K6PC-5 as a sphingosine kinase activator induces both keratinocyte differentiation and fibroblast proliferation in skin

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Sincerely,

Jong-Kyung Youm

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Sphingosine-1-phosphate (S1P), a bioactive sphingolipid metabolite, regulates multiple cellular responses such as Ca^{2+} signaling, growth, survival and differentiation. Because sphingosine kinase (SK) is the enzyme directly responsible for the production of S1P, many factors have been identified that regulate its activity and subsequent S1P levels. In this study, it was demonstrated that a newly-synthesized SK activator, K6PC-5, induces both keratinocyte differentiation and fibroblast proliferation in skin through intracellular Ca^{2+} signaling in keratinocytes and fibroblasts. K6PC-5, a hydrophobic compound chemically named N-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide, induced intracellular Ca^{2+}

concentration ($[Ca^{2+}]_i$) oscillations in HaCaT cells and human fibroblasts. Both dimethylsphingosine (DMS) and dihydroxysphingosine (DHS), SK inhibitors, and transfection of small interfering RNA for SK1 (SK1-siRNA) blocked the K6PC-5-induced $[Ca^{2+}]_i$ increases. The K6PC-5-induced $[Ca^{2+}]_i$ oscillations were dependent on thapsigargin-sensitive Ca^{2+} stores and Ca^{2+} entry, but independent of classical phospholipase C-mediated pathway. The Ca^{2+} responses of K6PC-5 in both keratinocytes and fibroblasts showed the same results. Next, to study the effect of K6PC-5 on keratinocyte differentiation, HaCaT keratinocytes *in vitro* and hyperproliferative murine model *in vivo* were used. K6PC-5 enhanced the expression of involucrin and filaggrin, specific differentiation-associated marker proteins in HaCaT cells, while transfection of siRNA-SK1 blocked the increase of involucrin. Topical K6PC-5 also enhanced the expression of involucrin, loricrin, filaggrin, and keratin 5, and induced Ca^{2+} mobilization, in intact murine epidermis. K6PC-5 inhibited epidermal hyperplasia induced by repeated tape stripping. The increase of both epidermal thickness and PCNA-positive keratinocytes was inhibited by topical K6PC-5. These results suggest that K6PC-5 acts to regulate both differentiation and proliferation of keratinocytes via $[Ca^{2+}]_i$ responses through S1P production. Finally, to study the effect of K6PC-5 on fibroblast proliferation, human neonatal fibroblasts *in vitro* and intrinsically-aged murine model *in vivo* were used. K6PC-5 promoted fibroblast proliferation and procollagen production in a dose-dependent manner in human fibroblasts. Topical application of K6PC-5 for 2 weeks to intrinsically-aged

hairless mice (56 weeks old) enhanced fibroblast proliferation, collagen production, and eventually increased dermal thickness. K6PC-5 also promoted specific epidermal differentiation marker proteins, including involucrin, loricrin, filaggrin, and keratin 5, without any alterations in epidermal barrier function. These results suggest that K6PC-5 acts to regulate fibroblast proliferation via $[Ca^{2+}]_i$ responses through intracellular S1P production, and can further promote keratinocyte differentiation. Thus, these results reveal that K6PC-5 induces paradoxical effects on the epidermis and dermis through SK1-mediated S1P production, and suggest that S1P regulation may represent a novel approach for the treatment of skin disorders such as atopic dermatitis, psoriasis, and skin aging.

Key words : sphingosine kinase, sphingosine-1-phosphate, Ca^{2+} signaling, keratinocyte differentiation, fibroblast proliferation

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I. INTRODUCTION

Sphingosine-1-phosphate (S1P) is derived from sphingosine, and represents a potent bioactive sphingolipid metabolite. S1P acts as a multifunctional mediator of a variety of cellular responses including Ca^{2+} mobilization, proliferation, survival, motility, and differentiation (reviewed by Spiegel et al.)[1]. S1P functions as both an extracellular ligand for a family of five G protein-coupled receptors (GPCRs) and an intracellular second messenger. S1P receptors regulate many physiological and pathophysiological processes, such as vascular maturation, cardiac development,

angiogenesis, immunity, and cell migration[1, 2]. S1P also shows a universal Ca^{2+} signaling role through receptor-dependent and -independent pathways[3]. Cellular levels of S1P are tightly regulated in a spatio-temporal manner both through synthesis, catalyzed by sphingosine kinases (SKs), and degradation by S1P lyase and specific S1P phosphohydrolases[4]. Two SK isoforms, SK1 and SK2, are known to regulate the relative levels of S1P, sphingosine, and ceramide in the sphingolipid metabolic pathway[4~6].

As SK is directly responsible for the production of S1P, many factors have been identified that alter its activity and regulate subsequent S1P levels[7, 8]. For example, SK is activated by ligands of GPCRs[9~16], including S1P itself[17], agonists of growth factor receptor tyrosine kinases[18~21], cross-linking of immunoglobulin receptors[22~24], and the endogenous ganglioside GM1[25]. Although in some cases the mechanisms are unknown, many other biologically active agents also activate SK including estrogen[26], TNF-α[27], vitamin D_3[28], phorbol ester[29, 30], aluminum fluoride ($AlF4^-$)[13], serum[8], and oxidized low density lipoprotein[31]. To date, there are no reports to demonstrate a chemically-induced, direct activation of SK. Although 12-O-tetradecanoylphorbol-13-acetate (TPA) showed direct effect on SK activation in total lysate and fractionated lysates of Balb/c 3T3 fibroblasts, it is unclear whether this upregluation of SK occurs through direct SK activation by the phorbol ester, or through an enhanced transcription of SK[29]. In this study, it is suggested that a newly synthesized K6PC-5 shows a direct SK activation and S1P-mediated $[Ca^{2+}]_i$ regulation in both

keratinocytes and fibroblasts.

S1P is an evolutionally-conserved Ca^{2+} signaling molecule in yeast, plants, and mammals, that uses specific Ca^{2+} signaling to initiate diverse cellular responses[1]. Acting as an agonist at GPCR, S1P increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through the classical phospholipase C (PLC)-dependent pathway, as well by PLC-independent pathways such as SK. Intracellular S1P also mobilizes Ca^{2+} from intracellular stores by an as yet unknown mechanism that might involve a novel Ca^{2+} channel[3]. These PLC-independent pathways do not include G-protein-coupled S1P receptors, and $[Ca^{2+}]_i$ signaling by many agonists requires SK-mediated S1P production. This study suggests that a direct activator of SK may help to resolve these outstanding issues regarding the relationship of intra- and extra-cellular S1P targets. In mammalian skin, Ca^{2+} serves as a regulator for keratinocyte proliferation and differentiation as well as a signaling molecule for epidermal permeability barrier homeostasis. It is well established that Ca^{2+} can potentially induce keratinocyte differentiation and repress cell growth[32, 33]. In addition, Ca^{2+} is an important signal that facilitates the repair of barrier function following acute injury[34].

As S1P is an important lipid mediator that has been implicated in many biological processes including cell growth, survival, and differentiation[1, 2], this study has been performed to investigate the potential benefits of S1P in skin disorders. Manggau et al. reported that 1α,25-dihydroxyvitamin D_3 protects keratinocytes from apoptosis induced by ceramides, ultraviolet irradiation, or TNF-α, and additionally this

cytoprotection is mediated via the formation of S1P[35]. Vogler et al. reported that all five S1P receptors ($S1P_{1-5}$) are expressed in keratinocytes, and that S1P enhances keratinocyte differentiation and exerts anti-proliferative effects by protecting cells from programmed cell death[36]. Conversely, S1P also enhances proliferation of primary fibroblasts, increases matrix protein formation including fibronectin and PAI-1[36], and is involved in cytoprotective actions of calcitriol in human fibroblasts[37]. Furthermore, it is well established that S1P regulates cell growth in a variety of fibroblast cell lines[18, 38~40].

To investigate the potential benefits of S1P in modifying skin disorders, this study has focused on the key enzymatic step in its synthesis, SK. It is anticipated that activation of SK and subsequent elevation of S1P level will induce diverse cellular responses in the dermis and epidermis for treatment of skin disorders. However, to date there are no reports that have demonstrated chemically-induced, direct activation of SK. Most factors related to SK activation regulate the activity of SK and the production of S1P by extracellular stimuli targeting plasma membrane receptors. Recently it is reported that a newly synthesized compound, K6PC-5, as a SK activator, activates SK obtained from C57BL/6 murine blood[41].

In this study, investigating was performed to find out whether a newly-synthesized SK activator, K6PC-5, induces both keratinocyte differentiation and fibroblast proliferation in skin through $[Ca^{2+}]_i$ signaling in keratinocytes and fibroblasts. K6PC-5 was synthesized as a short chain pseudoceramide which induces keratinocyte

differentiation[42], but the activity of SK was found. $[Ca^{2+}]_i$ signaling in keratinocytes and fibroblasts was investigated to confirm SK activity induced by K6PC-5, and a hyperproliferative murine model and intrinsically aged mice were used to elucidate the potency of K6PC-5 on skin disorders.

II. MATERIALS AND METHODS

1. Materials

Ciglitazone was purchased from Cayman Chemical Laboratories (Ann Arbor, MI, USA); troglitazone was from Biomol Laboratories (Plymouth, PA, USA). Affinity-purified rabbit primary antibody specific for human sphingosine kinase 1 was purchased from BabCo (Richmond, CA, USA); mouse filaggrin, loricrin, and involucrin were from Covance Research (Berkeley, CA, USA); keratin 5, PCNA, matrix metalloproteinase 13 (MMP-13) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); collagen type I was from Calbiochem (San Diego, CA, USA). Secondary goat anti-rabbit IgG-horseradish peroxidase and diaminobenzidine were from Dakoctomation (Glostrup, Denmark). All reagents for RT-PCR were from Takara Korea Biomedical (Otsu, Japan). All reagents for protein isolation and western immunoblotting were from Pierce Biotechnology (Rockford, IL, USA). All reagents for immunohistochemistry were from DakoCytomation. Fluorescein-FragEL™

apoptosis detection kit was from Oncogen Research Products (San Diego, CA, USA). Sphingosine-1-phosphate (S1P), thapsigargin, 2-aminoethoxydiphenyl borate (2-APB), U73122, U73343, G-418 and pertussis toxin (PTX) were purchased from Sigma; RPMI 1640, penicillin, streptomycin, Dulbecco's Modified Eagle Medium (DMEM), and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA); Fura2-acetoxymethyl ester (Fura-2, AM) was purchased from Teflabs (Austin, TX, USA). All other chemicals were of reagent grade. Adult female hairless mice, 8~10 weeks of age and 56 weeks of age, were purchased from the animal laboratory of Yonsei University (Seoul, Korea). The use of animals was approved by the Institutional Review Board of Yonsei University College of Medicine.

2. Cell culture

HaCaT cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI 1640 supplemented with 2 mM glutamine, 25 mM glucose, 100 μg/ml penicillin, 25 ng/ml streptomycin and 10% FBS.

Fibroblasts were cultured primarily from human foreskin (Chungnam University, Daejeon, Korea) in DMEM. The primary cultured fibroblasts were used within 10 passages for $[Ca^{2+}]_i$ measurements, cell proliferation and collagen synthesis.

For $[Ca^{2+}]_i$ measurements, the cells were seeded on 60 mm culture dishes and maintained at 37°C in humidified 5% CO_2 and 95% air and were used at 80%

confluence. The cells were serum-starved for 24h before each experiment.

For the other experiments, the cells were cultured in DMEM supplemented with 5% FBS. For treatment with K6PC-5 and S1P, approximately 1×10^6 cells were seeded on 100 mm culture dishes at approximately 80% confluence. Cells were starved of serum for 24 h, and treated with K6PC-5 or S1P in serum-free medium.

3. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurement

Cells were loaded with 3.5 μM fura-2, AM for 1hr at room temperature (RT) in physiologic salt solution (PSS) containing: 140 mM NaCl; 5 mM KCl; 1.2 mM $MgCl_2$; 1 mM $CaCl_2$; 10 mM HEPES; and 10 mM glucose, titrated to pH 7.4 with NaOH. Ca^{2+}-free PSS contained 1 mM EDTA and 1 mM ethyleneglycol-bis-(β-aminoethylether)-N, N, N´, N´-tetra acetic acid without $CaCl_2$. The fura-2, AM-loaded cells were mounted on the chamber of an inverted microscope (Nikon, Tokyo, Japan) for Ca^{2+} imaging. The excitation wavelength was alternated between 340 nm and 380 nm and the emission wavelength was monitored at 510 nm with a CCD camera using MetaFlour system (Universal Imaging Co., PA, USA). Fluorescence images were obtained at 3s intervals. Background fluorescence was subtracted from the raw intensity at each excitation wavelength before calculating the fluorescence ratio as follows: Ratio = F340/F380.

