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INVESTIGATIVE REPORT

Thromboxane A_2 is Involved in Itch-associated Responses in Mice with Atopic Dermatitis-like Skin Lesions

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To investigate the mechanisms underlying itching in atopic dermatitis, we examined whether thromboxane (TX) A_2, an arachidonic acid metabolite, is involved in spontaneous scratching, an itch-related response, in NC mice with atopic dermatitis-like skin lesions. The TXA_2 receptor (TP) antagonist ONO-3708 inhibited the spontaneous scratching. The mRNA expression of TX synthase (TXSyn) distributed mainly in epidermis and the concentration of TXB_2, a metabolite of TXA_2, were increased in lesional skin. Scratching caused by the PAR2 agonist SLIGRL-NH_2 was suppressed by ONO-3708. SLIGRL-NH_2-induced scratching decreased approximately 75% in TP-deficient mice, compared to wild-type mice. In primary cultures of mouse keratinocytes, SLIGRL-NH_2 induced the production of TXA_2, as evidenced by the increased TXB_2, which was inhibited by the TXSyn inhibitor sodium ozagrel and a PAR2-neutralizing antibody. Taken together, these results suggest that epidermal TXA_2, which may be produced via PAR2 activation, is involved in itching in atopic dermatitis. ***Key words: itch; thromboxane A_2; TP receptor; thromboxane synthase; atopic dermatitis; proteinase-activated receptor 2.***

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Atopic dermatitis (AD) is a severe, chronic inflammatory skin disease and itching constitutes its major diagnostic criterion (1–3). In AD patients, scratching induced by the itch sensation aggravates dermatitis and increases the frequency of itching (4). Therefore, itch control is very important for improving quality of life and treating AD. Although H_1 histamine receptor antagonists often serve as the drug of first choice for treating itching, AD-derived itching responds poorly to these drugs (5), suggesting that histamine is not essential for AD itching. Therefore, a comprehensive understanding of the mechanisms and mediators underlying AD itching is limited.

Glucocorticoids attenuate itching in chronic skin diseases including AD (6–8). The mechanisms underlying the pharmacological action of glucocorticoids including the inhibition of phospholipase A_2 (9) and the regulation of the expression of cyclooxygenase (COX)-2, but not COX-1 (10), have been elucidated. These findings suggest that arachidonic acid metabolites, which are regulated by glucocorticoids, are involved in the itch sensation.

Thromboxane (TX) A_2 is an arachidonic acid metabolite produced by catalysis with COX and TX synthase (TXSyn) and is altered spontaneously to form inactive TXB_2. Additionally, TXA_2 exerts its pharmacological activity (e.g., platelet aggregation) via the thromboxane TXA_2 receptor (TP), a G-protein coupled receptor (11) also involved in allergic skin inflammation (12). In patients with pruritic diseases, the serum concentration of TXB_2, a metabolite (and therefore an indicator) of TXA_2, is increased (13–16). Moreover, intradermal injection of TXA_2 elicits scratching, an itch-associated response, in mice (17). These results suggest that TXA_2 plays an important role in the itching sensation that accompanies several pruritic skin diseases. However, it remains unclear whether TXA_2 is involved in AD-associated itching.

NC mice that are maintained for a long time in a conventional environment develop chronic dermatitis, hyperplasia, dry skin, increased serum IgE levels, mast cells in the skin and spontaneous scratching, an itch-related behavior (18, 19); these features are similar to those of AD patients (1–3). In this study, we investigated whether TXA_2 is involved in spontaneous itching in NC mice with AD-like chronic dermatitis.

MATERIALS AND METHODS

Animals

Male NC/Nga mice (14–21 weeks old), purchased from Japan SLC (Hamamatsu, Japan), were used in this study. The mice were bred in a specific pathogen-free environment at the Division of Animal Resource and Development, Life Science Research Center, University of Toyama, and in a conventional environment in the experimental animal room in the Department of Applied Pharmacology, University of Toyama. All mice, except for the healthy controls, were kept together with mite-infected mice with chronic dermatitis for 2 weeks at 4–5 weeks of age in a conventional environment in the experimental animal

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room (19). These mice exhibited severe dermatitis after one and two months. In one series of experiments, TP receptor-deficient mice (TP-$R^{-/-}$) mice with a C57BL/6 genetic background and wild-type mice were used at 11 weeks of age (20). The mice were housed in a room with controlled temperature (21–23°C), humidity (45–65%), and light cycle (lights on from 08:00 to 20:00 h). Food and water were freely available. All animal experiment procedures were approved by the Committee for Animal Experiments at the University of Toyama and Asahikawa Medical University and were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

Agents (see Appendix S1[1])

Primary cultures of mouse keratinocytes (see Appendix S1[1])

Behavioral experiments (see Appendix S1[1])

Real-time PCR (see Appendix S1[1])

Reverse transcription and PCR (see Appendix S1[1])

