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Sphingosylphosphorylcholine down-regulates filaggrin gene transcription through NOX5-based NADPH oxidase and cyclooxygenase-2 in human keratinocytes

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ABSTRACT

Sphingosylphosphorylcholine (SPC) mediates various inflammatory and behavioral responses in atopic dermatitis. Recent studies have shown that dysfunction of the epidermal permeability barrier itself plays a primary role in the etiology of atopic dermatitis. However, the effects of SPC on major proteins essential to the development of the epidermal permeability barrier such as filaggrin, loricrin, involucrin, keratin 1, keratin 10 and small proline-rich proteins are still unclear. In this study, we demonstrated that SPC significantly reduces filaggrin gene transcription, implying that SPC plays a pivotal role in impairment of the epidermal permeability barrier in atopic dermatitis lesional skin. In cultured normal human keratinocytes (NHKs), SPC increases the intracellular level of reactive oxygen species (ROS) and up-regulates NADPH oxidase 5 (NOX5) gene transcription. SPC also stimulates prostaglandin (PG) E_2 production by increasing cyclooxygenase (COX)-2 expression in NHK. The effects of the prostanoid EP receptor agonists, limaprost, butaprost, and sulprostone on filaggrin gene expression in NHK suggest that the prostanoid EP2 receptor plays a significant role in the PGE_2-mediated filaggrin down-regulation. In contrast, limaprost and butaprost do not affect NOX5 expression in NHK, implying that the NOX5-regulated ROS pathway stimulated by SPC may be upstream of the COX-2 pathway. We propose that the increase in SPC levels further aggravates dermatological symptoms of atopic dermatitis through SPC-induced down-regulation of filaggrin in NHK.

1. Introduction

The association of filaggrin gene mutations with atopic dermatitis indicates that a defective epidermal permeability barrier in the stratum corneum is a primary etiological factor of this disease [1–3]. The maturation of filaggrin from pro-filaggrin in the granular cell layer of the epidermis is an essential step in generating a normal cornified cell envelope, an insoluble protein structure underneath the plasma membrane [3,4]. In addition to filaggrin, various cornified cell envelope-associated proteins such as involucrin, loricrin, small proline-rich proteins, and keratins are important in the development of the epidermal permeability barrier. Therefore, genetic defects in genes encoding these cornified cell envelope-associated proteins are associated with various inherited dermatological abnormalities. For instance, mutations or polymorphisms of the loricrin gene are associated with ichthyosis [5,6]. Allelic variants of keratin 1 (KRT1) and keratin 10 (KRT10) are linked to epidermolytic hyperkeratosis and/or ichthyosis [7–9]. Moreover, post-translational processing of the cornified cell envelope-associated proteins by serine proteases, caspases, and transglutaminases provides structural integrity to the epidermal permeability barrier during epidermal differentiation [10]. Allelic variants of these processing enzymes have also been reported to be linked to dermatological skin conditions [11,12]. For instance, a single-nucleotide polymorphism (SNP) of the kazal-type 5 serine protease inhibitor (SPINK5) is associated with atopic dermatitis [11] and genetic variants of transglutaminase 1 (TGM1) are linked to lamellar ichthyosis [12]. Thus, the regulation of the cornified cell envelope-associated proteins during epidermal differentiation is important in the generation of an intact epidermal permeability barrier.

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In the stratum corneum, cornified keratinocytes are embedded in a lipid-enriched intercellular matrix within a multilamellar membrane structure to give rise to the physiological epidermal permeability barrier [13,14]. The homeostatic regulation of the epidermal permeability barrier depends on the integrity of both the cornified envelope structure and the intercellular lipid composition of the stratum corneum [4,13]. Recent studies have demonstrated that abnormal sphingolipid metabolism in atopic dermatitis leads to changes in the lipid composition of the cornified layer of the epidermis [15]. The levels of ceramides both in lesional and non-lesional skin of atopic patients are significantly reduced in the stratum corneum compared to normal healthy skin [16–19]. In atopic dermatitis patients, the activity of sphingomyelin deacylase, an enzyme that converts sphingomyelin into sphingosylphosphorylcholine (SPC), is significantly higher than in healthy individuals [20]. As a consequence, increased SPC levels have been shown to accompany the reciprocal decrease in ceramide levels in the atopic stratum corneum [19,21]. A number of previous studies have examined the role of SPC in the inflammatory responses and/or the behavioral symptoms of atopic dermatitis. For example, SPC promotes pro-inflammatory responses of human keratinocytes by inducing tumor necrosis factor-α (TNFα) and intercellular adhesion molecule (ICAM)-1 expression [22]. An intradermal injection of SPC induces scratching behavior in mice, suggesting a link between SPC and pruritic behavior, a cardinal symptom of atopic dermatitis [23,24].

In spite of the scientific evidence for an association between SPC and atopic dermatitis, little is known about the effects of SPC on the homeostasis of the epidermal permeability barrier. Previous reports have demonstrated a connection between the significant calcium (Ca^{2+}) concentration gradient that exists from the basal layer of the epidermis to the stratum corneum and the maturation of the epidermal permeability barrier [10,13,14]. Correspondingly, SPC is also known to induce changes in the intracellular Ca^{2+} concentration and its distribution in mammalian cells [25–27]. In addition, SPC increases the activity and mRNA levels of transglutaminase-1 (TGM1), a Ca^{2+}-sensitive enzyme that cross-links various cornified cell envelope-associated proteins during epidermal differentiation [26,27]. Therefore, there is a clear link between SPC and Ca^{2+} mobilization in human keratinocytes during epidermal development. However, the effects of SPC on the regulation of cornified cell envelope-associated proteins, such as filaggrin and loricrin, important in epidermal permeability barrier formation, remain largely unknown.

