K6PC-5, a Direct Activator of Sphingosine Kinase 1, Promotes Epidermal Differentiation Through Intracellular Ca^{2+} Signaling

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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 (SphK) is the enzyme directly responsible for production of S1P, many factors have been identified that regulate its activity and subsequent S1P levels. Here we synthesized a previously unidentified SphK activator, K6PC-5, and have studied its effects on intracellular Ca^{2+} signaling in HaCaT cells and epidermal differentiation in murine skin. K6PC-5, a hydrophobic compound chemically named *N*-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide, activated SphK (obtained from C57BL/6 murine blood and F9-12 cell lysates) in a dose-dependent manner. K6PC-5 induced both intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillations in HaCaT cells and Ca^{2+} mobilization in hairless mouse epidermis. Both dimethylsphingosine (DMS) and dihydroxysphingosine (DHS), SphK inhibitors, and transfection of SphK1-siRNA blocked K6PC-5-induced increases in $[Ca^{2+}]_i$. The K6PC-5-induced $[Ca^{2+}]_i$ oscillations were dependent on thapsigargin-sensitive Ca^{2+} stores and Ca^{2+} entry, but independent of the classical phospholipase C-mediated pathway. In addition, K6PC-5 enhanced the expression of involucrin and filaggrin, specific differentiation-associated marker proteins in HaCaT cells, whereas transfection of SphK1-siRNA blocked the increase of involucrin. Topical K6PC-5 also enhanced the expression of involucrin, loricrin, filaggrin, and keratin 5 in intact murine epidermis. Finally, topical K6PC-5 inhibited epidermal hyperplasia by exerting antiproliferative effects on keratinocytes in murine epidermis. These results suggest that K6PC-5 acts to regulate both differentiation and proliferation of keratinocytes via $[Ca^{2+}]_i$ responses through S1P production. Thus, regulation of S1P levels may represent a novel approach for treatment of skin disorders characterized by abnormal differentiation and proliferation, such as atopic dermatitis and psoriasis.

Journal of Investigative Dermatology (2008) **128,** 2166–2178; doi:10.1038/jid.2008.66; published online 3 April 2008

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 and Milstien, 2003). S1P functions as both an extracellular ligand for a family of five G-protein-coupled receptors and an intracellular second messenger. S1P receptors regulate many physiological and pathophysiological processes, such as vascular maturation, cardiac development, angiogenesis, immunity, and cell migration (Spiegel and Milstien, 2003; Sanchez and Hla, 2004). S1P also shows a universal Ca^{2+} signaling role through receptor-dependent and independent pathways (Meyer zu Heringdorf, 2004). Cellular levels of S1P are tightly regulated in a spatio-temporal manner both through synthesis, catalyzed by sphingosine kinases (SphKs), and degradation by S1P lyase and specific S1P phosphohydrolases (Stunff *et al.*, 2004). Two SphK isoforms, SphK1 and SphK2, are known to regulate the relative levels of S1P, sphingosine, and ceramide in the sphingolipid metabolic pathway (Kohama *et al.*, 1998; Liu *et al.*, 2000; Stunff *et al.*, 2004).

As SphK is directly responsible for production of S1P, many factors have been identified that alter its activity and regulate subsequent S1P levels (Maceyka *et al.*, 2002;

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Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; FBS, fetal bovine serum; PCNA, proliferating-cell nuclear antigen; PSS, physiological salt solution; S1P, sphingosine-1-phosphate; SC, stratum corneum; siRNA, small interfering RNA; SphK, sphingosine kinase; Tg, thapsigargin

Received 2 March 2007; revised 17 January 2008; accepted 27 January 2008; published online 3 April 2008

Taha *et al.*, 2006). For example, SphK is activated by ligands of G-protein-coupled receptors (Meyer zu Heringdorf *et al.*, 1998; Alemany *et al.*, 1999, 2000; Gordon *et al.*, 2000; Young *et al.*, 2000; Blaukat and Dikic, 2001; Misasi *et al.*, 2001; van Koppen *et al.*, 2001), including S1P itself (Meyer zu Heringdorf *et al.*, 2001), agonists of growth factor-receptor tyrosine kinases (Olivera and Spiegel, 1993; Edsall *et al.*, 1997; Meyer zu Heringdorf *et al.*, 1999; Shu *et al.*, 2002), cross-linking of immunoglobulin receptors (Melendez *et al.*, 1998; Prieschl *et al.*, 1999; Chuang *et al.*, 2000), and the endogenous ganglioside GM1 (Wang *et al.*, 1996). Although in some cases the mechanisms are unknown, many other biologically active agents also activate SphK, including estrogen (Sukocheva *et al.*, 2003), tumor necrosis factor-α (Xia *et al.*, 1998), vitamin D_3 (Kleuser *et al.*, 1998), phorbol ester (Mazurek *et al.*, 1994; Buehrer *et al.*, 1996), aluminum fluoride (AlF4−) (Alemany *et al.*, 1999), serum (Taha *et al.*, 2006), and oxidized low-density lipoprotein (Augé *et al.*, 1999). To date, there are no reports to demonstrate a chemically induced, direct activation of SphK. Although 12-*O*-tetradecanoylphorbol-13-acetate showed direct effect on SphK activation in total lysate and fractionated lysates of Balb/c 3T3 fibroblasts, it is unclear whether this upregulation of SphK occurs through direct SphK activation by the phorbol ester, or through an enhanced transcription of SphK (Mazurek *et al.*, 1994). In this study, we suggest that a newly synthesized K6PC-5 exerts direct SphK activation and subsequent S1P-mediated Ca^{2+} regulation in keratinocytes.

S1P is an evolutionarily conserved Ca^{2+}-signaling molecule in yeast, plants, and mammals, that uses specific Ca^{2+} signaling to initiate diverse cellular responses (Spiegel and Milstien, 2003). Acting as an agonist at G-protein-coupled receptor, 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 intracellularly activated SphK. Intracellular S1P also mobilizes Ca^{2+} from intracellular stores by an as yet unknown mechanism that might involve a novel Ca^{2+} channel (Meyer zu Heringdorf, 2004). These PLC-independent pathways do not include G-protein-coupled S1P receptors, and Ca^{2+} signaling by many agonists requires SphK-mediated S1P production. We suggest that a direct activator of SphK may help to resolve these outstanding issues regarding the relationship of intra- and extracellular 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 (Hennings *et al.*, 1980; Hennings and Holbrook, 1983). In addition, Ca^{2+} is an important signal that facilitates repair of barrier function following acute injury (Lee *et al.*, 1992). Manggau *et al.* (2001) reported that 1α, 25-dihydroxyvitamin D_3 protects keratinocytes from apoptosis induced by ceramides, UV irradiation, or tumor necrosis factor-α, and additionally this cytoprotection is mediated via formation of S1P. More recently, Vogler *et al.* (2003) reported that all five S1P receptors are expressed in keratinocytes, and that S1P enhances differentiation of keratinocytes and protects the cells from programmed cell death.

Most factors related to SphK activation regulate the activity of SphK and the production of S1P by extracellular stimuli targeting plasma membrane receptors. Recently, we synthesized a new compound, K6PC-5, as an SphK activator, that directly activates SphK. In this study, we investigate intracellular Ca^{2+} signaling by K6PC-5 through its direct effects on SphK activity in HaCaT cells, and determine its effects on epidermal differentiation in murine skin.

