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Vitamin C Stimulates Epidermal Ceramide Production by Regulating Its Metabolic Enzymes

Kun Pyo Kim[1,†], Kyong-Oh Shin[2,†], Kyungho Park[3,†], Hye Jeong Yun[1], Shivtaj Mann[4], Yong Moon Lee[2] and Yunhi Cho[1,*]

[1]Department of Medical Nutrition, Graduate School of East-West Medical Science, Kyung Hee University, Yongin 446-701, [2]College of Pharmacy and MRC, Chungbuk National University, Cheongju 361-763, Republic of Korea, [3]Department of Dermatology, Northern California Institute for Research and Education (NCIRE)-VA Medical Center, University of California, San Francisco (UCSF), San Francisco, California 94158, USA, [4]Nova Southeastern College of Medicine, Fort Lauderdale, Florida 33314, USA

Abstract

Ceramide is the most abundant lipid in the epidermis and plays a critical role in maintaining epidermal barrier function. Overall ceramide content in keratinocyte increases in parallel with differentiation, which is initiated by supplementation of calcium and/or vitamin C. However, the role of metabolic enzymes responsible for ceramide generation in response to vitamin C is still unclear. Here, we investigated whether vitamin C alters epidermal ceramide content by regulating the expression and/or activity of its metabolic enzymes. When human keratinocytes were grown in 1.2 mM calcium with vitamin C (50 μg/ml) for 11 days, bulk ceramide content significantly increased in conjunction with terminal differentiation of keratinocytes as compared to vehicle controls (1.2 mM calcium alone). Synthesis of the ceramide fractions was enhanced by increased *de novo* ceramide synthesis pathway *via* serine palmitoyltransferase and ceramide synthase activations. Moreover, sphingosine-1-phosphate (S1P) hydrolysis pathway by action of S1P phosphatase was also stimulated by vitamin C supplementation, contributing, in part, to enhanced ceramide production. However, activity of sphingomyelinase, a hydrolase enzyme that converts sphingomyelin to ceramide, remained unaltered. Taken together, we demonstrate that vitamin C stimulates ceramide production in keratinocytes by modulating ceramide metabolic-related enzymes, and as a result, could improve overall epidermal barrier function.

Key Words: Ceramide, Ceramide metabolic enzymes, Vitamin C, Calcium, Keratinocyte differentiation, Epidermal barrier

INTRODUCTION

Mammalian epidermis consists of four different layers, stratum basale (SB), stratum spinosum (SP), stratum granulosum (SG), and stratum corneum (SC) (Elias and Menon, 1991; Uchida, 2014). Since the SC is the outermost layer and directly faces the external environment, it functions to serve as a barrier (Elias, 2005). Multiple factors are involved in maintaining epidermal barrier function, of which ceramide is of particular importance (Uchida, 2014). Precursors of ceramide are synthesized during keratinocyte (KC) differentiation and are packaged into lamellar bodies (LB) and eventually secrete their contents at the SG-SC interface (Uchida *et al*., 2001a; Uchida *et al*., 2001b). In turn, ceramide is incorporated into the SC, comprising nearly 50% of its lipid mass (Elias and Menon, 1991). In the SC certain species of ceramide, particularly ω-OH-ceramide, covalently attach to the cornified envelope (CE) to form the corneocyte lipid-bound envelope (CLE), a structure that is essential for epidermal barrier function (Wertz and Downing, 1986). As a result, pathologies that affect ceramide content in the SC contribute to a number of barrier abnormalities, including excess water loss (Imokawa *et al*., 1991; Motta *et al*., 1994). Considering this, ceramide content is a critical factor for epidermal homeostasis.

While the overall level of ceramide in the epidermis is regulated by several metabolic enzymes, *e.g*., serine palmitoyltransferase (SPT), ceramide synthase (CerS), sphingomyelinase (SMase), and sphingosine-1-phosphate phosphatase

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***Corresponding Author**
E-mail: choyunhi@khu.ac.kr
Tel: +82-31-201-3817, Fax: +82-31-201-3715
[†]The first three authors contributed equally to this work.