Reactive oxygen species (ROS) and various pro-inflammatory mediators, such as PGE_2, LTB_4, TNFα, and interleukin-4 (IL-4), are significantly increased in lesional and peri-lesional skin in atopic dermatitis [28,29]. Both ROS and these pro-inflammatory mediators can regulate the expression of cornified cell envelope-associated proteins [29,30]. While, SPC was reported to increase ROS in endothelial cells in a NOX-dependent manner [31,32], it is unclear whether SPC alters in ROS levels in human keratinocytes and little is known about the mechanisms by which this occurs. Recently, we reported that the T helper cell (Th) cytokines interferon γ (IFNγ), IL-4, and IL-17A inhibit filaggrin and loricrin gene transcription in human epidermal keratinocytes in culture [30]. However, the effects of pro-inflammatory mediators like PGE_2 on the expression of cornified cell envelope-associated proteins have not been studied systematically with regard to their influence on the epidermal permeability barrier.

In the present study, we evaluated the effects of SPC on the expression of cornified cell envelope-associated proteins to address the role of SPC in epidermal permeability barrier dysfunction in atopic dermatitis. In normal human keratinocytes (NHK), SPC decreased mRNA expression of filaggrin, suggesting SPC plays a direct role in the integrity of the cornified cell envelope structure in the stratum corneal layer. We also found that SPC increased intracellular ROS levels in NHK in a NOX5-dependent manner, which may be further related to the concomitant up-regulation of COX-2. Moreover, prostanoid receptor agonists, such as limaprost and butaprost, directly decreased filaggrin gene expression, indicating that the increase in PGE_2 may underlie the SPC-induced down-regulation of filaggrin in NHK.

2. Materials and methods

2.1. Cell culture and treatment with SPC and pharmacological reagents

Normal human keratinocytes (NHK) from neonatal foreskin were purchased from Lonza (Basel, Switzerland) and cultured in KBM medium with KGM2 growth supplements that containing insulin, human epidermal growth factor, bovine pituitary extract, hydrocortisone, epinephrine, transferrin, and gentamicin/amphotericin B (Sigma, St Louis, MO, USA). Cells were serially passaged at 70–80% confluence, and experiments were conducted with subconfluent cells at passage two or three when cells were actively proliferating. NHK cells were starved for 24 h in keratinocyte KBM medium containing transferrin, followed by preincubation with indomethacin (1 μM, Sigma, St. Louis, MO, USA), AH6809 (10 μM, Cayman Chemical Co., Michigan, USA), NAC (500 μM, Sigma, St. Louis, MO, USA) and Y27632 (5 μM, Sigma, St. Louis, MO, USA) for 1 h. For inhibitor experiments, cells were maintained in the same media as above but supplemented with various inhibitor compounds. Cells were stimulated with D-erythro-sphingosylphosphorylcholine (SPC, Matreya Inc., PA, USA), butaprost (5, 10 μM, Cayman Chemical Co. MI, USA), limaprost (5, 10 μM, Cayman Chemical Co., MI, USA), or sulprostone (5, 10 μM, Cayman Chemical Co., MI, USA) for 48 h.

2.2. Cell viability tests

The cell viability of NHK in the presence of SPC was evaluated using the WST-1 assay according to manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). The cell viability detecting reagent 4-3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate (WST-1; 10 μM pure solution) was added to NHK in culture, and cells were incubated for 2 h in a humidified atmosphere. The absorbance at A450 nm was measured, and the absolute optical density was expressed as a percentage of the control value.

2.3. RNA extraction and quantitative real-time RT-PCR (Q-RT-PCR)

Total RNA was isolated using TRIzol™ (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically, and the integrity of the RNA was assessed using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Two micrograms of RNA were reverse-transcribed into cDNA using SuperScript®III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and aliquots were stored at −20 °C. Quantitative real-time TaqMan RT-PCR technology (Q-RT-PCR) (Applied Biosystems, Foster City, CA, USA) was used to determine the expression level of selected target genes. The cycling conditions included a denaturing step at 95 °C for 10 min and 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The TaqMan probes (Applied Biosystems, Foster City, CA, USA) used in the Q-RT-PCR analysis were: filaggrin, Hs00856927_g1; loricrin, Hs01894962_s1; involucrin, Hs00846307_s1; SPRR1B, Hs00824893_m1; KRT1, Hs00196158_m1; KRT10, Hs01043110_g1; DSG3, Hs00170075_m1; transglutaminase 1, Hs01070316_m1; β-actin (ACTB), Hs99999903_m1; COX-1,

Hs00924803_m1; COX-2, Hs01573477_m1; NOX1, Hs00246589_m1; NOX4, Hs00276431_m1; NOX5, Hs00225846_m1; DUOX1, Hs00213694_m1; and DUOX2, Hs00204187_m1. Human GAPDH (4333764F, Applied Biosystems, Foster City, CA, USA) was also amplified to normalize variations in cDNA levels across different samples.

2.4. Enzyme immunoassay for PGE_2

NHKs were cultured in 60 mm plates and were treated with SPC for 8 h. The PGE_2 concentration in the supernatant was measured using an enzyme immunoassay (EIA) kit (Cayman Chemical Co., MI, USA) according to the manufacturer's instructions.

2.5. Western blots

NHK cells were lysed in a cell lysis buffer (RIPA buffer, Sigma, St. Louis, MO, USA) containing protease inhibitors (Sigma, St. Louis, MO, USA). The lysate was then subjected to centrifugation at 15,000 × *g* for 10 min, and the supernatant was used for Western blot analysis. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard. Proteins (40 μg per well) were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.15% Tween-20) for 1 h at room temperature and subsequently probed overnight at 4 °C with an anti-COX-2 antibody (Cayman Chemical Co., Ann Arbor, MI, USA). Blots were washed three times with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) at room temperature for 1 h. Detection was performed with the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The relative protein levels were analyzed using ImageMaster 2D Elite software (Amersham Biosciences, Buckinghamshire, UK). β-Actin (Santa Cruz Biotechnology, Santa Barbara, CA, USA) was used as a loading control.

