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Short communication

Characterizations of sphingosylphosphorylcholine-induced scratching responses in ICR mice using naltrexon, capsaicin, ketotifen and Y-27632

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Abstract

Sphingosylphosphorylcholine (SPC) is upregulated in the stratum corneum of atopic dermatitis patients by sphingomyelin deacylase. We conducted an investigation, both to confirm that intradermal injection of SPC elicits scratching in mice, and to elucidate the detailed mechanism of the SPC-induced itch–scratch response. Intradermal administration of SPC increased the incidence of scratching behavior in a dose-dependent manner. SPC-induced scratching could be suppressed, significantly, by the μ-opoid receptor antagonist, naltrexon, the vaniloid receptor agonist, capsaicin, and the histamine H_1 receptor antagonist ketotifen. D-*erythro* SPC, one of the SPC stereotypes, could elicit the scratch response, but not L-*threo* SPC. Y-27632 (1 mg/kg, an inhibitor of Rho-associated protein kinase (ROCK)), was found to suppress SPC-induced scratching. Both the stereospecificity of SPC and the involvement of the Rho/ROCK pathway suggested that SPC-induced scratching is related to the receptor.

Keywords: SPC [sphingosylphosphorylcholine]; Itch; Scratching; D-*erythro* SPC; Rho-associated protein kinase

1. Introduction

Pruritus (itching) can be defined as an unpleasant sensation that provokes a desire to scratch (Weisshaar et al., 2003) accompanying various skin diseases (e.g. atopic eczema, contact dermatitis, and urticaria) as well as several systemic disorders (e.g. chronic renal failure and cholestasis) (Wahlgren, 1991). Many endogenous chemicals (e.g. amines, proteases, growth factors, neuropeptides, opioids, cytokines and eicosanoids) are locally pruritogenic when injected into the skin. However, the details of the relevant mechanism and the relationship between the disease and the itch-inducing substance remain unclear.

Particularly, the molecular basis of pruritus in atopic eczema remains largely unexplained. Certainly, an itch–scratch vicious cycle exists in atopic patients, in which scratch irritation enhances itch (Yosipovitch et al., 2003).

Atopic dermatitis results from an interaction between susceptible genes, the host's environment, pharmacological abnormalities, skin barrier defects, and immunological factors (Leung and Bieber, 2003). Recently, new data from skin barrier defects has emphasized the roles of the skin barrier in atopic dermatitis. Filaggrin is a key protein involved in skin barrier functioning; indeed, mutations in the filaggrin gene have been shown to be important predisposing factors for atopic dermatitis (Palmer et al., 2006). Disturbed skin barrier function in atopic dermatitis is at least partly related to a disturbed lipid composition of the stratum corneum. A significant reduction in ceramides has been found in lesional forearm skin in atopic dermatitis patients as well as in non-lesional skin (Imokawa et al., 1991). A novel enzyme, glucosylceramide/sphingomyelin deacylase, which cleaves the N-acyl linkage of sphingomyelin and glucosylceramide, has been found in atopic dermatitis patients. Due to this enzyme activity, the level of sphingosylphosphorylcholine (SPC) in the stratum

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corneum of atopic dermatitis patients is high in comparison with that in normal skin (Hara et al., 2000). SPC, a sphingomyelin-breakdown product, effects diverse biological processes (Hannun and Linardic, 1993; Meyer et al., 1997). Therefore, SPC is suspected of being involved in several pathophysiological events. For instance, SPC is known to accumulate in Niemann–Pick disease type A (Rodriguez-Lafrasse and Vanier, 1999), in the stratum corneum of atopic dermatitis patients (Okamoto et al., 2003) and in the ascitic fluids of ovarian cancer patients (Xiao et al., 2001), but its role remains unknown.

Several orphan G-protein-coupled receptors, including GPR-4, OGR-1, and GPR-12 have been reported as putative SPC receptors (Zhu et al., 2001; Xu et al., 2000; Ignatov et al., 2003). However, there has been controversy over the question of whether SPC is the high-affinity endogenous ligand for those orphan G-protein-coupled receptors. For instance, an earlier report (Xu et al., 2000) that SPC is the ligand for GPR-68, also known as OGR1, was recently retracted. Still, however, some data suggests that SPC might act as the ligand of GPR-4 and GPR-12 (Kim et al., 2005; Ignatov et al., 2003).