4. SiRNA down regulation of SK1 expression

Small interfering RNA for SK1 (siRNA-SK1) was constructed using the psiSTRIKE vector system (Promega Corporation, Madison, WI, USA). The siRNA-SK1 target specific sequences were: sense 5'-ACCGCGGGTCGAGGTTATGGATTT CAAGAGAATCCATAACCTCGACCCGCTTTTTC-3'; reverse 5'-GCAGAAAAAG CGGGTCGAGGTTATGGATTCTCTTGAAATCCATAACCTCGACCCG-3'. Cells were co-transfected with siRNA-SK1 (1 μg/ml) and green fluorescent protein (eGFP-N1) (1 μg/ml) using Lipofectamine 2000 reagent (Invitrogen), and cultured for 48 h in serum-free RPMI 1640 including opti-MEM; cells were selected using G-418 (Invitrogen). In keratinocytes, depletion of endogenous SK1 by siRNA was confirmed by RT-PCR, western blotting and eGFP fluorescence.

5. Reverse transcriptase-PCR in keratinocytes

Total RNA was extracted from cultured HaCaT cells using Trizol (Invitrogen) according to the manufacturer's protocol. Equal amounts of RNA (2 μg) were reverse-transcribed using a RNA PCR kit (AMV) (Takara). Semiquantitative PCR was performed using the following specific primers: human SK1 (5'-ATGGATCCAG TGGTCGGTTG-3' and 5'-TCTTCATTGGTGACCTGCT-3'); human involucrin (5'-GGACGGACAACTAAAACAT-3' and 5'-AGCGGACCCGAAATAAG-3'); human filaggrin (5'-GTTCACATTTATTGCCAAAAGA-3' and 5'-GAGCCAACTTGAATA CCAT-3'); and GAPDH as an internal control (5'-TGAGCTGAACGGGAAG-3' and 5'-CTGTAGCCAAATTCGTTGT-3'). cDNAs, SK1, involucrin, filaggrin, GAPDH,

and β-actin, were amplified using 35, 35, 40, 30, 25, and 30 cycles, respectively. Reaction products were separated in 2% agarose gels and visualized with ethidium bromide.

6. Western immunoblotting

Cells were lysed in protein extraction reagent (Pierce Biotechnology Inc., Rockford, IL, USA). Lysates were centrifuged at 12,000×g for 15 min, and supernatants were collected for western immunoblotting. Protein concentrations were determined using the BCA assay (Pierce). Equal protein amounts (20 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes. Membranes were subsequently blocked with 5% skim milk in TBS-T (20 mM Tris-HCl, pH 7.6; 137 mM NaCl; 0.1 % Tween 20) and incubated sequentially with primary antibody (3 h), followed by horseradish peroxidase-conjugated secondary antibody (2 h) at RT. Blotting proteins were visualized by enhanced chemiluminescence (Pierce).

7. Fibroblast proliferation and collagen synthesis

Primary cultures of human dermal fibroblasts were prepared from neonatal foreskins. All experiments were performed with 3~7 passage cultures. The effect of K6PC-5 on the fibroblast proliferation was determined by MTT assay. Fibroblasts (1×10^4 cells/well) were seeded into 96-well plates in DMEM with 5% FBS for 48h, and incubated with K6PC-5 in DMEM serum-free media for 24h at 37°C. After

addition of 20 μl/well of MTT solution (10 mg/ml in phosphate buffered saline), plates were incubated for 4h at 37°C. Supernatants were then removed and the formazan crystals were dissolved in 100 μl of dimethylsulfoxide. Optical density was determined at 540 nm using a microplate reader (SpectraMax 340PC; Molecular Device Co., Sunnyvale, CA, USA). Collagen production in cultured fibroblasts was determined using procollagen type I c-peptide ELISA kit (Takara Shuzo, Otsu, Japan). The collagen (procollagen type 1) and collagenase (MMP-13) expression were determined in murine skin by western immunoblotting. Dermal skin was lysed using protein extraction reagent (Pierce Biotechnology Inc., Rockford, IL, USA). Lysates were centrifuged at 12,000×g for 15 min, and supernatants were collected for western blotting. Protein concentration of samples was determined by BCA assay according to the supplier's protocol (Pierce Biotechnology). Equal amounts of protein (20 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes. Membranes were subsequently blocked with 5% skim milk in TBS/T (20 mM Tris-HCl [pH 7.6]), 137 mM NaCl, 0.1% Tween 20) and incubated sequentially with primary antibody (3h), followed by an HRP-conjugated secondary antibody (2h) at room temperature. Blotting proteins were visualized by enhanced chemiluminescence (ECL) using SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biothechnology Inc., Rockford, IL, USA).

8. Animal model for epidermal hyperplasia

Epidermal hyperproliferation was achieved by repeated applications of D-squames® to the flanks of hairless mice (8~10 weeks of age) twice daily for 5 days until transepidermal water loss reached 40 mg/cm^2/h as determined with Tewameter (Courage and Khasaki, Cologne, Germany)[43]. After each tape stripping sequence, animals (n = 5 in each group) were treated with 1.0% K6PC-5, or 1 mM S1P dissolved in propylene glycol:ethanol (7:3 ratio), or vehicle alone to one flank, twice daily, for the last 3 days. A third control group consisted of non-tape-stripped and non-treated animals (n=3). Harvesting of tissue samples was achieved 6 h after the last treatment.

9. Animal model for intrinsically aged skin

Intrinsically aged female hairless mice (56 weeks of age) were housed in a standard environment, with temperature maintained at 22 ± 0.5 °C, relative humidity at 60 ± 5 % and a 12h/12h light and dark cycle. Seventeen aged mice were randomly divided into 3 groups and treated topically twice daily for two weeks with: i) group K (n=6, 1% K6PC-5); ii) group V (n=6, vehicle (PEG:EtOH=7:3)); iii) group C (n=2, non-treated).

10. Immunohistochemistry

Paraffin-embedded sections of hairless mice skin biopsies were used for immunohistochemistry with specific antibodies for involucrin, loricrin, filaggrin and

keratin 5. After deparaffination and rehydration, sections were incubated with Peroxidase Blocking Reagent (DakoCytomation, Glostrup, Denmark) for 30 min to block endogenous peroxidase activity. Nonspecific antibody activity was blocked by incubation with serum-free Protein block (DakoCytomation, Glostrup, Denmark) for 15 min at RT, the primary antibodies (anti-involucrin, anti-loricrin, anti-filaggrin,1:500 [Covance Research], and anti-K5, 1:500 [Santa Cruz biotechnology]) were applied for 30 min at 37°C, followed by horseradish peroxidase-conjugated secondary antibody (30 min) at RT. Staining for these proteins was detected by diaminobenzidine (DAB) as the substrate. Proliferating keratinocytes were visualized with anti-PCNA antibodies, following the same method to detect proliferating cells. PCNA-positive cells were counted in the basal and supra-basal layers in five images from each section (200× magnification).

11. Ca^{2+} mobilization in murine epidermis

The effect of K6PC-5 on Ca^{2+} mobilization in epidermis was visualized using a method reported previously[44]. An agarose gel membrane (2%) containing 10 μg/ml Calcium Green 1 (Molecular Probes, Eugene, OR, USA) was formed on the slide glass, and a frozen section of whole skin (8 μm thick) was placed directly on the gel membrane. A fluorescence photomicrograph was taken within 2 h with excitation wavelength 546 nm.

12. Microscopy and imaging

Skin samples were obtained from anesthetized animals or after euthanasia, and fixed in 10% formalin. Six-μm thick paraffin sections were stained with hematoxylin and eosin (H&E) and five images (200×) were taken of each section. Fibroblast numbers were counted for each photograph and calculated for each sample (cells per 3×3 cm^2). Epidermal thickness was determined as the distance from the basal layer to the stratum granulosum/stratum corneum (SC-SG) junction, and dermal thickness as the distance from the subcutaneous fat to dermal-epidermal junction, respectively. Thickness was measured in each photograph at random sites (10 sites for epidermis; 3 sites for dermis)[45]. The collagen fibers were visualized using Masson-trichrome staining and analyzed using Photoshop software.

13. Electron microscopy

After biopsy, murine skin samples were fixed in modified Karnovsky's fixative overnight, and post-fixed with 2% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide, as described previously[46]. After further counterstaining with lead citrate, ultrathin sections were examined under an electron microscope (Joel, Japan) operating at 80kV. Corneodesome density measured CD length at random from the first and second cell layers of the lower SC. The ratio of the total length of intact CD to the total length of cornified envelopes was determined by planimetry, as described[47].

14. Functional studies

Transepidermal water loss (TEWL), skin surface capacitance and skin pH were measured using MPA-5 (Courage & Khazaka, Cologne, Germany) after two week topical application of test samples. Recovery of permeability barrier function was determined by measuring TEWL immediately after (t=0) and 3, 6h after barrier disruption by tape stripping. SC integrity was determined by measurement of TEWL after 5 sequential strippings with D-Squame tape[48].

15. Statistics

Statistical analyses were performed using InStat 3 software (GraphPad Software Inc., San Diego, CA, USA). Non-paired two-tailed student's t-tests or ANOVA test were performed to calculate the statistical significance. Values are reported as means ± SEM.

III. RESULTS

1. K6PC-5 induces $[Ca^{2+}]_i$ oscillations in keratinocytes and fibroblasts.

Before determining whether SK-mediated S1P production is responsible for the $[Ca^{2+}]_i$ signaling, the $[Ca^{2+}]_i$ signals induced by K6PC-5 were characterized in intact HaCaT keratinocytes and primary neonatal fibroblasts. First, $[Ca^{2+}]_i$ oscillations were induced by K6PC-5 concentrations ($\geq$25 μM) in HaCaT cells (Figure 1.a). In addition, the percentage of responding cells was dependent on the K6PC-5 concentrations: i.e., 10.3 ± 1.5 % at 25 μM (n = 9); 47.9 ± 15.6 % at 50 μM (n = 9); and 94.4 ± 7.95 % at 100 μM (n = 12) (Figure 1.c). However, the amplitude of the spikes was unaffected by the K6PC-5 concentration in the range of 25 to 100 μM, while a spike-plateau response was observed at a K6PC-5 concentration of 100 μM. In addition, the K6PC-5-induced $[Ca^{2+}]_i$ oscillations were reversible (Figure 1.b). The average lag time between K6PC-5 exposure and the generation of $[Ca^{2+}]_i$ responses was 1.5 ± 1.0 min. Since 50 μM K6PC-5 did not induce cell death and generally produced reliable $[Ca^{2+}]_i$ oscillations in HaCaT cells, this concentration was used to analyze the mechanism by which K6PC-5 induced $[Ca^{2+}]_i$ signaling.

Next, $[Ca^{2+}]_i$ responses were induced by K6PC-5 concentrations ($\geq$5 μM) in human fibroblasts (Figure 2.a). In addition, the percentage of responding cells was dependent on the K6PC-5 concentration; i.e., 31.3 ± 3.34 % at 5 μM (n = 4); 74.1 ± 3.34 % at 10 μM (n = 4); 84.7 ± 4.67 % at 25 μM (n = 4); and 97.9 ± 2.68 % cells at

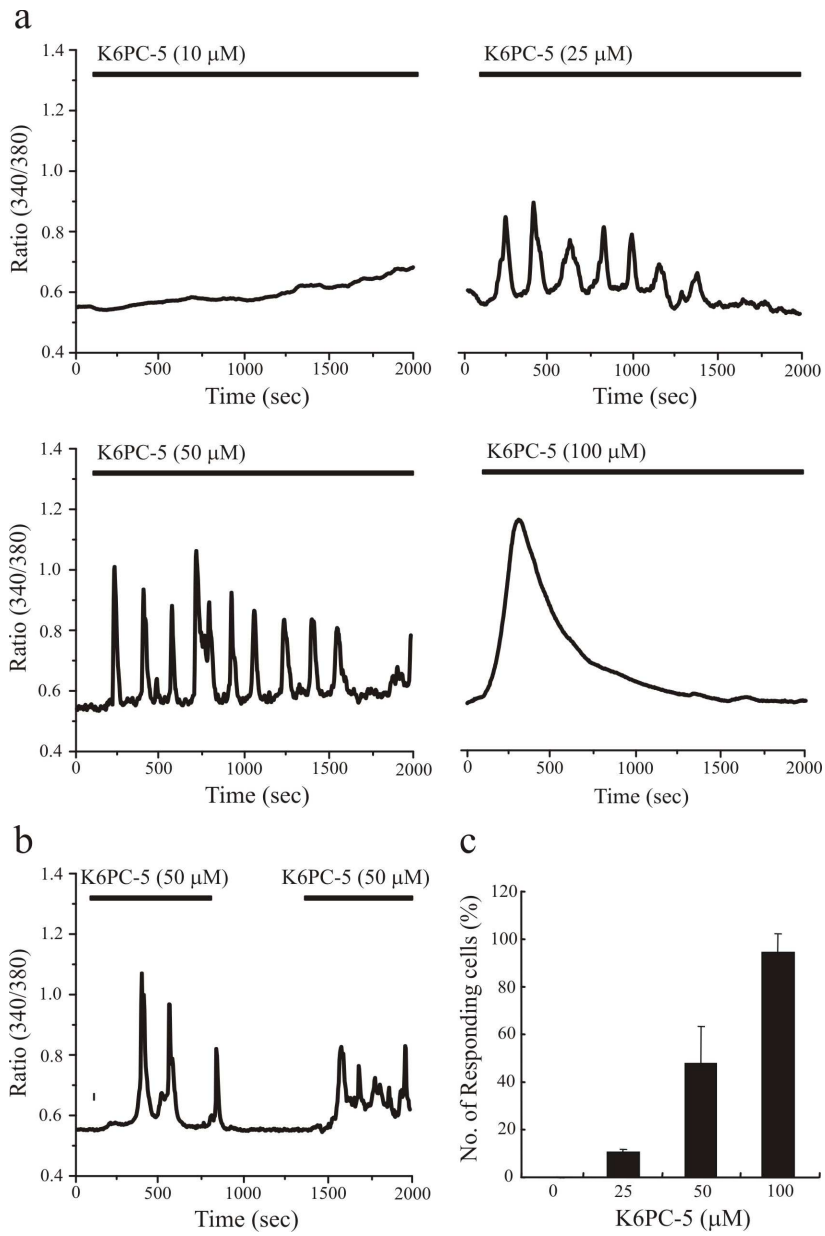

Figure 1. K6PC-5 induced $[Ca^{2+}]_i$ oscillations in HaCaT cells. HaCaT cells were loaded with fura-2 as described under "materials and methods", and changes in $[Ca^{2+}]_i$ were measured using ratiometric fluorescence imaging. Cells were exposed to 10, 25, 50, or 100 μM K6PC-5 (a). 50 μM K6PC-5 was applied, washed out with PSS, and re-applied (b). The percentage of responding cells to K6PC-5 was calculated (c). Results are depicted as mean ± S.E.

