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Leukotriene B_4 Mediates Sphingosylphosphorylcholine-Induced Itch-Associated Responses in Mouse Skin

Tsugunobu Andoh[1], Ayumi Saito[1] and Yasushi Kuraishi[1]

In atopic dermatitis, the concentration in the skin of sphingosylphosphorylcholine (SPC), which is produced from sphingomyelin by sphingomyelin deacylase, is increased. In the present study, we investigated the itch-eliciting activity of SPC and related substances and the mechanisms of SPC action in mice. An intradermal injection of SPC, but not sphingomyelin and sphingosine, induced scratching, an itch-associated response, which was not suppressed by a deficiency in mast cells or the H_1 histamine receptor antagonist terfenadine. The action of SPC was inhibited by the μ-opioid receptor antagonist naltrexone. SPC action also was inhibited by the 5-lipoxygenase inhibitor zileuton and the leukotriene B_4 antagonist ONO-4057, but not by the cyclooxygenase inhibitor indomethacin. Moreover, SPC action was inhibited by the antiallergic agent azelastine, which suppresses the action and production of leukotriene B_4. Administration of SPC to the skin and to primary cultures of keratinocytes increased leukotriene B_4 production. SPC increased intracellular Ca^{2+} ion concentration in primary cultures of dorsal root ganglion neurons and keratinocytes. These results suggest that SPC induces itching through a direct action on primary afferents and leukotriene B_4 production of keratinocytes. Sphingomyelin deacylase and SPC receptors may be previously unreported targets for antipruritic drugs.

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INTRODUCTION

Pruritus is an essential symptom of atopic dermatitis, and the suppression of itching (and scratching) is important in its treatment. Although H_1 histamine receptor antagonists are often the drug of first choice for the treatment of itch, the pruritus of atopic dermatitis responds poorly to them (Wahlgren, 1991). The precise mechanisms and mediators of itch in atopic dermatitis are unclear.

Dryness and impaired barrier function of the skin are clinical signs of patients with atopic dermatitis. The amount of ceramides is markedly reduced in the stratum corneum of these patients (Imokawa *et al.*, 1991; Okamoto *et al.*, 2003). Changes in the metabolism of ceramides have been believed to be involved in dry skin and impaired barrier function in atopic dermatitis. In healthy skin conditions, the amount of ceramide in the stratum corneum is regulated by a balance of the rate-limiting enzymes of sphingolipid synthesis, such as serine-palmitoyl transferase, sphingomyelinase, and ceramidase. Ceramide is synthesized from sphingomyelin by sphingomyelinase in the stratum corneum (Menon *et al.*, 1986). In the stratum corneum of atopic dermatitis patients, sphingomyelin hydrolysis is due mainly to sphingomyelin deacylase, which converts sphingomyelin into sphingosylphosphorylcholine (SPC) and free fatty acid, rather than into sphingomyelinase (Murata *et al.*, 1996). The activity of sphingomyelin deacylase is markedly higher in patients with atopic dermatitis than in healthy individuals (Murata *et al.*, 1996; Hara *et al.*, 2000), and SPC is increased in the stratum corneum of atopic dermatitis (Okamoto *et al.*, 2003). However, there are conflicting reports regarding the role of SPC in inflammation (Ceballos *et al.*, 2007; Murch *et al.*, 2008), and the role of SPC in atopic dermatitis is unclear.

An intradermal injection of SPC elicits scratching, an itch-associated response, in mice (Kim *et al.*, 2008). SPC-induced scratching was shown to be inhibited by an antagonist of G-protein-coupled receptor 4, which had been reported to be one of the SPC receptors (Sakai *et al.*, 2005). Thereafter, however, reports on the identification of G-protein-coupled receptor 4 and ovarian cancer G-protein-coupled receptor 1 as SPC receptors (Xu *et al.*, 2000; Zhu *et al.*, 2001) were retracted and an SPC receptor has not yet been identified. The precise mechanisms of SPC-induced scratching remain unclear, although the involvement of mast cells, capsaicin-sensitive primary afferents, and Rho-associated protein kinase

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Abbreviations: DRG, dorsal root ganglion; SPC, sphingosylphosphorylcholine; TRPV1, transient receptor potential vanilloid 1

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has been suggested (Kim *et al.*, 2008). SPC acts on the epidermal keratinocytes (Wakita *et al.*, 1998). Recent studies on peripheral mechanisms of itch have shown the important role of keratinocytes and their itch mediators, including leukotriene B_4 (Andoh *et al.*, 2001, 2004, 2007). Therefore, this study investigated the involvement of leukotriene B_4 and histamine in SPC-induced scratching in mice.