2.6. Measurement of ROS with flow cytometry

2′,7′-Dichloridi-hydrofluorescein diacetate (DCFH-DA) was used to detect intracellular ROS levels in NHK cells. Briefly, cells at 70% confluence were pre-incubated with 20 μM DCFH-DA in phenol free DMEM at 37 °C for the indicated times. Cell were then stimulated with SPC (10, 20 μM) for 30 min and analyzed using in a FACSCalibur instrument from Becton Dickinson (excitation wavelength, 488 nm; emission wavelength, 515–545 nm) [33].

2.7. Statistical analyses

All statistical analyses were performed using the Student's *t*-test feature of the Microsoft Excel data analysis program.

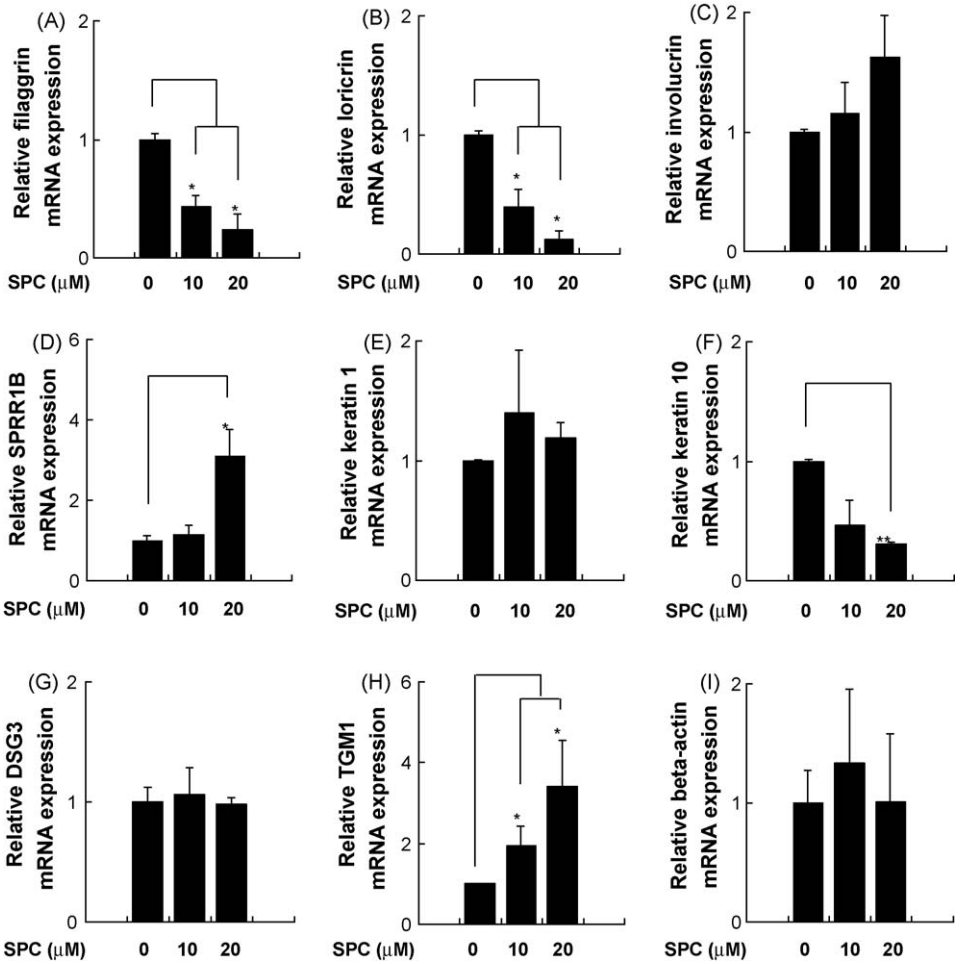

Fig. 1. Effects of SPC on mRNA expressions of cornified envelope-associated genes in NHK. NHK were cultured and then stimulated with SPC (10 or 20 μM) for 48 h. Total RNA was extracted, and Q-RT-PCR was performed for (A) filaggrin, (B) loricrin, (C) involucrin, (D) SPRR1B, (E) KRT 1, (F) KRT 10, (G) DSG3, (H) TGM1 and (I) β-actin. Values represent the mean expression ± SE of the mRNA of the various genes relative to human GAPDH expression (filaggrin: $n = 6$, other genes: $n = 3$). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

Experiments were repeated independently. Values are expressed as the mean ± SE.

3. Results

3.1. The effects of SPC on the transcription of markers of terminal differentiation in human keratinocytes

To determine the role of SPC in the impairment of the epidermal permeability barrier in atopic dermatitis, we first measured the levels of mRNA expression of the major cornified cell envelope-associated proteins following SPC treatment of NHK (Fig. 1). We examined the levels of filaggrin, loricrin, involucrin, small proline-rich protein 1B (SPRR1B), KRT1, KRT10, desmoglein 3 (DSG3) and transglutaminase 1 (TGM1). SPC did not affect NHK cell viability when cells were treated with up to 20 μM for 48 h as measured using a WST-1 assay (data not shown). SPC treatment of NHK induced changes in the mRNA levels of filaggrin, loricrin, SPRR1B, KRT10 and TGM1 (Fig. 1A, B, D, F, and H). While transcription of filaggrin, loricrin and KRT10 were significantly decreased in NHK treated with 20 μM SPC, SPRR1B and TGM1 mRNA levels were significantly increased (Fig. 1). The SPC-induced increase in TGM1 gene expression was consistent with a previous report by Higuchi et al. [27] (Fig. 1H). In spite of the down-regulation of KRT10, KRT1 transcription, which is typically co-expressed with KRT10 [8,9], showed no significant change following SPC treatment (Fig. 1E). In addition, the mRNA levels of involucrin and DSG3 did not show any significant change following SPC treatment compared to the control (Fig. 1C and G).

