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Improvement by sodium dl-α-tocopheryl-6-O-phosphate treatment of moisture-retaining ability in stratum corneum through increased ceramide levels

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ABSTRACT

Sodium dl-α-tocopheryl-6-O-phosphate (**1**), a water-soluble derivative of vitamin E (dl-α-tocopherol, **2**), exhibits protective effects against various type of skin damage. As reported herein, we found that topical application of **1** improves hygroscopicity and water holding capacity in the stratum corneum of hairless mice in vivo by increasing the ceramide content. In normal human epidermal keratinocytes, treatment with **1** increases ceramide levels and enhances gene expression of serine palmitoyltransferase, which catalyzes the first step of ceramide synthesis in vitro. In addition, **1** increases gene expressions of differentiation markers (transglutaminase 1, cytokeratin 10, involucrin and loricrin), and intracellular Ca^{2+} concentrations. These results suggest that **1** could be an excellent agent for improving skin moisture-retention by enhancing ceramide synthesis through the induction of differentiation.

1. Introduction

One of the major functions of the epidermis is to serve as a permeability barrier limiting excess water loss. In particular, the stratum corneum (SC), which is the end of product of terminal differentiation of the epidermis, plays a role in barrier function.[1] Hypoactive SC is frequently formed by internal or external factors, such as aging and stress that lend to the skin becoming rough from drying. Therefore, it is important to regulate the SC to maintain healthy skin. The SC comprises corneocytes and intercellular lipids with a lamellar structure. Intercellular lipids are mainly composed of ceramides, cholesterol and free fatty acids which are biosynthesized in the epidermis.[2] Ceramides account for roughly one half of intracellular lipids.[3,4] Ceramide content is high in differentiated keratinocytes as compared to undifferentiated keratinocytes.[5,6] Ceramides play a role in the water-holding properties of SC[7–9] and in skin barrier function.[10] Loss of ceramide lending to a hypoactive SC, is commonly associated with atopic dermatitis,[11–14] psoriasis,[15] and xerosis.[11,12] Intracellular ceramides are boosted by supplementation or by the enhancement of biosynthesis. Several ceramide mimics have been synthesized for the purpose of supplementation.[16] Effective ingredients that enhance biosynthesis of intercellular lipids, including nicotinamide[17] and lactic acid,[18] improve the function of SC.

Sodium dl-α-tocopheryl-6-O-phosphate (**1**) is hydrophilic derivative of dl-α-tocopherol (**2**). Compound **1** bears a sodium phosphoryl group on the chroman ring of **2** (Fig. 1). Since **1** is water-soluble and stable against oxidation, it can be easily incorporated in a variety of pharmaceutical formulations. Compound **1** is a quasi drug for the treatment of rough skin, due to its anti-inflammatory and antioxidative effects, and its ability to suppress lipid peroxide and ultraviolet radiation-damage.[19,20] During studies, we observed that treatment with **1** is helpful in moisturizing and smoothing the skin. These findings let us to investigate whether **1** can improve moisture-retaining properties and to evaluate its mechanism of action. We measured skin surface hydration levels and examined the effects of **1** on the biosynthesis of ceramide.

2. Results

2.1. Effects of 1 on relative hygroscopicity and water holding capacity in SC of hairless mouse skin

Initially, we investigated whether topical application of **1** improved skin hygroscopicity and water holding capacity of SC in hairless mice. Water sorption–desorption analysis was performed using mouse skin tissues administered with none, placebo and 1% and 2% of **1** for 4 weeks. As shown in Figure 2A, hygroscopicities

Abbreviations: **1**, sodium dl-α-tocopheryl-6-O-phosphate, TPNa; **2**, dl-α-tocopherol, Toc; SC, stratum corneum.

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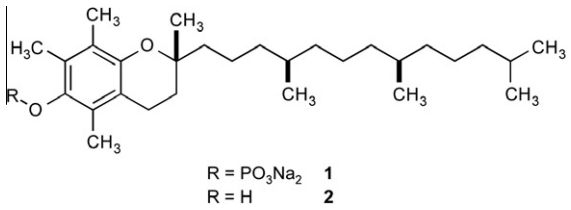

Figure 1. Chemical structures of **1** and **2**.