(SPPase), calcium (Ca^{2+}) and vitamin C (Vit C) are known to regulate both KC differentiation and ceramide content (Uchida *et al*., 2001a; Kim *et al*., 2011; Uchida, 2014). In particular, prior studies have shown that Vit C treatment significantly increases CerS activity in high Ca^{2+}-supplemented KC, accounting for increased levels of epidermal ceramide (Uchida *et al*., 2001a).

Although previous studies have provided evidence for the importance of epidermal ceramide in maintaining normal barrier function, quantitative analysis looking into specific levels of metabolic enzymes responsible for ceramide synthesis in KC following Vit C is still unclear. In this study, we show that high Ca^{2+} (1.2 mM) and Vit C treatment stimulates production of ceramide. This stimulation is mediated by altered activity/expression of metabolic enzymes involved in ceramide synthesis.

MATERIALS AND METHODS

Cell culture

Human primary KC from Life Technologies (Carlsbad, CA, USA) were maintained in serum-free KC growth medium containing 0.07 mM Ca^{2+}, as described previously (Park *et al*., 2013b). Early stage of KC differentiation was induced by a switch in extracellular Ca^{2+}concentration from low (0.07 mM) to high (1.2 mM). Moreover, late/terminal differentiated KC were generated by culturing cells in medium containing high Ca^{2+}, along with Vit C (50 μg/ml) treatment for up to 11 days, as described previously (Uchida *et al*., 2001a; Kim *et al*., 2011).

Quantitative reverse transcription polymerase chain reaction (*q*RT-PCR)

*q*RT-PCR was performed using 30 ng cDNA prepared from total RNA fraction of cell lysates, as we described previously (Park *et al*., 2011). The following primer sets were used: SPPase, 5'-CCATTTCTATGGTCCTCCTCACCT-3' and 5'-CAATCAGGTCCACAAATGGATAGAA-3'; human glyceraldehyde-3-phosphate dehydro-genase (GAPDH), 5'-GTCAACGGATTTGGTCGTATTG-3' and 5'-GCCATGGGTGGAATCATATTG-3'. mRNA expression was normalized to levels of GAPDH.

Western blot analysis

Western blot analysis was performed as previously described (Park *et al*., 2011). Cell lysates, prepared in radio-immunoprecipitation assay (RIPA) buffer, were resolved by electrophoresis on a 8 to 10% SDS-PAGE gel. The resultant bands were blotted onto nitrocellulose membranes, probed with anti-human β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), keratin 5 (Bioworld, St. Louis Park, MN, USA), keratin 10 (Santa Cruz Biotechnology), loricrin (Abcam, Cambridge, MA, USA), involucrin (Santa Cruz Biotechnology), SPT (Santa Cruz Biotechnology), CerS (Santa Cruz Biotechnology), and SPPase (Abcam), and detected by chemiluminescence reagent (SurModics, Eden Prairie, MN, USA).

Measurement of intracellular levels of ceramide

To assess the levels of cellular ceramide, human KC were incubated with Vit C and washed with phosphate-buffered saline followed by extracting total lipids, as we reported previously (Shin *et al*., 2012b; Park *et al*., 2013a). Ceramides were 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 (Shin *et al*., 2012b; Park *et al*., 2013a). Ceramides are expressed as pmol per mg protein.

Enzyme activity assay for serine palmitoyltransferase

SPT activity was determined as previously described with a minor modification (Rutti *et al*., 2009; Shin *et al*., 2012a). Briefly, cells were suspended in assay buffer (50 mM HEPES buffer containing 1 mM EDTA, 0.1% sucrose monolaurate, 5 mM L-serine, 50 μM palmitoyl-CoA, and 20 μM pyridoxal 5′-phosphate). The SPT enzymatic reaction was initiated by placing the cell suspension at 37°C for 60 min, followed by the addition of $NaBH_4$ (5 mg/mL) to terminate the reaction. The activity of SPT was analyzed by HPLC system equipped with a fluorometrical detector system (JASCO) as described previously (Shin *et al*., 2012a).