Enzyme immunoassay for TXB_2

Animals were anesthetized with sodium pentobarbital (80 mg/kg) and transcardially perfused with phosphate-buffered saline (PBS). The skin was isolated using an 8-mm-diameter punch, immediately shredded with scissors, and added to 1 ml of ice-chilled ethanol containing 10 μM indomethacin and 10 μM zileuton to inhibit cyclooxygenase and 5-lipoxygenase, respectively. Following homogenization, each sample was centrifuged at 600 × g at 4°C for 10 min. The supernatant (0.8 ml) was mixed with 5 ml of distilled water (pH 3.0) and applied to a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA) equilibrated with methanol. The cartridges were washed with hexane followed by distilled water and the lipids were then eluted with methyl formate. The eluted fraction was then evaporated and the resulting residue was suspended in an enzyme immunoassay buffer (Cayman Chemical, Ann Arbor, MI, USA) for the assay of TXB_2. The amount of TXB_2 was determined using an enzyme immunoassay kit (Cayman Chemical). In the case of cultured keratinocytes, the culture medium was changed to mouse keratinocyte growth medium (CnT-Prime basal medium; CELLnTEC Advanced Cell Systems AG), as the assay medium. Samples for the assay of TBX_2 were obtained from the supernatant 5 min post SLIGRL-NH_2 administration. Following collection of culture medium, the cultured keratinocytes were treated with 1% Triton X-100 to solubilize the cell proteins. Total protein concentration was determined using a Bio-Rad Protein Assay Kit (Hercules, CA, USA) and TXB_2 concentration was measured using an enzyme immunoassay kit (Cayman Chemical). The concentration of TXB_2 in keratinocytes was normalized to a skin section (8-mm diameter) and total protein concentration, respectively.

Immunostaining

Under anesthesia with sodium pentobarbital (80 mg/kg, intraperitoneal), the animals were transcardially perfused with PBS following by with 4% paraformaldehyde (PFA). The skin of the rostral back was isolated, post-fixed with 4% PFA and then immersed in 30% sucrose solution for 2 days. The tissue was then embedded in Tissue-Tek1 O.C.T. Compound (Sakura Fineteck Co., Ltd., Tokyo, Japan) and the frozen samples were sectioned at 16 μm with a cryostat (Leica, Wetzlar, Germany). The sections were washed with PBS and then treated with Protein Block® (DAKO Co., Hamburg, Germany) followed by 0.3% Triton X-100 in PBS. The sections were treated with anti-TXSyn antibody (Cayman Chemical) at 4°C overnight. For some experiments, sections were treated with the antibody (Cayman Chemical) pre-incubated with its specific antigen prior to staining. Subsequent to washing with PBS, the preparations were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA) for 1 h at room temperature. Fluorescence signals were observed using a fluorescence microscope (BX-61/DP70, Olympus, Tokyo, Japan). Fluorescent intensity was analyzed with NIH Image software (National Institute of Health, Bethesda, MD, USA).

Statistical analysis

Data are presented as mean ± S.E.M. The statistical significance of differences between group means was analyzed using the Student's *t*-test (two groups) or one-way analysis of variance (ANOVA) followed by a *post-hoc* Holm–Šidák test (3 or more groups). A p-value < 0.05 was considered statistically significant.

RESULTS

Spontaneous scratching in NC mice with chronic dermatitis

Fig. 1A shows the number of spontaneous bouts of hind paw scratching towards any region of the body in individual healthy and chronic dermatitis NC mice. Although the healthy mice did not exhibit spontaneous scratching, most age-matched chronic dermatitis mice exhibited apparent spontaneous scratching. In the following experiments, we used healthy NC mice that were given intradermal injections of SLIGRL-NH_2. In other experiments, healthy and chronic dermatitis NC mice were compared.

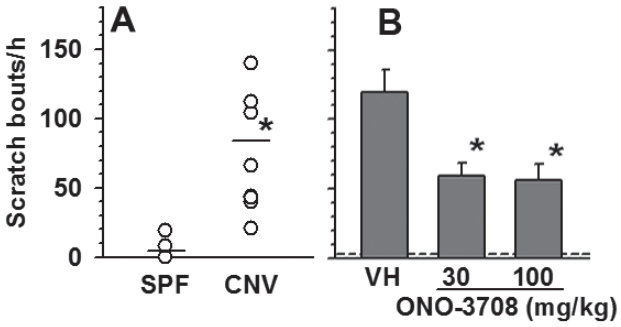

Fig. 1. (A) Comparison of spontaneous scratching between healthy and dermatitis mice. Spontaneous hind paw scratching towards any region of the body was counted for 1 h at 21 weeks of age. Healthy mice were kept under specific pathogen-free (SPF) conditions and dermatitis mice were transferred to a conventional environment (CNV) at 4–5 weeks of age. Open circles represent the values for individual animals; the horizontal bars represent the means (n = 10 each). *p < 0.05 (Student's *t*-test). (B) Effects of ONO-3708 on spontaneous scratching in NC mice with chronic dermatitis. Either ONO-3708 or the vehicle (VH) was administered orally 1 h prior to observation. The broken line represents the mean number of scratch bouts in healthy mice. Values represent the mean and S.E.M. (n = 16). *p < 0.05, as compared with VH-treated mice (Holm–Šidák test).

