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Sphingosine kinase 1 activation enhances epidermal innate immunity through sphingosine-1-phosphate stimulation of cathelicidin production

Se Kyoo Jeong[a,e], Young Il Kim[b,c,d], Kyong-Oh Shin[e], Bong-Woo Kim[a], Sin Hee Lee[a], Jeong Eun Jeon[a], Hyun Jong Kim[f], Yong-Moon Lee[e], Theodora M. Mauro[b,c,d], Peter M. Elias[b,c,d], Yoshikazu Uchida[b,c,d,*], Kyungho Park[b,c,d,*]

[a] CRID center, NeoPharm Co., Ltd., Daejeon 305-510, South Korea
[b] Department of Dermatology, School of Medicine, University of California, San Francisco, CA 94158, USA
[c] Department of Veterans Affairs Medical Center, San Francisco, CA 94121, USA
[d] Northern California Institute for Research and Education, San Francisco, CA 94121, USA
[e] College of Pharmacy, Chungbuk National University, Cheongju 361-763, South Korea
[f] Department of Dermatology and Atopy Clinic, Seoul Medical Center, Seoul 131-865, South Korea

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ABSTRACT

Background: The ceramide metabolite, sphingosine-1-phosphate (S1P), regulates multiple cellular functions in keratinocytes (KC). We recently discovered that production of a key innate immune element, cathelicidin antimicrobial peptide (CAMP), is stimulated *via* a NF-κB-dependent mechanism that is activated by S1P when S1P is generated by sphingosine kinase (SPHK) 1.
Objective: We investigated whether pharmacological modulation of SPHK1 activity, using a novel synthetic SPHK1 activator, (S)-methyl 2-(hexanamide)-3-(4-hydroxyphenyl) propanoate (MHP), stimulates CAMP expression.
Methods: MHP-mediated changes in both S1P and CAMP downstream mediators were analyzed in normal cultured human KC by *q*RT-PCR, Western immunoblot, ELISA, confocal microscopy for immunohistochemistry, HPLC and ESI–LC/MS/MS, and microbial pathogen invasion/colonization in a human epidermal organotypic model.
Results: Treatment with MHP directly activated SPHK1 and increased cellular S1P content in normal cultured human KC. Because MHP did not inhibit S1P lyase activity, which hydrolyses S1P, augumented S1P levels could be attributed to increased synthesis rather than blockade of S1P degradation. Next, we found that exogenous MHP significantly stimulated CAMP mRNA and protein production in KC, increases that were significantly suppressed by *si*RNA directed against SPHK1, but not by a scrambled control *si*RNA. NF-κB activation, assessed by nuclear translocation of NF-κB, occurred in cells following incubation with MHP. Conversely, pretreatment with a specific inhibitor of SPHK1 decreased MHP-induced nuclear translocation of NF-κB, and significantly attenuated the MHP-mediated increase in CAMP production. Finally, topical MHP significantly suppressed invasion of the virulent *Staphylococcus aureus* into murine skin explants.
Conclusion: MHP activation of SPHK1, a target enzyme of CAMP production, can stimulate innate immunity.
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Abbreviations: AMP, antimicrobial peptide; CAMP, cathelicidin antimicrobial peptide; ER, endoplasmic reticulum; KC, keratinocytes; K6PCN-5, *N*-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide MHP, (S)-methyl 2-(hexanamide)-3-(4-hydroxyphenyl) propanoate; SPHK, sphingosine kinase; S1P, sphingosine-1-phosphate; *Staphylococcus aureus*, *S. aureus*; VDR, vitamin D receptor.

* Corresponding authors at: Department of Dermatology, Veterans Affairs Medical Center, UCSF 1700 Owens Street, San Francisco, CA 94158, USA. Fax: +1 415 750 2106.
E-mail addresses: uchiday@derm.ucsf.edu (Y. Uchida), parkk@derm.ucsf.edu (K. Park).

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1. Introduction

Epidermis deploys multiple diverse barriers, including permeability, oxidative stress, ultraviolet irradiation (UV), and antimicrobial barriers [1]. Antimicrobial peptides (AMPs), which show broad-spectrum antimicrobial activities, are critical components of the antimicrobial barrier and epidermal innate immunity [2], as well as contributors to the permeability barrier [3]. Cathelicidin antimicrobial peptide (CAMP) is a major epidermal AMP [4] that increases following not only microbial pathogen colonization and/or invasion, but also external perturbations, such as permeability barrier disruption, UVB exposure or oxidative stress [5,6]. Our recent studies demonstrate that different, unrelated types of external perturbations, if subtoxic, induce endoplasmic reticulum (ER) stress that increases production of ceramide and levels of one of its metabolites, sphingosine-1-phosphate (S1P). The latter in turn stimulates CAMP production *via* NF-κB activation, independent of the well-known, vitamin D receptor (VDR)-dependent transcriptional regulation of CAMP [7,8], which is suppressed under ER-stressed conditions. In addition to activating CAMP, S1P signaling modulates cell proliferation, migration, differentiation, and apoptosis [9,10].