RESULTS

Behavioral effects of SPC, sphingomyelin, and sphingosine

An intradermal injection of SPC (100 nmol per site) increased scratching of the injected site by the hind paw, as compared with vehicle; the effect had almost subsided by 60 minutes (Figure 1a). SPC effects were dose dependent in the range of 10–100 nmol per site (Figure 1b). Intradermal injections of sphingomyelin and sphingosine did not significantly increase scratching (Figure 1b).

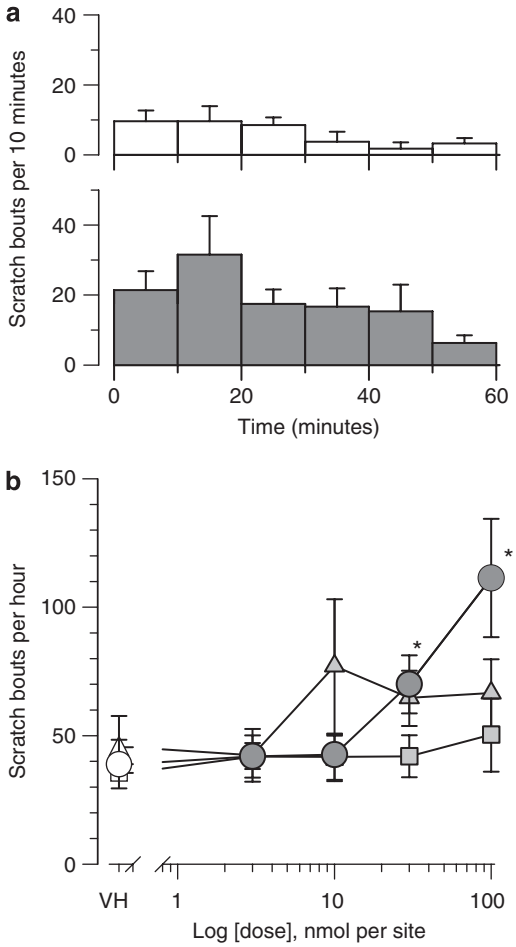

Figure 1. Scratching after intradermal injections of sphingosylphosphorylcholine (SPC) and related substances. Mice were given an intradermal injection and scratching was counted for 60 minutes. (**a**) Time course of scratching after vehicle (VH, upper panel) and SPC (lower panel, 100 nmol per site) injection. (**b**) Dose–response curves for SPC (circles), sphingomyelin (squares), and sphingosine (triangles). Values represent the means ± SEM for eight animals. *$P<0.05$ when compared with VH (Dunnett's multiple comparisons).

Effects of various agents on SPC-induced scratching

Effects of systemic pretreatment with several agents on scratching induced by an intradermal injection of SPC (100 nmol per site) were examined. The μ-opioid receptor antagonist naltrexone hydrochloride (1 mg kg^{-1}) significantly inhibited SPC-induced scratching (Figure 2). The antiallergic agent azelastine hydrochloride (3–30 mg kg^{-1}) produced a dose-dependent inhibition of SPC-induced scratching, but the H_1 histamine receptor antagonist terfenadine (30 mg kg^{-1}) was without effect (Figure 3). The 5-lipoxygenase inhibitor zileuton (30 and 100 mg kg^{-1}) and the leukotriene B_4 receptor antagonist ONO-4057 (30 and 100 mg kg^{-1}) produced a dose-dependent inhibition of SPC-induced scratching, but the cyclooxygenase inhibitor indomethacin (3 and 10 mg kg^{-1}) was without effect (Figure 4).