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Effects of TP antagonist on spontaneous scratching in NC mice with chronic dermatitis

The TP receptor antagonist ONO-3708 (30 or 100 mg/kg) significantly inhibited spontaneous scratching in NC mice with chronic dermatitis, compared with the vehicle-treated group (Fig. 1B).

Effects of the TP receptor antagonist and TP receptor-deficiency on SLIGRL-NH_2-induced scratching

PAR2 is involved in spontaneous scratching in NC mice with chronic dermatitis (24). Intradermal injection of SLIGRL-NH_2 (100 nmol/site), a PAR2 agonist peptide, elicited scratching in healthy NC mice (Fig. 2). ONO-3708 (30 and 100 mg/kg) significantly inhibited scratching induced by SLIGRL-NH_2 compared with the vehicle-treated group (Fig. 2 A). While SLIGRL-NH_2 (100 nmol/site) induced scratching in wild-type mice, the scratching was decreased by approximately 75% in TP-deficient mice, compared with that in wild-type mice (Fig. 2B). The mRNA expressions of TP receptor, TXSyn and PAR2 in the skin and DRG in wild-type and TP-deficient mice are shown in Fig. S1[1].

Expression of TXSyn mRNA and TXB_2 concentration in NC mouse skin

The expression of TXSyn mRNA (Fig. 3A) and TXB_2, a stable degradation product of TXA2 (Fig. 3B), were significantly higher in the lesional skin of NC mice with chronic dermatitis than in the skin of healthy NC mice.

Distribution of TXSyn in the skin of NC mice

In contrast to healthy skin, the lesional skin of NC mice with chronic dermatitis exhibited epidermal hyperplasia (Fig. 4). Immunohistochemical staining and fluorescent

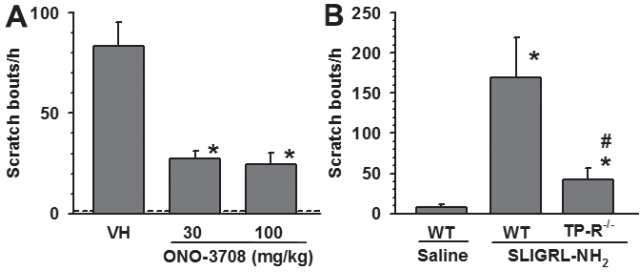

Fig. 2. Effects of the thromboxane (TX) A_2 receptor (TP) antagonist ONO-3708 on SLIGRL-NH_2-induced scratching. (A) SLIGRL-NH_2 (100 nmol/site) was injected intradermally into the rostral back of healthy NC mice. ONO-3708 or the vehicle (VH) was administered orally 1 h prior to SLIGRL-NH_2 injection. The broken line represents the mean number of scratch bouts in the saline-injected group. Values represent the mean and S.E.M. ($n = 8$). *$p < 0.05$, as compared with VH-treated group (Holm–Šidák test). (B) SLIGRL-NH_2 (100 nmol/site) or saline was injected intradermally into the rostral back of TP-deficient (TP-$R^{-/-}$) or the wild-type (WT) mice. Values represent the mean and S.E.M. ($n = 6$–8). *$p < 0.05$, as compared with saline-injected group (WT), #$p < 0.05$, as compared with SLIGRL-injected group (WT) (Holm–Šidák test).

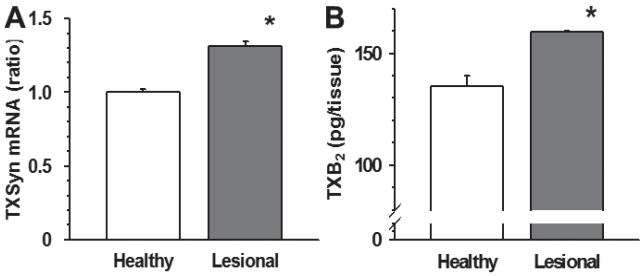

Fig. 3. (A) Expression level of TXSyn mRNA in the skin of healthy (lesional) or dermatitis mice (NC). The mRNA levels were determined in 8-mm diameter punch skin of the rostral back by real-time PCR. The mRNA levels were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase in each sample and then to the mean level observed in the healthy control mice. Values represent the means and S.E.M. ($n = 9$). *$p < 0.05$ (Student's *t*-test). (B) TXB_2 concentration in the skin of healthy or dermatitis (lesional) mice. The TXB_2 concentration in the rostral back skin of healthy or dermatitis mice was measured by an enzyme immunoassay. Values represent the mean and S.E.M. ($n = 10$). *$p < 0.05$ (Student's *t*-test).