Ceramide is hydrolyzed by ceramidase to sphingosine, which then is phosphorylated by sphingosine kinase (SPHK) to S1P [11]. We previously synthesized a SPHK1 activator, *N*-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide (K6PC-5), based on molecular design technology [12]. K6PC-5 improves epidermal barrier function through stimulation of keratinocyte differentiation, while also stimulating dermal fibroblast proliferation and collagen synthesis [12,13]. Because K6PC-5 exhibits poor solubility in water/DMSO binary mixtures and organic solvents, we recently synthesized (S)-methyl 2-(hexanamide)-3-(4-hydroxyphenyl) propanoate (MHP), a derivative of K6PC-5 that markedly improved solubility in hydrophilic solvents. Here, we demonstrate that MHP activates SPHK1, which also stimulates CAMP production and enhances epidermal antimicrobial defense *via* the recently identified S1P-dependent mechanism. Consistent with our prior studies [8], these studies further indicate that SPHK1 activity is a key determinant in the regulation of CAMP production.

2. Materials and methods

2.1. Cell culture

Primary cultured human keratinocytes (KC) isolated from human neonatal foreskins were grown, as described previously [8] under an Institutional Review Board-approval protocol (University of California San Francisco; NeoPharm Co., Ltd.). Cell viability and cytotoxicity were determined using MTT assay kit in accordance with the manufacturer's instructions.

2.2. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Quantitative real-time polymerase chain reaction (*q*RT-PCR) was performed using 30 ng of cDNA prepared from total RNA fraction of cell lysates, as described previously [7]. The following primer sets were used: CAMP, 5′-CACAGCAGTCACCAGAGGATTG-3′ and 5′-GGCCTGGTTGAGGGTCACT-3′; human glyceraldehyde3′-phosphate dehydrogenase (GAPDH), 5′-GGAGTCAACG-GATTTGGTCGTA-3′ and 5′-GCAACAATATCCACTTTACCAGAGTTAA-3. mRNA expression was normalized to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.3. Western immunoblot analysis

Western immunoblot analysis was performed, as described previously [7]. Briefly, cell lysates (25 μg), prepared in RIPA buffer, were resolved by electrophoresis on 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA). Resultant bands blotted onto nitrocellulose membranes were probed with anti-CAMP (LifeSpan BioSciences, Seattle, WA), anti-human β-actin (Sigma–Aldrich, St. Louis, MO), and detected using enhanced chemiluminescence (Thermo Scientific, Waltham, MA).

2.4. ELISA for CAMP quantifications

CAMP content of cell lysates of KC previously incubated with (S)-methyl 2-(hexanamide)-3-(4-hydroxyphenyl) propanoate (MHP) is determined by ELISA kit (Hycult Biotech, Plymouth Meeting, PA) in accordance with the manufacturer's instructions.

2.5. Immunofluorescence

Immunofluorescence was performed, as described previously, NF-κB [14] and murine CAMP (mCAMP) [15]. KC were treated with MHP or vehicle for 30 min. NF-κB distribution was assessed using anti- NF-κB p65 (Santa Cruz Biotechnology, Dallas, TX) and anti-rabbit IgG conjugated with fluorescein isothiocyanate (Invitrogen). Cells were counterstained with the nuclear marker histone H4 (Vector Laboratories, Burlingame, CA). Sections fixed with formalin (5 μm) were incubated for 1 hour in blocking buffer (4% BSA, 0.5% cold water fish gelatin in PBS), and then incubated with anti-mCAMP (LifeSpan Biosciences) overnight at 4 °C. The sections were then incubated for 1 h at room temperature with goat anti-rabbit Alexa Fluor 488 (Invitrogen) followed by counterstaining with propidium iodide. Images were viewed under a fluorescence microscope (Carl Zeiss, Thornwood, NY).

2.6. si*RNA and transfections*

KC were transfected with 20 nM *si*RNA for SPHK1 or non-targeted, control *si*RNA (Dharmacon, Lafayette, CO), using siLentFect (Bio-Rad, Hercules, CA), as previously described [7,8].

2.7. Measurement of intracellular levels of sphingosine-1-phosphate

To assess the levels of cellular S1P, KC were incubated with MHP and washed with phosphate-buffered saline followed by extraction of total S1P, as we reported previously [16]. S1P was derivatized with *o*-phthalaldehyde (OPA) reagent and then quantitated using an HPLC system equipped with a fluorometrical detector system (JASCO, Tokyo, Japan), as described previously [8,17]. S1P levels were expressed as pmol per mg protein.

2.8. Enzyme activity assays for sphingosine kinase 1

SPHK1 activity was assessed as described previously [18,19]. Briefly, recombinant SPHK1 (Sigma–Aldrich, St. Louis, MO), (2 μg) was incubated with sample reaction buffer (10 mM ATP, 200 mM $MgCl_2$, 200 μM C_{17}-Sphingosine, 5 mM NaF, Na_3VO_4, 5% Triton X-100) for 30 min. The reaction was terminated by the addition of $CHCl_3$/MeOH/HCl (8:4:3, v/v/v) with 100 pmol C_{17}-sphinganine-t1-phosphate as an internal standard. The organic phage separated by addition of $CHCl_3$ was dried using a vacuum system (Vision, Seoul, Korea). The dried residue was re-dissolved in MeOH and then injected into the LC–ESI–MS/MS system (ABCIEX, Toronto, Canada). The HPLC column effluent was introduced onto an API 3200 Triple quadruple mass (ABCIEX) and analyzed using electrospray ionization in positive mode with multiple reaction

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