Effect of mast cell deficiency on SPC-induced scratching

Deficiency in mast cells did not affect scratching induced by an intradermal injection of SPC (100 nmol per site); scratch

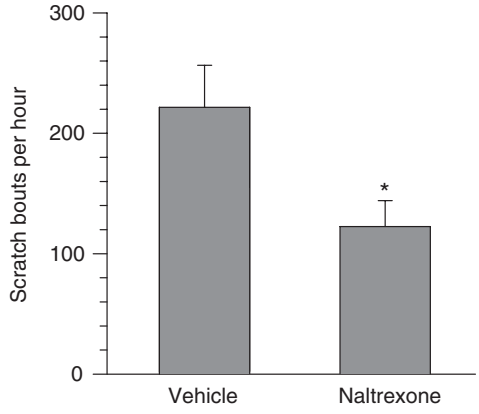

Figure 2. Suppression by naltrexone of sphingosylphosphorylcholine (SPC)-induced scratching. Mice were given an intradermal injection of SPC (100 nmol per site) and scratching was counted for 1 hour. Naltrexone hydrochloride (1 mg kg^{-1}) or vehicle (saline) was injected subcutaneously 15 minutes before intradermal injection. Values represent the means ± SEM for eight animals. *$P<0.05$ when compared with vehicle (Student's *t*-test).

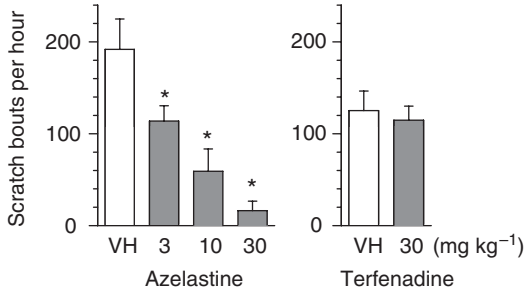

Figure 3. Effects of azelastine and terfenadine on scratching induced by sphingosylphosphorylcholine (SPC). Mice were given an intradermal injection of SPC (100 nmol per site) and scratching was counted for 1 hour. Azelastine hydrochloride, terfenadine, and vehicle (VH) were administered orally 1 hour before SPC injection. Values represent the means ± SEM for eight animals. *$P<0.05$ when compared with VH (Dunnett's multiple comparisons or Student's *t*-test).

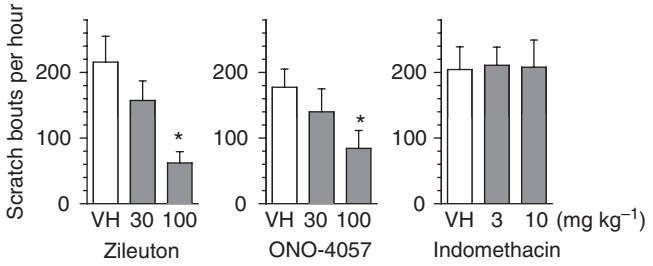

Figure 4. Effects of inhibition of eicosanoid systems on scratching induced by sphingosylphosphorylcholine (SPC). Mice were given an intradermal injection of SPC (100 nmol per site) and scratching was counted for 1 hour. Zileuton, ONO-4057, indomethacin, and vehicle (VH) were administered orally 1 hour before SPC injection. Values represent the means ± SEM for eight animals. *$P<0.05$ when compared with VH (Dunnett's multiple comparisons).

bouts for 1 hour were 348 ± 32 and 354 ± 69 (mean ± SEM, $n=8$ each) in WBB6F1 *W/W*^{v} and the control WBB6F1 +/+ mice, respectively.

SPC-induced production of leukotriene B_4

An intradermal injection of SPC (100 nmol per site) increased the concentration of leukotriene B_4 in the treated skin, which was almost abolished by zileuton (100 mg kg^{-1}) (Figure 5a). A bath application of SPC at a concentration of 200 μM, but not 2–20 μM, to primary cultures of mouse keratinocytes significantly increased the production of leukotriene B_4 (Figure 5b). The effect of SPC (200 μM) was almost abolished by zileuton (10 μM) (Figure 5c).

SPC-induced increase in intracellular Ca^{2+} concentration in dorsal root ganglion neurons and keratinocytes

Sphingosylphosphorylcholine at a concentration of 200 μM, but not 20 μM, markedly increased the concentration of intracellular Ca^{2+} ions in the cultured keratinocytes; the effect peaked around 20 seconds after administration and had almost subsided by 40 seconds (Figure 6a). In primary cultures of dorsal root ganglion (DRG) neurons, SPC at 20 and 200 μM produced a marked concentration-dependent increase in the concentration of intracellular Ca^{2+} ions; the effect peaked around 20 seconds after administration and had almost subsided by 120 seconds (Figure 6b).