3.2. SPC up-regulates COX-2 expression and increases PGE_2 synthesis in NHK

PGE_2 is a major pro-inflammatory autacoid in atopic dermatitis lesional skin [34]. In order to investigate the relationship between SPC and the increases in PGE_2 in atopic dermatitis, we tested whether SPC regulated the PGE_2 production pathways in NHK. When cells were treated with SPC, a significant concentration dependent increase in PGE_2 was observed in cell culture supernatants (Fig. 2A). When NHK were treated with SPC for 8 h, no change was observed in COX-1 mRNA levels (Fig. 2B), whereas the COX-2 mRNA level significantly increased relative to the control (Fig. 2C). Western blot analysis showed that the level of COX-2 protein was also increased in NHK treated with 20 μM SPC for 8 h (Fig. 2D). Although there was no notable change in COX-2 mRNA or protein levels at 10 μM SPC, PGE_2 was significantly increased at following treatment with either 10 μM or 20 μM SPC (Fig. 2A and D).

3.3. The prostanoid receptor agonists limaprost and butaprost decreases filaggrin and loricrin gene transcription in NHK

To examine the effects of SPC-induced PGE_2 on gene expression of terminal NHK differentiation markers such as filaggrin, loricrin, KRT1, and KRT10, we treated NHK with three prostanoid EP receptor agonists, limaprost, butaprost, and sulprostone (Fig. 3). Limaprost, a non-specific prostanoid EP receptor agonist, significantly decreased filaggrin and loricrin mRNA levels compared to the non-treated control (Fig. 3A and B). Butaprost, a prostanoid EP2 receptor agonist, also down-regulated filaggrin and loricrin

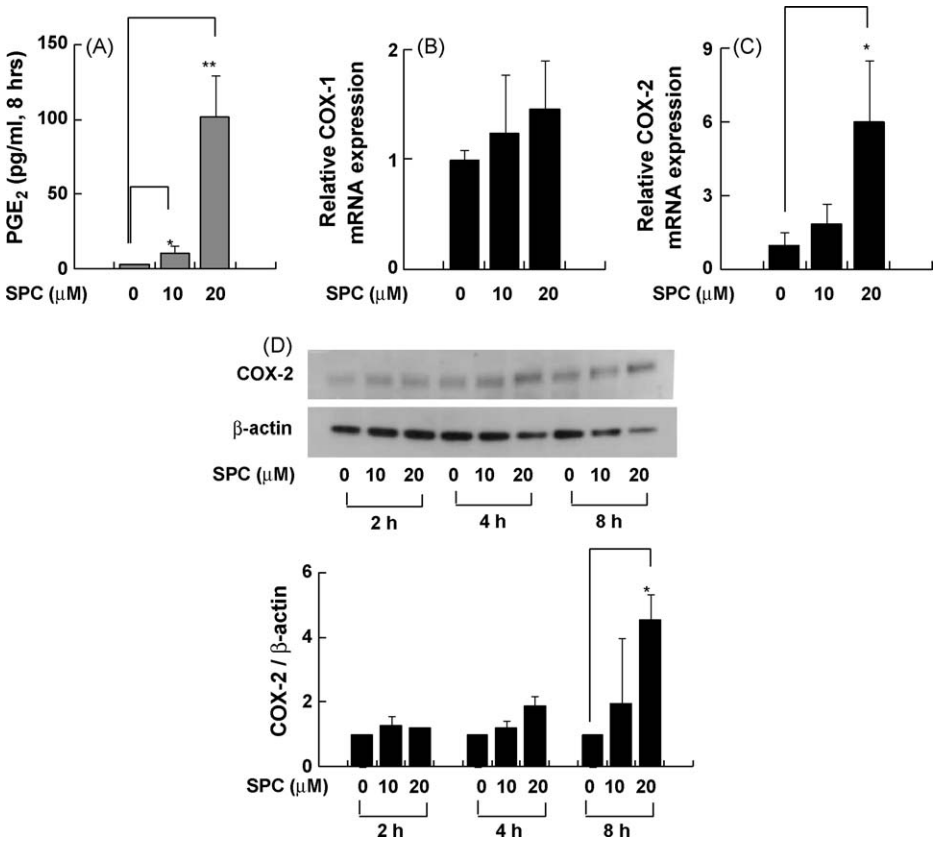

Fig. 2. SPC increases PGE_2 through COX-2 in NHK. NHK were cultured and then stimulated with SPC (10, 20 μM) for 48 h. The PGE_2 concentration in the supernatants of NHK cells stimulated with SPC for 8 h was measured using an EIA kit. Values represent the mean concentration of PGE_2 (pg/ml) (A). To determine the levels of COX-1 (B) and COX-2 (C) mRNA, total RNA was extracted from NHK treated with SPC and Q-RT-PCR was performed. Values represent the mean expression ± SE of the mRNA of the various genes relative to human GAPDH expression. (D) The expression of the COX-2 protein was measured by Western blot analysis. Error bars on the average values of COX-2 protein expression relative to β-actin represent SE of three independent measurement ($n = 3$). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