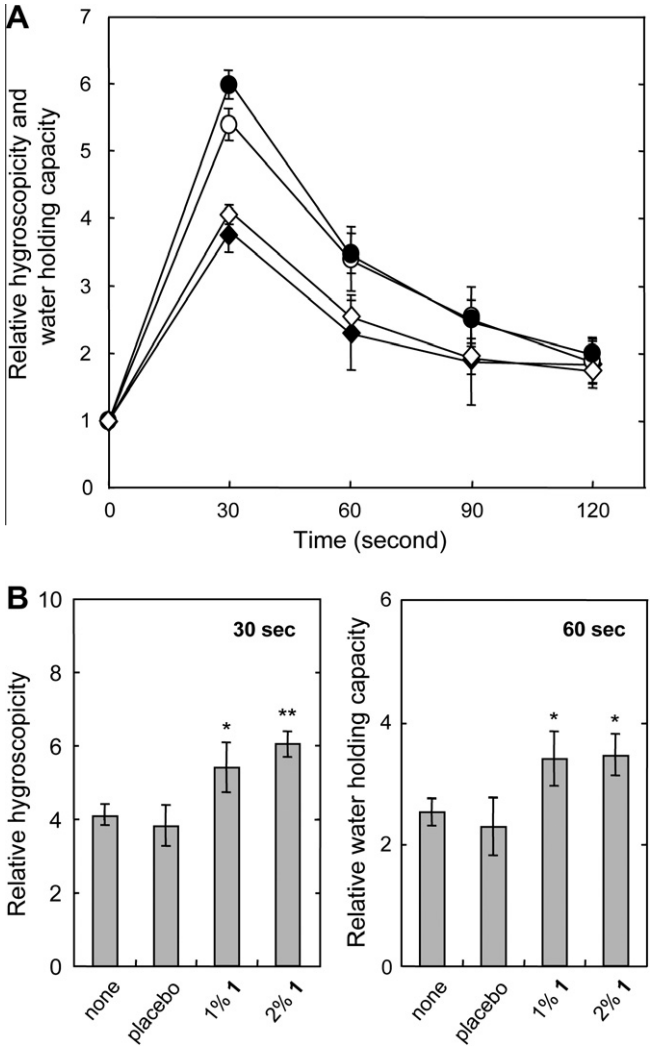

Figure 2. Effects of **1** on hygroscopicity and water holding capacity in mouse SC. Mouse skin was treated without or with **1** topically once daily, five times a week. After 4 weeks, water sorption–desorption tests were performed as described in Section 4. (A) Overall view of the water sorption–desorption test, (B) Skin surface hydration as hygroscopicity at a time point of 30 s (left panel) or water holding capacity at a time point of 60 s (right panel) after loading water onto skin. Each bar represents the mean ± SE. $^{*}p$ <0.05 and $^{**}p$ <0.01 versus placebo compared by Student's *t*-test. None (◇), placebo (♦), 1% **1** (○), 2% **1** (●)

in skin tissues treated without and with placebo increased approximately fourfold after 30 s as compared to $t = 0$, and decreased gradually in time-dependent manner to achieve a half-maximal value at 120 s. In contrast, 1% and 2% **1** treatments increased hygroscopicities approximately sixfold, reaching a peak at 30 s and then decreasing. The water holding capacity in untreated SC at 120 s was the same as **1**-treated SC. Hygroscopicities at 30 s (Fig. 2B. left panel) and water holding capacity at 60 s (Fig. 2B, right panel) in the presence of 1% and 2% of **1** were approximately 1.5-fold higher than those in untreated and placebo-treated SC (Fig. 2B). These results indicate that topical application of **1** improves the hygroscopicity and water holding capacity of the SC.

2.2. Effects of 1 on the amount of ceramide in SC of hairless mouse skin

The finding that **1** might increase water holding capacity let us to investigate whether the content of ceramide in skin SC of hairless mice, was increased by topical application of **1**. Ceramide content in SC stripped by tape was measured by the Kisic method after Bligh–Dyer extraction.

As shown in Figure 3, the amounts of ceramide in the SC treated with 1% and 2% of **1** for 4 weeks were approximately 1.7-fold higher than those in untreated and placebo-treated SC. These results indicate that topical application of **1** might improve water-retaining function by increasing the ceramide content in SC of hairless mice.