SPC has also been shown to stimulate the expression of intercellular adhesion molecule I (ICAM-1) in keratinocytes (Imokawa et al., 1999), the production of interleukin (IL)-6 in fibroblasts (Suhr et al., 2003), and the activation of dendritic cells stimulating the production of IL-12 (Ceballos et al., 2007).

To our knowledge, there is only one report on the effect of SPC in vivo related to pruritus, which report concerns a patent filed by Japanese researchers. They showed that SPC as a ligand of GPR-4 can induce the scratching response (Mayumi et al., 2005) and that the agent blocking GPR-4 can inhibit SPC-induced scratching. Their report, however, is deficient in two respects. First, and most seriously, they have since retracted their main hypothesis, that SPC is the ligand of GPR-4 (Zhu et al., 2001). Second, details of their experiments relating to their SPC-induced scratching mice model are not sufficiently evident in their report.

In the present report, we endeavored to study the SPC-induced itch–scratch response in detail, investigating the characterization of SPC-induced scratching using several chemicals, including capsaicin, naltrexon, ketotifen, and Y-27632.

2. Materials and methods

All of the experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Korean Pharmacological Society.

2.1. Materials

SPC and lysophosphatidic acid (LPA) were obtained from Avanti Polar Lipids (Alabaster, Ala., USA). Additional reagents included the following: ketotifen fumarate (Aldrich, St. Louis, Mo., USA, Inagaki et al., 1998), naltrexone (Sigma, St. Louis, USA, Bienkowski et al., 1999). Capsaicin (Wako Pure Chemical, Osaka, Japan, Holzer 1988), D-*erythro* and L-*thero* SPC (Matreya Int., Pleasant Gap, PA, USA), Y-27632 ((R)-(+)-trans-*N*-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, 2HCl, Calbiochem-Novabiochem, Darmstadt, Germany, Chitaley et al., 2001), SPC, LPA, ketotifen fumarate, naltrexone, D-*erythro* SPC, L-*threo* SPC and Y-27632 were dissolved in saline solution. Capsaicin was dissolved in polyethyleneglycol 400/ethanol.

2.2. Behavioral observations

Male ICR mice 8–9 weeks of age were used in the experiments (Charles River Laboratories, MA, USA). They were housed under controlled temperature (20–26 °C) and light (lights on from 08:00 to 20:00), with food and water freely available. Before the experiments, the mice, the hair on the rostral part of their backs having been clipped, were put into an acrylic cage (140 × 32 × 12 cm) comprising 20 cells for at least 1 h acclimation. Immediately after intradermal injection, the mice were each put back into the same cell and videotaped with no one present. Scratching responses to the injected site with the hind paws were enumerated as an itch-response index (Kuraishi et al., 1995).

2.3. Statistics

All data are presented as means and S.D. Statistical significance was analyzed using two sample *t* tests and a two-way ANOVA test; $P<0.05$ was considered significant.

3. Results

To determine whether SPC induces scratching behavior, SPC (dissolved in saline) was injected intradermally, and LPA, a lysophospholipid, which is reported to induce the scratching response in mice, was used as a positive control (Hashimoto et al., 2004). Fig. 1A indicates the 30 min time-courses of the post-SPC (50 nmol/site) and post-LPA-injection (50 nmol/site) scratching behaviors, respectively. Scratching was first observed within 1 min after injection in all of the mice examined, and then reappeared intermittently. In each case, the number of scratching responses gradually decreased and nearly subsided over the 30 min time course. The number of SPC-induced scratching was similar to that induced by LPA.

Fig. 1B illustrates the dose–response relationship in SPC-induced scratching. The bar graph shows the peaks from 5 to 150 nmol/site. The highest peaks on induction of scratching in the ICR mice appear at 50 and 150 nmol/site. No changes in gross behaviors other than scratching were observed after these doses of SPC or LPA.

In order to evaluate the characteristics of SPC-induced scratching in the transmission of itch signaling, we tested whether capsaicin or naltrexon inhibited scratching induced by SPC. Capsaicin, a well-known vanilloid receptor agonist (0.005 and 0.05%; Bevan and Szolcsanyi, 1990) was applied topically after the injection of SPC, and naltrexone was administered orally 1 h prior.