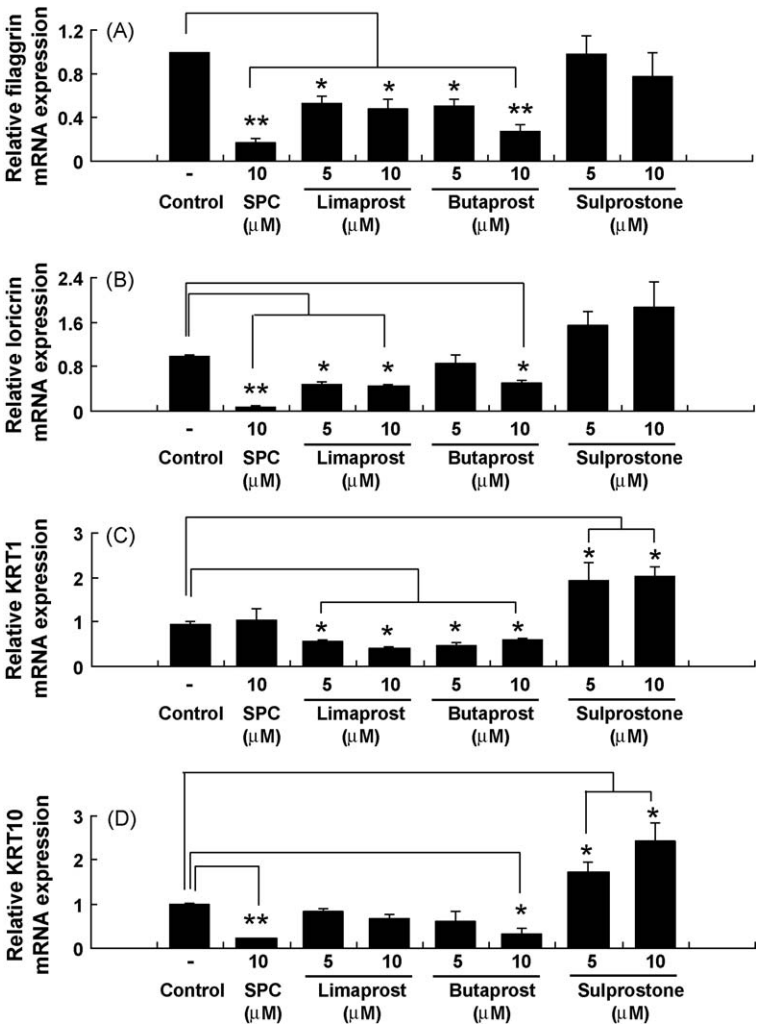

Fig. 3. The effects of prostanoid EP receptor agonists limaprost, butaprost and sulprostone on expression of cornified cell envelope-associated proteins in NHK. NHK were cultured and then stimulated with SPC (10 μM), limaprost (5, 10 μM), butaprost (5, 10 μM) and sulprostone (5, 10 μM) for 48 h. Total RNA was extracted and Q-RT-PCR was performed for (A) filaggrin, (B) loricrin, (C) KRT1 and (D) KRT10. Values represent the mean expression ± SE of the mRNA of the various genes relative to human GAPDH expression ($n = 3$). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

transcription (Fig. 3A and B). However, filaggrin and loricrin gene transcription was unaffected by treatment with sulprostone, a prostanoid EP1/EP3 receptor agonist (Fig. 3A and B). Interestingly, the response profiles of KRT1 and KRT10 to prostanoid receptor agonist treatment of NHK were different from the responses to SPC (Fig. 3C and D). While KRT1 gene transcription was unaffected by SPC treatment (Fig. 1E), NHK treatment with both limaprost and butaprost resulted in the down-regulation of KRT1 gene expression (Fig. 3C). Similar to SPC, butaprost decreased KRT10 gene transcription whereas limaprost had no effect (Fig. 3D). Moreover, sulprostone significantly increased KRT1 and KRT10 gene transcription in NHK (Fig. 3C and D).

To further confirm that SPC-induced PGE_2 up-regulation plays a direct role in the down-regulation of filaggrin and loricrin in NHK, we co-treated NHK with SPC and indomethacin, a non-selective cyclooxygenase inhibitor, or AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid), a prostanoid EP1/EP2 antagonist. Indomethacin partially inhibited the SPC-induced decrease in filaggrin gene transcription (Fig. 4A). Indomethacin also slightly blocked SPC-induced down-regulation of loricrin transcription, but the change was not statistically significant (Fig. 4B). Although AH6809 is known to block PGE_2-induced responses [35], there was no change in the SPC-mediated down-regulation of filaggrin and loricrin in NHK when cells were co-treated with this compound (Fig. 4). These results suggest the possibility that alternative molecular pathways may underlie SPC-induced down-regulation of filaggrin and loricrin transcription.

3.4. NOX5 up-regulation plays a role in SPC-induced increases in reactive oxygen species (ROS) in NHK

The levels of ROS are significantly increased in the lesional skin of atopic dermatitis patients [29]. We thus next evaluated the effect of SPC on ROS levels in NHK by flow cytometry (Fig. 5). When DCFH-DA-loaded NHK were treated with SPC, an increase in ROS was observed within 15 min (Fig. 5A), and this increase occurred in a dose-dependent manner (Fig. 5B). In order to examine whether a ROS-related molecular mechanism induces the up-regulation of COX-2 and subsequent increase in PGE_2 synthesis, we measured the mRNA levels of NOX and DUOX subtypes in SPC-treated NHK. In Q-RT-PCR, NOX1, NOX4, NOX5, DUOX1 and DUOX2 mRNAs were detected in NHK but NOX2 and NOX3 mRNAs were not measurable (data not shown). In NHK, SPC significantly increased NOX5 gene transcription (Fig. 6A). However, no prostanoid receptor agonist affected NOX5 gene transcription in NHK, suggesting that the effects on the prostanoid EP receptor pathway, including COX-2, may be secondary to SPC-induced changes in ROS and Ca^{2+}-dependant NOX5 expression in NHK (Fig. 6B).

