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 H₁ histamine receptor antagonist (terfenadine). Furthermore, a 5-lipoxygenase inhibitor (zileuton), but not a cyclooxygenase inhibitor (indomethacin), and a leukotriene B₄ (LTB₄) receptor antagonist (CUMVA) attenuated the action of IL-31. IL-31 receptor-immuno-reactivity 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 LTB₄ in mouse keratinocytes. These results suggest that IL-31 elicits itch not only through direct action on primary sensory neurones, but also by inducing LTB₄ production in keratinocytes.

Key words: itch; leukotriene B₄; interleukin-31; keratinocytes; scratching; dorsal root ganglia.

Accepted May 16, 2017; Epub ahead of print May 17, 2017

Corr: Tsugunobu Andoh, Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan. E-mail: andoht@pha.u-toyama.ac.jp

Itch (or pruritus), an unpleasant skin sensation that evokes the desire to scratch, is the most common symptom of dermatitis (e.g. atopic dermatitis) and certain systemic disorders (e.g. cholestasis). Histamine from the mast cells has been thought to play an essential role in itch. H₁ histamine receptor antagonists are the drugs of first choice for the treatment of itch. However, many severe pruritic diseases, except acute urticaria, respond poorly to H₁ histamine receptor antagonists (1–3). Therefore, the underlying mechanisms and mediators of itch in most pruritic diseases are unclear.

Interleukin-31 (IL-31), a cytokine released from T cells (especially, CD4⁺Th2 cells), signals through a receptor complex comprising IL-31RA and oncostatin M receptor β (OSMRβ) (4, 5). IL-31 plays a major role in the induction of chronic inflammation in diseases such as dermatitis, allergic rhinitis and asthma (6). In particular, the expression of IL-31 mRNA is elevated in the skin of patients with atopic dermatitis (7, 8) as well as in animal models of atopic dermatitis (9) and contact dermatitis (10). IL-31 induces skin inflammation (5) and scratching, an itch-associated response (11). However, in animal models of atopic dermatitis, the administration of anti-IL-31 antibody inhibited spontaneous scratching, but not dermatitis (12). These findings suggest that IL-31 is a mediator of itch.

The peripheral mechanisms of IL-31-induced pruritus are not completely clear. A recent study showed that IL-31 increases the intracellular Ca²⁺ ion concentration in the primary cultures of mouse dorsal root ganglia (DRG) neurones, suggesting that IL-31 directly activates primary afferents and induces itch sensation (13). However, IL-31 receptor complex is expressed not only in DRG neurones, but also in several immune cells (e.g. activated monocytes, macrophages, eosinophils, and basophils) and keratinocytes (4). A recent study showed that IL-31 elicits its pruritic effect indirectly via keratinocytes and secondary mediators rather than through the stimulation of its receptors on cutaneous nerves (14). In addition, our previous studies on the peripheral mechanisms of itch have shown the important role of keratinocytes and their itch mediators, including leukotriene B₄ (LTB₄) (15–18). Therefore, this study investigated the involvement of LTB₄ in peripheral mechanism of IL-31-induced itch.

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 (on 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-
The fluorescence signals were observed using a confocal laser-Technologies, Carlsbad, CA, USA) for 1 h at room temperature. and Alexa Fluor 488-conjugated anti-goat IgG antibodies (Life were incubated with Alexa Fluor 594-conjugated anti-rabbit IgG of Wight, UK) at 4°C overnight. After washing, the preparations of gene product 9.5 antibody (RA95101; 1:2000, Ultraclone Ltd, Isle cryostat (Leica, Wetzlar, Germany). After being washed with PBS until use. The frozen samples were sectioned at 20 μm with a collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) 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’-tctgtctcggctgaatga-3’; IL-31RA (antisense) 5’-caagtcaccaagacacg-3’, OSMRβ (sense) 5’-tataccagctgcctgctca-3’, OSMRβ (antisense) 5’-aagttttcaccggt-gag-3’, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense) 5’-ccaggtctcagcagcaac-3’; GAPDH (antisense) 5’-tactcagctggtctcgtgatct-3’. The reaction product was separated on 2% agarose gel and stained with ethidium bromide. 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 sulfoxide, 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. 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.