DISCUSSION

Sphingomyelin is metabolized to SPC and ceramide by sphingomyelin deacylase and sphingomyelinase, respectively. Ceramide is further metabolized to sphingosine by ceramidase. In this study, SPC, sphingomyelin, and sphingosine were administered to the mouse skin and only SPC significantly elicited scratching, an itch-related behavior, suggesting that SPC, but not sphingomyelin and sphingosine, is an endogenous itch mediator. As the activity of sphingomyelin deacylase is very low in healthy skin (Hara *et al.*, 2000), substantial amounts of SPC might not be produced from sphingomyelin after its intradermal administration.

SPC-induced scratching was inhibited by the μ-opioid receptor antagonist naltrexone. This result is similar to the

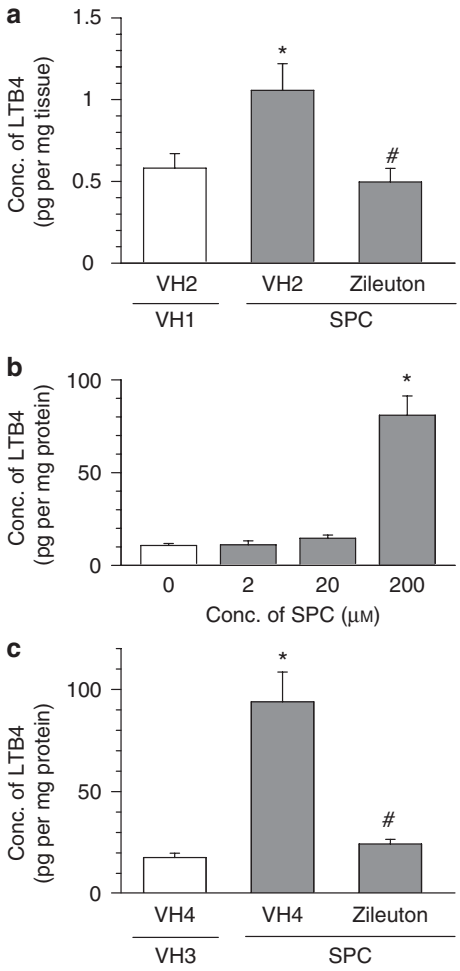

Figure 5. Sphingosylphosphorylcholine (SPC)-induced production of leukotriene B4 in the skin and cultured keratinocytes. (**a**) LTB_4 production in the skin. Mice were given an intradermal injection of SPC (100 nmol per site) or vehicle (VH1: 1% ethanol in saline) and the concentration of LTB_4 in the treated skin was determined 10 minutes later. Zileuton (100 mg kg^{-1}) and vehicle (VH2: 0.5% carboxymethyl cellulose) were administered orally 1 hour before SPC injection. Values represent the means ± SEM for eight animals. *$P<0.05$ when compared with VH1 + VH2. $^{\#}P<0.05$ when compared with VH2 + SPC. (**b**) Concentration dependence of SPC-induced LTB_4 production of the keratinocytes. SPC was administered to cultured keratinocytes, and LTB_4 concentration in the cultured medium was determined 10 minutes later. *$P<0.05$ when compared with control. (**c**) Inhibition by zileuton of SPC-induced LTB_4 production of the keratinocytes. LTB_4 concentration was determined 10 minutes after the administration of SPC (200 μM) and vehicle (VH3: 0.1% dimethyl sulfoxide). Zileuton (10 μM) and vehicle (VH4: 0.1% dimethyl sulfoxide) were applied 1 hour before SPC administration. Values represent the means ± SEM for six wells. *$P<0.05$ when compared with VH3 + VH4. $^{\#}P<0.05$ when compared with VH4 + SPC. Statistical significance was determined by Dunnett's multiple comparisons.

observation by another group (Kim *et al.*, 2008). Opioid antagonists have been shown to inhibit scratching induced by several pruritogens (Andoh *et al.*, 1998b, 2004; Yamaguchi *et al.*, 1999), immediate allergy (Ohtsuka *et al.*, 2001), chronic dermatitis (Yamaguchi *et al.*, 2001), and xerosis (Miyamoto *et al.*, 2002). Opioid antagonists have also been shown to alleviate the itch of patients with pruritic diseases