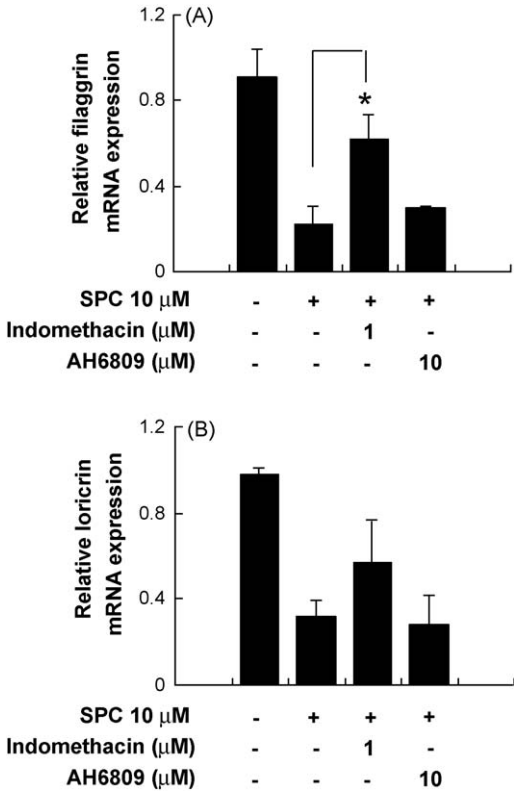

Fig. 4. The effects of indomethacin and AH6809 on SPC-induced down-regulation of filaggrin and loricrin in NHK. NHK were cultured, pre-incubated with indomethacin (1 μM) or AH6809 (10 μM) for 1 h, followed by stimulation with SPC (10 μM) for 48 h. Total RNA was then extracted and Q-RT-PCR was performed for (A) filaggrin and (B) loricrin. Values represent the mean expression ± SE of the mRNA of the various genes relative to human GAPDH expression ($n = 3$). $^*P \leq 0.05$.

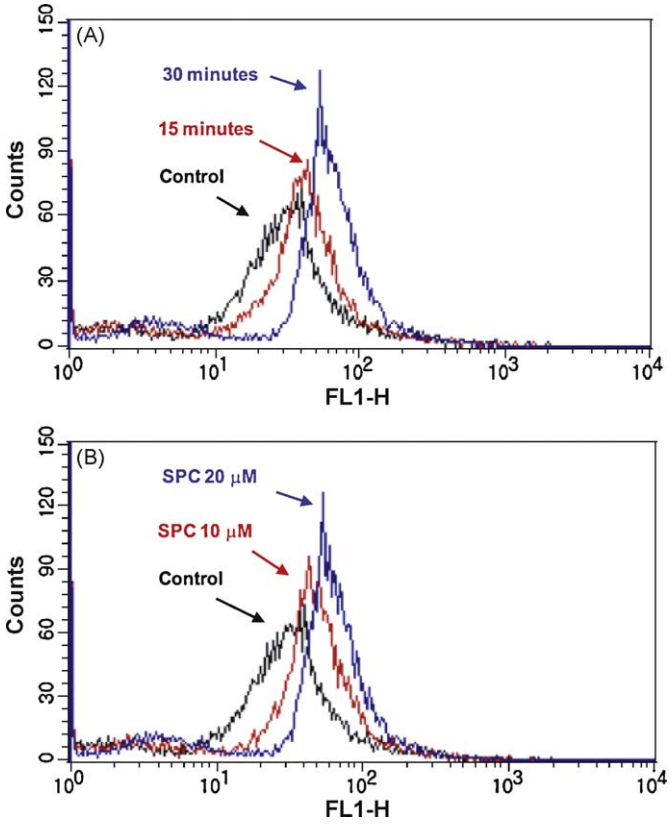

Fig. 5. SPC increases ROS in NHK. NHK were cultured, stained with DCF-DA, and stimulated with SPC (10 μM) for 30 min. The fluorescence was analyzed with a FACSCalibur (Becton Dickinson) with excitation at 488 nm and emission at 530 nm. (A) SPC increases the levels of intracellular ROS in NHK and (B) SPC dose-dependently increases the levels of intracellular ROS.

3.5. Y27632 inhibits SPC-induced down-regulation of filaggrin and loricrin gene transcription

To test whether ROS mediated SPC-induced responses in NHK, cells were treated with an anti-oxidant, N-acetyl-cysteine (NAC). NAC treatment significantly blocked SPC-induced down-regulation of filaggrin and loricrin, indicating that ROS are critical for the effects of SPC in these cells (Fig. 7). We previously demonstrated that Y27632, a specific inhibitor of the Rho-associated serine/threonine kinases (Rho/ROCK), dose-dependently decreased the SPC-induced itch-scratching response in mice [24]. Therefore, in addition to testing the effect of NAC, we also evaluated whether Y27632 regulated the SPC-induced decrease in filaggrin and loricrin gene transcription in NHK. Consistent with the animal studies [24], Y27632 significantly inhibited SPC-induced filaggrin and loricrin down-regulation in NHK (Fig. 7).

4. Discussion

The level of SPC, an abnormal sphingolipid metabolite, is increased in the stratum corneum of patients with atopic dermatitis [19–21]. In the present study, we first analyzed the effects of SPC on cornified cell envelope-associated proteins in human keratinocytes to address the role of SPC in the epidermal permeability barrier defects that occur in atopic dermatitis. In cultured human keratinocytes, we demonstrate that SPC decreased expression of filaggrin, loricrin, and KRT10, while it increased transcription of SPRR1B and TGM1 (Fig. 1). We also show that the changes in filaggrin and loricrin gene transcription are partially associated with the expression of NOX5 and COX-2 in NHK. Although the cause and effect relationship between SPC and atopic dermatitis is still unclear, these results suggest that SPC plays an important role in the impairment of epidermal development and thereby aggravates the pathological symptoms of atopic dermatitis.