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 4°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 dissocation. 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), were 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)₁₄ primer and Reverscript III® (Wako Pure Chemical Industries Ltd). PCR

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 sulfoxide, 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–Šidák 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, terfenadine (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-lipoxygenase 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). Naltrexone 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 spontaneous 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-lipoxygenase 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...
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 CMHV A. 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

Fig. 4. Distribution of interleukin-31 receptor A (IL-31RA) in the mouse skin and the expression of IL-31 receptors mRNA. (A) The typical examples of the distribution of IL-31 receptor A (IL-31RA) and primary afferent neurones in the mouse skin. IL-31RA (green) and PGP-9.5 (red, a marker of neurone) were immunostained in the rostral back skin. Arrowheads indicate primary sensory neurones, and the dotted lines indicates the boundary between the dermis and epidermis. Scale bar = 100 μm. (B) The expression of IL-31 receptor (IL-31RA and OSMRβ) mRNA in the mouse skin, dorsal root ganglion (DRG), and primary cultures of DRG neurones and keratinocytes. IL-31RA, OSMRβ, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were determined using RT-PCR.

Fig. 5. Interleukin (IL)-31-induced leukotriene B₄ (LTB₄) production in the primary cultures of keratinocytes. (A) IL-31-induced production of LTB₄ and effect of 5-lipoxygenase inhibitor zileuton on LTB₄ production. Zileuton (10 μM) or the vehicle (VH2) was treated 60 min before IL-31 or the vehicle (VH2) application. The concentration of LTB₄ in the tested medium was determined 10 min after IL-31 application. Data are presented as mean and standard error of the mean (SEM) (n = 18). *p < 0.05 compared with that of VH1+VH2 (Holm–Šidák test). (B) Effect of anti-IL-31RA on IL-31-induced LTB₄ production. Anti-31RA antibody (anti-31RA, 10 μg/ml) or non-specific immunoglobulin G (IgG) (nIgG, 10 μg/ml) were treated 30 min before IL-31 (2 μg/ml) or VH2. The concentration of LTB₄ in the tested medium was determined 10 min after IL-31 application. Data are presented as mean and SEM (n = 6). *p < 0.05 compared with that of nIgG+VH2 (Holm–Šidák test).
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 remain 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 LT1 receptors, BLT1 and BLT2, have high and low binding affinities for LTB4, respectively (51). The LT1 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-31RA(+) TRPV1(+) neurones (13). In addition, IL-31-induced scratching is attenuated in TRPV1-deficient mice (13). Our previous reports have shown that TRPV1-deficiency or -desensitization in primary sensory neurones abolish the scratching induced by pruritogens (e.g. substance P and serotonin) (27, 28) and allergy (e.g. mosquito allergy) (53). Taken together, TRPV1-positive sensory neurones play an important role in the itch signalling induced by IL-31 and LTB4. IL-31 acts not only on IL-31RA(+) TRPV1(+) neurones, but also on IL-31RA(+) transient receptor channel potential cation channel ankyrin subtype 1 (TRPA1)(+) neurones (13). Fernandes et al. (52) showed that LTB4-induced scratching is attenuated in both TRPV1- and TRPA1-deficient mice. Therefore, it is suggested that TRPA1-positive sensory neurones also play an important role in IL-31- and LTB4-induced scratching.

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

ACKNOWLEDGEMENTS

The authors thank Chugai Pharmaceutical Co., Ltd for providing the mouse IL-31. This work was supported by a research grant from Chugai Pharmaceutical Co. Ltd., and partially supported by the Platform Project for Supporting in Drug Discovery and Life Science Research Platform for Drug Discovery, Informatics, and Structural Life Science from the Japan Agency for Medical Research and Development (AMED).

REFERENCES

11. Arai I, Tsujii T, Takeda H, Akiyama N, Saito S. A single dose of interleukin-31 (IL-31) causes continuous itch-associated...