RESULTS

K6PC-5 activates SphK in both murine blood and F9-12 cells

K6PC-5 is a hydrophobic compound containing a ketone group, two hydroxy groups, two short alkyl groups, and an amide linkage, characterized as a ''pseudo-ceramide'' backbone with a chemical name of *N*-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide. The origin of K6PC-5 is a bioactive short-chain pseudoceramide that we synthesized and selected for its effects on keratinocyte differentiation (Kwon *et al.*, 2007). K6PC-5 directly activated SphK obtained from mouse blood in a dose-dependent manner (Figure 1a). C17-sphingosine (C17-Sph) was used as the substrate for SphK, and the production of C17-S1P was analyzed by high-performance liquid chromatography with fluorescence detection. Whole blood showed significant SphK activity as reported previously (Venkataraman *et al.*, 2006), with K6PC-5 enhancing production of C17-S1P by 30% compared with the control. Venkataraman *et al.* (2006) also demonstrated non-detectable SphK activity in SphK1-knockout mouse plasma, with approximately a corresponding 65% reduction in S1P levels. Together, these results suggest that K6PC-5 has a direct effect on SphK1 activity.

K6PC-5 also activated SphK in lysates from F9-12 mouse embryonic carcinoma cells in a dose-dependent manner (Figure 1b). The F9-12 cells lack S1P lyase and overexpress SphK1 (Kariya *et al.*, 2005). K6PC-5-induced SphK activation was significantly inhibited by *N,N*-dimethylsphingosine (DMS), an SphK inhibitor. These results also suggest that K6PC-5 has a direct effect on SphK1 activity since possible effects of blood factors on SphK activation are excluded by the F9-12 cell lysates.

K6PC-5 induces $[Ca^{2+}]_i$ oscillations in HaCaT cells

To confirm whether SphK-mediated S1P production by K6PC-5 induces $[Ca^{2+}]_i$ signaling as a result of the action mechanism, we characterized the Ca^{2+} signals induced by 10–100 μM K6PC-5 in intact HaCaT keratinocytes. $[Ca^{2+}]_i$ oscillations were induced by K6PC-5 concentrations $\geqslant 25$ μM (Figure 2a). In addition, the percentage of responding cells was dependent on K6PC-5 concentrations, that is, $10.3 \pm 1.5\%$ at 25 μM ($n = 9$); $47.9 \pm 15.6\%$ at 50 μM ($n = 9$); and $94.4 \pm 7.95\%$ at 100 μM ($n = 12$) (Figure 2c). However, the amplitude of the spikes was unaffected by the K6PC-5 concentration in the range of 25–100 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 2b). The average lag time between

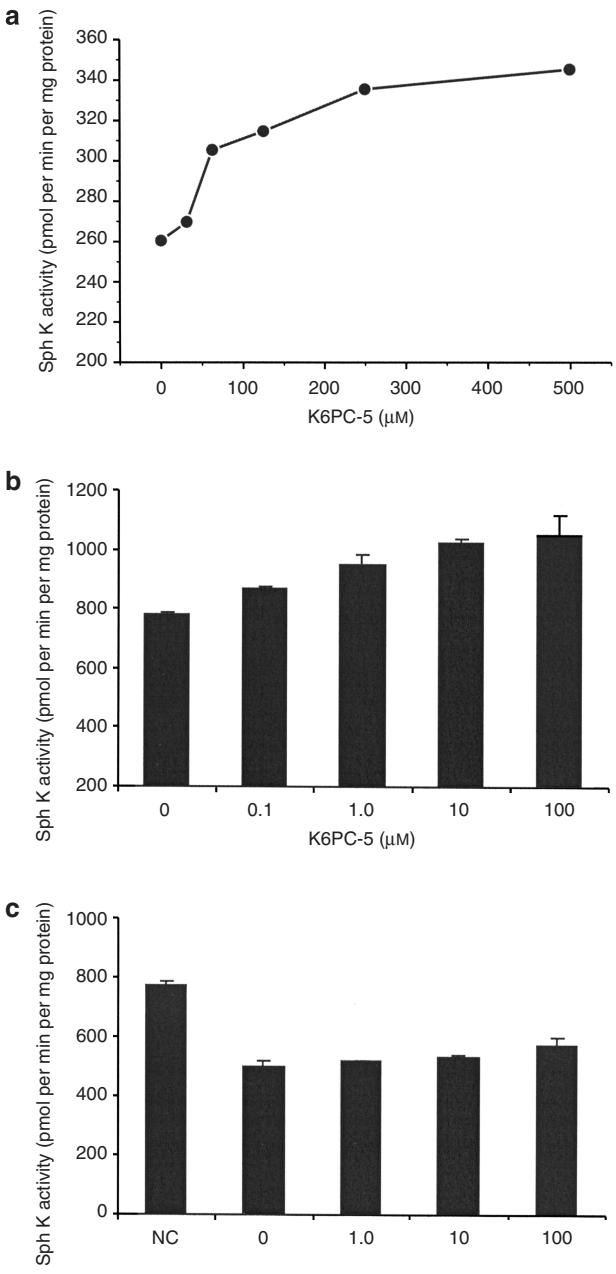

Figure 1. K6PC-5 activated SphK in mice blood and F9-12 cells. Whole blood of C57BL/6 mice was incubated with C17-Sph and K6PC-5 was added under diluting in half from 500 μM. The black circles in order are 0 μM (vehicle only), 33.25, 62.5, 125, 250, 500 μM K6PC-5 (**a**). F9-12 cell lysates were incubated with C17-Sph and K6PC-5 (**b**). 50 μM DMS was incubated with K6PC-5 in to confirm the inhibitory effect on SphK in F9-12 cell lysates and C17-Sph system (**c**). NC, normal control as the vehicle. All data have significant *P*-values less than 0.001 compared with the vehicle control.

K6PC-5 exposure and the generation of $[Ca^{2+}]_i$ responses was 1.5 ± 1.0 minutes. Since 50 μM K6PC-5 did not induce cell death (data not shown) and generally produced reliable $[Ca^{2+}]_i$ oscillations, this concentration was used to analyze the mechanism by which K6PC-5 induced $[Ca^{2+}]_i$ signaling. Exogenous S1P also induced $[Ca^{2+}]_i$ oscillations in a dose-dependent manner (data not shown). These results reveal that K6PC-5 induces $[Ca^{2+}]_i$ oscillations in HaCaT cells.

K6PC-5-induced $[Ca^{2+}]_i$ oscillations involve SphK1 activation in HaCaT cells

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 family (Johnson *et al.*, 2003). Intracellular S1P is generated by SphK-dependent phosphorylation of sphingosine. Although intracellular targets for S1P have yet to be identified, the production of intracellular S1P, but not IP_3, mobilizes intracellular $[Ca^{2+}]_i$ (Meyer zu Heringdorf *et al.*, 2003). To clarify the possibility that K6PC-5-activated $[Ca^{2+}]_i$ oscillations and intracellular S1P levels are related, we used potent SphK inhibitors, DMS and DL-*threo*-dihydrosphingosine (DHS). Both DMS and DHS partially inhibited K6PC-5-mediated $[Ca^{2+}]_i$ oscillations (Figure 3a, $n = 6$).

The Ca^{2+}-signaling mechanism of K6PC-5 was further investigated by using small interfering RNA (siRNA) to examine the functional consequences of depletion of SphK1. Transfection of siRNA-psiSTRIKE-SphK1 suppressed the expression of mRNA and the protein level of SphK1 (Figure 3b, $n = 3$). mRNA levels of transfected cells were double checked on both the first and sixth selections with G-418. We determined the protein level for SphK1 in cells after the sixth round of selection (Figure 3b), because there was no distinguishable change of protein levels of SphK1 in the first selection. In addition, $[Ca^{2+}]_i$ responses were measured in cells from the third to the sixth selection. As shown in Figure 3c, siRNA-psiSTRIKE-SphK1 blocked $[Ca^{2+}]_i$ responses (44 cells, $n = 6$, third selection), whereas $[Ca^{2+}]_i$ oscillations persisted in control (scrambled)-psiSTRIKE-SphK1-transfected cells (31 cells, $n = 6$, third selection). To confirm these results, we measured the $[Ca^{2+}]_i$ responses in hMGFP-transfected cells. siRNA-hMGFP-SphK1 transfected cells were also blocked the $[Ca^{2+}]_i$ responses (data not shown). Therefore, the effects of K6PC-5 on Ca^{2+} oscillations are S1P-dependent, through activity of SphK1.