Enzyme activity assay for ceramide synthase

CerS activity assay was performed as described previously (Kim *et al*., 2012). Briefly, cell lysates, prepared in assay buffer (20 mM HEPES, pH 7.4, 25 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT, 0.1% (*w/v*) fatty acid-free BSA, and 50 μM fatty acid-CoA), were incubated with 10 nmol of C_{17}-sphinganine for 30 min at 37°C. Total lipids were extracted by the addition of $CHCl_3$: MeOH (1:2, *v/v*), and 100 pmol of C_{12} ceramide as the internal standard, and applied onto LC-ESI-MS/MS (ABCIEX, Framingham, MA, USA), as described previously (Kim *et al*., 2012). The activity of CerS is expressed as pmol (dihydroceramide production) per mg protein per min.

Enzyme activity assay for sphingomyelinases

Activities of SMases were assessed as described previously (Loidl *et al*., 2002). Briefly, cells suspended in appropriate SMases assay buffers (acdic SMase buffer: 250 mM sodium-acetate, 0.2% Triton X-100, pH 4.5 or neutral SMase buffer: 20 mM HEPES, 0.2% Triton X-100, pH 7.4) were incubated with 5 nmol of NBD-sphingomyelin (SM) for 1 h at 37°C. The reaction was stopped by the addition of $CHCl_3$: CH_3OH (2:1, *v/v*) and the organic phases were removed under N_2 gas. The residues then were resuspended in MeOH and applied onto HPLC system equipped with a fluorometrical detector system (JASCO). The activities of both SMases are expressed as pmol (NBD-ceramide) per mg protein per min.

Enzyme activity assays for sphingosine-1-phosphate phosphatase

SPPase activity was determined as previously described with a minor modification (Johnson *et al*., 2003). Briefly, cells were suspended in assay buffer (1 mM EDTA, 0.1% BSA, 10 μM C_{17} Sphingosine-1-phosphate (S1P) as substrate) and incubated at 37°C for 20 min. The reaction was terminated by the addition of MeOH containing 100 pmoles of dihydrosphingosine as the internal standard and the activity was analyzed by LC-ESI-MS/MS (ABCIEX), as described previously (Johnson *et al*., 2003). The activity of SPPase is expressed as pmol (C_{17} sphingosine (SO)) per mg protein per min.

Statistical analysis

Statistical analyses used the unpaired Student *t* Test. The *p* values were <0.01 in all cases.

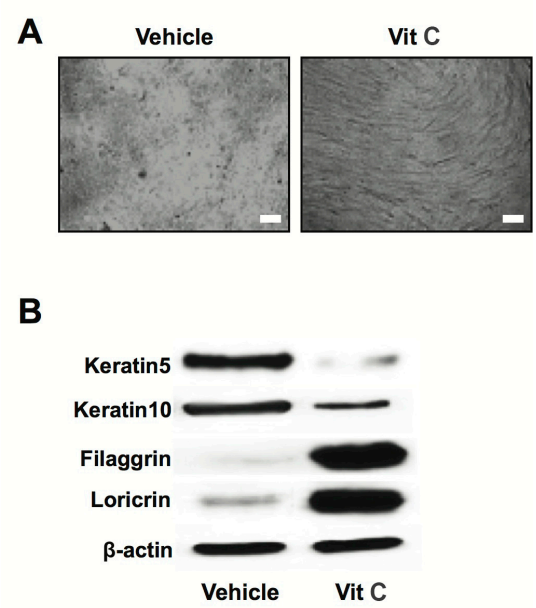

Fig. 1. Altered levels of differentiation-associated genes during Vit C-induced keratinocyte differentiation. Human primary keratinocytes (KC) were cultured in high Ca^{2+}-supplemented medium with or without Vit C, as described in materials and methods. Phage contrast images of early or terminally differentiated KC (A). Protein expression of differentiation-associated genes was determined by western immunoblot analysis (B). Scale bar=20 μm.

RESULTS

Changes in expression levels of differentiation-associated genes in keratinocyte following Vit C