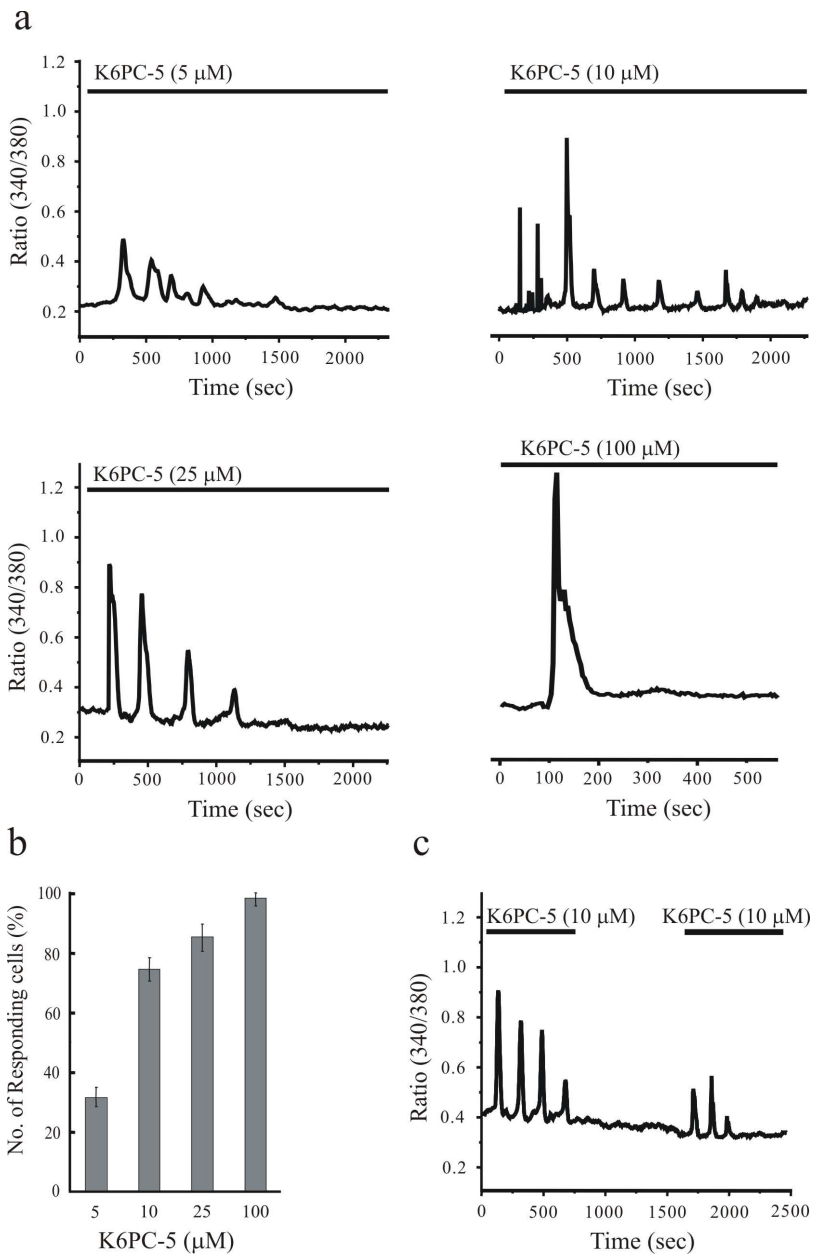

Figure 2. K6PC-5 induced $[Ca^{2+}]_i$ oscillations in fibroblast cells. Human fibroblasts were loaded with fura-2 as described under "Materials and Methods", and changes in $[Ca^{2+}]_i$ were measured using ratiometric fluorescence ratio. Cells were exposed to 5, 10, 25, or 100 μM K6PC-5 (a). Data are calculated as the percentage of responding cells (b). Results are depicted as mean ± S.E. 10 μM K6PC-5 was applied, washed out with PSS, and re-applied (c).

100 μM (n = 4) (Figure 2.b). However, the amplitude of the spikes was unaffected by the K6PC-5 concentration in the range of 5 to 100 μM, while a spike-plateau response was observed at a K6PC-5 concentration of 100 μM. In addition, the K6PC-5-induced $[Ca^{2+}]_i$ oscillations were reversible (Figure 2.c). The average lag time between K6PC-5 exposure and the generation of $[Ca^{2+}]_i$ responses was ~1 min. Since 10 μM K6PC-5 did not induce cell death and generally produced reliable $[Ca^{2+}]_i$ increase in human fibroblasts, this concentration was used to analyze the mechanism by which K6PC-5 induced $[Ca^{2+}]_i$ signaling.

These results reveal that K6PC-5 induces $[Ca^{2+}]_i$ oscillations in both keratinocytes and fibroblasts, and that the fibroblasts are more sensitive than on keratinocytes to the K6PC-5-induced $[Ca^{2+}]_i$ responses when compared to prior study (Figure 1.).

2. K6PC-5-induced $[Ca^{2+}]_i$ oscillations involve SK1 activation.

S1P is a highly bioactive lipid that exerts numerous biological effects both intracellularly as a second messenger and extracellularly by binding to specific G-protein-coupled receptors of the endothelial differentiation gene (EDG) family[49]. Intracellular S1P is generated by SK-dependent phosphorylation of sphingosine. Although intracellular targets for S1P have yet to be identified, the production of intracellular S1P, but not IP_3 (inositol 1,4,5-trisphosphate), mobilizes intracellular Ca^{2+} [50]. To clarify the possibility that the K6PC-5-activated $[Ca^{2+}]_i$ oscillations and intracellular S1P levels are related, we used potent SK inhibitors, N,N-

dimethylsphingosine (DMS) and DL-threo-dihydrosphingosine (DHS). Both DMS and DHS inhibited the K6PC-5-mediated $[Ca^{2+}]_i$ oscillations in both HaCaT cells (Figure 3.a) and human fibroblasts (Figure 4.a).

The $[Ca^{2+}]_i$ signaling mechanism of K6PC-5 was further investigated by using siRNA to examine the functional consequences of depletion of SK1. Transfection of siRNA-SK1 suppressed the expression of mRNA and protein level of SK1 in HaCaT cells. In addition, shown in Figure 3.b, siRNA-SK1 blocked the $[Ca^{2+}]_i$ responses whereas $[Ca^{2+}]_i$ oscillations persisted in empty vector-transfected cells. SiRNA-SK1 also inhibited the $[Ca^{2+}]_i$ oscillations in human fibroblasts whereas empty vector did not (Figure 4.b).

Therefore, the effects of K6PC-5 on $[Ca^{2+}]_i$ oscillations are S1P-dependent, through activity of SK1, in both HaCaT cells and human fibroblasts.

3. K6PC-5-induced $[Ca^{2+}]_i$ oscillations do not involve PLC/IP_3-related pathway.

Numerous studies also have shown that S1P-induced increases in $[Ca^{2+}]_i$ signals involve a G_i-PLC-mediated pathway[51, 52]. To determine whether K6PC-5-induced $[Ca^{2+}]_i$ responses involve a G_i-PLC-mediated pathway, cells were treated with U73122, a PLC inhibitor, and as control its inactive analogue U73343. First, in HaCaT cells (Figure 5.a), U73122, but not U73343, prevented only the exogenous S1P-evoked $[Ca^{2+}]_i$ oscillations, with no effect on the K6PC-5-induced events. HaCaT

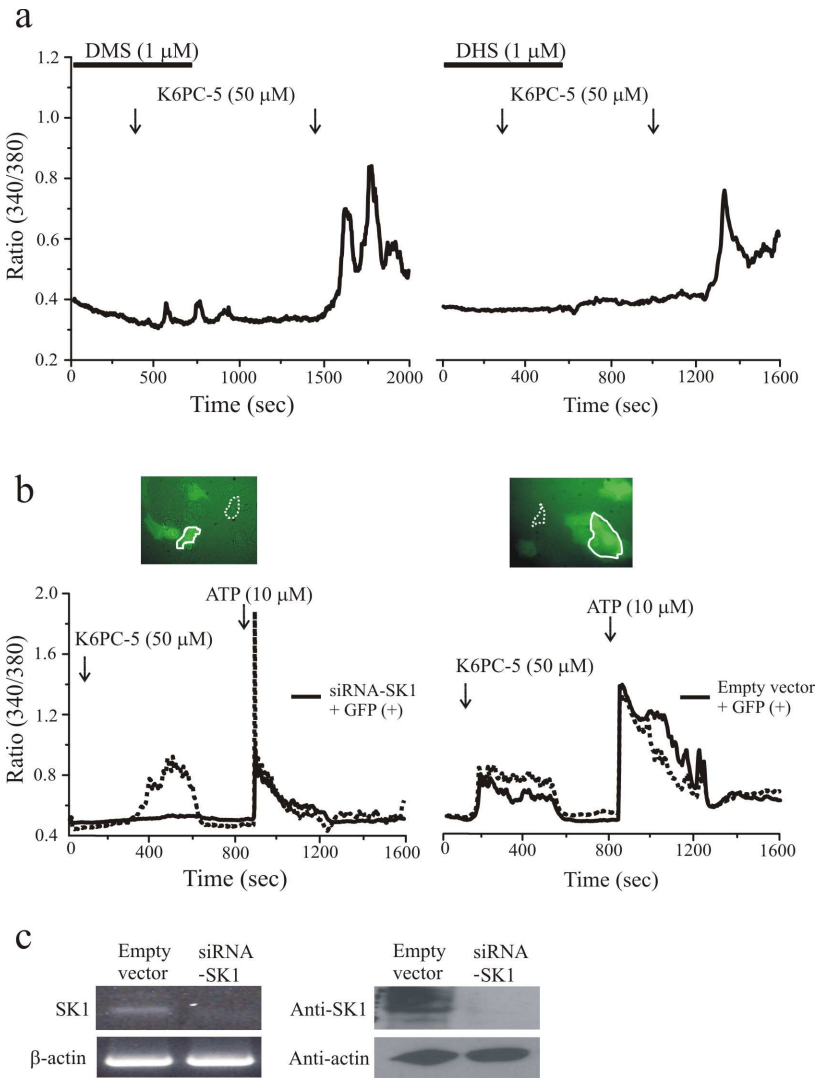

Figure 3. K6PC-5-induced $[Ca^{2+}]_i$ oscillations involved SK1 activation in HaCaT cells. After pretreatment of 1 μM DMS or 1 μM DHS, HaCaT cells were exposed 50 μM K6PC-5 (a). $[Ca^{2+}]_i$ responses of transfected cells with siRNA of SK1 (siRNA-SK1, left) or psiSTRIKE (empty vector, right) were measured (b), and mRNA (left) and protein (right) levels of SK1 were also measured by RT-PCR and western blotting (c). eGFP expressed cells (transfected cells, dotted) and non-transfected cells (solid) were measured $[Ca^{2+}]_i$ signal with the treatment of 50 μM K6PC-5 and 10 μM ATP. The arrow means the point of chemicals treatment.