Numerous studies also have shown that S1P-induced increases in $[Ca^{2+}]_i$ signals involve a G_i-PLC-mediated pathway (Muraki and Imaizumi, 2001; Young and Nahorski, 2002). To determine whether K6PC-5-induced $[Ca^{2+}]_i$ responses involve a G_i-PLC-mediated pathway, cells were pretreated with pertussis toxin, a G_i inhibitor (El-Shewy *et al.*, 2006). Pertussis toxin inhibited the Ca^{2+} responses induced by exogenous S1P, whereas the K6PC-5-induced $[Ca^{2+}]_i$ responses were not affected (Figure 3d, $n = 4$). Together, these results suggest that K6PC-5-induced $[Ca^{2+}]_i$ oscillations involve generation of intracellular S1P by activation of SphK1, corresponding with the direct effect of K6PC-5 on SphK1 activity in mice blood (cf. Figure 1).

K6PC-5-induced $[Ca^{2+}]_i$ oscillations are dependent on both thapsigargin-sensitive Ca^{2+} stores and Ca^{2+} entry, but not the PLC/IP_3 pathway, in HaCaT cells

G-protein-coupled agonists induce two types of $[Ca^{2+}]_i$ oscillations, that is, those acutely dependent on Ca^{2+} influx and those largely involving Ca^{2+} release and reuptake into the endoplasmic reticulum, with minimal contribution

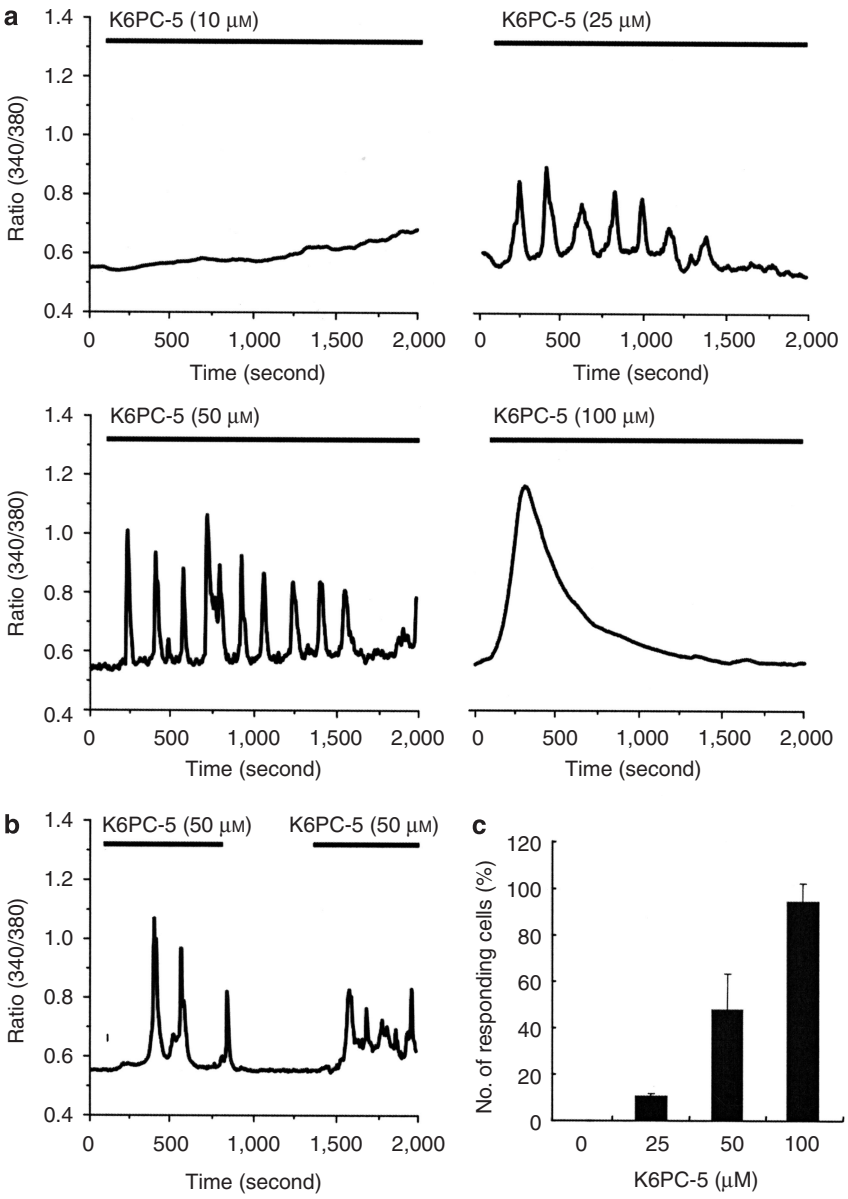

Figure 2. K6PC-5 induced $[Ca^{2+}]_i$ oscillations in HaCaT cells. HaCaT cells were loaded with fura-2 as described under Materials and Methods, and changes in $[Ca^{2+}]_i$ were measured by ratiometric fluorescence imaging. Cells were exposed to 10, 25, 50, or 100 μM K6PC-5 (**a**). 50 μM K6PC-5 was 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 ± SE.

of Ca^{2+} influx (Kiselyov *et al.*, 2003). To identify the source of $[Ca^{2+}]_i$ mobilization by K6PC-5, Ca^{2+} was removed from physiological salt solution (PSS). Depletion of intracellular Ca^{2+} by thapsigargin (Tg), a specific inhibitor of the sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump (Hong *et al.*, 2004), prevented K6PC-5-induced $[Ca^{2+}]_i$ oscillations (Figure 4a; $n=25$). In a paired experiment, we found that depletion of the endoplasmic reticulum by stimulation with 100 μM K6PC-5 in the absence of external Ca^{2+} prevented the Ca^{2+} influx caused by 1 μM Tg ($n=25$). These results indicate that intracellular Ca^{2+} stores responsible for K6PC-5-induced $[Ca^{2+}]_i$ oscillations are Tg-sensitive. Treatment of cells with 100 μM K6PC-5, with or without external Ca^{2+}, revealed that K6PC-5-induced Ca^{2+} amplitude decreased approximately 12% in the absence of Ca^{2+} ($n=5$). To further delineate the source of $[Ca^{2+}]_i$ increase, cells were first treated with 50 μM K6PC-5 for 10 minutes in the presence of external Ca^{2+}, followed by Ca^{2+}-free PSS for 15 minutes. Re-addition of Ca^{2+} failed to yield $[Ca^{2+}]_i$ oscillations in these cells (Figure 4b). These results suggested that extracellular Ca^{2+} also is a source for K6PC-5-induced $[Ca^{2+}]_i$ oscillations. To examine whether K6PC-5-induced $[Ca^{2+}]_i$ signal occurs through plasma membrane Ca^{2+} channels, cells were exposed to 75 μM 2-aminoethoxydiphenyl borate (2-APB) and 1 μM gadolinium (Gd^{3+}) (non-selective Ca^{2+} channel blockers). Both 2-APB ($n=17$) and