Previous studies have shown high Ca^{2+} and/or Vit C to alter gene expression associated with KC differentiation (Eichner *et al*., 1984; Simon and Green, 1985; Mehrel *et al*., 1990). To evaluate our culture system for KC differentiation induction, we first measured expression of previously identified differentiation marker genes *e.g*., keratins, involucrin and loricrin, in two distinct KC differentiation stages: 1) incubation with high Ca^{2+} (early differentiation stage) and 2) incubation with high Ca^{2+} and Vit C (late/terminal differentiation stage). Our western blot analysis showed that a high Ca^{2+} environment induced expression of keratin 5 and 10 (Fig. 1B). This increased expression was not seen in KC incubated in high Ca^{2+} in combination with Vit C (Fig. 1B). Furthermore, our data shows that an environment of high Ca^{2+} and Vit C, representing a late KC differentiation stage, also induced expression of both involucrin and loricirin (Fig. 1B). Expression of these latter proteins was not seen in KC incubated solely in a high Ca^{2+} environment (Fig. 1B). Moreover, KC at the early differentiation stage induced by high Ca^{2+} grew as a monolayer (Fig. 1A). However, cells cultured in high Ca^{2+} and Vit C grew as multilayered sheets with thicker cell layer, which is morphological features of terminal differentiated KC (Fig. 1A). Together, these results are consistent with previous studies, which showed a reduction in keratin expression and an increase in expression of involucrin and loricrin as KC become terminally differentiated (Eichner *et al*., 1984; Simon and Green, 1985; Mehrel *et al*., 1990).

Table 1. Increased ceramide content in human KC following Vit C

Treatment	Ceramide (pmol/mg protein ± SD)
Vehicle	1,086.01 ± 92.14
Vit C	1,431.34 ± 118.44*

All value are mean ± SD.
*Significantly different from Vehicle. $p<0.01$ (n=3).

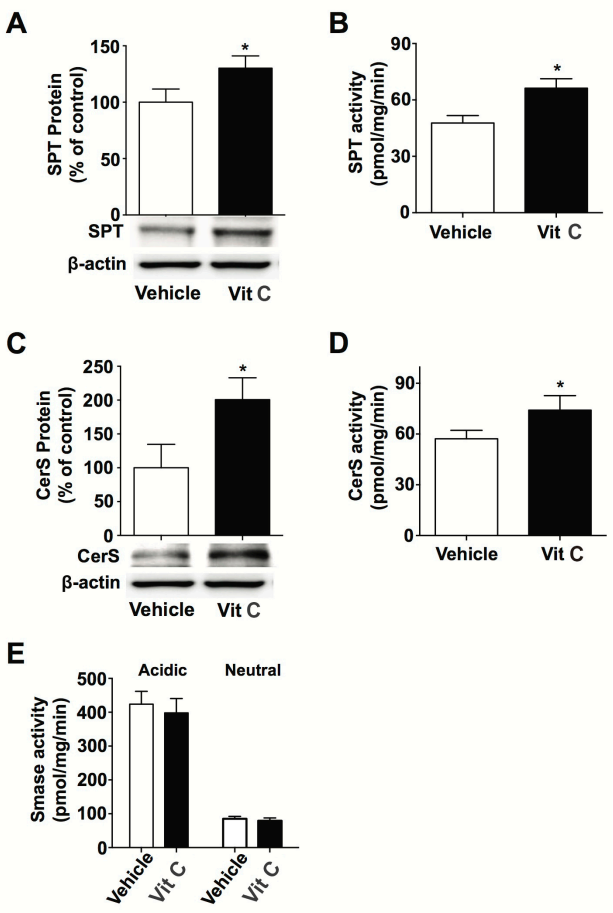

Fig. 2. Vit C treatment alters expression and activity of ceramide metabolic enzymes. Human primary keratinocytes (KC) were cultured in high Ca^{2+}-supplemented medium with or without Vit C, as described in materials and methods. Protein expressions of serine palmitoyltransferase (SPT) (A) and ceramide synthase (CerS) (C) were measured by western immunoblot analysis. Enzyme activities of SPT (B), CerS (D), and sphingomyelinases (SMases) (E) were analyzed by either HPLC or ESI-LC/MS/MS systems. Data are means ± SD (n=3). *$p<0.01$ *versus* vehicle control.