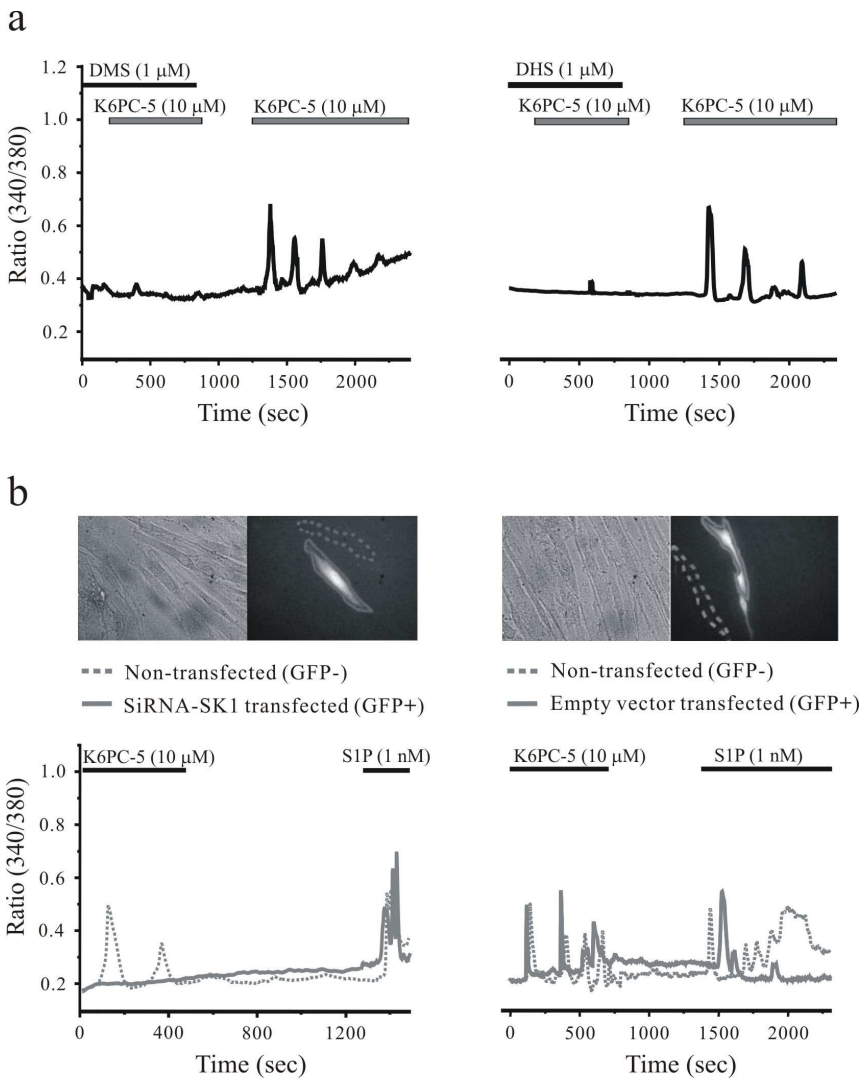

Figure 4. K6PC-5-induced $[Ca^{2+}]_i$ oscillations involved SK1 activation in fibroblasts. After pretreatment of 1 μM DMS or 1 μM DHS, human fibroblasts were exposed 10 μM K6PC-5 (a). In (b), siRNA of SK1 (siRNA-SK1, left) or psiSTRIKE (empty vector, right) was transfected into the cells with eGFP to measure $[Ca^{2+}]_i$ changes. In eGFP expressed cells, $[Ca^{2+}]_i$ changes were measured with the treatment of 10 μM K6PC-5 and 1 nM S1P.

cells were also pretreated with pertussis toxin (PTX), a G_i inhibitor[53]. PTX inhibited the $[Ca^{2+}]_i$ responses induced by exogenous S1P, whereas the K6PC-5-induced $[Ca^{2+}]_i$ responses were not affected (Figure 5.b). Next, in human fibroblasts (Figure 5.c), neither 1 μM U73122 nor 1 μM U73343 prevented the K6PC-5-evoked $[Ca^{2+}]_i$ oscillations.

These results suggest that K6PC-5-induced $[Ca^{2+}]_i$ oscillations are independent of the PLC/IP_3-related pathway in both HaCaT cells and human fibroblasts.

4. K6PC-5-induced $[Ca^{2+}]_i$ oscillations are dependent on Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores and Ca^{2+} entry.

G protein-coupled agonists induce two types of $[Ca^{2+}]_i$ oscillations; i.e., those acutely dependent on Ca^{2+} influx and those largely involving Ca^{2+} release and reuptake into the ER with minimal contribution of Ca^{2+} influx[54]. To identify the source of the $[Ca^{2+}]_i$ mobilization by K6PC-5, Ca^{2+} was removed from physiologic salt solution (Ca^{2+}-free PSS). Depletion of intracellular Ca^{2+} by thapsigargin (Tg), a specific inhibitor of the sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump[55], prevented the K6PC-5-induced $[Ca^{2+}]_i$ oscillations in both HaCaT cells (Figure 6.a; n = 25) and human fibroblasts (Figure 7.a; n=4). In a paired experiment, it was found that depleting the ER by means of stimulation with 100 μM K6PC-5 in the absence of external Ca^{2+} prevented the Ca^{2+} influx caused by 1 μM Tg. These results indicate that the intracellular Ca^{2+} stores responsible for the K6PC-5-induced

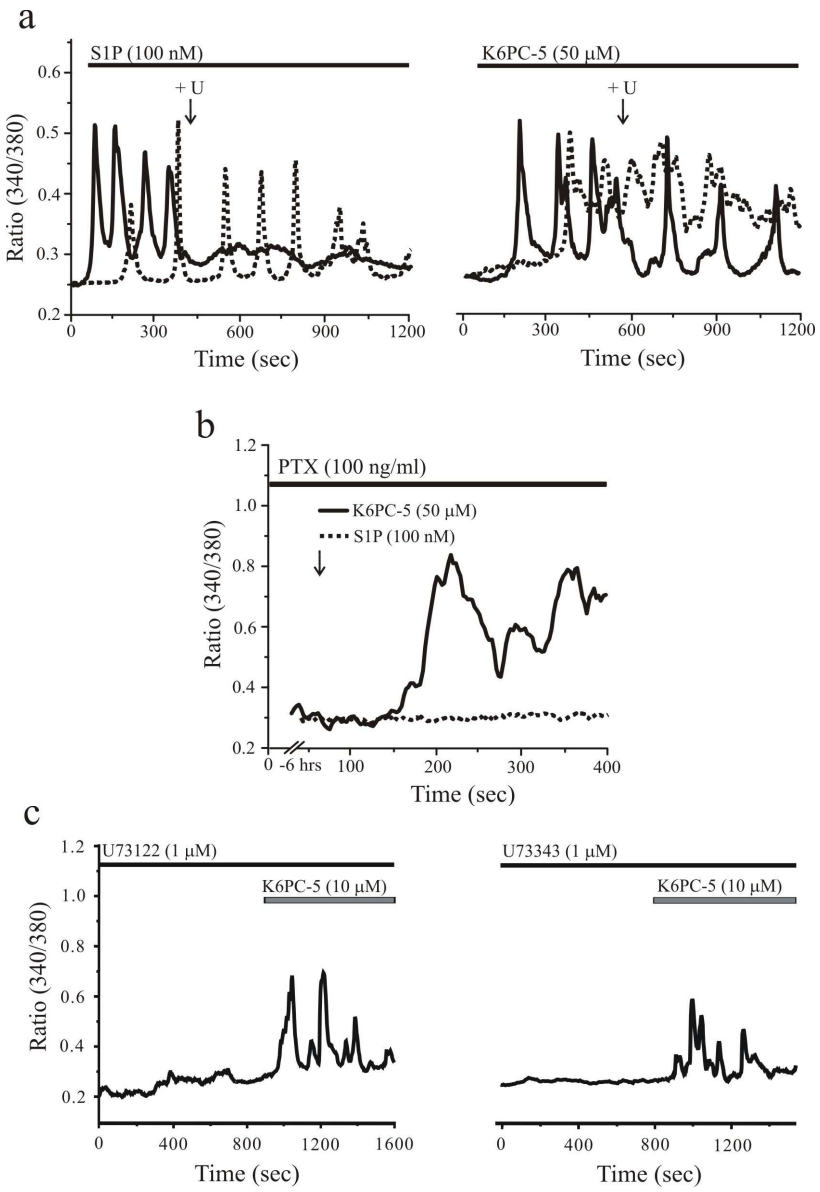

Figure 5. K6PC-5-induced $[Ca^{2+}]_i$ oscillations did not involve PLC/IP_3-related pathway. S1P- or K6PC-5-treated HaCaT cells were treated with 1 μM U73122 (solid) or 1 μM U73343 (dotted) at the point of the arrow (U) (a). After pretreatment of 100 ng/ml pertussis toxin (PTX) for 6 h, HaCaT cells were applied 100 nM S1P (dotted) or 50 μM K6PC-5 (solid) (b). Human fibroblasts were also exposed to 1 μM U73122 or 1 μM U73343, and treated with 10 μM K6PC-5 (c).

$[Ca^{2+}]_i$ oscillations are Tg-sensitive in both cells. Treatment of cells with 100 μM K6PC-5 with or without external Ca^{2+}, revealed that the K6PC-5-induced Ca^{2+} amplitude decreased in the absence of Ca^{2+} (n=5) (Figure 6.b, Figure 7.b). To further delineate the source of the $[Ca^{2+}]_i$ increase, cells were first treated with K6PC-5 in the presence of external Ca^{2+}, followed by Ca^{2+}-free PSS; re-addition of Ca^{2+} yielded $[Ca^{2+}]_i$ oscillations again in both cells (Figure 6.b; n = 5, Figure 7.b; n = 4). These results suggest that extracellular Ca^{2+} also is a source for K6PC-5-induced $[Ca^{2+}]_i$ oscillations in both HaCaT cells and human fibroblasts. To examine whether the K6PC-5-induced $[Ca^{2+}]_i$ signal occurs through plasma membrane Ca^{2+} channels, cells were exposed to 75 μM 2-APB and 1 μM Gadolinium (Gd^{3+}) (non-selective Ca^{2+} channel blockers). Both 2-APB (n=17) and Gd^{3+} (n =16) prevented the generation of $[Ca^{2+}]_i$ oscillations by K6PC-5 in both HaCaT cells (Figure 6.c). Gd^{3+} also prevented the generation of $[Ca^{2+}]_i$ oscillations by K6PC-5 in human fibroblasts (Figure 7.c; n = 4).

These results reveal that Tg-sensitive Ca^{2+} stores and Ca^{2+} entry are each responsible in part for the observed K6PC-5-induced $[Ca^{2+}]_i$ signaling in both HaCaT cells and human fibroblasts, which is independent of the PLC/IP_3 pathway.

5. K6PC-5 increases differentiation-related marker proteins in keratinocytes.

To determine whether K6PC-5 as a SK activator affects keratinocyte differentiation through SK-mediated S1P production involving $[Ca^{2+}]_i$ signaling, reverse

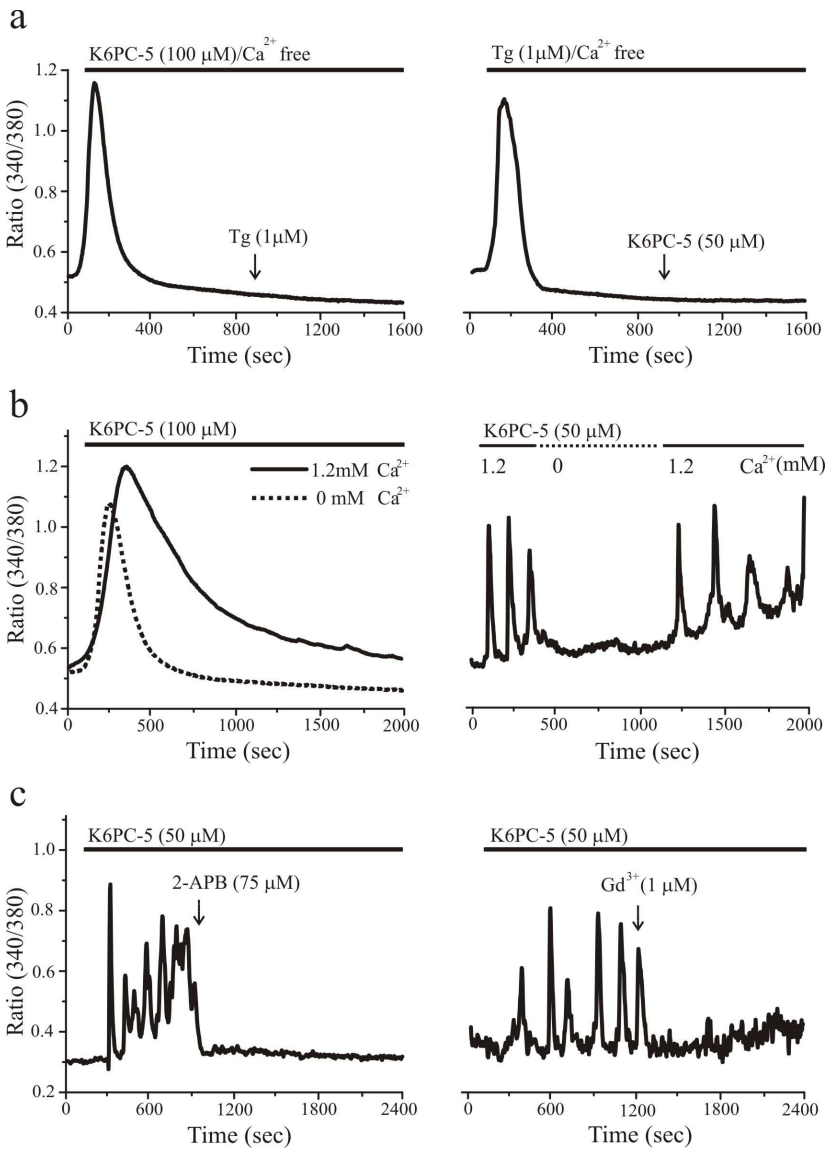

Figure 6. K6PC-5-induced $[Ca^{2+}]_i$ oscillations were dependent on both thapsigargin-sensitive Ca^{2+} stores and Ca^{2+} entry in HaCaT cells. HaCaT cells were exposed to 1 μM thapsigargin (Tg) followed by 100 μM K6PC-5 in a nominally Ca^{2+}-free PSS (a, left). In a complementary experiment, 1 μM Tg applied for depleting the ER (a, right). 50 μM K6PC-5 applied to the cells with 1.2 mM (solid line) or 0 mM Ca^{2+} (dotted line) (b). In the presence of 50 μM K6PC-5, cells were treated with 75 μM 2-APB (c, left) and 1 μM Gd^{3+} (c, right).