2.3. Effects of 1 on ceramide production in NHEK

Our finding that ceramide levels in SC of hairless mouse skin treated with **1** is high as compared with control mouse SC, let us to examine whether treatment of primary normal human epidermal keratinocytes (NHEK) with **1** could also increase ceramide content. As shown in Figure 4A, the amount of ceramide in NHEK was increased following treatment with **1** in a concentration-dependent manner. Compound **1** at concentrations of 10 and 50 μM resulted in approximately 1.5- and 1.8-fold, respectively increased ceramide levels as compared with untreated cells. These results indicate that treatment with **1** also increased ceramide concentrations in NHEK.

2.4. Effects of 1 on mRNA expression of serine palmitoyltransferase and ceramidase in NHEK

The increase in ceramide levels by the treatment with **1** in hairless mice in vivo and in NHEK in vitro might be due either to

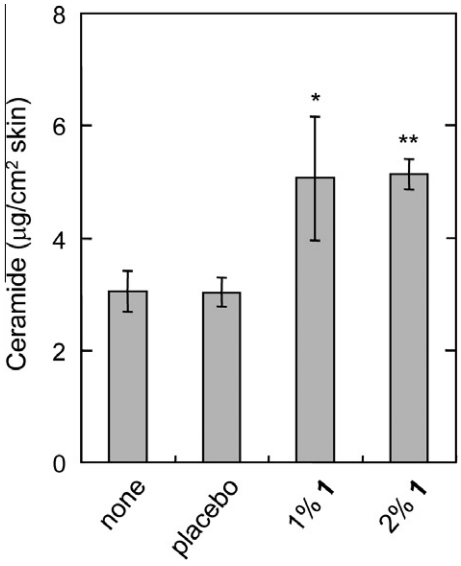

Figure 3. Effects of **1** on the amount of ceramide in mouse SC. The SC of mouse skin treated without or with **1** topically for 4 weeks was stripped using adhesive tapes. Lipids on the adhesive tapes were extracted and ceramide was determined as described in Section 4. Each bar represents the mean ± SE. $^{*}p$ <0.05 and $^{**}p$ <0.01 versus placebo compared by Student's t-test.

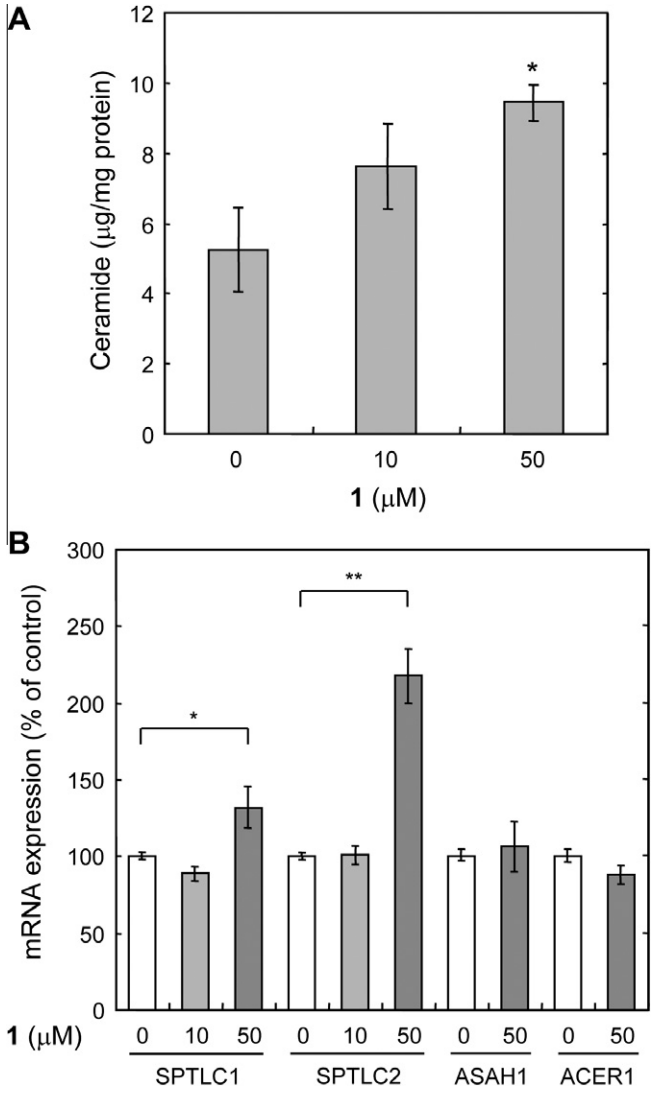

Figure 4. Effects of **1** on ceramide levels or mRNA expression of serine palmitoyltransferase (SPTLC1, SPTLC2) and ceramidase (ASAH1, ACER1) in NHEK. NHEK (10^5 cells/ml) cultured to semiconfluent (80%) were incubated without (control) and with **1** at concentrations of 10 or/and 50 μM for 24 h. (A) Cells were harvested, and the amount of ceramide was determined as described in Section 4. (B) Total RNA was isolated from cells and mRNA expression levels of SPTLC1, SPTLC2, ASAH1 or ACER1 were determined by real-time PCR analysis as described in Section 4. Each bar represents the mean ± SE. $^{*}p$ <0.05 and $^{**}p$ <0.01 versus control compared by Student's *t*-test.