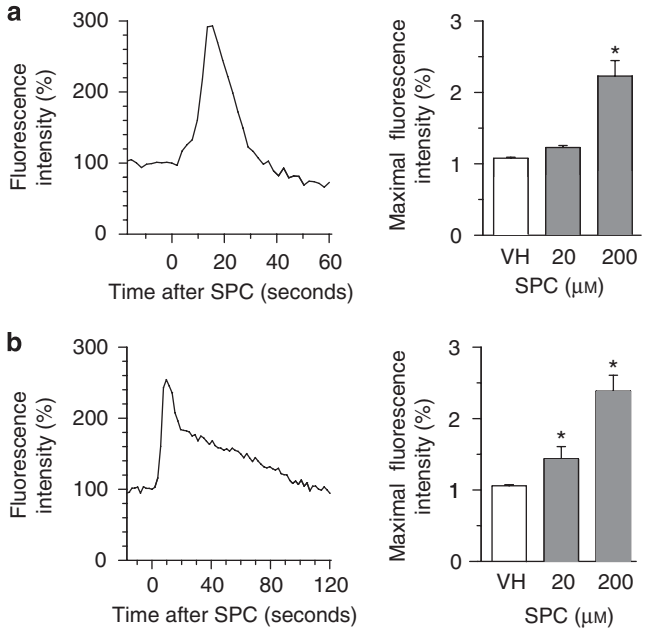

Figure 6. Sphingosylphosphorylcholine (SPC) acts on keratinocytes and dorsal root ganglion neurons to increase intracellular Ca^{2+} ions. (**a**) Keratinocytes and (**b**) dorsal root ganglion neurons. Left graphs, typical examples of changes after the administration of SPC (200 μM). Right graphs, maximal change after SPC administration in the keratinocytes ($n = 16$–23) and dorsal root ganglion neurons ($n = 8$–29). The primary cultures were preloaded with fluo-3. Values represent the means ± SEM. *$P < 0.05$ when compared with vehicle (VH).

such as cholestasis, chronic urticaria, and atopic dermatitis (Monroe, 1989; Bergasa *et al.*, 1995). Opioid antagonists may inhibit itching and scratching through action on μ-opioid receptors in the central nervous system, especially in the lower brainstem (Thomas *et al.*, 1993; Tohda *et al.*, 1997; Andoh *et al.*, 2008; Kuraishi *et al.*, 2008). With these findings being taken into account, it is suggested that SPC-induced scratching is an itch-associated response.

SPC-induced scratching was inhibited by the 5-lipoxygenase inhibitor zileuton and the leukotriene B_4 antagonist ONO-4057, but not by the cyclooxygenase inhibitor indomethacin. Considering the findings that an intradermal injection of leukotriene B_4, but not prostaglandin E_2, elicits scratching in mice (Andoh and Kuraishi, 1998), the present results suggest that leukotriene B_4 has an important role in SPC-induced scratching. The idea is supported by the results that a pruritogenic dose of SPC increased the cutaneous concentration of leukotriene B_4, which was blocked by zileuton. Azelastine (3–30 mg kg^{-1}) produced a marked inhibition of SPC-induced scratching. This effect may be mediated by the blockade of leukotriene B_4 action, because the same doses exert an inhibitory effect on leukotriene B_4-induced scratching (Andoh and Kuraishi, 2002). Because azelastine suppresses the production of leukotriene B_4 (Taniguchi *et al.*, 1990; Shimizu *et al.*, 1995; Andoh and Kuraishi, 2002), this suppression may be partly involved in the inhibition of SPC-induced scratching.

Administration of SPC to cultured keratinocytes increased the production of leukotriene B_4, suggesting that epidermal keratinocytes are important for SPC-induced leukotriene B_4 production in the skin. In the stratum corneum of atopic dermatitis, sphingomyelin is hydrolyzed to SPC with sphingomyelin deacylase rather than to ceramide with sphingomyelinase (Murata *et al.*, 1996). In the stratum corneum of lesional skin, the activity of sphingomyelin deacylase is markedly higher in the lower layers than in the upper layers (Murata *et al.*, 1996) and is higher in the whole epidermis than in the stratum corneum (Hara *et al.*, 2000). Taken together, these findings suggest that the activity of sphingomyelin deacylase and the production of SPC are increased in the epidermal keratinocytes of the lesional skin of atopic dermatitis. Thus, keratinocytes may be important sources of SPC, and SPC may act in an autocrine or paracrine manner to produce itch-associated mediators such as SPC itself and leukotriene B_4.