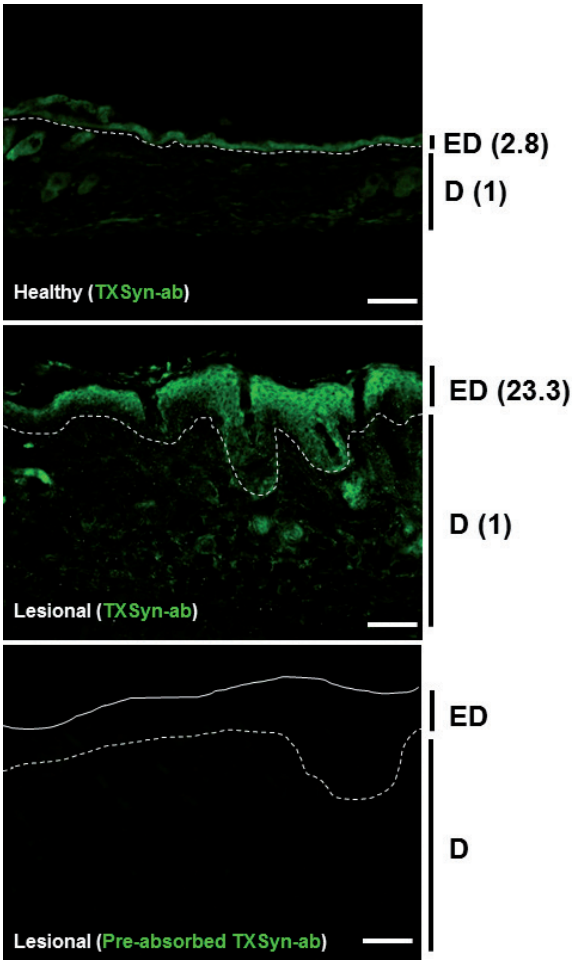

Fig. 4. Thromboxane synthase (TXSyn). Representative images of TXSyn (green) immunostaining in the skin of healthy (Top) and dermatitis (Middle) NC mice. Skin samples of dermatitis NC mice were also immunostained with the pre-absorbed TXSyn antibody (Bottom). Dotted lines indicate the boundary between the dermis (D) and epidermis (ED). The values in parenthesis indicate the fluorescence intensity ratio in the ED and D (ED/D); scale bar = 100 µm.

intensity analysis in the epidermis and dermis revealed that TXSyn is predominantly expressed in epidermal keratinocytes (Fig. 4). Staining was eliminated when pre-absorbed antibody was used (Fig. 4 bottom).

The production of TXA_2 induced by SLIGRL-NH_2 in primary cultures of mouse keratinocytes

Both TXSyn and PAR2 mRNA were expressed in primary cultures of mouse keratinocytes (Fig. 5A).

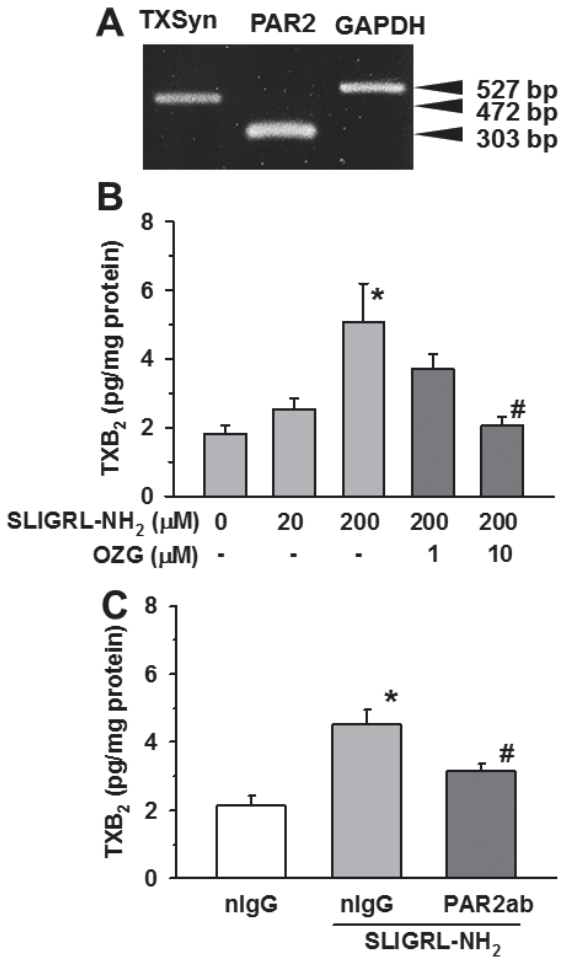

Fig. 5. The production of thromboxane (TX)A_2 induced by SLIGRL-NH_2 in primary cultures of mouse keratinocytes. (A) The expression levels of TX synthase (TXSyn) and proteinase-activated receptor 2 (PAR2) mRNA were determined using reverse transcription (RT)-PCR. Typical examples of the bands of RT-PCR products for TXSyn, PAR2 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA are seen in the gel. (B) Effects of the TXSyn inhibitor sodium ozagrel on SLIGRL-NH_2-induced TXA_2 production. Sodium ozagrel was applied 5 min prior to the application of SLIGRL-NH_2. Since TXA_2 is altered spontaneously to form inactive TXB_2, the TXB_2 content in the assay medium was measured using an enzyme immunoassay. Values represent the mean and S.E.M. ($n=6$). *$p<0.05$, as compared with assay medium alone, #$p<0.05$, as compared with SLIGRL-NH_2 (200 μM) alone (Holm–Šidák test). (C) Effect of PAR2-neutralized antibody on SLIGRL-NH_2-induced TXA_2 production. All cultures were exposed to Non-specific IgG (nIgG) (1:500) or PAR2-neutralized antibody (PAR2ab, 1:500) 10 min prior to the application of SLIGRL-NH_2 (200 μM). Values represent the means and S.E.M. ($n=6$). *$p<0.05$, as compared with nIgG alone, #$p<0.05$, as compared with nIgG+SLIGRL-NH_2 (Holm–Šidák test).