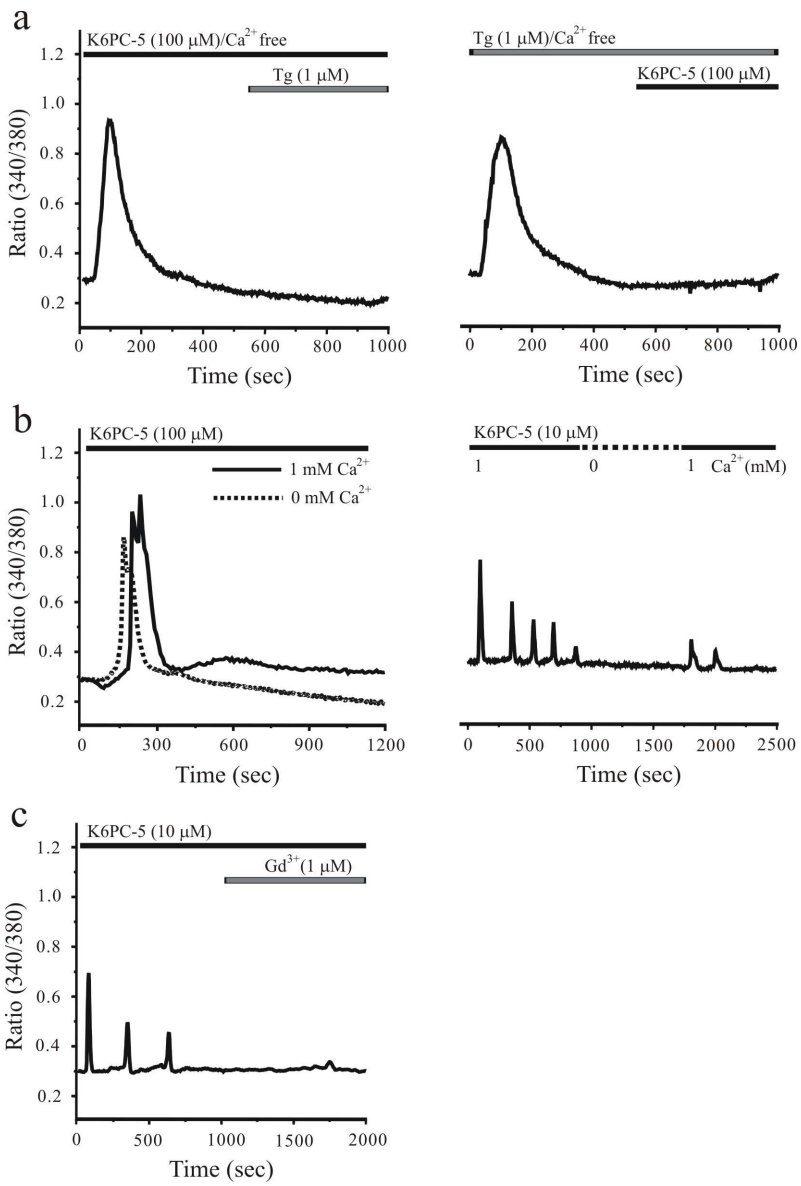

Figure 7. K6PC-5-induced $[Ca^{2+}]_i$ oscillations were dependent on thapsigargin-sensitive Ca^{2+} stores and extracellular Ca^{2+} entry in fibroblasts. Human fibroblasts were exposed to 1 μM thapsigargin (Tg) followed by 100 μM K6PC-5 in a nominally Ca^{2+}-free solution (a). In a complementary experiment, 1 μM Tg applied for depleting the ER. The absence of $[Ca^{2+}]_i$ increased after K6PC-5 application indicates that K6PC-5 induced Ca^{2+} from a Tg-sensitive Ca^{2+} store. 10 μM K6PC-5 applied to the cells with 1 mM (solid line) or 0 mM Ca^{2+} (dotted line) (b). In the presence of 10 μM K6PC-5, cells were exposed to 1 μM Gd^{3+} (c).

transcriptase PCR and western blotting were performed. K6PC-5 and exogenous S1P increased the mRNA levels of keratinocyte differentiation-associated marker proteins including involucrin and filaggrin in HaCaT cells (Figure 8.a). The expression levels of these proteins were also promoted by K6PC-5 and S1P (Figure 8.b).

K6PC-5-induced keratinocyte differentiation was further investigated by using siRNA-SK1-transfected cells to confirm the functional consequences of SK-mediated S1P production by K6PC-5. SiRNA-SK1 blocked the expression of involucrin as a differentiation marker protein, while empty vector did not alter expression (Figure 8.c). Interestingly, extracellular S1P-induced differentiation also was blocked in siRNA-SK1-transfected cells, suggesting that extracellular S1P induces SK1-mediated keratinocyte differentiation. These results suggest that the effects of K6PC-5 are dependent on an S1P-related mechanism through SK1, correlating with the $[Ca^{2+}]_i$ responses induced by siRNA-SK1 (c.f. Figure 4.b).

6. K6PC-5 enhances production of differentiation marker proteins in murine epidermis *in vivo*, and also induces Ca^{2+} mobilization.

To elucidate the potency of K6PC-5 in vivo, we used a well-established hyperproliferative murine model, as reported previously[43]. Again, both K6PC-5 and S1P accelerated the expression of differentiation-associated specific proteins including involucrin, loricrin, filaggrin, and keratin 5 (Figure 9.). These results correlate well with the in vitro results above (c.f. Figure 8.), and suggest that K6PC-5

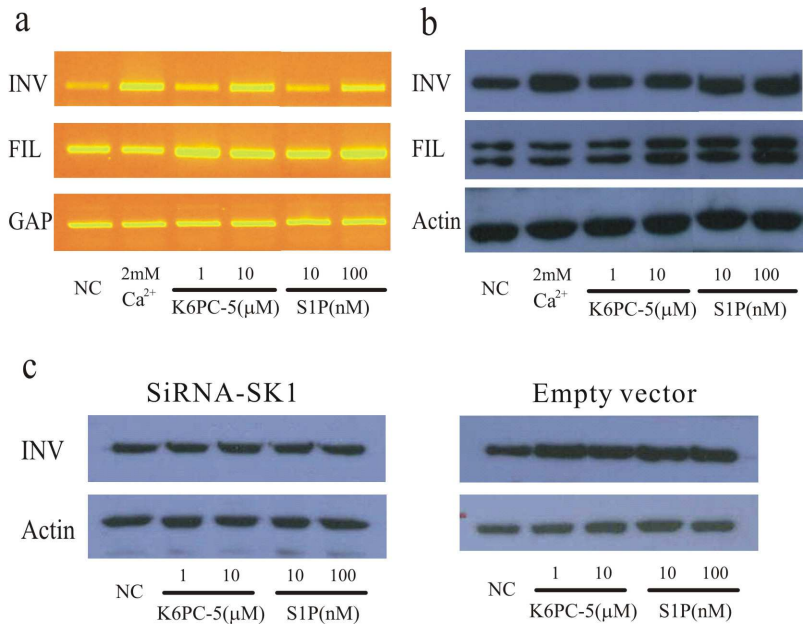

Figure 8. K6PC-5 increased differentiation-related marker proteins in HaCaT cells. mRNA (a) and protein (b) levels of Involucrin (INV) and filaggrin (FIL), as marker proteins of keratinocyte differentiation, induced by K6PC-5 and S1P, were measured by reverse transcriptase PCR and western blotting, respectively. NC; normal control. The expression of involucrin in siRNA-SK1-transfected cells was measured by western blotting (c).

and S1P can promote epidermal differentiation in intact skin. Topical treatment of K6PC-5 in normal hairless mice skin yielded similar results (data not shown). Next, Ca^{2+} signaling was also studied in vivo by topical treatment of intact hairless mice skin with K6PC-5 and S1P. K6PC-5 increased the Ca^{2+} mobilization, visualized by calcium green-1 fluorescence (Figure 10.), an increase that was eliminated by 3 h. S1P also yielded an equivalent result, although the effect was less intense than that of K6PC-5. The difference of time and intensity of K6PC-5 and S1P may reflect the relative hydrophobicity of K6PC-5, which affects its penetration through the intact stratum corneum (SC).

7. K6PC-5 decreases epidermal hyperplasia by inhibiting keratinocyte proliferation.

Prior studies have shown that repeated removal of either the whole SC or the SC intercellular lipids results in an increased epidermal thickness, stemming from increased keratinocytes proliferation[43]. To elucidate the possible activity of K6PC-5 in certain skin disorders, we used this hyperproliferative murine model. Both K6PC-5 and S1P attenuated the increase in epidermal thickness induced by sequential tape-stripping (Figure 11.a), and correspondingly inhibited the increase of PCNA-positive and proliferating keratinocytes (Figure 11.c). The H&E images confirmed these results (Figure 11.b, d). Together, these results suggest that K6PC-5 and S1P decrease epidermal hyperplasia by inhibiting keratinocytes proliferation.

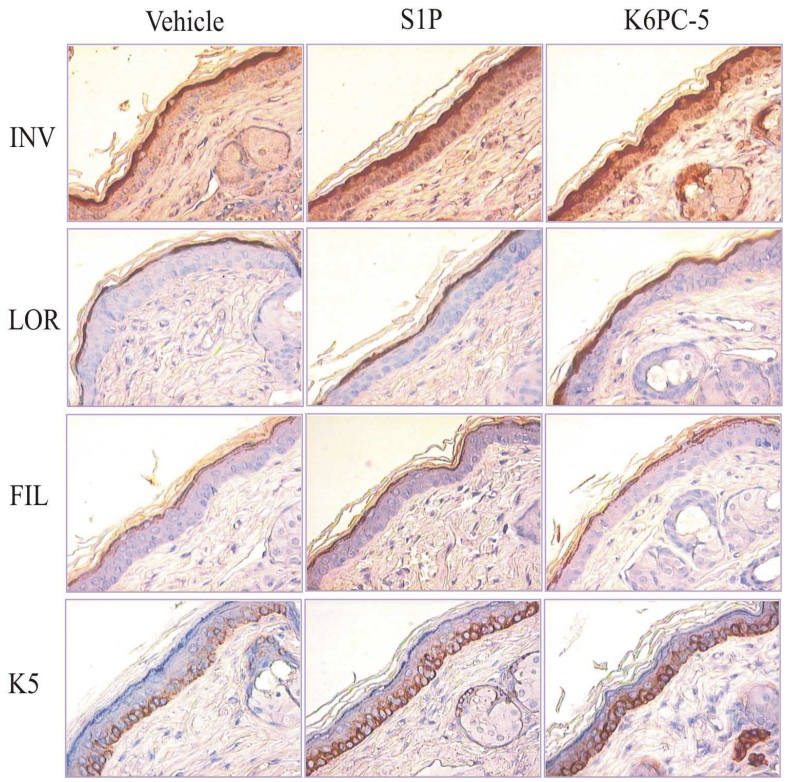

Figure 9. K6PC-5 enhanced the production of differentiation marker proteins in murine epidermis *in vivo*. The expression levels of differentiation-associated proteins induced by topical applications of 1% K6PC-5 and 1 mM S1P on hairless mouse skin were analyzed by immunohistochemistry. Involucrin (INV), loricrin (LOR), filaggrin (FIL) and keratin 5 (K5) were used as marker proteins of epidermal differentiation.