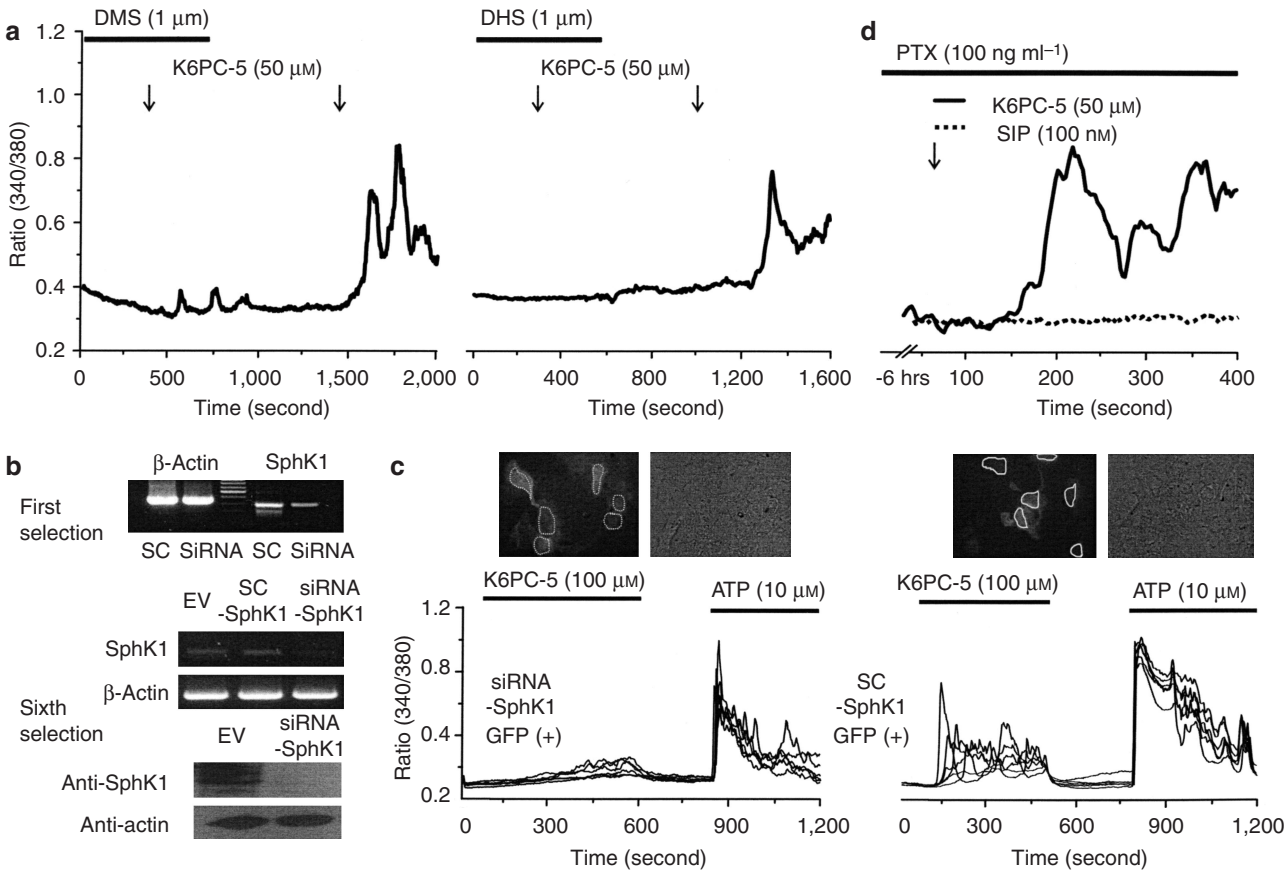

Figure 3. K6PC-5-induced $[Ca^{2+}]_i$ oscillations involved SphK1 activation in HaCaT cells. After treatment of 1 μM DMS or 1 μM DHS, 50 μM K6PC-5 was applied (**a**). mRNA (first and sixth selections) and protein (sixth selection) levels of SphK1 after transfection were measured (**b**). EV, empty vector; SC, scrambled. Ca^{2+} responses of cells transfected (GFP(+)) with siRNA of SphK1 (dotted) or scrambled (SC, solid) were measured. 100 μM K6PC-5 and 10 μM ATP were applied (**c**). After treatment of 100 ng ml^{-1} pertussis toxin for 6 hours, 100 nM S1P (dotted) or 50 μM K6PC-5 (solid) was applied (**d**). The arrow indicates the point of treatment(s) with indicated compounds.

Gd^{3+} ($n=16$) prevented the generation of $[Ca^{2+}]_i$ oscillations by K6PC-5 (Figure 4c). To determine the role of PLC in K6PC-5-mediated $[Ca^{2+}]_i$ oscillations, we next tested the effect of U73122, a PLC blocker, and as control its inactive analog U73343 ($n=5$). As shown in Figure 4d, U73122, but not U73343, prevented only exogenous S1P-evoked $[Ca^{2+}]_i$ oscillations, with no effect on K6PC-5-induced events. 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, which is independent of the PLC/IP_3 pathway.

K6PC-5 increases differentiation-related marker proteins in keratinocytes

To determine whether K6PC-5 as a SphK activator affects keratinocytes differentiation through SphK-mediated S1P production involving Ca^{2+} signaling, reverse 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 5a). The expression levels of these proteins were also promoted by K6PC-5 and S1P (Figure 5b).

K6PC-5-induced keratinocyte differentiation was further investigated by using siRNA for SphK1 (siRNA-SphK1)-transfected cells to confirm the functional consequences of SphK-mediated S1P production by K6PC-5. SiRNA-SphK1 blocked the expression of involucrin as a differentiation marker protein, whereas empty vector did not alter expression. Interestingly, extracellular S1P-induced differentiation also was blocked in siRNA-SphK1-transfected cells (fourth selection), suggesting that extracellular S1P induces SphK1-mediated keratinocyte differentiation. These results suggest that the effects of K6PC-5 are dependent on a S1P-related mechanism through SphK1, correlating with the $[Ca^{2+}]_i$ responses induced by siRNA to SphK1 (cf. Figure 3).

K6PC-5 enhances production of differentiation marker proteins in murine epidermis *in vivo*

To elucidate the potency of K6PC-5 *in vivo*, we used a well-established hyperproliferative murine model, as reported previously (Denda *et al.*, 1996). Again, both K6PC-5 and S1P accelerated the expression of differentiation-associated specific proteins, including involucrin, loricrin, filaggrin, and keratin 5 (Figure 6a). These results correlate well with the *in vitro* results above (cf. Figure 5), and suggest that K6PC-5

