Increasing effect of ceramides in skin by topical application of sphingosine

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Abstract

Introduction and objective: Ceramidase metabolizes ceramide and generates sphingoid and fatty acid. Alkaline ceramidase has been involved in reduction of the ceramide level in atopic dermatitis or in aged dry skin. We hypothesized that the ceramide content can be increased by inhibition of the ceramidase activity. In this study, we aimed to examine the effect of alkaline ceramidase inhibitor on the amount of the ceramide in mice skin and in three-dimensional cultured epidermis model.

Material and methods: 4-Nitrobenzo-2-oxa-1,3-diazole-labeled ceramide was used as a substrate and was incubated with skin homogenate at 37°C for 1 or 10 h. Fluorescent-labeled fatty acid, enzymatic reaction product was detected by fluorescence high performance liquid chromatography to determine the alkaline ceramidase activity. The ceramide content was quantitatively analyzed by high performance thin layer chromatography.

Results: 50% Inhibitory concentration of sphingosine for alkaline ceramidase in mice skin and three-dimensional cultured epidermis model was >70-fold lower than the corresponding oleoylthanolamidine. Ceramide [AS] and ceramide [AP] contents of sphingosine-applied group of mice stratum corneum were significantly higher compared with the normal group. CER [NDS] in three-dimensional cultured epidermis model was also significantly increased in sphingosine-applied group.

Conclusion: This study demonstrated that sphingosine inhibited alkaline ceramidase in mice skin and in three-dimensional cultured epidermis model and ceramide contents was increased by application of sphingosine. These results suggest that sphingosine was beneficial compound to increase skin ceramide, though sphingosine may also be affected by another pathway.
NBD-CER was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Oleoyl ethanolamide (OEA) was obtained from Enzo Biochem, Inc. (New York, NY, USA). D-Erythro-sphingosine was obtained from Wako Pure Chemical Industries (Osaka, Japan). CE determination for quantification was obtained from Matranya, LLC (Pleasant Gap, PA, USA). Eton Industries (Essen, North Rhine-Westphalia, Germany), and Takasago International Corporation (Tokyo, Japan). Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were kindly donated by Nippon Fine Chemical (Osaka, Japan). Other reagents were obtained from Wako Pure Chemical Industries, Daichi Sankyo Co., Ltd. (Tokyo, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Alfa Aesar (Heysham, Lancashire, UK) and Sigma-Aldrich Co., LLC. (St. Louis, MO, USA).

Preparation of homogenate

Mice were killed by cervical dislocation and dorsal skin was obtained. Subcutaneous tissue was removed by scissors. After weighing, the skin was minced using scissors for 3 min on ice and was homogenized in 0.25 M sucrose, supplemented with 1% Triton X-100 using Polytron PT 1200E homogenizer (Kinematica AG, Luzernerstrasse, Lucern, Switzerland) at 25,000 rpm for 1 min on ice. The homogenate was centrifuged at 500×g at 4°C for 1 min and the supernatant was stored at −30°C until use. Three-dimensional cultured epidermal models were collected from transwell by spatula and homogenized, as described above. Protein concentration was determined by a modified Lowry method.