SPC increased intracellular Ca^{2+} ions in primary cultures of mouse keratinocytes. There are at least two possible pathways for keratinocytes to produce leukotriene B_4 after SPC stimulation. One is the activation of phospholipase A_2 through the increase in intracellular free Ca^{2+} ions induced by SPC (Clark *et al.*, 1990; Shirao *et al.*, 2002). Another pathway is Rho kinase-mediated phospholipase A_2 activation (Chen *et al.*, 2008). Scratching induced by intradermal SPC is inhibited by LY-2732, a Rho kinase inhibitor (Kim *et al.*, 2008), which may be partly due to the inhibition of Rho kinase–mediated production of leukotriene B_4, because Rho kinase has no effect on leukotriene B_4 receptor–mediated signaling (Sabirsh *et al.*, 2004).

Two leukotriene B_4 receptors, BLT1 and BLT2, have been cloned (Yokomizo *et al.*, 1997, 2000). These receptors have higher and lower binding affinities for leukotriene B_4, respectively, and are both G-protein-coupled (Yokomizo *et al.*, 1997, 2000). BLT1, but not BLT2, receptor is present in primary sensory neurons (Andoh and Kuraishi, 2005). Many BLT1-positive neurons express transient receptor potential vanilloid 1 (TRPV1) channels and respond to capsaicin (Andoh and Kuraishi, 2005). Capsaicin-sensitive primary afferents have an important role in itch signaling (Andoh *et al.*, 1998b; Yamaguchi *et al.*, 1999; Nakano *et al.*, 2008). BLT1 receptor activation increases the intracellular concentration of inositol 1,4,5-triphosphate, which leads to Ca^{2+} release from intracellular stores and then Ca^{2+} influx (Yokomizo *et al.*, 1997; Sabirsh *et al.*, 2004). This intracellular signaling may sensitize TRPV1 channels (Clapham *et al.*, 2005). Although efficacy is low, leukotriene B_4 directly activates TRPV1 channels (Hwang *et al.*, 2000). Taken together, these findings suggest that TRPV1 channels play a role in leukotriene B_4-associated itch. BLT1, but not BLT2, receptor mRNA is expressed in the skin (Andoh and Kuraishi, 2005), and leukotriene B_4 acts on the epidermal keratinocytes (Kragballe *et al.*, 1985). Although it remains unclear whether leukotriene B_4 acts on the keratinocytes to produce and/or release itch-associated mediators, such as nitric oxide (Andoh and Kuraishi, 2003), thromboxane A_2 (Andoh *et al.*, 2007), and nociceptin (Andoh *et al.*, 2004), it is possible that

keratinocytes are also involved in leukotriene B_4–associated itching.

SPC-induced scratching was not inhibited by the H_1 histamine receptor antagonist terfenadine at the dosage that inhibits scratching induced by histamine and mast cell degranulation (Inagaki *et al.*, 1999; Ohtsuka *et al.*, 2001). In addition, SPC-induced scratching was not affected by deficiency in mast cells. These results suggest that the mast cell and its mediator histamine do not have a key role in SPC-induced scratching. SPC-induced scratching was, however, shown to be inhibited by ketotifen at an intravenous dose of $1\,mg\,kg^{-1}$ (Kim *et al.*, 2008). These findings are not incompatible with the above idea, because the oral dose ($1\,mg\,kg^{-1}$) of this agent markedly inhibits plasma extravasation induced by histamine and passive cutaneous reaction (Ueno *et al.*, 1998), but it does not inhibit SPC-induced scratching (Kim *et al.*, 2008). The inhibition effect of ketotifen may be due partly to the suppression of leukotriene B_4 production (Matsushita *et al.*, 1998).

The frequency of SPC-induced scratching in ICR mice was roughly similar between these experiments and those of another group (Kim *et al.*, 2008), but the frequency of scratching in WBB6F1 mice was clearly higher than that in ICR mice. One possible explanation for the strain difference is that the expression levels of SPC receptors in the keratinocytes and itch-associated primary afferents are higher in WBB6F1 mice than in ICR mice. However, SPC receptors have not been identified, as mentioned in the Introduction, and verification should wait for the clear identification of the SPC receptor. In contrast to SPC, scratch responses to histamine and serotonin were clearly larger in ICR mice than in WBB6F1 mice (Inagaki *et al.*, 2001). The frequency of scratching induced by substance P is similar between ICR and WBB6F1 mice (Andoh *et al.*, 1998b). Therefore, the difference in behavioral response to SPC may not be due to strain differences in behavioral reactivity.