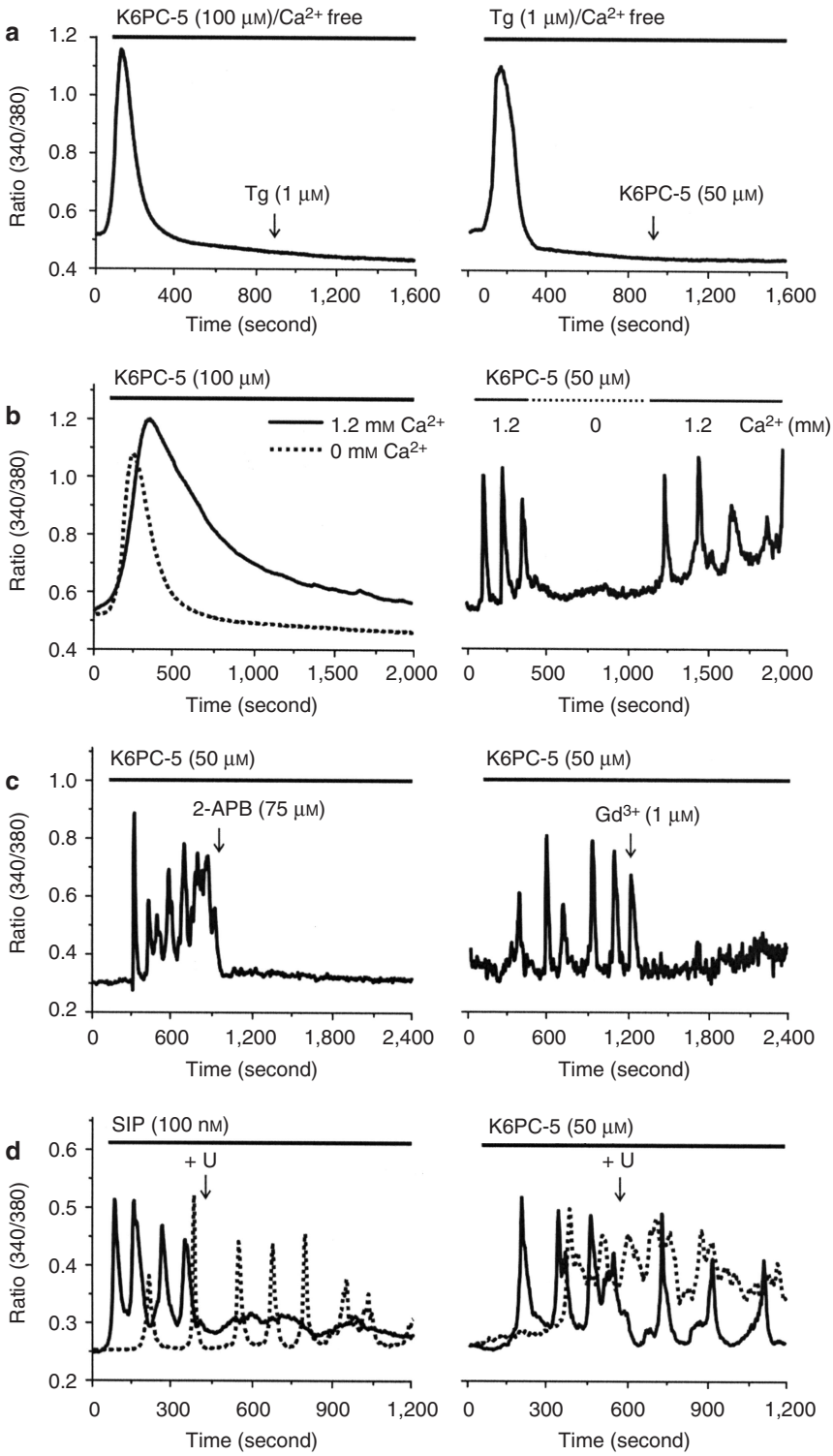

Figure 4. K6PC-5-induced $[Ca^{2+}]_i$ oscillations were dependent on both Tg-sensitive Ca^{2+} stores and Ca^{2+} entry, but not the PLC/IP_3 pathway, in HaCaT cells. Cells were exposed to 1 μM Tg followed by 100 μM K6PC-5 (**a**, left). A complementary experiment was performed (**a**, right). 50 μM K6PC-5 containing 1.2 mM (solid) or 0 mM Ca^{2+} (dotted) was applied (**b**). After treatment with 50 μM K6PC-5, 75 μM 2-APB (**c**, left) and 1 μM Gd^{3+} (**c**, right) were applied. S1P-/K6PC-5-treated cells were also treated with 1 μM U73122 (solid) or 1 μM U73343 (dotted) as indicated by the arrow (U) (**d**).

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

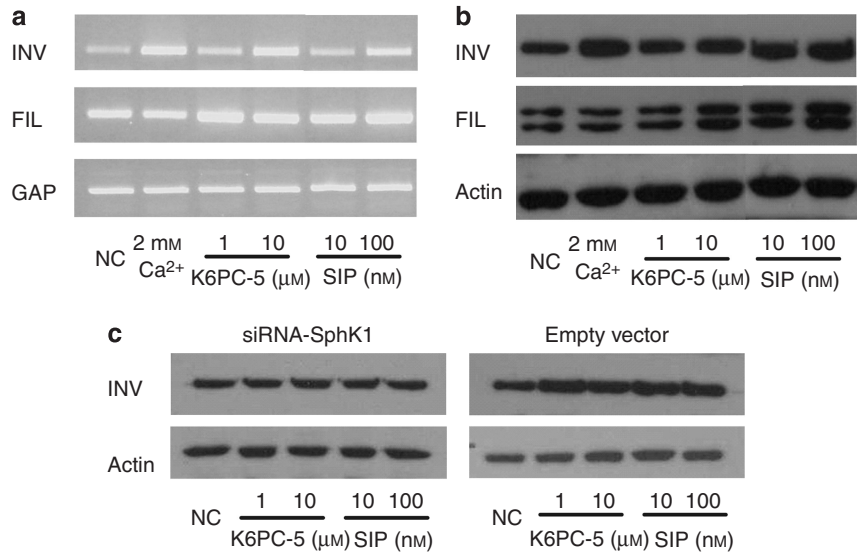

Figure 5. K6PC-5 increased differentiation-related marker proteins in HaCaT cells. mRNA (**a**) and protein (**b**) levels of Involucrin (INV) and filaggrin (FIL), as marker proteins of keratinocyte differentiation, induced by K6PC-5 and S1P, were measured by reverse transcriptase–PCR and western blotting, respectively. NC, normal control. The expression of involucrin in siRNA-SphK1-transfected cells (fourth selection) was measured by western blotting (**c**).

(Figure 6b), an increase that was eliminated by 3 hours. 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).

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 (Denda *et al.*, 1996). 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 7a), and correspondingly inhibited the increase of proliferating-cell nuclear antigen (PCNA)-positive and proliferating keratinocytes (Figure 7c). The hematoxylin and eosin images confirmed these results (Figure 7b and d). We also performed viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays in HaCaT cells to confirm the antiproliferative effect of K6PC-5 and S1P *in vitro*. K6PC-5 and S1P both significantly inhibited keratinocyte proliferation in a dose-dependent manner in HaCaT cells (Figure 7e). Together, these results suggest that K6PC-5 and S1P decrease epidermal hyperplasia by inhibiting keratinocytes proliferation.

DISCUSSION

We report results with a novel SphK1 activator, K6PC-5, on Ca^{2+} signaling in human keratinocytes as well as epidermal differentiation and proliferation in both cultured cells and murine skin. The activation of SphK by K6PC-5 was revealed while new chemicals derived from bioactive and short-chain pseudoceramides were being developed to promote keratinocyte differentiation (Kwon *et al.*, 2007). However, K6PC-5 represents a compound with novel activities not anticipated from its simple short-chain pseudoceramide structure. Thus, this study is the first to report K6PC-5 as a small-molecule SphK activator. K6PC-5 activated SphK obtained both from C57BL/6 murine blood and from F9-12 cell lysates in a dose-dependent manner, inducing conversion of C17-Sph to C17-S1P. Since SphK1-knockout mice show no SphK activity in whole blood (Venkataraman *et al.*, 2006), and F9-12 cells overexpress SphK1 (Kariya *et al.*, 2005), these results reveal K6PC-5 to be a direct activator of SphK1. In addition, K6PC-5-induced $[Ca^{2+}]_i$ oscillations in HaCaT cells were blocked by SphK inhibitors, DMS, and DHS. Moreover, HaCaT cells transfected with siRNA to SphK1 did not respond to K6PC-5. These results further reveal that K6PC-5 is a direct activator of SphK, especially SphK1. Distinct Ca^{2+} mobilization by K6PC-5 also was evident in murine epidermis. In addition, pertussis toxin and U73122 did not prevent $[Ca^{2+}]_i$ oscillations by K6PC-5, but both of these compounds inhibited the S1P-induced effects. These findings suggest that K6PC-5 can enhance the role of S1P as an intracellular second messenger. Although many activators of SphK have been identified, most of them involve upstream cell-membrane receptors (Taha *et al.*, 2006). However, we have no evidence that K6PC-5 induces cellular response through a membrane receptor to activate SphK. In fact, the hydrophobic property of K6PC-5 may allow easy passage into and through the cell membrane. We therefore suggest that K6PC-5 is a unique compound having direct effect(s) on SphK1 enzyme activity in human epidermal keratinocytes.