SLIGRL-NH_2 (20–200 μM) induced TXA_2 production, as determined by an increase in TXB_2 concentration in primary cultures of mouse keratinocytes (Fig. 5B). SLIGRL-NH_2-induced TXA_2 production was significantly inhibited by the TXSyn inhibitor sodium ozagrel (1–10 μM) (Fig. 5B). In addition, the PAR2-neutralized antibody (Fig. S2[1]) also attenuated the TXA_2 production inhibited by SLIGRL-NH_2 (Fig. 5C).

DISCUSSION

Responses to pruritogens differ among mouse species (25). An intradermal injection of histamine (10–1,000 nmol/site) did not induce scratching in healthy NC mice (Fig. S3[1]), suggesting that NC mice respond minimally to histamine. Thus, pruritogens other than histamine are involved in the observed spontaneous scratching in NC mice with chronic dermatitis. The results of the present study show that the TP receptor antagonist ONO-3708 inhibits spontaneous scratching in NC mice with chronic dermatitis. In healthy NC mice, an intradermal injection of the TXA_2 analogue U-46619 (1–100 nmol/site) elicited dose-dependent scratching (Fig. S3[1]). Additionally, U-46619-induced scratching was inhibited by ONO-3708 (Fig. S4[1]). ONO-3708 inhibits receptor binding of U-46619 but not TXB_2, leukotriene D_4, or prostaglandin D_2 and E_1 (21, 22), suggesting that the action of U-46619 is mediated via the TP receptor. Both TXSyn mRNA expression and TXA_2 production, as gauged by TXB_2 concentration, were increased in the skin of NC mice with chronic dermatitis. These results suggest that TXA_2 and its receptor are involved in spontaneous itching in NC mice with chronic dermatitis.

TXSyn is a key enzyme involved in the synthesis of TXA_2 from prostaglandin H_2, a metabolite of arachidonic acid (26). TXSyn was predominately expressed in epidermal keratinocytes in the skin of both healthy and dermatitis NC mice. Previously, we have also shown that keratinocytes express TXSyn in ICR mice, which are untreated healthy mice (17). Mast cells in the skin also express TXSyn and produce TXA_2 via immunological stimulation (27). However, the TXA_2 content is much lower than the contents of other arachidonic acids metabolites, such as prostaglandin D_2 and leukotriene C_4 (27). In addition, no immunoreactivity against TXSyn was observed in the dermis, where the mast cells are distributed, of either healthy or dermatitis NC mice. Therefore, these results do not support the involvement of mast cell TXA_2 in itching in NC mice with chronic dermatitis. However, it is still possible that mast cell-derived TXA_2 is involved in itching associated with dermatitis. Taken together, these findings indicate that epidermal keratinocytes constitute the main source of TXA_2 production in lesional skin.

In the skin of NC mice with chronic dermatitis, expression of TXSyn mRNA was increased. The AP-1

family of transcription factors plays an important role in the promotor activity for TXSyn (28). Increased immunoreactivity to AP-1-related transcription factors (e.g., Jun D, c-Fos, Fos B, Fra-1, and Fra-2) has been observed in the epidermis (29) of AD patients compared to that of healthy subjects. Jun D and c-Fos, in particular, exhibit strong cytoplasmic positivity in the epidermis of AD patients (29). Thus, the increase in AP-1 expression may be related to the elevated TXSyn expression in atopic dermatitis-like skin lesions.

The mechanisms underlying TXA_2 production in lesional skin are not completely understood. Previously, we have demonstrated that serine proteinase and PAR2 are involved in itch-related responses in NC mice with chronic dermatitis (24). In this study, the PAR2 agonist peptide SLIGRL-NH_2-induced itch-related responses were inhibited by a TP receptor antagonist in healthy NC mice and were decreased in TP receptor-deficient mice, suggesting that TXA_2 is involved in PAR2-related responses. PAR2 is predominately expressed in epidermal keratinocytes (24). In primary cultures of NC mouse keratinocytes, the application of a PAR2 agonist peptide induced the production of TXA_2, and this response was inhibited by the TX inhibitor and the PAR2-neutralizing antibody. Thus, PAR2 activation may be involved in the production of TXA_2 in keratinocytes. PAR2 is activated by trypsin-like serine proteases (e.g., tryptase and kallikrein 5) and a synthetic PAR2-tethered ligand known as PAR2 agonist peptide (30–32). In NC mice with chronic dermatitis, tryptase from mast cells (24) and kallikrein 5 from keratinocytes (33) are involved in itching behavior, suggesting that these serine-proteases play an important role in the production of TXA_2. There are at least two possible pathways by which keratinocytes produce TXA_2 following PAR2 stimulation. PAR2 is a G-protein-coupled receptor (30) and its activation increases the level of free intracellular Ca^{2+} ions (30, 34, 35). Thus, the activation of phospholipase A_2 via increased free intracellular Ca^{2+} ions (36) may promote TXA_2 production. Production may also occur via Rho kinase-mediated phospholipase A_2 activation (37); the activation of PAR2 stimulates Rho kinase activity (38). In addition to the increase in TXSyn expression, these two pathways may contribute to the production of TXA_2 in the lesional skin of NC mice with chronic dermatitis.