SPC increased intracellular Ca^{2+} ions in primary cultures of mouse DRG neurons, suggesting the direct action of SPC on primary afferents. SPC-induced scratching was slow in onset and long in duration, whereas the effect of SPC on the DRG neurons was relatively short in duration. Capsaicin-sensitive primary afferents expressing TRPV1 channels may play a role in itch signaling (Kim *et al.*, 2008; Nakano *et al.*, 2008). The sensitization of $G_{q/11}$-coupled receptors of primary sensory neurons (for example, by adenosine 5′-triphosphate and bradykinin) results in the sensitization of TRPV1 channels through the increase in intracellular Ca^{2+} ions and the activation of protein kinase C (Story, 2006). Thus, we speculate that SPC increases intracellular Ca^{2+} ions to sensitize TRPV1 channels of itch-signaling primary afferents.

Ceramides have an essential role in the barrier and water-holding functions of the stratum corneum (Imokawa *et al.*, 1989, 1994). In normal conditions, the ceramide content in the stratum corneum is regulated by the production from sphingomyelin and glucosylceramide by sphingomyelinase and β-glucocerebrosidase, respectively, and the degradation to sphingosine by ceramidase (Imokawa, 2001). Sphingomyelin hydrolysis activity and ceramides are markedly increased and decreased, respectively, in the stratum corneum of atopic dermatitis (Imokawa *et al.*, 1991; Murata *et al.*, 1996). The increase in the sphingomyelin hydrolysis activity is due mainly to the increase in metabolism of sphingomyelin to SPC by sphingomyelin deacylase (Hara *et al.*, 2000), which probably leads to the decrease in the production of ceramides from sphingomyelin. This study showed that SPC, but neither sphingomyelin nor sphingosine, induced itch-related behaviors in mice. Thus, alterations in sphingomyelin metabolism, namely, decrease in ceramides and increase in SPC, cause dysfunction of barrier and water-holding of the skin and itching, respectively. SPC may induce an itch through action on the keratinocytes to produce leukotriene B_4 and on the primary afferents. Leukotriene B_4, increased in the skin by atopic dermatitis (Fogh *et al.*, 1989), also acts on the primary afferents and keratinocytes (Andoh and Kuraishi, 2005). The autocrine or paracrine actions of SPC and leukotriene B_4 may augment itching of atopic dermatitis.

MATERIALS AND METHODS

Animals

Male ICR mice (5–9 weeks old or neonatal) were used, except in one experiment in which mast cell–deficient WBB6F1 *W/W^v* mice (6 weeks old) and the normal WBB6F1 +/+ littermates were used, all purchased from Japan SLC (Shizuoka, Japan). The mice were housed in a room under controlled temperature ($22 \pm 1\,^{\circ}C$), humidity ($55 \pm 10\%$), and light (lights on from 0700 to 1900 hours). Food and water were freely available. Procedures used in the animal experiments were approved by the Committee for Animal Experiments at the University of Toyama.

Materials

For *in vivo* experiments, SPC, sphingomyelin, and sphingosine (all purchased from Sigma, St Louis, MO) were dissolved in physiological saline containing 1% ethanol. These agents were injected intradermally into the interscapular region in a volume of 50 μl. For *in vitro* experiments, SPC, zileuton, and indomethacin were dissolved in mouse keratinocyte growth medium containing 0.1% DMSO. Naltrexone hydrochloride (Sigma) was dissolved in physiological saline and injected subcutaneously 15 minutes before SPC injection. Azelastine hydrochloride was dissolved in tap water and administered orally 30 minutes before SPC injection. Tefenadine, tranilast, zileuton, indomethacin, and ONO-4057 (5-[2-(2-carboxyethyl)-3-[6-(4-methoxyphenyl)-5E-hexenyl]oxyphenoxy] valeric acid) were dissolved in 0.5% sodium carboxymethyl cellulose (Wako Pure Chemical Industries, Osaka, Japan). These agents were administered orally 1 hour before SPC injection.