Measurement of the alkCDase and aCDase activity

The CDase activity was measured by the method of Houben et al. [1]. NBD-Cer was used as substrate for CDase. An appropriate amount of NBD-Cer, with final concentration of 250 μM, was dissolved in methanol and allowed to dry under nitrogen gas. The substrate was redissolved by sonication (50°C, 10 min) in 12.5 μl of detergent mixture containing 40 μg/ml Triton X-100, 16 mg/ml Tween 80, and 25 μl of 250 mM Tris-HCl buffer (for alkCDase, pH 8.5) or acetate buffer (for aCDase, pH 4.2). Mice skin or three-dimensional cultured epidermis model homogenate was preincubated at 37°C for 2 min. Enzymatic reactions were initiated by the addition of 12.5 μl of 60 ng protein/ml homogenate, and the final concentration was 15 ng protein/ml to the substrate solution at 37°C. Incubation time was 1 h for mice skin or 10 h for three-dimensional cultured epidermis model. The reaction was stopped by addition of 300 μl of chloroform/methanol (2:1) that contained 0.26 μM NBD-Cer as internal standard for HPLC analysis. The reaction mixture was dried under nitrogen gas and dissolved in 100 μl of methanol. HPLC system (SHIMADZU Prominence HPLC system, SHIMADZU Corp., Kyoto, Japan), equipped with a reversed phase L-Column 2 ODS (5 μm, 2.1×150 mm; Chemicals Evaluation and Research Institute, Tokyo, Japan), and a fluorescence detector (RF-10AXL, SHIMADZU) set to excitation and emission wavelengths of 460 and 534 nm, respectively, were used to determine NBD-Cer in the enzymatic reaction sample. Column was maintained at 30°C. The mobile phases were 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). A binary mobile phase at a flow rate of 0.4 ml/min was used under linear gradient conditions as follows: 0 min, 30% A; 0–5 min, 30%–100% A; 5–14.5 min, 100% A; 14.5–15.1 min, 30% A; 14.51–30 min, 30% A. Standard curve of peak height ratio (NBD-Cer vs. internal standard) was used to determine NBD-Cer that was formed during the enzymatic reaction. To correct the CDase activity values, the NBD-Cer content in an incubated reaction mixture without protein was subtracted from that in a reaction mixture with protein. To assess inhibitory effect, SPH dissolved in methanol was added to the substrate and dried. The inhibitory activity was measured as described above. OEA, which has the inhibitory activity for alkCDase and aCDase [1], was used as a positive control of CDase inhibitor.

Application of SPH and extract of SC lipids in mice

SPH and OEA were dissolved in propylene glycol/ethanol (7:3; concentration, 40 mM) [13]. Samples of 50 μl were applied twice a day for three days to the dorsal skin (area, 5–6 cm²) of mice. Control group was exposed to propylene glycol/ethanol (7:3). SC sheet was collected by stripping the dorsal skin area with applied sample with cyanoacrylate resin and was stored frozen at −80°C until use. Lipids were extracted by the method of Imokawa et al. [4]. SC sheets were immersed in hexane/ethanol (95:5) and sonicated for 20 min. Solutions of extracted lipids were filtered (MILLEX-GV Syringe Driven Filter Unit, Merck Millipore, MA, USA) and transferred to test tubes. Organic solvent was dried under nitrogen gas stream at 50°C. Lipid in test tube was dissolved in chloroform/methanol (2:1) and sonicated at 37°C for 5 min. Extract was transferred to a 1.5 ml tube and dried. SC lipid samples were stored frozen at −80°C until use.

Application of SPH and extraction of epidermal lipids for three-dimensional cultured epidermis model

Liposome structured SPH/DPPC/DPPG (molar ratio 4:4:1) was prepared by the thin layer method previously described [14]. SPH concentration in a liposome was 10 mM. Three-dimensional cultured epidermis model was cultured at 37°C under 5% CO₂. A 50 μl solution of liposome containing SPH was applied to the SC side of three-dimensional cultured epidermis model for 6 days and was changed every 2 days. Medium was changed every day for 6 days and was supplied with 25 μg/ml ascorbic acid on the seventh day. After 24 h, three-dimensional cultured epidermis model was collected from transwell by scalpel. Control group was applied with SPH-free liposome (DPPC/DPPG, 4:1). Three-dimensional cultured epidermal samples were immersed in chloroform/methanol (2:1) and sonicated by SONIFIRE 250 (Branson, Danbury, CT, USA) on ice. Lipid collection was conducted as described above.