In the skin, TP receptors are predominantly expressed in keratinocytes and primary afferent neurons (17) (Fig. S3[1]). In addition, TP receptors are expressed in small-size neurons in the dorsal root ganglion (17); these have chiefly unmyelinated C-fibers (39, 40), which play an important role in itch signaling (41, 42). The application of the TXA_2 analogue U-46619 increases the concentration of free intracellular Ca^{2+} ions, which is inhibited by the TP receptor antagonist, in primary cultures of dorsal root ganglion neurons (17). The action of U-46619 is also inhibited by L-type calcium channel blockers, suggesting that the activation of TP receptors elicits membrane depolarization (43). These observations suggest that TXA_2 acts on primary afferent neurons or through both autocrine and paracrine mechanisms in keratinocytes. Additionally, intra-epidermal neurites increase in the skin of NC mice with chronic dermatitis (44) and patients with atopic dermatitis (45, 46). These findings also support the hypothesis that TXA_2 released from keratinocytes acts directly on primary afferent neurons.

In conclusion, we demonstrated that TXA_2 produced by epidermal keratinocytes is involved in spontaneous itch-related behavior in NC mice with AD-like skin lesions. Moreover, we found that the serine protease-PAR2 system partially contributes to the production of TXA_2. PAR2, TXSyn and TP receptor may constitute potential new targets for the development of antipruritic drugs for AD.

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The authors declare no conflicts of interest.

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Appendix S1

Agents

The TP receptor antagonist (Z)-7-[(1R,3S,4S,5S)-3-[[(2R)-2-cyclopentyl-2-hydroxyacetyl]amino]-6,6-dimethyl-4-bicyclo[3.1.1]heptanyl]hept-5-enoic acid (ONO-3708; Ono Pharmaceutical Co. Ltd., Osaka, Japan) (21, 22) was dissolved in tap water and administered orally in a volume of 0.1 ml per 10 g body weight using a feeding needle. ONO-3708 was administered 1 h prior to observations of spontaneous scratching or pruritogen injections. The proteinase-activated receptor 2 (PAR2) agonist SLIGRL-NH_2 (Ser-Leu-Ile- Gly-Arg-Leu-NH_2; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in physiological saline and injected intradermally into the rostral back in a volume of 50 µl per site using a 19-gauge needle.

For *in vitro* experiments, the TXSyn inhibitor sodium ozagrel (Wako Pure Chemical Ind., Osaka, Japan) was dissolved in culture medium and applied 5 min prior to the application of SLIGRL-NH_2. Non-specific IgG (prepared by the authors) and the anti-PAR2 antibody targeting the second extracellular loop of PAR2 (designed by the authors and manufactured by Sigma-Aldrich) were dissolved in culture medium and applied 10 min prior to the application of SLIGRL-NH_2.

Primary cultures of mouse keratinocytes

The skin was removed from neonatal mice and washed with phosphate-buffered saline (PBS) containing 100 units/ml penicillin (Nacalai Tesque, Kyoto, Japan) and 100 µg/ml streptomycin (Nacalai Tesque). The washed skin was 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 Ind.), 0.01% penicillin G (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and 0.006% kanamycin (Wako Pure Chemical Ind.) at 4°C overnight. The epidermal sheets were removed from the dermis and the isolated keratinocytes were cultured in keratinocyte growth medium (CnT-Prime medium; CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) in the 24-well plates and experiments were conducted when cells reached 80–90% confluence in each well.

Behavioral experiments

Hair was removed from the rostral back of healthy control mice 2 days prior to the experiments for an intradermal injection of SLIGRL-NH_2. For the evaluation of spontaneous scratching, the mouse hair was not removed. The animals were housed individually in acrylic cages composed of 4 equally sized compartments (13 × 9 × 35 cm) for at least 1 h to allow them to acclimate. Following the acclimation period, the spontaneous scratching behavior of the mice was recorded using a digital video camera (HDC-TM25; Panasonic Co., Osaka, Japan) for 1 h without the presence of personnel in the observation room. To observe evoked scratching, the animals were given an intradermal injection after a 1-h acclimation period and immediately returned to the same compartments. The behavior of these mice was also recorded using a digital video camera for 1 h without the presence of personnel in the observation room. Playback of the video was used to determine hind-paw scratching as an index of itching (23). Hind paw scratching toward any region of the body was recorded in mice with dermatitis, while in SLIGRL-NH_2-injected mice, hind paw scratching aimed at the injection site was recorded. A series of several scratching movements of approximately 1 s was considered as a single bout of scratching.