S1P has been shown to operate via both PLC-dependent and -independent pathways to increase Ca^{2+} signaling (Meyer zu Heringdorf, 2004). Blom *et al.* (2005) also reported recently that IP_3 is the major Ca^{2+}-releasing second

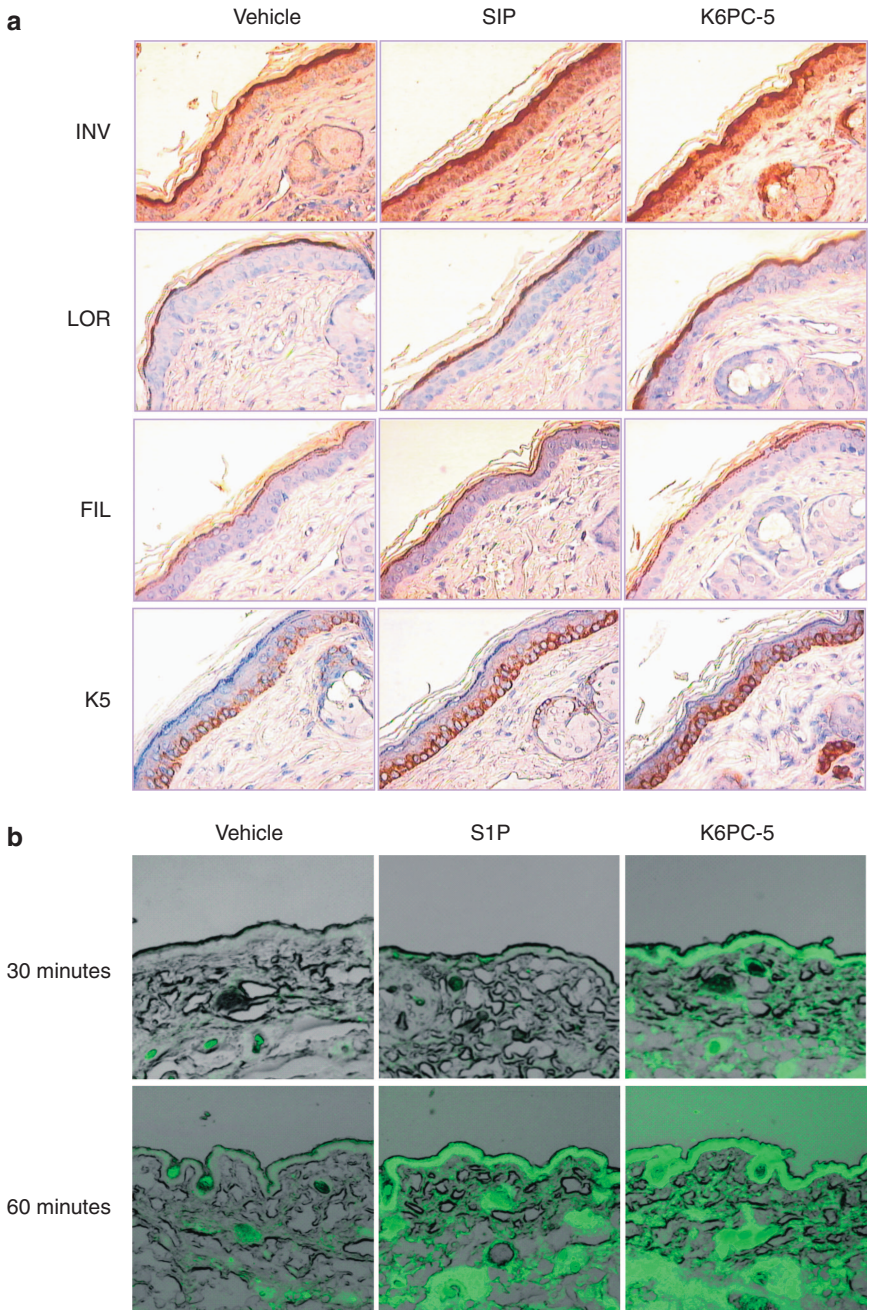

Figure 6. K6PC-5 enhanced production of differentiation marker proteins in murine epidermis *in vivo*. The expression levels of differentiation-associated proteins induced by topical applications of 1% K6PC-5 and 1 mM S1P on hairless mouse skin were analyzed by immunohistochemistry (**a**). Involucrin (INV), loricrin (LOR), filaggrin (FIL), and keratin 5 (K5) were used as marker proteins of epidermal differentiation. The image of K6PC-5-induced Ca^{2+} mobilization in murine epidermis was measured by Calcium Green-1 (**b**). 1% K6PC-5 and 1 mM S1P were applied topically on hairless mouse skin, and tissue biopsies were performed after application in 30 and 60 minutes, respectively.

messenger in an exogenous S1P-stimulating system, effects that are dependent on both PLC and the subsequent generation of intracellular S1P. Additionally, endogenous S1P also affects $[Ca^{2+}]_i$ mobilization in human embryonic kidney-293 cells. 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 (Ghosh *et al.*, 1994; Meyer zu Heringdorf *et al.*, 2003). However, the mechanism of which intracellular S1P mobilizes Ca^{2+} from intracellular stores also is unknown, although a novel Ca^{2+} channel has been reported (Young and Nahorski, 2001). Current studies reveal another possible mechanism for these effects. Specifically, since K6PC-5-induced Ca^{2+} response is dependent on both Tg-sensitive Ca^{2+} stores and extracellular Ca^{2+}, and since both 2-APB and Gd^{3+} prevented $[Ca^{2+}]_i$ oscillations by K6PC-5,