Quantification of the CER

The CER was quantified by the method of Imokawa et al. [4]. Extracted lipids were redissolved in chloroform/methanol (2:1) and 10 μl of extract was spotted by capillary on the high-performance thin-layer chromatography (HPTLC) Silica gel 60, measuring 20×10 cm (Merck Millipore). The CER was isolated by chloroform/methanol/ acetic acid (190:91 to 9 cm) and was developed twice. The HPTLC plate was sprayed with color reagent (10% cupric sulfate, 8% phosphoric acid) and heated at 180°C for 10 min by TLC Plate Heater III (CAMAG, Muttenz, Switzerland). HPTLC plate was imaged by LAS1000-plus (Fujifilm Corporation, Tokyo, Japan) and the CER was quantitated from band density by Multi Gauge Version 3.0 (Fujifilm Corporation).

Data analysis

Data were expressed as the mean ± S.D. (n=4) and compared by Turkey’s test, Dunnett’s test, or Student’s t-test using SAS version 9.2 (SAS Institute ltd., Cary, NC, USA).
Results

The CDase activity in mice skin

Activity of alkCDase in the presence of 10 mM SPH on mice skin homogenate was measured. Compared with the normal group, the alkCDase activity was significantly reduced by 46% in OEA (positive control) group (p<0.05) and 88% in SPH group (p<0.001). The alkCDase activity in the SPH group was significantly decreased by 78% (p<0.05) relative to the OEA group (Figure 1A). Similarly, the acDase activity in the SPH group had a tendency to be lower than that in normal group, in spite of no significant (Figure 1B). Subsequently, the inhibitory activity of SPH on the alkCDase was determined. SPH and OEA dose-dependently inhibited the alkCDase activity (Figure 2). The IC_{50} value of SPH was significantly lower than that of OEA (p<0.001) (Table 1).

The CER content in mice SC

The CER content in mice SC was measured after application of 40 mM SPH or OEA. CER [NS] and CER [NP] contents were not changed by the application of OEA or SPH (Figure 3A and 3B). In contrast, CER [AS] in the SPH group was significantly increased relatively to the normal group (p<0.05) (Figure 3C). CER [AP] content was also significantly increased by application of SPH when compared with the normal group (p<0.001) (Figure 3D).

The alkCDase activity in three-dimensional cultured epidermis model

Activity of alkCDase in the presence of 10 mM SPH on the three-dimensional cultured epidermis model homogenate was measured. Compared with the normal group, the alkCDase activity was significantly decreased by 56% in OEA group and 83% in SPH group (p<0.001). The alkCDase activity in the SPH group was significantly reduced by 62% (p<0.01) relatively to the OEA group (Figure 4). SPH and OEA inhibited alkCDase in a dose-dependent manner (Figure 5). In agreement with the animal experiment, the IC_{50} value of SPH for alkCDase was significantly reduced compared with that of OEA (p<0.01) (Table 2).

The CER content in three-dimensional cultured epidermis model

The CER content was measured after application of liposome containing 10 mM SPH. SPH did not change the amounts of CER [NS], CER [NP], CER [AS], and CER [AP] (Figure 6A-6D). However, CER [NDS] was significantly increased in the SPH group in comparison with the normal group (p<0.001) (Figure 6E).