The SPC-induced down-regulation of filaggrin gene transcription we observed in NHK was one of the most important cellular changes, especially with regard to the pathophysiological mechanisms involved in atopic dermatitis. Recent genetic studies regarding the association of filaggrin genetic polymorphisms or mutations with both atopic dermatitis and ichthyosis vulgaris suggest that filaggrin is an important mediator during terminal epidermal differentiation [1–3]. The major function of filaggrin during epidermal differentiation is to initiate the aggregation of keratin intermediate filaments to form a tightly organized matrix of aligned and cross-linked filaments [4]. In addition to modulating epidermal permeability barrier structure, filaggrin plays an important role in the retention of water molecules in the stratum corneum. At the final stage of epidermal development, filaggrin is proteolytically processed and further post-translationally modified into hygroscopic amino acids and their derivatives, such as pyrrolidone carboxylic acid (PCA) and *trans*-urocanic acid (UCA), which are major components of natural moisturizing factor (NMF) [36]. Both increased trans-epidermal water loss and skin dryness are major characteristics of clinically unaffected non-lesional skin in atopic dermatitis [37]. Although it is still unclear whether alteration in SPC levels is a causal factor or a pathological outcome of the disease, SPC-induced down-regulation of filaggrin may contribute to the aggravation of atopic symptoms by both inhibiting the development of the cornified envelope structure

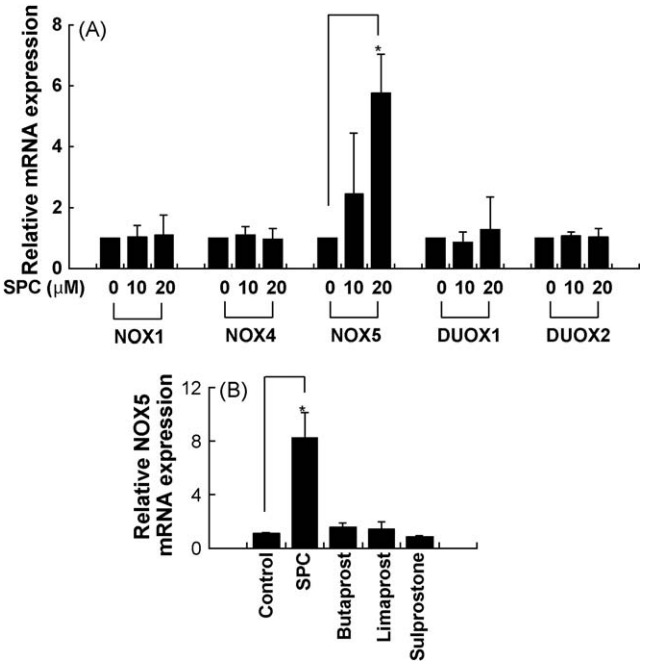

Fig. 6. SPC increases NOX5 gene transcription in NHK. (A) NHK were cultured and then stimulated with SPC for 48 h. Total RNA was extracted and Q-RT-PCR was performed for NOX1, NOX4, NOX5, DUOX1 and DUOX2. (B) NHK were treated with SPC (20 μM), limaprost (10 μM), butaprost (10 μM) and sulprostone (10 μM) for 48 h and total RNA was extracted. Q-RT-PCR was performed for NOX5. Values represent the mean expression ± SE of the mRNA of the various genes relative to human GAPDH expression ($n = 3$). $^{*}P \leq 0.05$.

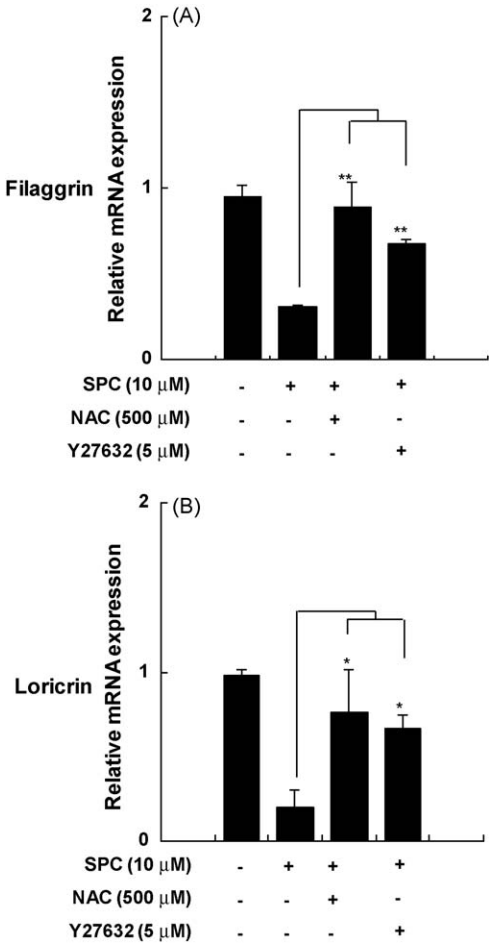

Fig. 7. The effects of NAC and Y27632 on SPC-induced down-regulation of filaggrin and loricrin in NHK. NHK were pre-incubated with NAC (500 μM) or Y27632 (5 μM) for 1 h and followed by the treatment with SPC (10 μM) for 48 h. Total RNA was extracted and Q-RT-PCR was performed for (A) filaggrin and (B) loricrin. Values represent the mean expression ± SE of the mRNA of the various genes relative to human GAPDH expression ($n = 4$). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

and decreasing NMF, resulting in a defective epidermal permeability barrier.

The SPC-induced down-regulation of loricrin and up-regulation of SPRR1B we observed in NHK (Fig. 1B and D), is in accordance with the expression profiles of these genes reported in a microarray study that compared atopic dermatitis lesional skin with skin from a healthy control [38]. Loricrin and small proline-rich proteins (SPRs), like SPRR1B, are recruited to re-enforce the cornified cell envelope structure beneath the plasma membrane of terminally differentiated keratinocytes and form covalent cross-links with envoplakin, periplakin, and involucrin [39]. Although frame-shift mutations in the loricrin gene were reported to be significantly linked to Vohwinkel's syndrome and progressive symmetric erythrokeratoderma, diseases in which the phenotypes suggest defective skin barrier function [5,6,40], no significant phenotypic change has been observed in loricrin gene knockout adult mice [41,42]. This result suggests the possibility that redundant mechanisms compensate for the loss of loricrin during epidermal development. Moreover, a genetic defect in SPRs that causes a severe dermatological symptom has not been reported thus far. Interestingly, it is well known that the sum of loricrin and SPR levels remains constant in the cornified cell envelope, although their relative amounts are varying in different skin regions [43]. It has been suggested that SPRs can compensate for the structural role that loricrin plays during development of the epidermal permeability barrier [13,42,43]. In this regard, it may be possible to postulate that the SPC-induced increase in SPRR1B functions as a compensatory mechanism for the SPC-induced down-regulation of loricrin in NHK. However, it is an still open question whether changes in the normal ratio of loricrin to SPRs in one skin region can affect the integrity of the epidermal permeability barrier in that specific skin area. These issues should be examined in the future.