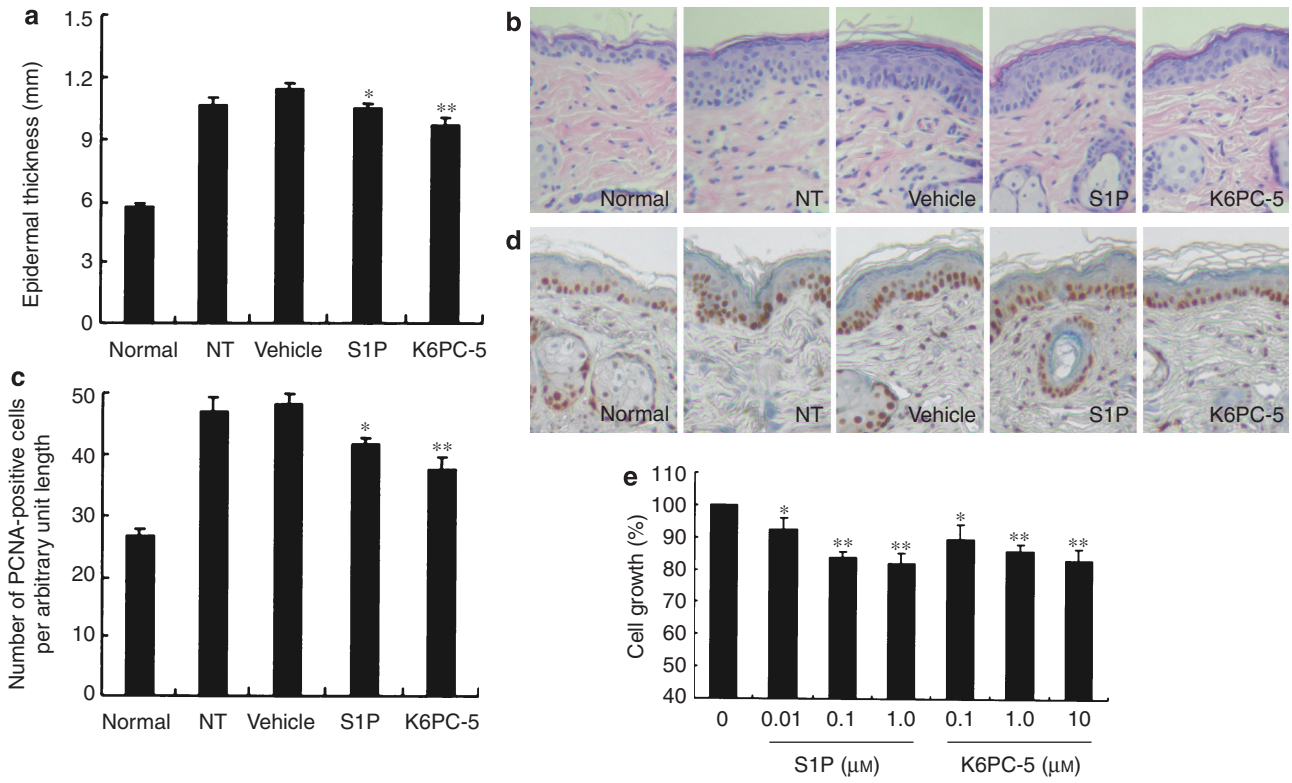

Figure 7. K6PC-5 decreased epidermal hyperplasia by inhibiting keratinocytes proliferation. Epidermal hyperplasia was induced by repeated tape-stripping. Epidermal thickness after topical application of 1% K6PC-5 and 1 mM S1P on hairless mouse skin was measured as described under Materials and Methods (**a**). Proliferating keratinocytes were measured by PCNA-positive cells per centimeter on images obtained randomly at ×200 magnification ($n=5$). The image of hematoxylin and eosin-stained sections (**b**) and PCNA-stained sections (**d**) is also shown. The effect of K6PC-5 and S1P on keratinocyte viability (**c**) also was determined using MTT assay in HaCaT cells (**e**). *$P<0.01$, **$P<0.001$; NT, non-treatment after barrier disruption.

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+} into the cytoplasm. In fact, this effect may reflect indirect Ca^{2+} signaling of intracellular S1P, which Blom *et al.* (2005) previously suggested to explain results in their system.

The study of S1P-mediated events is challenging as it functions both as an extracellular ligand for a family of five G-protein-coupled receptors and an intracellular second messenger (Spiegel and Milstien, 2003). Exogenous S1P affects its receptors on the cell membrane, as its structural characteristics preclude its direct penetration through cell membranes. Conversely, since, as we have shown here, K6PC-5 can readily penetrate cell membranes to activate SphK1 and increase endogenous S1P levels, this novel compound should be useful for studying the intracellular roles and Ca^{2+} signals induced by S1P.

Vogler *et al.* (2003) previously reported that S1P enhances differentiation of cultured keratinocytes and possesses anti-proliferative effect that protects cells from programmed cell death. Kim *et al.* (2004) also showed that S1P inhibits human keratinocyte proliferation via Akt/protein kinase B inactivation. Therefore, we anticipated similar effects of K6PC-5 on keratinocytes via its induction of S1P levels. We show that K6PC-5 and S1P increased mRNA and protein levels of involucrin and filaggrin as specific differentiation markers in HaCaT cells, and that siRNA-SphK1 blocked the expression of involucrin induced by K6PC-5 and S1P. Their respective protein expression is elevated by K6PC-5 topical treatment in murie 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 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 a skin disease 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 (Jensen *et al.*, 2004). 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.

MATERIALS AND METHODS

Materials

Adult female hairless mice, 8–10 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. Ciglitazone was purchased from Cayman Chemical Laboratories (Ann Arbor, MI); troglitazone was from Biomol Laboratories (Plymouth, PA). Affinity-purified rabbit primary antibodies specific for human SphK1 were purchased from BabCo (Richmond, CA); mouse filaggrin, loricrin, and involucrin were from Covance Research (Berkeley, CA); keratin 5 and PCNA were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary goat anti-rabbit IgG-HRP and diaminobenzidine were from Dako-Cytomation (Glostrup, Denmark). All reagents for reverse transcriptase–PCR were from Takara Korea Biomedical (Otsu, Japan). Fluorescein-FragEL apoptosis detection kit was from Oncogen Research Products (San Diego, CA). S1P, thapsigargin, 2-APB), U73122, U73343, G-418, and pertussis toxin were purchased from Sigma (St Louis, MO); RPMI 1640, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA); fura2-acetoxymethyl ester was purchased from Teflabs (Austin, TX). C17-Sph and C17-S1P were obtained from Avanti Polar Lipid Inc. (Alabastar, AL). *o*-Phthalaldehyde was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of reagent grade.

SphK activity

Mouse blood from C57BL/6 mice was used as the source of SphK. The mice (4–6 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). Whole blood was collected in acid-citrate buffer (0.1 ml ml^{-1} of blood) and heparin (50 U ml^{-1} blood) via cardiac puncture following avertin anesthesia, and diluted with 900 µl of 4 × SphK assay buffer (0.5 M HEPES–KOH, 1 M NaF, 1 M β-glycerophosphate, 1 M orthovanadate, 10% Triton X-100, 1 M $MgCl_2$) to a total volume of 1.0 ml (Dil-blood). A 1-µl volume of K6PC-5 from the stock solution (dissolved in DMSO) was added to each tube (Dil-blood 50 µl, 4 × SphK assay buffer 25 µl, 1 mM C17-Sph 1 µl, 10 mM ATP 1 µl, water 22 µl) for 30 minutes at 37 °C to measure SphK activity. The phosphorylation reaction was terminated by addition of 200 µl of MeOH and solutions were stored at −80 °C until S1P analysis. Quantification of C17-S1P was performed using high-performance liquid chromatography with fluorescence detection, as previously described (Min *et al.*, 2002). The SphK activity is reported as the picomoles of C17-S1P formed per minute per mg of protein.

SphK activity was determined in F9-12 cells according to the method reported previously(Jin *et al.*, 2006). Briefly, F9-12 cell lysates were prepared and 30 µg of cell lysate were incubated 20 minutes at 37 °C with 100 µM C17-Sph in 5% Triton X-100, 10 µM ATP solution, and K6PC-5, and terminated by addition of 1 N HCl and 0.8 mM $CHCl_3$/methanol/HCl (100:200:1, v/v) at 4 °C. Samples were vigorously vortexed and centrifuged after adding 200 pmol C17-S1P as an internal standard. The $CHCl_3$ phase was extracted with 2 M NaCl, followed by H_2O phase extraction with 3 N NaOH to obtain C17-S1P. The alkaline aqueous phase containing C17-S1P was mixed thoroughly with dephosphorylating reaction buffer (200 mM Tris-HCl (pH 7.4), 75 mM $MgCl_2$ in 2 M glycine buffer, pH 9) and 50 U of alkaline phosphatase, and incubated for 1 hour at 37 °C. Dephosphorylated C17-Sph was extracted with $CHCl_3$, washed with alkalinized water (pH 10), and dried. For high-performance liquid chromatography analysis, the residue was dissolved in EtOH and incubated with the *o*-phthalaldehyde reagent (50 mg of *o*-phthalaldehyde, 1 ml of ethanol, 100 µl of 2-mercaptoethanol, and 50 ml of 3% (w/v) boric acid solution) for 10 minutes at 50 °C. After the derivatization process, the *o*-phthalaldehyde derivative of C17-Sph was used for high-performance liquid chromatography analysis of C17-S1P. SphK activity was calculated from newly synthesized C17-S1P and expressed as pmol mg^{-1} min^{-1}.

Cell culture

Mouse embryonic carcinoma F9-12 cells were kindly supplied by Hokkaido University, Japan. Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin for SphK activity measurement.