As shown in Fig. 1C, capsaicin at doses of 0.005% or 0.05% and naltrexone at 1 mg/kg or 10 mg/kg suppressed SPC-induced scratching (*$P<0.05$ when compared with the SPC-only group).

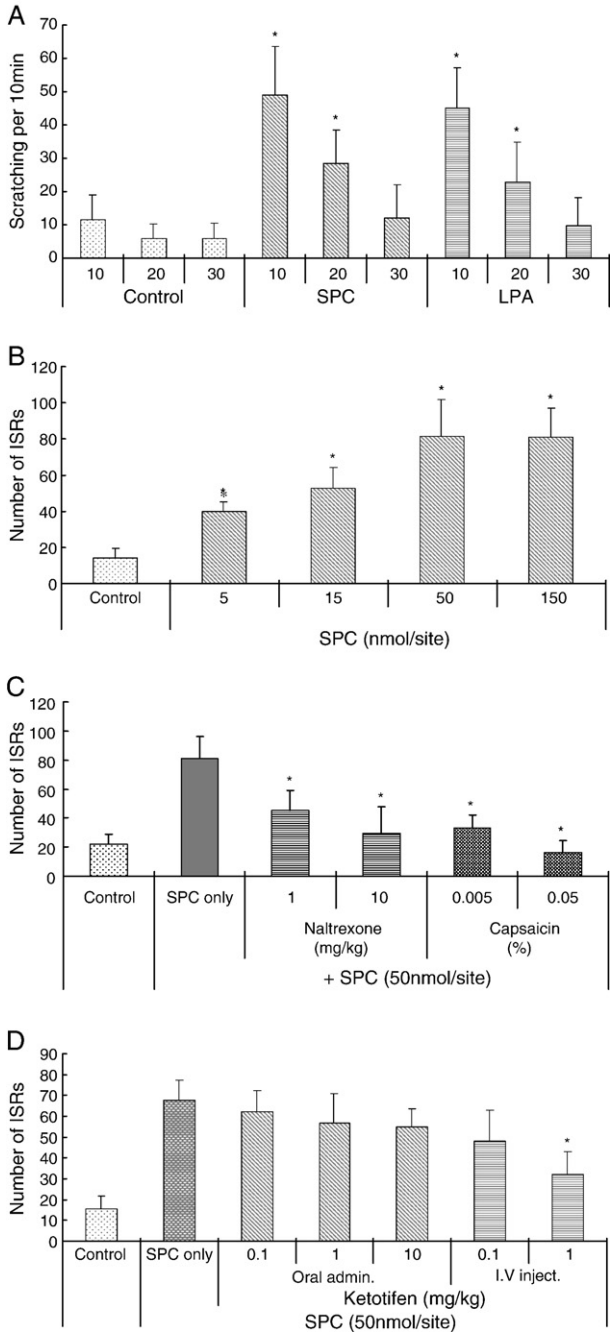

Fig. 1. A. Time-course graph for SPC-induced scratching in mice. SPC and LPA (50 nmol/site) were each administered intradermally into the rostral region of the back. B. Dose-dependent scratch-inducing effect of SPC. C. Effects of naltrexon and capsaicin on the SPC-induced scratching in mice. Naltrexon (1, 10 mg/kg) was administered orally 1 h prior to SPC administration. 0.005 and 0.05%(w/v) of capsaicin was administered topically 5 min prior to SPC (50 nmol/site) injection. D. Effects of ketotifen on SPC-induced scratching in mice. Ketotifen (0.1, 1, and 10 mg/kg) was administered orally 1 h prior to SPC administration, and 0.1, 1 mg/kg of ketotifen was injected intravenously. Each control group was injected with saline. A, B. Each value represents the means and S.D. ($n=8$–10) *: a significant difference from the control group was $P<0.05$. Fig. 1. C. D. Each value represents the means and S.D. ($n=8$)*: a significant difference from the SPC-only group was $P<0.05$.