Behavioral experiments

The day before the experiment, the hair was clipped over the rostral part of the back of 5- or 9-week-old mice. Before behavioral observation, the animals were put individually in an acrylic cage composed of four cells ($13 \times 9 \times 35\,cm$) for at least 1 hour for acclimation. Immediately after intradermal or subcutaneous injection, the animals were put back into the same cells and their behaviors were videotaped for 1 hour with personnel kept out of the

observation room. Playback of the videotape served for the determination of scratching of the rostral back or licking of the hind paw (Kuraishi *et al.*, 1995).

Cell culture

The bilateral DRG at the C1–T13 levels were removed from 6-week-old mice. The cells were dissociated with 0.25% collagenase (Wako Pure Chemical Industries) and cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum and 2 μM cytosine arabinoside (Sigma), which kills glial cells, for at least 7 days.

To prepare keratinocytes, the skin was removed from neonatal mice and treated with 0.05% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) dissolved in mouse keratinocyte growth medium at 4 °C overnight. The keratinocyte samples were cultured in mouse keratinocyte growth medium containing 5 μg ml^{1} insulin, 0.5 μg ml^{1} hydrocortisone, 14.1 μg ml^{1} phosphorylethanolamine, 0.01 μg ml^{1} epidermal growth factor, 10 μg ml^{1} transferrin, and 0.1 mg ml^{1} bovine pituitary extract (Andoh *et al.*, 2007).

Measurement of intracellular Ca^{2+} concentration

Primary cultures of DRG neurons and keratinocytes were washed with the medium Opti-MEM (Invitrogen), incubated with 10 μM fluo-3/AM (Dojindo, Kumamoto, Japan) in Opti-MEM containing 0.05% poloxamer (Calbiochem, Darmstadt, Germany), and then washed with Opti-MEM. Intracellular Ca^{2+} concentration was measured fluorometrically at 488 nm excitation and 515–545 nm emission using a laser-scanning microscope system (Radiance 2100; Bio-Rad, Hercules, CA). Although SPC receptors have not been identified, the action of SPC is inhibited by pretreatment with capsaicin (Kim *et al.*, 2008), suggesting that SPC acts on capsaicin-sensitive primary sensory neurons. Most of the small and medium-sized neurons ($\leqslant$25 μm in diameter) are capsaicin-sensitive (Andoh *et al.*, 1998a). Therefore, DRG neurons $\leqslant$25 μm in diameter were used in this experiment. Neuronal diameter was determined using bright-field microscopy with an objective micrometer (Nikon, Tokyo, Japan). Trypan blue exclusion test confirmed the absence of any apparent damage to the cultured cells after 10-minute administration of SPC.

Enzyme immunoassay for LTB_4

The SPC-injected region of the skin (1.7 cm in diameter) was removed 10 minutes after injection. After being weighed, the skin was immediately shredded with scissors and put into 2 ml of ice-chilled ethanol containing 10 μM indomethacin and 10 μM zileuton. After being homogenized with a Polytron homogenizer, the sample was centrifuged at 2,000 r.p.m. at 4 °C for 5 minutes. One milliliter of the supernatant was mixed with 5 ml of double-distilled water and the pH was adjusted to 3.5 with HCl. The sample was then applied to C18 Sep-Pak cartridges (Waters, Milford, MA) equilibrated with methanol. After the cartridges were washed with hexane followed by double-distilled water, the lipids were eluted with ethanol. After evaporation of the eluate, the residue was suspended in enzyme immunoassay buffer (Cayman Chemical, Ann Arbor, MI) for the assay of LTB_4. In the case of cultured keratinocytes, the culture medium was changed to mouse keratinocyte growth medium before experimentation. Samples were taken from the supernatant 10 minutes after SPC administration and served for the assay of LTB_4. The amount of LTB_4 was determined using EIA kits (Cayman Chemical). After supernatant sampling, cultured keratinocytes were treated with 1% Triton X-100 to solubilize the cell proteins, the concentration of which was determined using a Bio-Rad protein assay kit (Bio-Rad). The concentration of LTB_4 in the skin and keratinocytes was normalized to skin weight and protein amount, respectively.

Data processing

Data are presented as means ± SEM. Statistical significance was analyzed using Dunnett's multiple comparisons or Student's *t*-test; $*P<0.05$ was considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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