Vit C increases ceramide content by the action of ceramide metabolic enzymes

We also assessed ceramide content in KC incubated with Vit C. Our lipid analysis data shows that Vit C treatment significantly increases ceramide content (Table 1). To investigate the metabolic step(s) at which Vit C exhibits its effects on ceramide synthesis, we next measured the expression and activity of key enzymes that are involved in ceramide

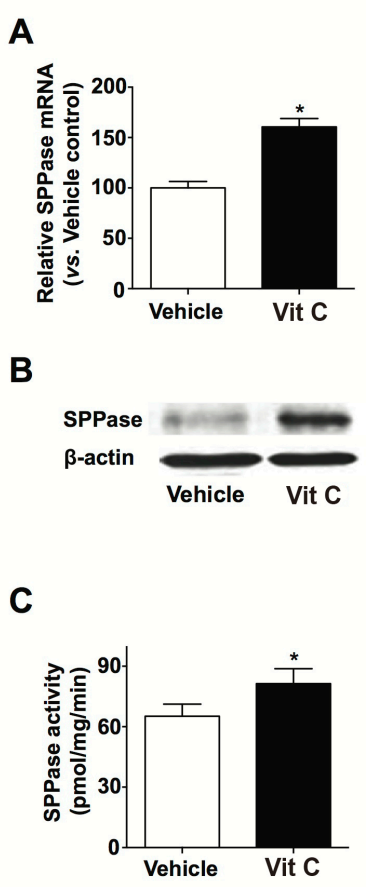

Fig. 3. Increased expression and activity of sphingosine-1-phosphate phosphatase in KC following Vit C. Human primary keratinocytes (KC) were cultured in high Ca^{2+}-supplemented medium with or without Vit C, as described in materials and methods. Sphingosine-1-phosphate phosphatase (SPPase) mRNA expression was determined by *q*RT-PCR (A). Protein expression of SPPase protein was determined by western immunoblot analysis (B). SPPase activity was analyzed by ESI-LC/MS/MS (C). Data are means ± SD (n=3). **p*<0.01 *versus* vehicle control.

metabolism, e.g., SPT, CerS, SMase, SPPase, following Vit C treatment. Western blot and enzyme assays showed that both expression and activity of SPT and CerS was significantly increased in cells treated with Vit C (Fig. 2A-2D). Whereas, acidic and neutral SMase activities were not affected by Vit C treatment (Fig. 2E). Furthermore, prior studies have suggested that SPPase expression is a critical factor in regulating ceramide levels. As such, we measured SPPase mRNA and protein expression in KC cultured in Vit C (Fig. 3). Our *q*PCR and western blot analyses showed that Vit C supplementation significantly increased both SPPase mRNA and protein expression. These results show that altered expression/activity of several ceramide metabolic enzymes account for increased ceramide production that is stimulated by Vit C.

DISCUSSION

Of the lipids enriched in the extracellular lamellar membranes, ceramide is the most dominant lipid, which ultimately comprise 50% of total lipid mass in the SC (Elias and Menon, 1991). Prior studies have highlighted the importance of the respective lipid in maintaining barrier function: i) epidermal ceramide synthesis is required for normal barrier recovery after barrier disruption (Holleran *et al*., 1991), ii) a decrease in content of epidermal ceramide occurs in multiple skin disorders that show barrier abnormalities, *e.g*., atopic dermatitis and psoriasis (Imokawa *et al*., 1991; Motta *et al*., 1994), iii) epidermal ceramide, in particular ω-OH-ceramide, is attached to the CE, forming CLE, a structure that is required for normal barrier function (Wertz and Downing, 1986). We show here that Vit C supplementation increases epidermal ceramide content *via* specifically increasing the enzymatic activity and expression of SPT, CerS, and SPPase.

It is well established that ceramide content rises in parallel with KC differentiation (Uchida *et al*., 2001a), the latter being directly modulated by Ca^{2+}. The data in our study is in line with these findings. However, we show that high Ca^{2+} supplementation (vehicle-treated) has no effect on expression of involucrin and loricrin. Expression of these proteins, which are vital to forming the CE and thus contribute greatly in maintaining the epidermal barrier (Eichner *et al*., 1984; Simon and Green, 1985; Mehrel *et al*., 1990), was instead stimulated by Vit C.