Our results also indicate that SPC increases PGE_2 production by inducing COX-2 in NHK (Fig. 2), which suggests that the up-regulation of PGE_2 is partially associated with the SPC-induced decrease in filaggrin and loricrin gene transcription. NHK have been shown to express four prostanoid receptors, EP1, EP2, EP3, and EP4 [44–46]. The EP1 receptor affects intracellular Ca^{2+} mobilization that is essential for keratinocytes differentiation [44]. Both the EP2 and EP4 receptors mediate PGE_2 signaling by increasing intracellular cAMP levels through the stimulatory G protein (Gs), whereas EP2 activation is known to promote keratinocyte proliferation [44,47]. In NHK, 1,2-diacylglycerol (DAG) has been shown to be a second messenger in EP3 receptor signaling [45]. Interestingly, intracellular ceramide levels are also affected by EP3 receptor stimulation, leading to the suggestion that EP3 receptor signaling may play a role in epidermal development [45]. In our results, similar to SPC, limaprost, a non-specific PGE_2 receptor agonist, significantly down-regulated filaggrin and loricrin gene transcription in NHK (Fig. 3A and B). In NHK, butaprost, an EP2 selective agonist, decreased gene expression of filaggrin and loricrin, whereas sulprostone, which is a known EP1/EP3 receptor agonist, did not induce any significant transcriptional change in either filaggrin or loricrin (Fig. 3A and B). Although our results cannot directly address how EP4 receptor signaling contributes to the effect of SPC-induced PGE_2 on NHK, the expression profile changes that occurred in response to PGE_2 prostanoid receptor agonists indicate that the EP2 receptor signaling pathway primarily mediates the effects of SPC-induced PGE_2 on filaggrin and loricrin gene transcription.

Although we demonstrated that stimulation of PGE_2 signaling pathways decreased mRNA levels of filaggrin and loricrin in NHK (Fig. 3A and B), the SPC-induced effects were not fully blocked by the non-selective COX inhibitor indomethacin (Fig. 4). In addition, there was no significant effect on SPC-induced down-regulation of filaggrin and loricrin by AH6809, an EP1/EP2 receptor antagonist (Fig. 4). These results suggest that SPC may stimulate another signaling pathway independent of PGE_2 in NHK. For example, SPC has been reported to increase TNFα in NHK [22], and TNFα increases the level of cellular ROS in mammalian cells in a NOX enzyme-dependent manner [48]. The NOX-dependent increase in ROS by SPC has also been reported in endothelial cells [32]. Among the three NAPDH-dependent oxidases, which can mediate Ca^{2+}-dependent ROS production, NOX5, DUOX1, and DUOX2 [31]. SPC increased NOX5 gene expression in NHK (Fig. 5). Because NOX5 gene expression was unaffected by prostanoid EP receptor agonists in NHK (Fig. 6B), SPC-induced ROS generation may occur upstream of the COX-2 induction pathway. NOX1-mediated ROS generation stimulates PGE_2 synthesis in UVA treated human keratinocytes [49], while in esophageal adenocarcinoma cells, NOX5 directly mediated acid-induced COX-2 expression through nuclear factor-kappa B (NF-κB) activation [50]. As shown in Fig. 7, NAC significantly blocked the SPC-induced effects on filaggrin and loricrin. Therefore, given that indomethacin only partially inhibited the SPC-induced effects (Fig. 4), NOX5-dependent ROS generation by SPC may trigger cellular pathways other than PGE_2 auto and/or paracrine signaling that lead to a decrease in filaggrin and loricrin gene expression. In this study, we did not directly address the molecular and cellular mechanisms that couple NOX5 to COX-2. In endothelial cells, SPC exploits NF-κB signaling to induce NOX-dependent ROS generation [32]. Since Y27632 blocked the SPC-induced effects on filaggrin and loricrin (Fig. 6), it is possible that the Rho/ROCK pathway is associated with NOX5-COX-2 coupling in NHK. Indeed, Rho/ROCK pathway-dependent regulation of keratinocyte differentiation has been reported [51–53], and, in kidney epithelial cells, the COX-2 pathway mediates Rho/ROCK regulated NF-κB activation [54]. Because NF-κB is a pleiotropic transcriptional activator which is sensitive to intracellular ROS, it will be interesting to examine the molecular and cellular mechanisms of the NOX5–ROS–COX-2 coupling in NHK, especially in the context of epidermal permeability dysfunction in inflammatory dermatological conditions like atopic dermatitis.

In conclusion, the present findings demonstrate that SPC plays an important role in the impairment of the epidermal permeability barrier and suggests the existence of a vicious cycle that aggravates atopic dermatitis, which may occur independent from defects in the epidermal permeability barrier. SPC down-regulates filaggrin expression in NHK through a NOX5–ROS–COX-2 mediated mechanism, which may be a central pathway through which SPC participates in epidermal permeability barrier dysfunction.

Conflict of interest

The authors, Hyun Choi, Shin Hyoung Kim, Hyoung-June Kim, Kwang-Mi Kim, and Minsoo Noh, are employees of AmorePacific Co.

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