Ketotifen (a histamine H_1 receptor antagonist) is used to check the involvement of histamine H_1 receptor and histamine release in the transmission of itch signaling induced by SPC. Fig. 1D represents the effects of ketotifen on SPC-induced scratching. The saline-treated and SPC (50 nmol/site)-induced scratching groups were used as controls. Ketotifen, a histamine H_1 receptor antagonist, did not significantly reduce the SPC-induced scratching when administrated orally. However, intravenous injection (0.1 or 1 mg/kg) significantly suppressed SPC-induced scratching. At 1 mg/kg of ketotifen via i.v., the scratching was decreased by 52.2% (32.3±10.7) compared with the SPC-only group. We examined the scratching response induced by the two SPC stereisomers, D-*erythro* SPC, which has an S-configuration amino group at the 2-position and an R-configuration hydroxy group at the 3-position, and its enantiomer, L-*threo* SPC. There was a significant difference in the enantiomerically pure SPC-induced scratching. The saline-treated and SPC (50 nmol/site) -induced scratching groups were again used as controls. The D-*erythro* and L-*threo* SPC were administered intradermally at 15, 50 and 150 nmol/site, respectively. The D-*erythro* form induced scratching very significantly, but the L-*threo* form did not elicit any scratching at all (Fig. 2A).

SPC is known to activate rho-associated protein kinase (Shirao et al., 2002, Somlyo, 2002), and it has been reported that rho-associated protein kinase is involved in histamine release from mast cells induced by LPA (Hashimoto et al., 2005). Therefore, to elucidate whether rho-associated protein kinase is

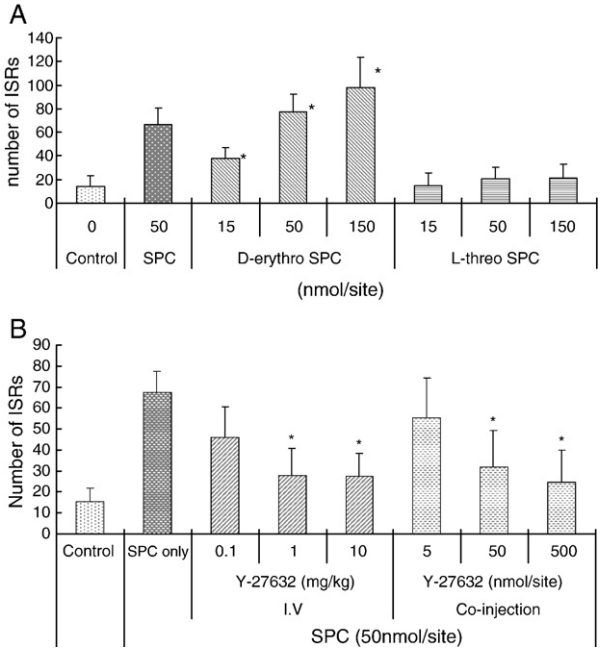

Fig. 2. A. Comparison of stereo-specificity between D-*erythro* and L-*threo* SPC-induced scratching in mice. D-*erythro* and L-*threo* SPC (15, 50 and 150 nmol/site, respectively.) were administered intradermally into the rostral region of the back. B. Effects of Y-27632 on SPC-induced scratching in mice Y-27632 (0.1, 1 and 10 mg/kg) was administered intravenously 5 min prior to SPC administration, and Y-27632 (5, 50 and 500 nmol/site) was co-injected with SPC at the same time. SPC (50 nmol/site) was administered intradermally into the rostral region of the back. The number of itch–scratch responses was observed and recorded for 30 min. Saline and SPC (50 nmol/site) were used as negative and positive controls, respectively. Each value represents the means and S.D. ($n=8$). A. *A significant difference from the control group was $P<0.05$. B. *A significant difference from the SPC-only group was $P<0.05$.

involved in SPC-induced scratching in ICR mice, we tested the effect of Y-27632 (an inhibitor of Rho-associated protein kinase). Y-27632 (1 mg/kg) was administered intravenously 5 min prior to SPC administration (Hashimoto et al., 2004), and significantly inhibited SPC-induced scratching (*$P<0.05$ when compared with the SPC-only group) (Fig. 2B).