Real-time PCR

Animals were euthanized by transcardiac infusion of PBS under sodium pentobarbital (80 mg/kg) anesthesia. The skin on the rostral back was isolated and stored at −80°C until assayed. Total RNA was extracted from the tissue samples using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) and then treated with DNase I (Takara Bio Inc., Otsu, Japan) in 50 µl of reaction buffer. Total RNA (1 µg) was reverse-transcribed in a 20-µl reaction mixture containing 50 pmol oligo $(dT)_{16}$ primer, 1 µl of ReverScript III (Wako Pure Chemical Ind.), 10 pmol of each deoxyribonucleotide (Takara Bio Inc.), and 20 U RNase inhibitor (Toyobo Co. Ltd., Osaka, Japan) at 37°C for 1 h. Quantitative PCR using an aliquot (2.5 µl) of the cDNA and specific primers was performed with a Mx3000PTM real-time PCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). The sequences of the primers used are as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCAAGCTCATCCATGACAAC-3′ (sense) and 5′-TTACTCCTTGGAGGCCATGT-3′ (antisense); TXSyn, 5′- CCCTGTCCTCTTCTGAGTGC-3′ (sense) and 5′-GCCTCTGCTGTGAACCTTTC-3′ (antisense). The mRNA expression levels of GAPDH and TXSyn were analyzed using Mx Pro QPCR software (Agilent Technologies, Inc.). PCR conditions consisted of 40 cycles of denaturation (30 s at 95°C), primer annealing (30 s at 55°C), and elongation (30 s at 72°C). The relative amount of TXSyn mRNA in each sample was first normalized to the level of GAPDH mRNA and then normalized toTXSyn mRNA expression levels in healthy controls.

Reverse transcription and PCR

Primary cultures of mouse keratinocytes were washed with PBS. Total RNA extraction and cDNA preparation were performed as described in the previous section. An aliquot (1 µl) of the cDNA was mixed with a PCR mix containing 1.5 mM $MgCl_2$, 1 × Green GoTaq® Flexi buffer, 2.5 U GoTaq® DNA Polymerase (Promega CO., Madison, WI, USA), and 50 pmol of the sense and antisense primer. The primer sequences used for reverse transcription (RT)-PCR are as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCAAGCTCATCCATGACAAC-3′ (sense) and 5′-TTACTCCTTGGAGGCCATGT-3′ (antisense); TXSyn, 5′- CCCTGTCCTCTTCTGAGTGC-3′ (sense) and 5′-GCCTCTGCTGTGAACCTTTC-3′ (antisense); PAR2, 5′- TTTTCCCTCATCCTCGATCA-3′ (sense) and 5′-AAGCCACAAATACAGGCAGT-3′ (antisense).

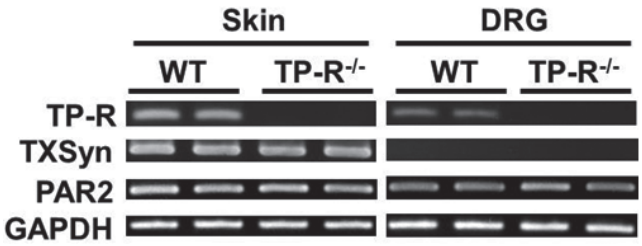

Fig. S1. mRNA expression of TP receptor (TP-R), thromboxane synthase (TXSyn) and proteinase-activated receptor 2 (PAR2) in the skin and dorsal root ganglion (DRG) of TP receptor deficient (TP-R$^{-/-}$) and the wild-type (WT) mice. mRNA expression of TP-R, TXSyn, PAR2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed using reverse-transcription PCR. The sequences of the specific primer pairs for TXSyn, PAR2 and GAPDH are shown in the Materials and Methods section. The primer sequences for TP-R are shown as follows: (sense) 5′- CCGCTACAGTGTGCAGTACC-3′ and (antisense) 5′-ATCATCTCCACCTCGCAGTC-3′ (predicted length: 213 bps).

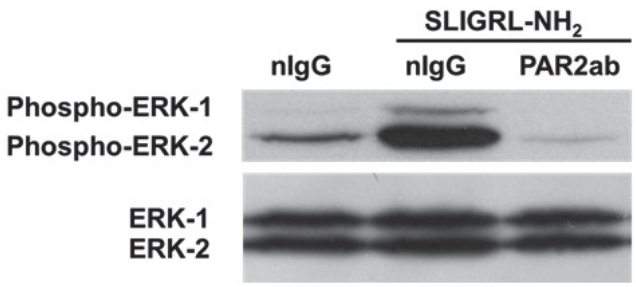

Fig. S2. Effect of PAR2-neutralized antibody on SLIGRL-NH_2-induced ERK phosphorylation in primary cultures of mouse keratinocytes. The photographs show typical examples of western blot bands. Non-specific IgG (nIgG) (1:500) or PAR2-neutralized antibody (PAR2ab, 1:500) was treated 10 min prior to the application of SLIGRL-NH_2 (200 μM). Cells were collected 5 min following the application of SLIGRL-NH_2. Western blot analysis was performed on total protein from the collected cells with specific antibodies for ERK and the phosphorylated ERK (Cell signaling Technology, Danvers, MA, USA).

