0.05 was considered statistically significant.

**RESULTS**

**Scratching behaviour induced by IL-31**

An intradermal injection of IL-31 (100 ng/site), but not vehicle, into the rostral back elicited hind-paw scratching directed towards the injection site (Fig 1A). This effect peaked during the first 10 min. Compared with the
treatment of vehicle (PBS) control, IL-31 (100 ng/site)-
induced scratching increased significantly from 10 min
after the injection, and the significant effects continued
for at least 50 min (Fig. 1A). The dose-response curve
for IL-31 was bell-shaped (Fig. 1B).

Effects of various drugs on IL-31-induced scratching
The effects of systemic pretreatment on the scratching
induced by an intradermal injection of IL-31 (100 ng/
site) were examined by using several agents. The μ-opioid
receptor antagonist naltrexone hydrochloride (1 and 10
mg/kg) significantly inhibited IL-31-induced scratching
(Fig. 2). The H1 histamine receptor antagonist, terfena-
dine (30 mg/kg), did not exhibit any effect on scratching
(Fig. 2). The anti-allergic agents tranilast (100 and 300
mg/kg; Fig. 2) and azelastine hydrochloride (30 mg/kg;
Fig. 2) inhibit IL-31-induced scratching. The 5-lipoxy-
genase inhibitor, zileuton (30 and 100 mg/kg), inhibited
IL-31-induced scratching, whereas the cyclooxygenase
inhibitor indomethacin (10 mg/kg) did not (Fig. 3). The
LTB4 receptor antagonist CMHVA (10, 30 and 100 mg/
kg) also attenuated IL-31-induced scratching (Fig. 3). Na-
trexone hydrochloride (10 mg/kg), tranilast (30 mg/kg),
azelastine hydrochloride (30 mg/kg), zileuton (100 mg/kg,
p.o.), and CMHVA (100 mg/kg) did not affect the spon-
taneous locomotor activity, suggesting that these drugs at
the dosage used do not have sedative effects (Fig. S1).

Distribution of IL-31 receptor A in the mouse skin, and
the expression of IL-31 receptor mRNA in the mouse
skin, dorsal root ganglion (DRG), and primary cultures
of DRG neurones and keratinocytes
IL-31RA-immunoreactivity was mainly localized in
the epidermis and PGP-9.5-immunoreactive primary afferents
(Fig. 4A).

The RT-PCR revealed the substantial expression of
IL-31RA and OSMRβ mRNA in the DRG and skin (Fig.
4B). In addition, the primary cultures of DRG neurones
and keratinocytes also expressed both IL-31RA and
OSMRβ mRNA (Fig. 4B).

IL-31-induced production of LTB4 in primary cultures
of mouse keratinocytes
A bath-application of IL-31 (0.2 and 2 μg/ml) to the
primary cultures of mouse keratinocytes significantly
increased the production of LTB4 (Fig. 5A). The effect
of IL-31 (2 μg/ml) was almost abolished by the 5-lipoxy-
genase inhibitor zileuton (10 μM; Fig. 5A). In addition,
anti-IL-31RA antibody (10 μg/ml) significantly inhibited
IL-31-induced LTB4 production (Fig. 5B).

DISCUSSION
This study aimed to demonstrate peripheral mechanisms
of IL-31-induced itch and found that IL-31-induced itch

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through the production of LTB₄ in epidermal keratinocytes.

An intradermal injection of IL-31 elicited scratching at the injection site. Next, it was examined whether scratching in mice was an itch-related behaviour. The scratching was inhibited by the μ-opioid receptor antagonist naltrexone. μ-Opioid receptor antagonists inhibit the scratching induced by several pruritogens (e.g. substance P, sphingosylphosphorylcholine, serotonin, gastrin-releasing peptide, and α-melanocyte-stimulating hormone) (18–20, 27, 28), dermatoses (e.g. skin allergy, atopic dermatitis and xerosis) in rodents (29–31), and itching/scratching in humans with pruritic diseases (e.g. cholestasis, urticaria and atopic dermatitis) (32, 33). μ-Opioid receptor antagonists suppress itch-associated behaviour without inhibiting the pain-related behaviour (34, 35). The opioid antagonists may inhibit itching/scratching by acting on the μ-opioid receptors in the central nervous system, especially in the lower brainstem (36–39). Thus, it can be suggested that IL-31-induced scratching is an itch-associated response in mice.