Discussion

First, the inhibitory activity of SPH for alkCDase was assessed by comparison with OEA (positive control). The IC_{50} values of SPH were lower than OEA (Tables 1 and 2). Mao et al. reported that the IC_{50} values of SPH were lower than OEA (Tables 1 and 2). Mao et al. reported that the IC_{50} values of SPH were lower than OEA (Tables 1 and 2). Mao et al. reported that the IC_{50} values of SPH were lower than OEA (Tables 1 and 2).
value of SPH for maCER1 (pH 8.0) was −0.08 mM [12]. The mice skin IC_{50} values of SPH and OEA were similar between mice skin and three-dimensional cultured epidermis model (Tables 1 and 2). These data indicated that SPH is a stronger inhibitor of alkCDase than OEA. Subsequently, mice SC CER after application of SPH or OEA was determined. SPH concentration of 40 mM was higher than the intracellular level. However, we considered that the SPH amount in the viable epidermis was low because SPH penetration was prevented by the SC barrier. CER in the SC was shown to be composed of 4 types of sphingoids; namely, SPH [S], dihydroxy sphingosine [DS], phytosphingosine [P], and 6-hydroxy sphingosine [H]; and 3 types of FAs; namely, non-hydroxy FA [N], α-hydroxy FA [A], and esterified ω-hydroxy FA [EO]. CER [AS] and CER [AP] were increased by application of SPH (Figure 3C and 3D). Mao et al. demonstrated that maCER1 (pH 8.0) had specificity for CER containing D-erythro-sphingosine, but CER had D-ribo-phytosphingosine and D-erythro-dihydroxy sphingosine was not hydrolyzed [12]. Thus, it is likely that degradation of CER [AS], which contains D-erythro-sphingosine, was suppressed by inhibition of alkCDase. However, CER [NS] that also contained D-erythro-sphingosine, was not increased (Figure 3A); whereas, CER [AP] that contained D-ribo-phytosphingosine was increased (Figure 3D). Therefore, we considered that a mechanism other than alkCDase inhibition is also related to the increase of the CER. Further, the CER content in three-dimensional cultured epidermis model after application of SPH was also quantitated. We prepared a liposome containing SPH for application to three-dimensional cultured epidermis model because SPH was a lipophilic compound. The CER content of the control group with SPH-free liposome (DPPC/DPPG, 4:1) was unchanged (Figure 6). Unlike the results on mice SC, CER [AS] and CER [AP] were unchanged; however, CER [NDS] was increased (Figure 6C-6E). Density of CER [NDS] in HPTLC analysis.
from mice SC was unchanged (data not shown). CER [NDS] density was increased, in spite of no significant, by application of liposome containing 1 mM SPH (data not shown). It was reported that CER [NP], CER [NH], and CER [AH] were popular classes (22.1, 14.5, and 10.8% respectively) among the total CER content of human skin and that CER [EOS] accounted for 6.5% [15]. Simotoyodome et al. also showed that CER [NP] and CER [NH] existed in abundance in human SC CER [16]. However, Liou et al. reported that CER [EOS] was dominant (approximately 75%) in nude skin CER [17]. These reports indicated that the generation profile of individual CER classes maybe different between human and mice. Hence, we thought that different CER classes were increased between mice SC and three-dimensional cultured epidermis model by the same compound. Duan et al. reported that the mRNAs of ceramide synthase (Cers) 2, CerS3, and CerS4 in human foreskin keratinocytes were upregulated by dihydrophosphogine, sphingosine, and 4,8-sphingadienine purified from maize glucosylceramide [18]. Shirakura et al. reported an increased mRNA expression of the enzyme related to CER production and that CER [EOS], CER [NS], and CER [NP] were increased by application of 4,8-sphingadienine and 4-hydroxy-8-sphinganine isolated from konjac glucosylceramide to three-dimensional cultured epidermis model [19]. Sigruener et al. also reported that sphingoids increased the mRNA level of sphingolipid metabolizes enzymes and the CER contents in human keratinocyte [20]. Thus, it is probable that de novo synthesis of CER was stimulated by SPH in our experiment. Sphingosine-1-phosphate (S1P) is generated from SPH by sphingosine kinase (SPHK) and induces keratinocyte differentiation [21]. Hong et al. demonstrated that specific differentiation-associated maker proteins in intact mice epidermis and HaCaT cells were increased by application of SPHK activator [22]. Thus, it is likely that SPH applied to mice or three-dimensional cultured epidermis model was metabolized to S1P and differentiation of keratinocyte was promoted; consequently, the CER was increased. It was known that a salvage pathway reconverted SPH to CER [23], but it was still unclear whether it contributed to skin barrier.

Conclusion

We revealed that SPH was a stronger inhibitor than OEA to increase the CER contents, indicating that SPH suppresses degradation of CER in SC by inhibition of alkCDase. However, SPH may also be affected by another pathway, like de novo synthesis. Thus, SPH may be a beneficial compound to increase skin CER.

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