an increase in ceramide synthesis or to a decrease in ceramide metabolism. Therefore we examined the levels of mRNA expression of ceramide synthetase (serine palmitoyltransferase: SPTLC1 and SPTLC2), and ceramidase (ASAH1 and ACER1) in NHEK without and following treatment with **1**.

In NHEK treated with 50 μM of **1**, SPTLC1 and SPTLC2 mRNA expression significantly increased approximately 1.2- and 2.2-fold, respectively as compared with untreated cells (Fig. 4B). On the other hand, ASAH1 and ACER1 mRNA expression in NHEK was unaffected by 50 μM of **1** (Fig. 4B). These results indicate that the increase in ceramide contents in NHEK following treatment with **1** might be due to up-regulation of the ceramide synthetases, SPTLC1 and SPTLC2, not to down-regulation of ceramidases, ASAH1 and ACER1. Therefore, **1** might improve hygroscopicity and water holding capacity of the SC through enhancement of ceramide synthesis.

2.5. Effects of 1 on mRNA expression of differentiation markers of keratinocyte

Next we examined whether treatment of NHEK with **1** influences gene expression levels of enzymes and proteins expressed during the differentiation process. As shown in Figure 5, mRNA expression of transglutaminase 1 (TGM1), cytokeratin 10 (KRT10), involucrin (IVL) and loricrin (LOR) in NHEK treated with 50 μM of **1** increased approximately 2.5-, 2.2-, 3.3- and 2.7-fold, respectively, as compared with untreated. In contrast, mRNA expression of cytokeratin 1 (KRT1) and filaggrin (FLG) were unaffected by treatment with **1**. These results suggest that treatment with **1** might induce differentiation of NHEK.

2.6. Effect of 1 on intracellular Ca^{2+} concentration

Since **1** might induce the differentiation of NHEK, we examined whether treatment with various concentrations of **1** for 1 h and 24 h could increase the intracellular Ca^{2+}, which is a differentiation-inducing factor.

As shown in Figure 6A, intracellular Ca^{2+} concentrations in NHEK increased in dose-dependent manner by treatment with **1** for 1 h. Analogue **1** at concentrations of 50 and 75 μM enhanced Ca^{2+} levels in NHEK significantly as compared with control. In addition, intracellular Ca^{2+} concentrations in NHEK treated with **1** for 24 h increased dose-dependently, being approximately 1.4-fold higher in NHEK treated with 50 μM of **1** than in untreated NHEK (Fig. 6B). In another human keratinocyte cell line, SVHK, **1** also increased intracellular Ca^{2+} concentrations in dose-dependent manner (data not shown). These results suggest that **1** might induce differentiation of NHEKs by increasing intracellular Ca^{2+} concentrations.

2.7. Uptake and conversion of 1 to 2 in NHEK

We investigated whether the conversion of **1** to **2** occurs in NHEK. As shown in Table 1, in NHEK treated with 10 and 50 μM of **1** for 24 h, approximately 4.8% and 3.1% of **1** was converted to **2**, respectively. The concentrations of **1** in NHEK were 20- to 31-fold higher than concentrations of **2**. These results indicate that the conversion of **1** to **2** occurs very slowly in NHEK.