HaCaT cells were purchased from American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 supplemented with 2 mM glutamine, 25 mM HEPES buffer, 100 µg ml^{-1} penicillin, 25 ng ml^{-1} streptomycin, and 10% FBS. For $[Ca^{2+}]_i$ measurements, cells were seeded in 60-mm culture dishes and maintained at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air to 80% confluence. The cells were serum-starved for 24 hours before each experiment.

For HaCaT differentiation, cells were cultured in DMEM supplemented with 10% 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 hours and treated with K6PC-5 or S1P in serum-free medium.

Keratinocyte proliferation

The effect of K6PC-5 or S1P on keratinocyte viability was determined using MTT assay. HaCaT cells (1×10^4 cells $well^{-1}$) were seeded in 96-well plates in DMEM with 5% FBS for 48 hours, and incubated with K6PC-5 or S1P in DMEM serum-free media for 24 hours at 37 °C. After addition of 20 µl $well^{-1}$ of MTT solution (10 mg ml^{-1} in phosphate-buffered saline), plates were incubated for 4 hours at 37 °C. Supernatants were then removed and 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).

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurement in HaCaT cells

Cells were loaded with 3.5 µM fura2-acetoxymethyl ester for 1 hour at room temperature in PSS containing 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, and 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*-tetraacetic acid, without $CaCl_2$. The fura2-acetoxymethyl ester-loaded cells were mounted into the chamber of an inverted microscope (Nikon, Tokyo, Japan) for calcium imaging. Excitation wavelength was alternated between 340 and 380 nm, and emission wavelength was monitored at 510 nm with a CCD camera using MetaFlour system

(Universal Imaging Co., Downingtown, PA). Fluorescence images were obtained at 3-second intervals. Background fluorescence was subtracted from the raw intensity at each excitation wavelength.

Ca^{2+} mobilization in murine epidermis

The effect of K6PC-5 on Ca^{2+} mobilization in epidermis was visualized using a method reported previously (Denda *et al.*, 2000). An agarose-gel membrane (2%) containing 10 μg ml^{-1} Calcium Green 1 (Molecular Probes, Eugene, OR) was formed on the slide, and a frozen section of whole skin (8-μm thick) was placed directly on the gel membrane. A fluorescence photomicrograph was taken within 2 hours with an excitation wavelength of 546 nm.

siRNA downregulation of SphK1 expression

siRNA-SphK1 was constructed using the psiSTRIKE vector system (Promega, Madison, WI). The siRNA-SphK1 target-specific sequences were sense, 5′-ACCGCGGGTCGAGGTTATGGATTTCAA GAGAATCCATAACCTCGACCCGCTTTTTC-3′ and reverse, 5′-GCA GAAAAAGCGGGTCGAGGTTATGGATTCTCTTGAAATCCATAACC TCGACCCG-3′. The scramble-SphK1 sequences were forward, 5′-ACCGGAGTCAGTCTGGGTATGGTTCAAGAGACCATACCCAGAC TGACTCCTTTTTC-3′ and reverse, 5′-TGCAGAAAAAGGAGTCAG TCTGGGTATGGTCTCTTGAACCATACCCAGACTGACTC-3′. Cells were co-transfected with siRNA-SphK1 (1 μg ml^{-1}) and green fluorescent protein (eGFP-N1) (1 μg ml^{-1}) using Lipofectamine 2000 reagent (Invitrogen), and cultured for 48 hours in serum-free RPMI 1640 including opti-MEM; cells were selected using G-418 (Invitrogen). Depletion of endogenous SphK1 by siRNA was confirmed by reverse transcriptase–PCR in first-/sixth-selected cells and Western blotting in sixth-selected cells. To check the functional effect of siRNA, $[Ca^{2+}]_i$ were measured in third-selected cells. To clarify the transfection of siRNA, same target specific sequences were re-constructed using the hMGFP-siRNA vector to measure $[Ca^{2+}]_i$ by the same manufacturer's directions (Promega). The hMGFP vector system used only to confirm with GFP fluorescence of transfected cells.

Reverse transcriptase–PCR

Total RNA was extracted from cultured HaCaT cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Equal amounts of RNA (2 μg) were reverse-transcribed using an RNA PCR kit (AMV) (Takara, Otsu, Japan). Semi-quantitative PCR was performed using the following specific primers: human SphK1 (5′-ATGGATCCAGTGGTCGGTTG-3′ and 5′-TCTTCATTGGTGACCTGCT-3′); human involucrin (5′-GGACG GACAACTAAAACAT-3′ and 5′-AGCGGACCCGAAATAAG-3′); human filaggrin (5′-GTTCACATTTATTGCCAAAAGA-3′ and 5′-GAG CCAACTTGAATACCAT-3′); and glyceraldehyde-3-phosphate dehydrogenase as an internal control (5′-TGAGCTGAACGGGAAG-3′ and 5′-CTGTAGCCAAATTCGTTGT-3′). cDNAs, SphK1, involucrin, filaggrin, glyceraldehyde-3-phosphate dehydrogenase, 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.

Western immunoblotting

Cells were lysed in protein extraction reagent (Pierce Biotechnology Inc., Rockford, IL). Lysates were centrifuged at 12,000 *g* for 15 minutes and supernatants were collected for western immunoblotting. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). Equal protein amounts (20 μg) were separated by 10% SDS-PAGE and blotted onto polyvinylidene difluoride membranes. Membranes were subsequently blocked with 5% skimmed 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 hours), followed by horseradish peroxidase-conjugated secondary antibody (2 hours), at room temperature. Blotting proteins were visualized by enhanced chemiluminescence (Pierce).

Animal model for epidermal hyperplasia

Epidermal hyperproliferation was achieved by repeated applications of D-squames to the flanks of hairless mice twice daily for 5 days until transepidermal water loss reached 40 mg cm^{-2} hour^{-1} as determined with Tewameter (Courage and Khasaki, Cologne, Germany) (Denda *et al.*, 1996). 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 group consisted of control non-tape-stripped, non-treated animals ($n=3$). Harvesting of tissue samples was performed 6 hours after the last treatment.

Microscopy and imaging

Skin samples were fixed in 10% formalin. Three-to-five images of 6-μm-thick paraffin sections stained with hematoxylin and eosin were obtained using a Zeiss Axioplan 2 microscope (Jena, Germany) at ×200 magnification. Epidermal thickness, considered as the distance from the basal layer to the stratum granulosum/SC junction, was randomly measured on 10 points in each image field, with mean values presented for each sample (Demerjian *et al.*, 2006).

Immunohistochemistry

Paraffin-embedded sections of hairless mice skin biopsies were used for immunohistochemistry, with specific antibodies for involucrin, loricrin, filaggrin, and keratin 5. After deparaffinization and rehydration, sections were incubated with peroxidase blocking reagent (DakoCytomation, Glostrup, Denmark) for 30 minutes to block endogenous peroxidase activity. Nonspecific antibody activity was blocked by incubation with serum-free protein block (DakoCytomation) for 15 minutes at room temperature; 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 minutes at 37 °C, followed by horseradish peroxidase-conjugated secondary antibody (30 minutes) at room temperature. Staining for these proteins was detected by diaminobenzidine 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).

Statistics

All data are expressed as mean ± SEM. Statistical significance was determined by two-tailed Student's *t*-test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by a grant from Regional Industrial Technology Development Program (10012509), which is managed by the Ministry of Commerce, Industry and Energy, Republic of Korea, and was supported by a Korea Science and Engineering Foundation (KOSEF) grants funded by the Korean government (MOST) (no. R01-2006-000-10478 and R11-2007-040-02003-0). We appreciate Dr Akio Kihara and Dr Yasuyuki Iagarashi at Hokkaido University, Japan, for providing us mouse embryonic carcinoma F9-12 cells. We thank Dr Walt Holleran at UC San Francisco for his critical review and discussions.

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