It has been reported that IL-31 acts directly on primary afferent neurones (13). In this study, although an intradermal administration of IL-31 elicited scratching with the effect peaking during the first 10-min period, the significant effect of IL-31 continued for another 50 min after the attainment of peak effect, suggesting that IL-31 affects not only directly, but also indirectly, primary afferent neurones.

The H₁ histamine receptor antagonist terfenadine (30 mg/kg) inhibited histamine-induced scratching in mice (30). However, the same dosage of terfenadine did not affect IL-31-induced scratching, suggesting that histamine may not play an important role in scratching. However, anti-allergic drugs (tranilast and azelastine) attenuated IL-31-induced scratching. Tranilast has an inhibitory action on the production of LTB₄ (40). In addition to H₁, histamine receptor antagonistic activity, azelastine has an inhibitory effect on LTB₄ production and LTB₄ receptor antagonistic activity (22). The IL-31-induced scratching was inhibited by 5-lipoxygenase inhibitor zileuton and LTB₄ receptor antagonist CMHAV. However, the cyclooxygenase inhibitor indomethacin did not show any effect on scratching. An intradermal injection of LTB₄, but not prostaglandin E₂, elicits scratching in mice (41). Taken together, these findings suggest that LTB₄ is involved in IL-31-induced scratching.

5-Lipoxygenase is a key enzyme involved in the production of LTB₄ (42). In the skin, 5-lipoxygenase is
expressed mainly in the keratinocytes (43) and mast cells (44). IL-31 receptors are expressed in the keratinocytes, but not in the mast cells (4, 6, 45; Fig. 4 and Fig. S2A). In addition, an intradermal injection of IL-31 elicited scratching in mast cell-deficient mice and the normal littermates (Fig. S2B). Therefore, it is suggested that the mast cells are not involved in IL-31-induced LTB₄ production. Our previous report showed that an intradermal injection of proteinase-activated receptor-2 (PAR2) agonist SLIGRL-NH₂ (peptidergic agonist) induced LTB₄ production (46). PAR2 is mainly expressed in the keratinocytes in the skin (25). These findings suggest that intradermal pruritogens including IL-31 act on the epidermal keratinocytes. In this study, IL-31 induced LTB₄ production in the primary cultures of mouse keratinocytes via IL-31 receptor. Thus, it can be suggested that cutaneous keratinocytes are the main LTB₄ producing cells. The mechanisms underlying IL-31-induced LTB₄ production in keratinocytes remain unclear. IL-31 activates 3 signalling pathways: Jak/STAT pathway, PI3K/AKT pathway, and MAPK pathway (47). However, whether these pathways are involved in the production of LTB₄ remains unknown. IL-31 also increases the intracellular Ca²⁺ concentration in keratinocytes (48). The increase in intracellular free Ca²⁺ ions activates phospholipase A₂, a key enzyme for the production of arachidonic acid (49). Arachidonic acid, a substrate of 5-lipoxygenase, is involved in the production of LTB₄. Thus, as a possible mechanism, IL-31-induced Ca²⁺ influx is involved in the production of LTB₄.

LTB₄ directly activates DRG neurones and induces scratching (41, 50). The LTB₁ receptors, BLT1 and BLT2, have high and low binding affinities for LTB₄, respectively (51). The LTB₁ receptor antagonist CMHV A has a similar affinity for both receptors (51). The DRG neurones and skin in the normal mice expressed BLT1 receptor mRNA, but not BLT2 receptors (50). Most (81%) of the BLT1-immunoreactive DRG neurones are transient receptor potential vanilloid 1 (TRPV1, a marker of unmyelinated C-fibre neurones) (50). Therefore, it is suggested that BLT1 receptor is involved in LTB₄-mediated IL31-induced scratching. A recent study showed that BLT2 antagonist, but not BLT1 receptor antagonist, inhibits LTB₄-induced scratching (52). However, our previous study has been shown that an intradermal injection of BLT2 receptor agonist (12(S)-hydroxy-5Z, 8E, 10E)-heptadecatrienoic acid and CAY10538) did not induce scratching in mice (data not shown). Therefore, further study on the role of BLT2 in scratching will be necessary.

In this study, IL-31 receptors were expressed in the DRG neurones/primary afferents, suggesting that IL-31 directly activates the primary afferents. Cevikbas et al. showed that IL-31 acts on IL-31α/IL31Rα/IL10RAα/IL10RBβ- and CAY10538-) did not induce scratching in mice (data not shown). Therefore, further study on the role of BLT2 in scratching will be necessary.

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In conclusion, IL-31 elicits itch not only through direct action on primary sensory neurones, but also by inducing LTB₄ production in keratinocytes.

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REFERENCES