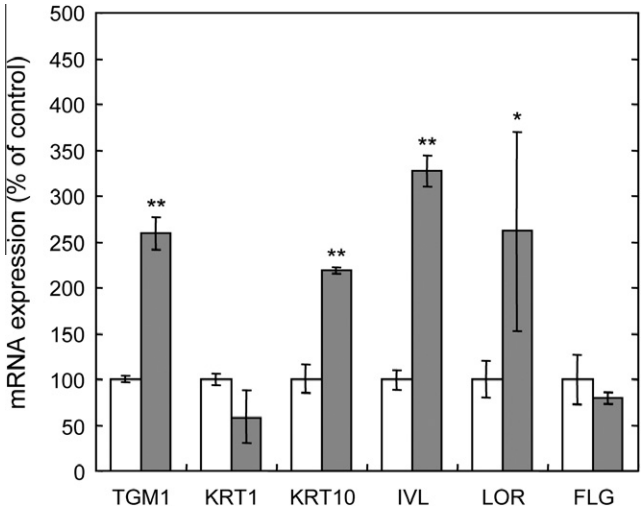

Figure 5. Effects of **1** on mRNA expression of differentiation markers in NHEK. NHEK (10^5 cells/ml) cultured to semiconfluent (80%) were incubated without (control) and with 50 μM of **1** for 24 h. Total RNA was isolated from the cells and mRNA expression levels of TGM1, KRT1, KRT10, IVL, LOR or FLG were determined by real-time PCR analysis as described in Section 4. Each bar represents the mean ± SE. $^{*}p$ <0.05 and $^{**}p$ <0.01 versus control compared by Student's *t*-test.

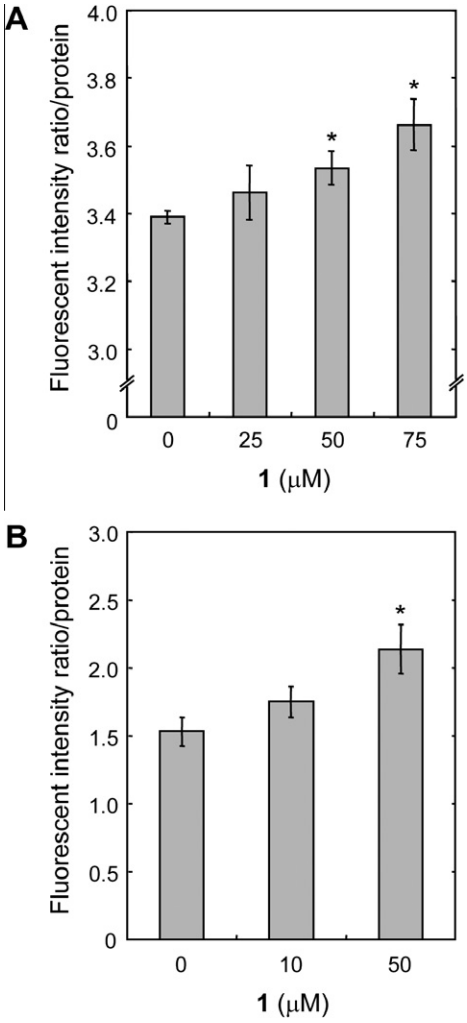

Figure 6. Effect of **1** on Ca^{2+} levels in NHEK. NHEK (10^5 cells/ml) cultured to semiconfluent (80%) were incubated without (control) and with various concentrations of **1** for 1 h (A) or 24 h (B), and then with Fura 2-AM for 1 h. Intracellular Ca^{2+} levels were determined as described in Section 4. Each bar represents the mean ± SE. $^*p$ <0.05 versus control compared by Student's t-test.

Table 1
Concentration of **2** and **1** in NHEK after 24 h administration[a]

Dose (μM)	**2**	**1**
0	ND	ND
10	1.7 ± 0.4 (4.8%)	34.0 ± 7.4 (95.2%)
50	4.7 ± 0.3 (3.1%)	147.8 ± 0.8 (96.9%)

[a] Concentration are given in nmol/mg protein.

3. Discussion

Skin moisture-retaining ability was significantly improved with topical application of **1**. Results in SC of hairless mouse showed that treatment with **1** resulted in increased hygroscopicity (Fig. 2B, left panel) and water holding capacity (Fig. 2B, right panel) and that this was accompanied by elevated ceramide levels (Fig. 3). In NHEK, **1** increased ceramide levels (Fig. 4A) and gene expression of ceramide synthetase, while not increasing the levels of ceramidase (Fig. 4B). In addition, mRNA expression of differentiation markers (TGM1, KRT10, IVL, and LOR) (Fig. 5) and intracellular Ca^{2+} concentrations (Fig. 6) were increased by treatment with **1** in NHEK. These results suggested that **1** exhibits hygroscopicity and water holding activity by increasing ceramide concentration due to the activation of ceramide synthesis. This is by the induction of differentiation via an increase in intracellular Ca^{2+} concentrations.