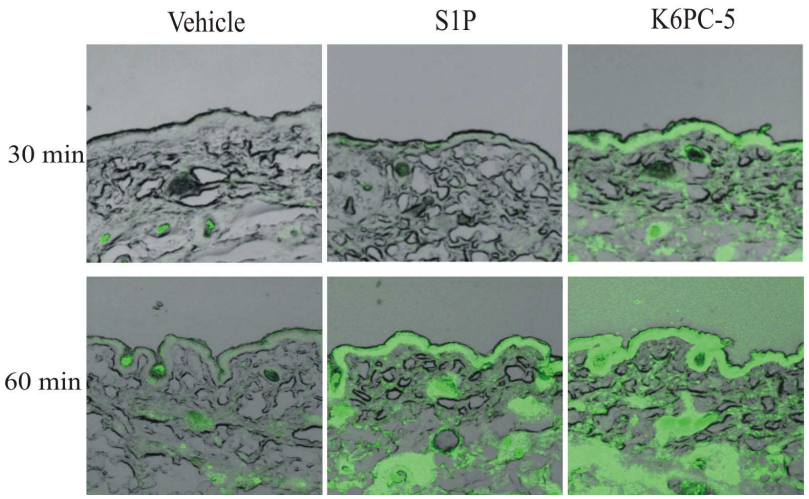

Figure 10. K6PC-5 induced Ca^{2+} mobilization in murine epidermis. The image of K6PC-5-induced Ca^{2+} mobilization in murine epidermis was measured by Calcium Green-1. 1% K6PC-5 and 1 mM S1P were applied topically on hairless mouse skin, and tissue biopsies were performed 30 min and 60 min after application, respectively.

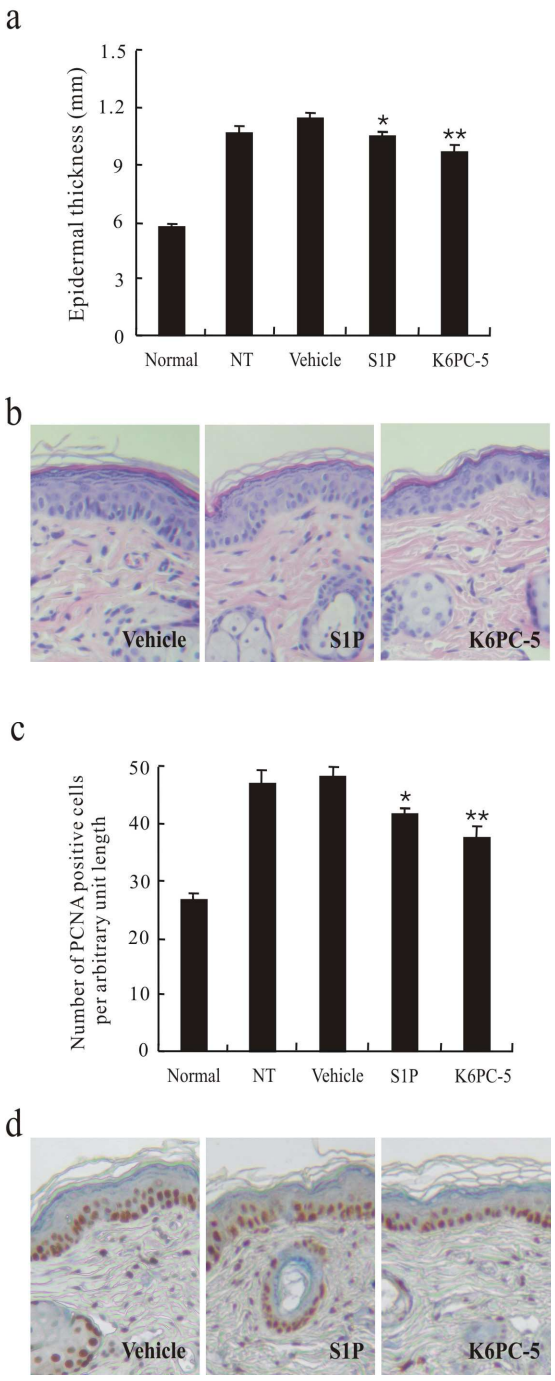

Figure 11. K6PC-5 decreased epidermal hyperplasia by inhibiting keratinocyte proliferation. Epidermal hyperplasia was induced by repeated tape-stripping. Epidermal thickness after topical application of 1% K6PC-5 and 1 mM S1P on hairless mouse skin was measured as described under "materials and methods" (a). Proliferating keratinocytes were measured by PCNA-positive cells per cm on randomly obtained images at a ×200 magnification (n=5). The photograph of H&E stained sections (b) and PCNA-stained sections (d) were also shown. * P<0.01, ** P<0.001; NT, non-treatment after barrier disruption.

8. K6PC-5 promotes proliferation and collagen synthesis in cultured human fibroblasts.

Extracellular S1P induced proliferation and matrix protein formation by primary fibroblasts obtained from juvenile foreskin[36]. Given that K6PC-5 has been shown to increase SK activity, the effect of K6PC-5 on human fibroblasts in vitro was studied. K6PC-5 promoted fibroblast proliferation in MTT dye assay (Figure 12.a); e.g., K6PC-5 (10 μM) induced approximately a 30% increase in optical density (Figure 12.a), consistent with increased proliferation. Since both decreased collagen production and collagen degradation are characteristic features of chronologically-aged and photo-damaged skin[56, 57], the effects of K6PC-5 on collagen levels was investigated. K6PC-5 promoted collagen synthesis, as measured by procollagen type I C-peptide (PICP), with approximately a 30% increase at 10 μM K6PC-5 (Figure 12.b). Consistent with these results, S1P similarly promoted fibroblast proliferation and collagen synthesis, effects that were more potent than K6PC-5, comparing by concentration. These results suggest that K6PC-5 and exogenous S1P induce similar effects on fibroblasts by activating SK.

9. K6PC-5 increases both collagen production in fibroblasts and dermal thickness of aged mouse skin.

To confirm the dermal effect of K6PC-5 in skin, aged hairless mice were used as an intrinsic model. The mice for the intrinsic model were reared for 56 weeks without

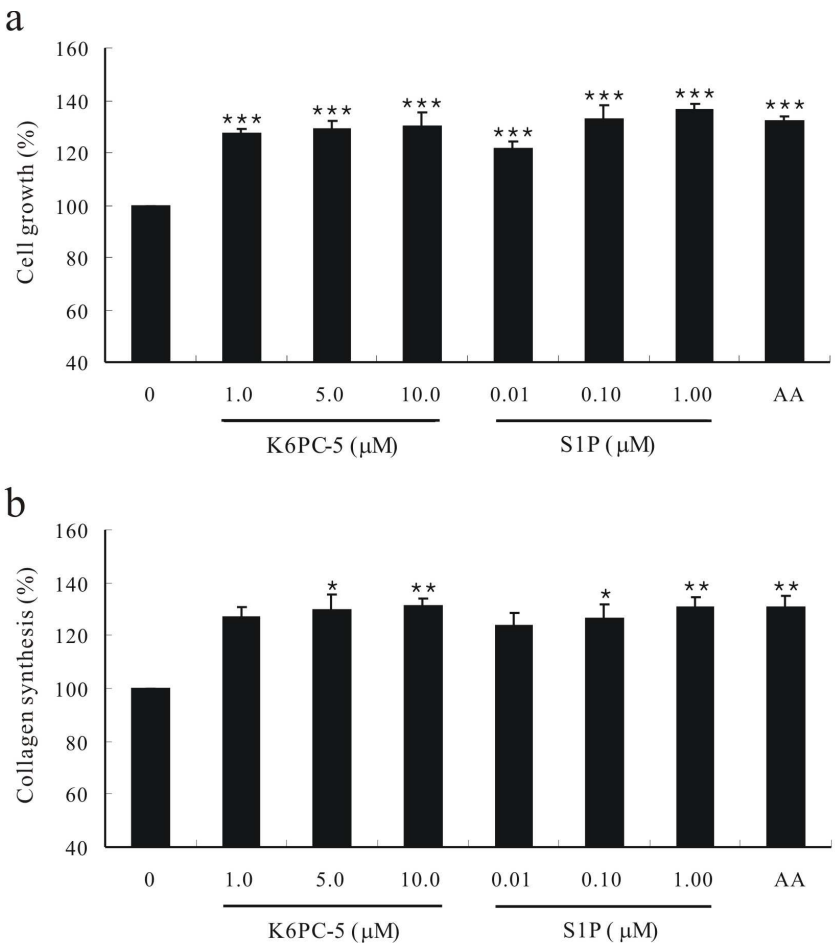

Figure 12. K6PC-5 promoted fibroblasts proliferation and collagen synthesis in vitro. After human fibroblasts were incubated with the indicated concentration of K6PC-5 and S1P for 24h, MTT solution was added. Optical density of MTT dye was determined as described (a). Collagen synthesis was determined by a procollgen type I c-peptite ELISA kit as described (b). 50 μg/ml vitamin C (AA) was used as positive controls. The value are presented as percentage of control (0 concentration) and are the mean ± SEM of results from at least four experiments, each run in triplicate. *p<0.05, **p<0.01, ***p<0.001

any UV light. 1% K6PC-5 was applied topically for 2 weeks. This K6PC-5 treatment significantly increased both the number of dermal fibroblasts (Figure 13.a) and collagen production (Figure 13.c, d, e), although it is unclear whether K6PC-5 inhibited matrix metalloprotease-3 (data not shown). As a consequence, dermal thickness also increased significantly (Figure 13.b). These results correlate well with the in vitro results above (c.f. Figure 12.), and suggest that S1P production by SK activation have positive effects on aged skin through the dermal fibroblast proliferation.

10. K6PC-5 enhances specific differentiation marker proteins in aged murine epidermis.

To confirm the epidermal effect of K6PC-5 on aged skin, immunostaining for various differentiation marker proteins was performed, including involucrin, loricrin, filaggrin, and keratin 5. As shown in Figure 14., K6PC-5 promoted the expression of differentiation-associated proteins. This result was coincident with the previous study in young mouse skin (c.f. Figure 9), and reveals that K6PC-5 also promotes differentiation in aged mouse epidermis. The prior study showed that topical K6PC-5 increased the Ca^{2+} mobilization, which was visualized by calcium green-1 fluorescence, depending on time (c.f. Figure 10). It is anticipated that the same mechanism will be operative in aged mouse skin.

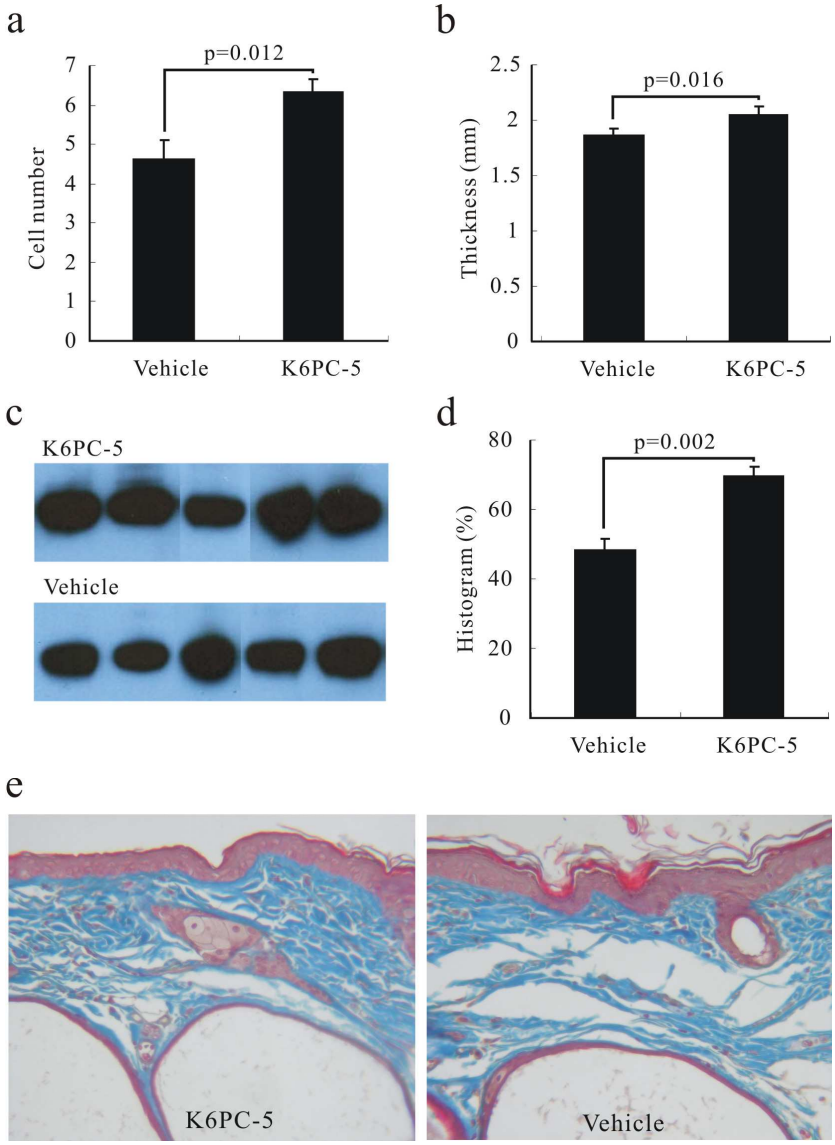

Figure 13. K6PC-5 increased fibroblasts, collagen production, and eventually dermal thickness in the dermis of aged mouse skin. After topical 2-week applications of 1% K6PC-5 and vehicle on hairless mouse skin, skin specimens were biopsized. Hematoxylin and eosin stain were used to measure fibroblast number (a) and dermal thickness (b), as described under "materials and methods". Collagen density was measured in Masson-trichrome stain (d, e-blue). Results shown are shown as mean ± SEM (n=5). The expression of type I procollagen in each experiment group was also measured by western blotting (c).