Prior studies have demonstrated that overall epidermal ceramide production is stimulated through *de novo* ceramide synthesis pathway by the action of key enzymes, *i.e*., SPT and CerS, respectively (Holleran *et al*., 1990; Uchida *et al*., 2001a). Consistent with these findings, Vit C-induced elevation in ceramide production appears to be regulated by increased expression/activity of these enzymes. In addition to *de novo* synthesis pathway, SM hydrolysis is also critical in epidermal production of ceramide (Uchida *et al*., 2000). SM in human skin is hydrolyzed to ceramide by at least two isoenzymes, a lysosomal type acid-pH optimum SMase (aSMase) and a non-lysosomal, magnesium-dependent neutral-pH optimum SMase (nSMase) (Uchida *et al*., 2000). However, in this study, Vit C treatment did not alter the activity of either aSMase or nSMase, suggesting that constitutive levels of SMases are likely suffice to stimulate ceramide production in the epidermis.

Moreover, we show here that Vit C-mediated increase in both expression and activity of SPPase, an enzyme which dephosphorylates S1P to SO, contributes, at least in part, to increased epidermal ceramide synthesis. Previous studies using HEK-293 (human embryonic kidney cells) and NIH 3T3 fibroblasts have provided evidence for the important role of SPPase in regulating ceramide levels; i) SPPase regulates ceramide levels in the endoplasmic reticulum (Le Stunff *et al*., 2004), ii) overexpression of SPPase in cells significantly increased ceramide levels (Le Stunff *et al*., 2002), iii) exogenous S1P treatment further increased all ceramide species in SPPase-overexpressing cells (Giussani *et al*., 2006). In agreement with these findings, our results indicate that both expression and activity of SPPase contribute in part to elevating ceramide production in KC following Vit C supplementation.

In conclusion, the present studies show that Vit C supplementation increases epidermal ceramide production by three pathways (Fig. 4): 1) increased *de novo* ceramide synthesis pathway due to activations of SPT and CerS; 2) SM hydrolysis *via* substrate regulation of constitutively expressed SMases;

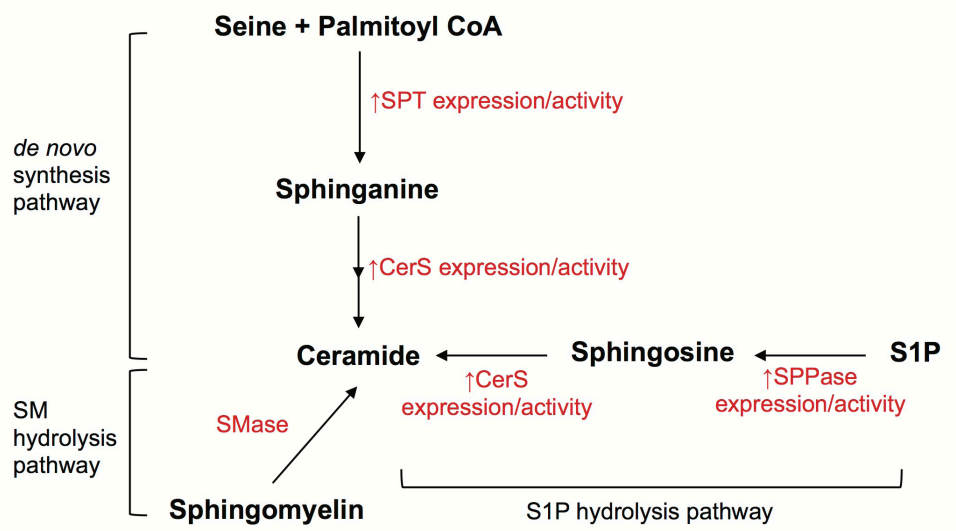

Fig. 4. Proposed mechanism of Vit C-mediated increase in ceramide production in human keratinocyte (KC). Vit C supplementation increases epidermal ceramide production by three pathways: i) increased *de novo* ceramide synthesis pathway due to activations of serine palmitoyltransferase (SPT) and ceramide synthase (CerS); ii) sphingomyelin (SM) hydrolysis *via* substrate regulation of constitutively expressed sphingomyelinases (SMases); iii) increased sphingosine-1-phosphate (S1P) hydrolysis by sphingosine-1-phosphate phosphatase (SPPase) to produce sphingosine (SO), followed by ceramide synthesis by CerS.

3) increased S1P hydrolysis by SPPase to produce SO, followed by ceramide synthesis by CerS. Taken together, our studies suggest that Vit C could be utilized to enhance epidermal barrier function in skin disorders like psoriasis and atopic dermatitis, which are characterized by a significant reduction in epidermal ceramide content.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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