Ceramide is a major lipid class in the SC, which plays significant roles in the skin barrier function.[21] Previous studies have shown that ceramide levels in SC increased following topical application of sake concentrate and ethyl α-D-glucoside (α-EG),[22] nicotiamide,[17] lactic acid,[18] eucalyptus extract,[23] Musk T (1,4-dioxacycloheptadecane-5,17-dione)[24] and ursolic acid (URA).[25] Ceramide content of SC from hairless mouse skin that had been treated with sake concentrate (10%) and α-EG (1% v/v) for 4 weeks or with Musk-T (1% w/v) for 30 h were approximately 1.6-, 1.1- or 1.3-fold greater than those of vehicle-treated mouse skin, respectively. Volunteers' dry skins that had been treated with nicotiamide (2%) and lactic acid (4%) for 4 weeks, had approximately 1.4-fold higher levels of ceramides than those treated with vehicle alone. Eucalyptus extract (1%) and URA (0.3%, 1%) increased ceramide levels approximately 1.8- and 1.3-fold as compared to vehicle-treated skins when applied for 4 and 11 days, respectively. Although these agents were effective at enhancing skin barrier functions, no data have shown regarding skin moisture-retaining functions. In the current study, topical application of 1% and 2% of **1** for 4 weeks increased ceramide concentrations approximately twofold in SC of hairless mouse skin (Fig. 3) as well as hygroscopicity and water holding capacity (Fig. 2).

On the other hand, in in vitro experiments, treatment using 1–30 μM nicotiamide,[17] 20 mM lactic acid,[18] 0.0001%, 0.0005%, and 0.001% eucalyptus extract[23] or 1% URA[25] increased ceramide content in keratinocytes, approximately 4.1- to 5.5-fold following 6 day treatment, and approximately 4.3-, 1.2- and 2.2- to 3-fold following treatment for 24 h, 3 and 6 days, respectively as compared with vehicle treatment (control). Treatment with 10 and 50 μM of **1** for 24 h increased ceramide content in NHEK approximately 1.5- and 1.8-fold, respectively (Fig. 4A). Among **2** and tocopheryl derivatives this is the first report that **1** increases ceramide synthesis in vivo and in vitro and enhances moisture-retaining function in skin tissues in vivo.

During keratinization, ceramides are synthesized and degraded. Ceramides are synthesized by various enzymes from ketosphinganine, a ceramide precursor that is produced by serine palmitoyltransferase (SPT), which consists of three subunits (LC1, LC2, LC3). Ceramides are degraded by ceramidases, including acid ceramidase and alkaline ceramidase. Nicotiamide treatment (10 μM, 4 days) increased gene expression for human SPT (LCB1 and LCB2) approximately 1.8-fold in neonatal human foreskin keratinocytes.[17] Treatment with macrocarpal, an active component of the eucalyptus extract (10 nM, 2 days) increased SPTLC1 gene expression approximately 1.08-fold, but it had no effect on gene expression of ceramidases in neonatal human foreskin keratinocytes.[23] In the current study, treatment with **1** (50 μM, 24 h) was found to increase gene expression for SPTLC1 and SPTLC2 approximately 1.2- and 2.2-fold, respectively, but it had no effect on ceramidase, in NHEK (Fig. 4B). It appears that **1** exhibits effects similar to these other materials.

The synthesis of ceramide is associated with differentiation of keratinocytes.[21] Ceramide is synthesized, transformed and resynthesized in the process of keratinocyte differentiation. It is covalently bound to cornified envelope proteins, in particular to involucrin, and it forms the backbone in SC. Ceramide serves as a main structural component of intercellular lipids in SC, where it functions as a moisture-retaining barrier by brick-and-mortar formations with horny cells in the skin. Based on the above, we speculated that **1** might induce the differentiation of keratinocytes. This was due in part to the up-regulation of ceramide synthetase and an increase in ceramide following treatment with **1**. It is was well known that Ca^{2+} functions as a keratinocyte differentiation-induc-

ing factors[26,27] and that keratinocytes incubated with high Ca^{2+} concentrations differentiate and have promoted gene expressions of various differentiation markers, including TGM1, KRT1, KRT10, IVL, LOR and FLG.[28,29] Our current study is the first report among derivatives of **2** that treatment with **1** increases intracellular ceramide content, the gene expression of ceramide synthetase and differentiation markers, and Ca^{2+} concentration in NHEK (Figs. 2–5). Gene expression levels of TGM1, KRT10, IVL or LOR and intracellular Ca^{2+} concentrations in NHEK increased approximately 2.6-, 2.2-, 3.3-, 2.7- and 1.4-fold, respectively, following treatment with 50 μM of **1** for 24 h (Figs. 5 and 6). It has been previously reported that lysophospholipids, in particular lysophosphatidic acid (LPA), have effects similar to **1**.[30] It is hard to compare the reported data with our data because experiments were performed under different conditions. However, we are in agreement that **1** and LPA play roles in skin moisture-retention and barrier function with up-regulation of ceramide synthesis due to the induction of differentiation.

