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

Jeong Hee Hong[1,6], Jong-Kyung Youm[2,3,6], Mi Jung Kwon[3], Byeong Deog Park[3], Yong-Moon Lee[4], Syng-Ill Lee[1], Dong Min Shin[1] and Seung Hun Lee[2,5]

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;

[1]*Department of Oral Biology, Brain Korea 21 Project, Oral Science Research Center, Center for Natural Defense System, Yonsei University College of Dentistry, Seoul, Korea;* [2]*Brain Korea 21 Project for Medical Science, Yonsei University, Seoul, Korea;* [3]*Neopharm Co. Ltd., Daejeon, Korea;* [4]*Chungbuk National University College of Pharmacy, Cheongju, Korea and* [5]*Department of Dermatology, Human Barrier Research Institute, Yonsei University College of Medicine, Seoul, Korea*

[6]*These authors contributed equally to this work, and shall be considered as co-first authors.*

Correspondence: Dr Seung Hun Lee, Department of Dermatology, Yongdong Severance Hospital, Yonsei University College of Medicine, no. 146-92, Dogok-dong, Kangnam-gu, Seoul 135-720, Korea. E-mail: ydshderm@yuhs.ac

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