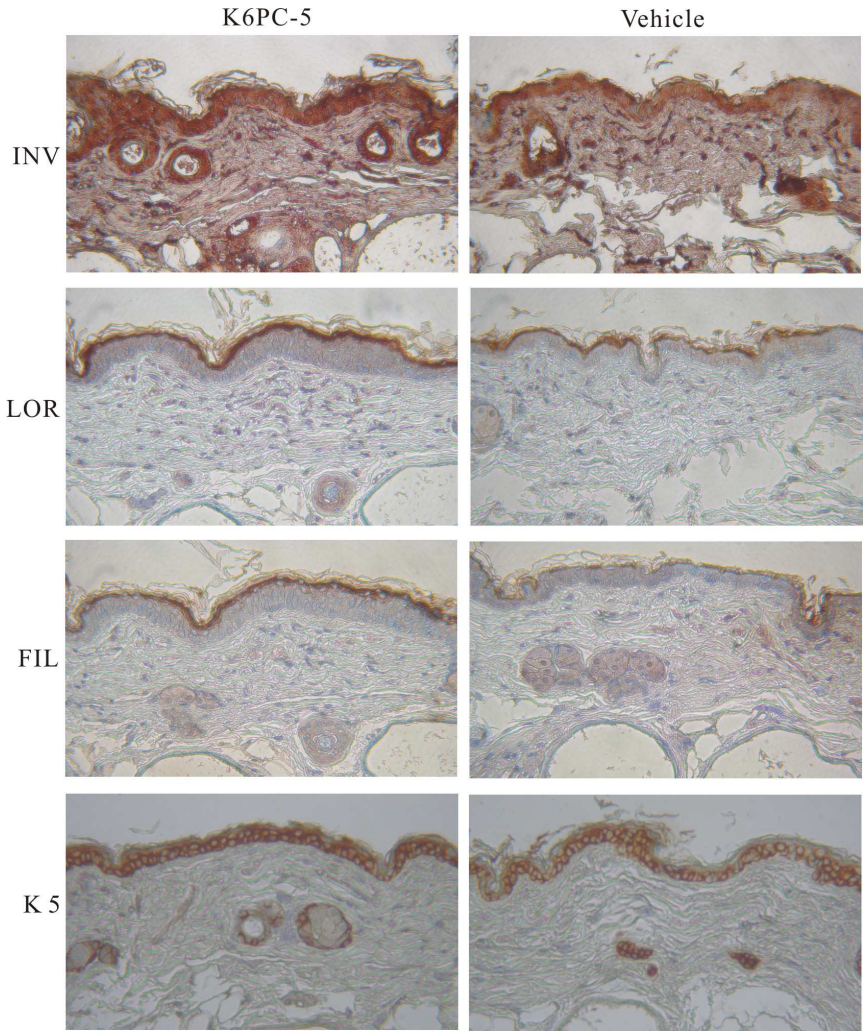

Figure 14. K6PC-5 increased the specific differentiation marker proteins in the epidermis of aged mouse skin. The expression levels of differentiation-associated marker proteins induced by topical applications of 1% K6PC-5 and vehicle for 2 weeks on hairless mouse skin were shown by immunohistochemstry. Involucrin (INV), loricrin (LOR), filaggrin (FIL), and keratin 5 (K 5) were used as specific marker proteins of epidermal differentiation.

11. K6PC-5 does not induce barrier alterations in aged mouse epidermis.

Functional studies of the skin barrier are indispensable for evaluating potential side-effects of topical agents that may induce skin irritation and/or barrier disturbance(s). To determine possible additional effects of K6PC-5 in intact epidermis, functional studies of the skin barrier were performed. K6PC-5 did not alter basal TEWL (Figure 15.a), skin hydration or skin pH (data not shown), and there was no difference in epidermal thickness and PCNA-positive keratinocytes between K6PC-5 and vehicle-treated skin (data not shown); there also was no difference in recovery rate for these animals following acute abrogation of the barrier by tape-stripping (Figure 15.b). However, stratum corneum (SC) integrity was increased in K6PC-5-treated skin (Figure 15.c), although no increase in corneodesmosome (CD) density was evident by electron-microscopy (Figure 15.d). K6PC-5 inhibited the attenuation of SC integrity by vehicle, but there was no difference with CD density, a major factor affecting SC integrity[48]. It is anticipated that the strengthened SC integrity by K6PC-5 might be due to the increased keratinocyte differentiation, a process that includes the cross-linking of proteins such as involucrin and loricrin to form the cornified envelope. Together, these results suggest that K6PC-5 does not induce barrier disturbance. As K6PC-5 induces epidermal differentiation through the paradoxical effect of S1P on intact skin, it is estimated that there will be few side-effects from this agent, contrasting with the well-known retinoid-induced epidermal hyperplasia[58].

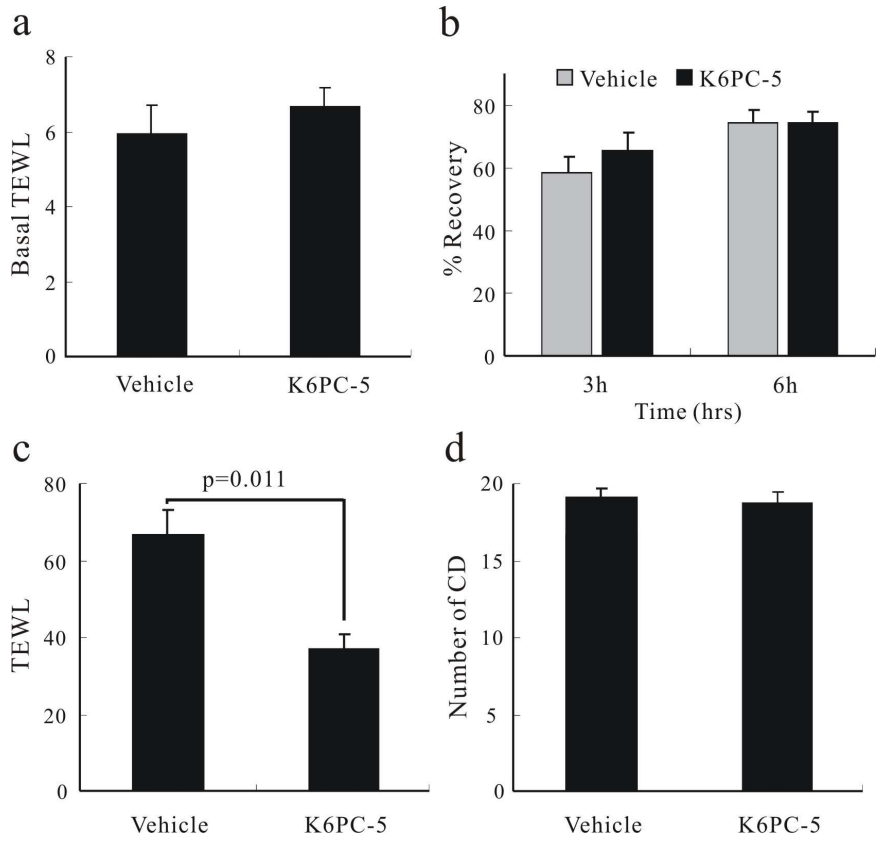

Figure 15. K6PC-5 did not induce barrier disturbance in the epidermis of aged mouse skin. After topical applications of 1% K6PC-5 and vehicle for 2 weeks on hairless mice skin, basal TEWL (a) was measured. Barrier recovery rate (b) and SC integrity (c) was determined by TEWL after tape stripping. Corneodesmosome (CD) density (d) was measured through electron microscope images, as described under “materials and methods”. Results shown are the mean ± SEM (n=5 for each experimental group).

IV. DISCUSSION

This study reports the results of a novel SK1 activator, K6PC-5, on Ca^{2+} signaling in human keratinocytes and fibroblasts as well as keratinocyte differentiation and fibroblast proliferation in both cultured cells and murine skin. SK-activating profile of K6PC-5 was found when new chemicals derived from bioactive and short-chain pseudoceramides were being developed to promote keratinocytes differentiation[42]. However, K6PC-5 represents a compound with novel activities not anticipated from its simple short-chain pseudoceramide structure. K6PC-5 activated SK in a dose-dependent manner, inducing the conversion of C17-sphingosine to C17-S1P[41]. Since SK1 knockout mice show no SK activity in whole blood[59], this result revealed K6PC-5 to be a direct activator of SK1.

In this study, K6PC-5-induced $[Ca^{2+}]_i$ oscillations in both HaCaT keratinocytes and human fibroblasts were blocked by the SK inhibitors, DMS and DHS. Moreover, both cells transfected with siRNA-SK1 did not respond to K6PC-5. These results further reveal that K6PC-5 is a direct activator of SK, especially SK1. Distinct $[Ca^{2+}]_i$ mobilization by K6PC-5 also was evident in murine epidermis. In addition, PTX and U73122 did not prevent $[Ca^{2+}]_i$ oscillations by K6PC-5, but both of these compounds inhibited the S1P-induced effects in HaCaT cells. These findings suggest that K6PC-5 can enhance the role of S1P as an intracellular second messenger. Although many activators of SK have been identified, most of them involve upstream cell membrane

receptors[8]. However, there is no evidence that K6PC-5 induces cellular response through a membrane receptor. In fact, the hydrophobic property of K6PC-5 may allow easy passage into and through the cell membrane. It is therefore suggested that K6PC-5 is a unique compound having direct effect(s) on SK1 enzyme activity in epidermal keratinocytes and dermal fibroblasts of skin.

S1P has been shown to operate via both PLC-dependent and -independent pathways to increase $[Ca^{2+}]_i$ signaling[3]. Blom et al. also reported recently that IP_3 is the major $[Ca^{2+}]_i$ releasing second messenger in an exogenous S1P-stimulating system, effects that are dependent on both PLC and the subsequent generation of intracellular S1P[60]. Additionally, endogenous S1P also affects $[Ca^{2+}]_i$ increases in HEK-293 cells[60]. Although an intracellular target(s) for S1P has/have yet to be identified, several investigations have shown direct $[Ca^{2+}]_i$ mobilizing effects of intracellular S1P[50, 61]. However, the mechanism of which intracellular S1P mobilizes $[Ca^{2+}]_i$ from intracellular stores is also unknown, although a novel Ca^{2+} channel has been reported[62]. The current studies reveal another possible mechanism for these effects. Specifically, since the K6PC-5-induced $[Ca^{2+}]_i$ response was dependent on both Tg-sensitive Ca^{2+} stores and extracellular Ca^{2+}, and since both 2-APB and Gd^{3+} prevented the $[Ca^{2+}]_i$ oscillations by K6PC-5, intracellular S1P produced by K6PC-5 appears to release Ca^{2+} from intracellular stores using an alternate pathway. The $[Ca^{2+}]_i$ signal induced by K6PC-5 is distinct from the classical PLC/IP_3 pathway because these K6PC-5 effects are independent of G_iPCR and PLCβ. Although K6PC-

5 induced S1P-evoked $[Ca^{2+}]_i$ signaling, there was no evidence that S1P binds directly to the IP_3 receptor to release intracellular Ca^{2+} to the cytoplasm. In fact, this effect may reflect indirect Ca^{2+} signaling of intracellular S1P, which Blom et al. previously suggested to explain results in their system[60].

The study of S1P-mediated events is challenging as it functions both as an extracellular ligand for a family of five GPCRs and an intracellular second messenger[1]. Exogenous S1P affects its receptors on cell membrane, as its structural characteristics preclude its direct penetration through cell membranes. Conversely, since, as shown here, K6PC-5 can readily penetrate cell membranes to activate SK1 and increase endogenous S1P levels, this novel compound should be useful for studying the intracellular roles and $[Ca^{2+}]_i$ signals induced by S1P.