Calcium ionophores, including ionomycine and A23187, are well known to form stable complexes with Ca^{2+} and act as a mobile-carriers. In increasing Ca^{2+} levels in NHEK (Fig. 6) **1** might act like a calcium ionophore. However, while ionomycine and A23187 are cytotoxic and irritating drugs, **1** is both mild and safe. We have shown that **1** is transdermally absorbed, and penetrates into keratinocytes, improving skin roughness and promoting anti-inflammatory activities.[20] It would be interesting to examine whether tocopheryl phosphate forms complexes with Ca^{2+} and can behave as a Ca^{2+}-carrier ionophore.

In conclusion, **1** induces differentiation of keratinocytes by increasing Ca^{2+} uptake, stimulating gene expression of ceramide synthetase, and increasing ceramide content in keratinocytes and SC. This results in improved skin moisture-retention. Compound **1** is chemically stable and it could potentially be useful as a provitamin E supplement or as a sole agent to treat skin roughness. Its anti-oxidative and anti-inflammatory properties might make it a useful therapeutic.

4. Experimental

4.1. Chemicals

Compound **1** was purchased from Showa Denko (Tokyo, Japan). The purity of **1** was greater than 98%, and the amount of **2** and other **2** derivatives in **1** was less than 2%. All other chemicals were of reagent grade.

4.2. Animals

Hairless mice (Hos: HR-1, 7 week old) were purchased from Hoshino Laboratory Animals Corp. (Bandoh, Ibaraki, Japan). Mice were kept under controlled conditions (ambient temperature, 22 ± 3 °C; relative humidity, 50 ± 20%; 12 h light/12 h dark cycle). Mice (female, 8.5 week of age) were fed commercial diet (Lab Diet #5002, PMI Nutrition International, Brentwood, MO, USA) and water ad libitum. Animal husbandry procedures were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments (June 1, 2006, Science Council of Japan).

4.3. Preparation of 1 and its topical application

Compound **1** for topical use was prepared at final concentrations of 1% and 2% in a oil in water emulsion containing water, polyol, silicone oil, thickener and polymer emulsifier.[31] Placebo consisted of emulsion alone. The emulsion (50 μl) without or with **1** was applied on the dorsal surface (2 × 3 cm^2) of hairless mouse skin, once daily and five times a week for 4 weeks.

4.4. Water sorption–desorption tests

Water sorption–desorption tests were performed according to the method of Tagami et al.[32] Skin surface hydration levels were measured with a skin surface hygrometer (Skikon-200, I.B.S. Ltd, Shizuoka, Japan) by determining high frequency conductance. Initially, conductance-values of skin tissues were measured to obtain values at prehydration levels. A drop of distilled water (500 μl) on nonwoven fabric was placed on an area of lumbar skin for 10 s. Immediately after removing the moist fabric, the water remaining on the skin was removed by a water-absorption sponge, and then after 30 s, hygroscopicity was measured. Subsequently, measurements were repeated intervals of 30 s for 2 min to obtain a value for the water holding capacity. Data were expressed as the relative hygroscopicity and relative water holding capacity as compared to values at 0 s. Relative hygroscopicities were calculated for individual mice as the amount at 30 s after water had been placed on skin tissues, divided by the amount at the prehydration state (the amount of 0 s). Relative water holding capacities for individual mice were calculated as the amount at 60, 90 and 120 s after placement of water, divided by the amount at the prehydration state (the amount of 0 s). Data from individual mice were averaged and analyzed by statistic methods.

4.5. Cell culture

Normal human epidermal keratinocytes (NHEK) (Kurabo, Osaka, Japan) were propagated in HuMedia-KG2 (Kurabo).