4. Discussion

SPC is formed by a novel enzyme called glucosylceramide/sphingomyelin deacylase, which cleaves the *N*-acyl linkage of sphingomyelin and glucosylceramide (Hara et al., 2000). The SPC level in the epidermal stratum corneum is increased in both the lesional and non-lesional skin of atopic dermatitis patients (Okamoto et al., 2003). SPC has been suggested to play a role in inflammatory reactions such as induction of adhesion protein-1 (Imokawa et al., 1999) and activation of dendritic cells that stimulate IL-12 (Ceballos et al., 2007). Much evidence indirectly supports the hypothesis that SPC exploits G protein-coupled receptors, such as the GPR-4- or GPR-12,-signaling pathways in performing these functions (Kim et al., 2005, Ignatov et al., 2003). The existence of several orphan receptors that have the potential to mediate the SPC signaling pathway implies that SPC might be one of the active effectors of the symptoms of atopic dermatitis, not just one of the products of the break-down of the skin barrier. This suggestion led us to investigate the SPC-induced itch–scratch response in ICR mice.

Japanese researchers have shown that SPC can induce scratching (Mayumi et al., 2005). They subcutaneously administered SPC to mice, inducing the scratching response in order to evaluate the GPR-4 receptor antagonists as anti-pruritic drugs. However, excessive amounts of GPR-4 receptor antagonists were used to inhibit the scratch response, in spite of their strong binding affinity for the GPR-4 receptor, and thus their hypothesis, based on SPC being the ligand of GPR-4, is now questionable. The experimental details were not well described either, and so more study is required in order to establish the SPC-induced itch–scratch mouse model. Accordingly, we evaluated, in detail, the active mechanism of SPC as a pruritogen in ICR mice.

The results of our study indicated that SPC induced scratching at 5 to 150 nmol/site in mice, showing a peak at 50 nmol/site (Fig. 1B). It was found that, at the same concentration, SPC elicits scratching to the same extent as histamine and LPA (Inagaki et al., 2001; Hashimoto et al., 2004). Incidentally, the reason that high amounts of SPC are required to provoke the scratch response in ICR mice might be the weak binding affinity of the putative SPC receptor in mice.

The SPC-induced scratch response might be at least partly mediated by capsaicin-sensitive primary afferents, and the opioids systems might be involved in transmission of itch signaling in the central nervous system, which would be consistent with our observation that capsaicin and naltrexone suppressed SPC-induced scratching (Fig. 1C).

Interestingly, in our study, ketotifen did not show any suppressive effect on SPC-induced scratching when orally administrated. However, injected intravenously at 1 mg/kg, it could significantly inhibit scratching (Fig. 1D). These results are similar to the inhibitory effects of ketotifen on LPA-induced scratching (Hashimoto et al., 2004). Toluidine bule stain showed an increase in mast cell degranulation in the SPC-treated group, and this degranulation was decreased as a result of the intravenous administration of 1 mg/kg of ketotifen prior to SPC injection (unpublished data).

SPC has two stereotypes, the D-*erythro* and the L-*threo* forms. Naturally occurring D-*erythro* SPC could elicit scratching response dose-dependently, but L-*threo*-SPC could not. The stereoselectivity of SPC-induced scratching is similar to that of a Ca^{2+} increase in intact cells by D-*erythro* SPC, which is sensitive to pertussis toxin. Ca^{2+} release by SPC in permeabilized cells is not stereoselective (Heringdorf et al., 1998). So, SPC-induced scratching in ICR mice is probably mediated by putative membrane SPC receptors. Currently it is not clear whether GPR4 or GPR12 is involved in the SPC-induced itch–scratch response, because the antagonists of GPR4 and GPR12 are not available. Further studies are ongoing to characterize the putative receptor.

Y-27632, an inhibitor of ROCK, inhibited SPC-induced scratching when administered at a 1 mg/kg dose (Fig. 2B). This finding was indicative of the involvement of the Rho/ROCK-mediated pathway in SPC-induced scratching. The result is consistent with the report that 1 mg/kg of Y-27632 inhibited LPA-induced scratching (Hashimoto et al., 2004). The relationships between SPC and the Rho/ROCK-mediated pathway with regard to the itch–scratch response need to be elucidated.

To summarize, we showed that SPC, which is formed by destruction of the skin barrier in atopic dermatitis patients, can act as a pruritogen, especially stereospecifically, and that the histamine and Rho/ROCK pathways are involved in SPC-induced scratching.

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