Vogler et al. previously reported that S1P enhances differentiation of cultured keratinocytes and possesses anti-proliferative effect that protects cells from programmed cell death[36]. Kim et al. also showed that S1P inhibits human keratinocyte proliferation via Akt/protein kinase B inactivation[63]. Therefore, similar effects of K6PC-5 can be anticipated on keratinocytes via its induction of S1P levels. This study shows that K6PC-5 and S1P increased mRNA and protein levels of involucrin and filaggrin as specific differentiation markers in HaCaT cells, and that siRNA-SK1 blocked the expression of involucrin induced by K6PC-5 and S1P. Their respective protein expression is elevated by K6PC-5 topical treatment in murine epidermis. Interestingly, exogenous S1P also yielded similar results. Moreover, using an

established *in vivo* model, the hyperplasia induced by tape stripping in hairless mouse epidermis was significantly inhibited by both K6PC-5 and S1P, with a corresponding inhibition of PCNA staining. These results reveal that K6PC-5 and S1P have anti-proliferative effect on the epidermis of murine skin under hyperproliferative conditions.

Although that the extent to which impaired epidermal proliferation plays a role in the pathogenesis of certain skin diseases remains a topic of debate, the present results suggest that the regulation of S1P levels may represent a useful, novel approach for the treatment for such skin diseases by regulating both epidermal differentiation and proliferation. Interestingly, K6PC-5 increased expression of specific differentiation-associated marker proteins which are downregulated in the skin of atopic patients including K5, K10, involucrin, and filaggrin[64]. Moreover, K6PC-5 decreases the epidermal thickness and PCNA-positive cells in a murine hyperproliferative model. Together, these findings suggest that S1P represents an attractive sphingolipid for the regulation of both differentiation and proliferation of epidermal keratinocytes.

Here, the role of S1P in aged skin is also investigated, again through topical application of K6PC-5 as a SK activator. K6PC-5 promoted cell proliferation and procollagen-I synthesis in primarily cultured human neonatal fibroblasts, in addition, K6PC-5 induced a significant increase in fibroblast number, collagen production, and dermal thickness in aged murine skin after two weeks of topical application. The depletion of collagen synthesis and a decrease of fibroblast numbers are well-

established for both chronologically-aged and photo-damaged skin. Isolated fibroblasts from aged human skin showed decreased proliferation and altered appearance[56]. Collagen degradation was also observed in both intrinsically-aged and photo-damaged skin[57]. As S1P up-regulates proliferation and survival of fibroblasts, it may be suggested that the effects of K6PC-5 on murine dermis are S1P-mediated results through specific activation of SK1.

K6PC-5 also elevated the expression of differentiation-specific marker proteins, including involucrin, loricrin, filaggrin, and keratin 5 in aged mouse epidermis. This effect is consistent with the prior study in young mouse skin, where K6PC-5 has paradoxical effects on epidermis and dermis. These paradoxical results of K6PC-5 in intrinsically aged skin (as an *in vivo* model) correlate well with the previous *in vitro* results[36]. S1P stimulates both keratinocyte differentiation and fibroblast proliferation, which account for the opposing effects on epidermis and dermis. It may be suggested that the paradoxical effect of S1P on epidermis and dermis may provide additional benefits to aged skin over other anti-aging agents. For example, retinoids are widely used in the treatment of skin aging to improve dermal function; i.e., to enhance both fibroblast proliferation and collagen production, and to decrease matrix metallo-proteinase-mediated extracellular matrix degradation[65]. However, retinoids also induce epidermal hyperplasia[58]. Although Rittie et al. demonstrated that epidermal growth factor receptor activation mediates retinoid-induced epidermal hyperplasia by specific induction of its ligands, heparin-binding EGF and amphiregulin, and

suggested that EGFR inhibitors can mitigate the retinoid-induced scaling[66], retinoid-induced scaling remains a major deterrent to its topical use. It was investigated whether K6PC-5 similarly induces epidermal hyperplasia, but no such effect was evident. Moreover, K6PC-5 did not increase epidermal thickness and PCNA-positive (proliferating) keratinocytes. These results suggest that K6PC-5 may be a useful treatment for skin aging without inducing epidermal hyperplasia and affecting barrier function. Although mechanism(s) responsible for the paradoxical effects of S1P is not known, these specific signals may provide many benefits when treating skin disorders as compared to other compounds, such as retinoids. Interestingly, K6PC-5 also inhibited the vehicle-induced attenuation of SC integrity as a result of elevated keratinocyte differentiation, although CD density was not affected. It is anticipated that this represents another potential benefit of K6PC-5 in aged skin.

It has also been previously reported that the topical application of a K6PC-5 cream improves eye-wrinkles of women volunteers in a clinical study[67]. K6PC-5 of 1% concentration cream, when used for 8 weeks, yielded a significant improvement of eye wrinkles as assessed by video analysis of skin replicas. This clinical result suggested that K6PC-5 could be useful for treatment of aged skin, including sun-exposed skin.

Recently, S1P is being recognized as an important sphingolipid, along with ceramide and sphingosine, each of which has diverse physiological functions, including regulation of cell growth and survival. The regulation and roles of S1P and

SK in many diseases including cancer, allgery, asthma, and the development of vascular and neuronal system have recently been reviewed[68]. However, in skin disorders, the roles of S1P and SK have not been adequately studied. Here, this study reveals that K6PC-5 induces paradoxical effects on the epidermis and dermis through SK-mediated S1P production, and suggests that S1P regulation may represent a novel approach for the treatment of skin disorders such as atopic dermatitis, psoriasis, and skin aging.

Finally, for further studies, it has to be investigated what signal transduction pathway(s) is/are involved in the intracellular Ca^{2+}-induced keratinocyte differentiation and fibroblast proliferation. In keratinocytes, it is well known that Ca^{2+} is an important regulator of keratinocyte differentiation. Li et al. suggested that intracellular Ca^{2+} stores are important for inhibition of DNA synthesis, while elevation of $[Ca^{2+}]_i$ stimulates the expression of differentiation-associated marker proteins in cultured mouse keratinocytes[69]. Efimova et al. reported that activation of involucrin transcription involves a pathway that includes protein kinase C, Ras, MEKK1, MEK3, and p38/RK[70]. Tu et al. also showed that the increase of the extracellular Ca^{2+} leads to a rapid increase in $[Ca^{2+}]_i$ and IP_3 production and, subsequently, to the expression of differentiation-related genes in cultured human keratinocytes[71]. In prior study, K6PC-5 showed that the phosphorylation of p42/44 extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) are downstream signaling events following the increase of Ca^{2+} in normal human

keratinocytes[42]. It is anticipated that K6PC-5-induced $[Ca^{2+}]_i$ also induces keratinocyte differentiation through a similar pathway in keratinocytes. In fibroblasts, but, the roles of intracellular and/or extracellular Ca^{2+} for fibroblast proliferation are little known. It has to be studied further including signal transduction pathway(s).

V. CONCLUSION

The aim of this study is to investigate the effect of K6PC-5 as a SK activator on keratinocyte differentiation and fibroblast proliferation in skin through $[Ca^{2+}]_i$ signaling.

1. K6PC-5 induces $[Ca^{2+}]_i$ oscillations through SK activation in human keratinocytes and fibroblasts. K6PC-5 induced intracellular Ca^{2+} signaling in intact HaCaT keratinocytes and primary neonatal fibroblasts, effects that are attenuated by the SK inhibitors, DMS and DHS, and by siRNA-SK1. K6PC-5-induced $[Ca^{2+}]_i$ oscillations were dependent on both thapsigargin-sensitive Ca^{2+} stores and Ca^{2+} entry, but not PLC/IP_3-related pathway.

2. K6PC-5 acts to regulate both differentiation and proliferation of keratinocytes through SK activation. K6PC-5 enhanced the expression of differentiation-associated marker proteins, including involucrin and filaggrin, in both cultured HaCaT keratinocytes and intact murine epidermis. K6PC-5 also inhibited epidermal hyperplasia induced by repeated tape stripping of hairless mouse epidermis. The increase of both epidermal thickness and PCNA-positive keratinocytes was inhibited by topical K6PC-5.

3. K6PC-5 acts to regulate fibroblast proliferation through SK activation. K6PC-5 promoted proliferation and procollagen production in human fibroblasts, and enhanced both fibroblast procollagen production and dermal thickness in intrinsically-aged hairless mice (56 weeks old) after two weeks topical application. K6PC-5 also promoted specific epidermal differentiation marker proteins without any alterations in epidermal barrier function.

These results demonstrated that K6PC-5 as a SK activator induces both keratinocyte differentiation and fibroblast proliferation in skin, and paradoxical effects on the epidermis and dermis via $[Ca^{2+}]_i$ responses through intracellular S1P production.

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K6PC-5가 스핑고신키나제 활성화제로서 피부 각질형성세포의 분화 및 섬유아세포의 증식에 미치는 효과

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염 종 경

스핑고신-1-포스페이트(S1P)는 생리활성 스핑고지질 대사물로서 세포의 칼슘신호, 증식, 생존 및 분화와 같은 다양한 세포 반응을 조절한다. 스핑고신 키나제(SK)는 S1P를 직접적으로 만드는 효소이기 때문에 그의 활성과 S1P의 양을 조절하는 많은 인자들이 확인되었다. 본 연구에서는 새로이 합성된 SK 활성화제인 K6PC-5가 각 세포 내 칼슘신호를 통해서 피부에서 각질형성세포의 분화 및 섬유아세포의 증식을 모두 유도함을 증명하였다. 친유성 화합물이며, 화학명이 N-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide인 K6PC-5는 먼저 HaCaT 각질형성세포 및 사람의 피부 섬유아세포에서 세포 내 칼슘 농도의 파동을 야기함을 확인하였다. SK 억제제인 디메칠스핑고신 및 디히드로스핑고신과 siRNA-SK1은 K6PC-5가 유도하는

세포 내 칼슘증가를 억제하였다. K6PC-5에 의해 야기되는 칼슘 파동은 탑시가르진에 민감한 칼슘 저장고를 이용하였다. 또한, K6PC-5에 의한 칼슘 증가는 1,4,5-이노시톨 포스페이트 경로에는 비의존적임을 확인하였다. 이러한 K6PC-5의 세포 내 칼슘 반응은 각질형성세포 및 섬유아세포에서 모두 동일하였다. 다음으로 HaCaT 각질형성세포와 표피 과증식 마우스 모델을 이용하여 K6PC-5가 각질형성세포의 분화에 미치는 영향을 평가하였다. K6PC-5는 HaCaT 세포에서 분화 관련 표지 단백질인 인보루크린 및 필라그린의 발현을 증가시켰다. 그러나, siRNA-SK1 는 K6PC-5에 의한 인보루크린의 증가를 억제하였다. K6PC-5를 피부에 도포한 경우에도 인보루크린, 로리크린, 필라그린, 케라틴5의 발현을 증가시켰고, 또한, 마우스의 표피에서 칼슘의 이동을 유도하였다. K6PC-5는 반복된 테이프 스트리핑에 의해 유도된 표피 과증식을 억제하였다. 즉, 표피 두께 및 PCNA로 염색되는 각질형성세포의 증가를 억제하였다. 이러한 결과들은 K6PC-5가 세포 내 S1P 생성을 통해 세포 내 칼슘 반응을 유도하여 각질형성세포의 분화 및 증식을 동시에 조절함을 제안한다. 끝으로 사람의 피부 섬유아세포와 내인성 노화 마우스 모델을 이용하여 K6PC-5가 섬유아세포의 증식에 미치는 영향을 평가하였다. K6PC-5는 먼저 섬유아세포의 증식과 콜라겐 생성을 농도의존적으로 촉진하였다. K6PC-5를 내인성 노화를 유도한 무모 생쥐(56주령)의 등부에 2주간 도포하였을 때, 진피에서 섬유아세포의 증식과 콜란겐 생성을 촉진하였고, 그 결과 진피의 두께도 유의하게 증가시켰다. K6PC-5는 또한 표피 분화 단백질인 인보루크린, 로리크린, 필라그린, 케라틴5을 증가

시켰고, 표피 장벽 기능의 이상을 유도하지 않았다. 이러한 결과들은 K6PC-5가 S1P 생성을 통해 세포 내 칼슘 반응을 유도하여 섬유아세포의 증식을 조절하고, 더 나아가 각질형성세포의 분화를 촉진함을 제안한다. 따라서, 이상의 연구 결과로부터 K6PC-5는 SK1 활성화에 의한 직접적인 S1P 생성을 통해 표피 및 진피에 역설적인 효과를 유도함을 밝혔고, 또한, S1P의 조절은 아토피 피부염, 건선, 피부 노화 등의 피부질환 치료를 위해 새로운 접근법임을 제안한다.

핵심되는 말 : 스핑고신키나제, 스핑고신-1-포스페이트, 칼슘 신호, 각질형성세포 분화, 섬유아세포 증식