4.6. Ceramide content in SC of mice and NHEK

The SC of mice (area: 2 × 1 cm) that had been subjected to topical application of emulsion or emulsion with **1** for 4 weeks were stripped using adhesive tape (P.P.S., Nichiban, Tokyo, Japan). The tapes were stored at −20 °C until analyzed. Lipids on the adhesive tapes were extracted by the Bligh and Dyer procedure.[33]

NHEK (10^5 cells/ml) in HuMedia-KG2 were incubated without and with 50 μM of **1** for 24 h, washed with D-PBS(−), and harvested using a scraper. Cells (10^6 cells) were homogenized by using a sonicator (Ohtake works, Tokyo, Japan) and cellular lipids were extracted by the Bligh and Dyer procedure.

Ceramide was determined by the method of Kisic et al.[34] Briefly, samples were hydrolyzed with 3 N hydrochlolic acid (0.05 ml) for 2 h at 100 °C, and then hydrochlolic acid was removed in vacuo. To the dry-residue, was added ethylacetate (0.6 ml) and 0.1 M acetate buffer pH 3.7 (0.75 ml) and the mixtures were stirred. To the mixtures were added an acetone solution (0.15 ml) of fluorescamine (7 mg/25 ml) and stirring was continued. Following centrifugation (2,000 × *g*, 3 min), the fluorescent intensity (excitation wavelength, 410 nm; emission wavelength, 490 nm) of fluorescamine in the ethylacetate layer was determined using a fluorescence detector (Infinite M200, Tecan, Mannedorf, Switzerland).

4.7. Realtime PCR analysis

Semi-confluent NHEK in HuMedia-KG2 were incubated without and with 10 μM and 50 μM of **1** for 24 h, and washed with D-PBS(−). Cells were harvested and frozen in RNAcell protect reagent (QIAGEN, Valencia, CA, USA). Total RNA was isolated from the cells using RNeasy Plus Mini (QIAGEN). Single-strand cDNA was synthesized using a PrimeScript™ RT Reagent Kit (Perfect Real Time, TAKARA BIO INC., Shiga, Japan). Real-time PCR was performed using a Roche LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) with SYBR Premix Ex TaqII (TAKARA BIO INC.) and primers specific for the genes of palmitoyltransferase (SPTLC1), acid ceramidase (ASAH1), alkaline ceramidase (ACER1), transglutamin-

ase 1 (TGM1), cytokeratin 1 (KRT1), cytokeratin 10 (KRT 10), involucrin (IVL), loricrin (LOR) and filaggrin (FLG), and housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) (Primer set ID: HA042580 for SPTLC1, HA121961 for SPTLC2, HA127474 for ASAH1, HA057790 for ACER1, HA032140 for TGM1, HA042147 for KRT1, HA093630 for KRT10, HA103446 for IVL, HA098302 for LOR, HA127774 for FLG and HA031578 for GAPDH, TAKARA BIO INC., Shiga, Japan). A single fluorescence reading at 530 nm was obtained for each sample at the extension step. Samples were analyzed using LightCycler software (Roche Applied Science, Indianapolis, IN, USA). Quantitative PCR results were normalized using GAPDH.

4.8. Measurement of Ca^{2+} levels in NHEK

(1) Semi-confluent NHEK in HuMedia-KG2 were washed with Buffer A (10 mM HEPES pH 7.4, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 10 mM glucose), and then incubated in Buffer A containing 0.05 mM Ca^{2+} without and with 25, 50 and 75 μM of **1** at 37 °C for 1 h. (2) Semi-confluent NHEK in HuMedia-KG2 were incubated without and with **1** at concentrations of 10 and 50 μM for 24 h. Subsequently, cells were washed with Buffer A, and treated with 3 μM Fura 2-acetoxymethyl ester (Fura 2-AM, Dojindo, Kumamoto, Japan) for 1 h at 37 °C. After washing with Buffer A, cells were lyzed with Buffer A containing 0.5% Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA). Intracellular Ca^{2+} levels were determined by measuring fluorescence intensity (excitation wavelength, 340 and 380 nm; emission wavelength, 510 nm) using a fluorescence detector (Infinite M200, Tecan, Mannedorf, Switzerland) and calculated as the ratio of 340/380 nm. Protein determinations were performed using DC Protein Assay (Bio-Rad, Hercules, CA, USA).

4.9. Statistics

The statistical significance of the data was evaluated by the Student's *t* test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were considered significant. Each experiment was performed at least three times and repeated.

5. Conflicts of interest

None declared.

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