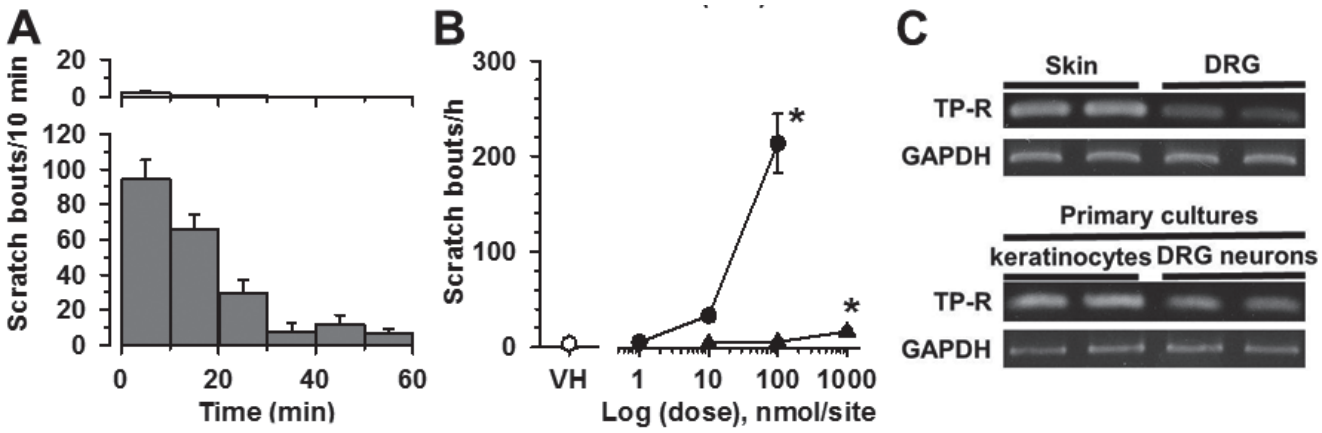

Fig. S3. Scratching following intradermal injections of the thromboxane (TX) A_2 receptor (TP-R) agonist U-46619 in healthy NC mice and TP-R mRNA expression in skin, dorsal root ganglion (DRG) and primary cultures (keratinocytes and DRG neurons). (A) Time course of scratching following vehicle (VH) (upper panel, 0.2% ethanol in saline) or U-466169 (lower panel, 100 nmol/site) injection. (B) Dose-response curves for scratching in response to U-46619 and histamine. Healthy NC mice were given intradermal injections of U-46619 (●), histamine (▲), or vehicle (VH; ○, 0.2% ethanol in saline; △saline). Values represent the means and S.E.M. ($n = 8$). *$p < 0.05$, as compared with VH-treated mice (Holm–Šidák test). (C) Expression of TP-R mRNA in NC mice. mRNA expression of TP-R in skin, DRG and primary cultures (keratinocytes and DRG neurons) was analyzed using RT-PCR. The methods of primary cultures of keratinocytes and DRG neurons are described in the Materials and Methods section and as previously published (17), respectively. The sequences of the specific primer pairs for TP-R and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are detailed in the legend of Fig. S1 and the Materials and Methods section, respectively.

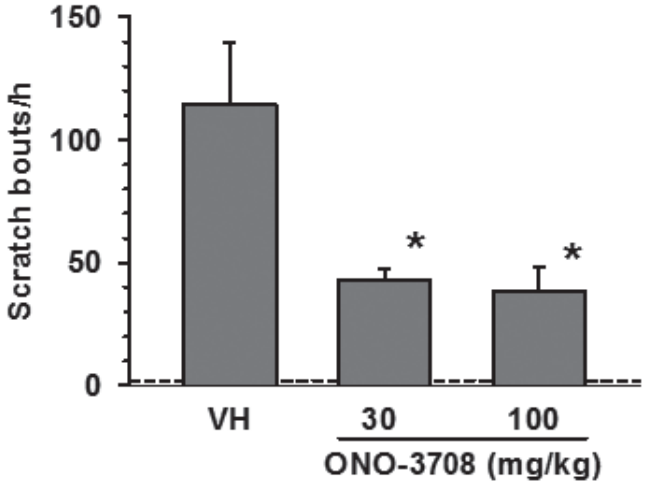

Fig. S4. Effects of the thromboxane (TX) A_2 receptor (TP) antagonist ONO-3708 on U-46619-induced scratching in healthy NC mice. U-46619 (100 nmol/site) was injected intradermally into the rostral back of healthy NC mice. ONO-3708 or the vehicle (VH, 0.2% ethanol in saline) was administered orally 1 h prior to U-46619 injection. The broken line represents the mean number of scratching bouts in the saline-injected group. Values represent the mean and S.E.M. ($n = 8$). *$p < 0.05$, as compared with VH-treated mice (Holm–Šidák test).