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Effect of galactosylceramide on stratum corneum intercellular lipid synthesis in a three-dimensional cultured human epidermis model

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ABSTRACT

Intercellular lipids in the stratum corneum (SC), such as ceramide (CER), free fatty acid (FFA), and cholesterol (CHOL), contribute to the formation of stable lamellar structures in the SC, making them important for skin barrier function. β-Galactosylceramide (GalCer) is a glycosphingolipid that is used in some cosmetics and quasi-drugs in anticipation of a moisturizing effect. GalCer promotes keratinocyte differentiation and increases CER production by increasing β-glucocerebrosidase (β-GCase) activity. However, few reports have described the mechanism of these effects, and detailed studies on the role of GalCer in intercellular lipid production in the SC have not been conducted. This study investigated the effect of GalCer on the metabolism and production of intercellular lipids in the SC in a three-dimensional cultured epidermis model. After reacting GalCer with a homogenate solution of three-dimensional cultured epidermis, GalCer was hardly metabolized. Treatment of the three-dimensional cultured epidermis with GalCer increased the expression of genes involved in the β-GCase metabolic pathway and promoted CER production. In addition, GalCer treatment reduced the expression of FFA metabolism-related genes as well as palmitic acid levels. In addition, transepidermal water loss, which is a barrier index, was reduced by GalCer treatment. These findings suggested that GalCer, which is hardly metabolized, affects the production of intercellular lipids in the SC and improves skin barrier function.

1. Introduction

The epidermis is the outermost layer of the skin. Skin barrier formation in mammalian epidermis requires the organization of a group of three lipids, ceramide (CER), free fatty acid (FFF), and cholesterol (CHOL), into extracellular lamellar membrane structures within the stratum corneum (SC) interstices [1,2]. Keratinocytes in the stratum granulosum contain lateral and lamellar lipid structures termed lamellar bodies, which store lipids such as glucosylceramide (GlcCer), sphingomyelin (SM), and phospholipids. These are precursors of the SC lipids, and they are enzymatically processed into their final constituents, namely CER and FFA. CER and FFA are, together with CHOL, the main lipid classes in the SC. Although the three key lipids are present in an approximately equimolar ratio, CER predominates by weight, accounting for approximately 50% of SC lipids [3,4]. CER is involved in water retention and skin barrier function. In addition, CER is implicated in the induction of cellular stress responses, including accelerated differentiation, apoptosis, and senescence [5–8]. The *de novo* biosynthesis of CER mainly occurs in the endoplasmic reticulum, and the synthesized CER is then transported to the Golgi apparatus, in which SM and GlcCer are synthesized. Thereafter, GlcCer and SM accumulate in lamellar granules. GlcCer and SM are transported and secreted to the interface of the stratum granulosum and SC, at which they are converted back to their CER species via hydrolysis by β-glucocerebrosidase (β-GCase) and acid sphingomyelinase (aSMase), respectively [9–11]. Through the action of ceramidases, CER is catabolized via deacylation to produce sphingosine and FFA. CER is a type of sphingolipid composed of a sphingoid base and fatty acids, and CER in human SC consists of 12 species generated through combinations of four sphingoid bases and three fatty acids [12]. All SC CER species can be derived from GlcCer, but only two SC CER species, N-nonhydroxyacyl sphingosine (NS) and N-alpha (α)-hydroxyacyl sphingosine (AS), are generated to a significant degree from SM [9,13]. Such various CER species contribute to the formation of stable lamellar structures in the SC together with CHOL and FFA.

Intracellular lipids in the SC such as CER and FFA are greatly involved in skin barrier function. Decreased levels of CER and FFA in the SC lead to skin barrier dysfunction. Impairment of skin barrier function can cause or aggravate skin disorders, including dry skin, psoriasis, atopic dermatitis, and ichthyosis [14–17]. The skin of patients with

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Abbreviations

GalCer	β-Galactosylceramide,
β-GCase	β-glucocerebrosidase
CER	ceramide,
COL:	cholesterol
FFA	free fatty acid

atopic dermatitis and psoriasis exhibits markedly decreased CER levels in the SC [18,19]. In addition, Gaucher disease with congenital β-GCase deficiency impairs skin barrier function by reducing CER production due to GlcCer accumulation [20]. CER levels in the SC fluctuate depending on factors such as aging and season [18,21]. In one study, barrier function was recovered by applying a CER-containing cream to the skin of a patient with atopic dermatitis [22]. Therefore, controlling CER content in the skin can be expected to improve skin diseases and skin barrier function. However, it is not easy to formulate CER because of its high hydrophobicity. Our group previously reported that the application of SM and GlcCer liposomes as precursors of CER to a three-dimensional cultured human epidermis model increased CER levels [23,24]. In addition to CER, FFA chain length in the SC is important for maintaining skin barrier function [25]. The reduction of FFA chain length is associated with reduced CER chain length, and reduced lipid chain length is correlated with a less dense lipid organization and decreased skin barrier function [26]. The skin of patients with atopic dermatitis exhibits elevated levels of FFA containing more than 24 carbon atoms, whereas the levels of C16 and C18 FFA are diminished [25]. In addition, FFA levels are reduced in the skin of patients with psoriasis [27].

β-Galactosylceramide (GalCer) is a glycosphingolipid with a lipophilic sphingosine and a hydrophilic galactose moiety attached via an ether linkage to sphingosine. GalCer is abundantly present in the myelin sheath of the central and peripheral nervous systems [28,29]. GalCer induces keratinocyte differentiation and enhances CER production by increasing the activity of β-GCase [30,31]. However, few reports described the mechanism of these effects, and no detailed study of CER production induced by GalCer has been reported. In addition, the effect of GalCer on the production of CHOL and FFAs is unknown. Therefore, the effects of GalCer on enzyme activity were evaluated in a three-dimensional cultured epidermis model, and the effects of GalCer on intercellular lipid production in the SC were investigated using the same model.

2. Materials and methods

2.1. Materials

GalCer from the horse spine was obtained from Zenyaku Kogyo Co., Ltd. (Tokyo, Japan). C12-6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino (NBD)-CER, C12-NBD-GalCer, C12-NBD-GlcCer, and C12-NBD-FFA were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Hydroxy ceramide and non-hydroxy ceramide were purchased from Matreya (Pleasant Gap, PA, USA). Ceramides NP and AP were obtained from Evonik (Essen, Germany). Conduritol B epoxide (CBE) was purchased from Santa Cruz Biotechnology Inc. (Delaware, CA, USA). L-Ascorbic acid phosphate magnesium salt n-hydrate was purchased from FUJIFILM Wako Pure Chemical Co. Ltd. (Osaka, Japan). alamarBlue™ Cell Viability Reagent was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Palmitic acid (PA) was purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA). RNAiso Plus, PrimeScript® RT reagent kits, and SYBR® Premix Ex Taq™ were purchased from Takara Bio Inc. (Kusatsu, Shiga, Japan). All other chemicals and solvents were of analytical grade.

2.2. Preparation of three-dimensional cultured skin epidermis homogenate

LabCyte EPI-MODEL 6D was obtained from Japan Tissue Engineering Co. Ltd. (Gamagori, Aichi, Japan). The three-dimensional cultured human epidermis was minced using scissors for 2 min on ice and homogenized in 0.25 M sucrose solution (dissolved in 0.6% sodium taurocholate and 0.25% Triton X-100 in 50 mM phosphate-citrate buffer [pH 5.0]) using Polytron PT 1200 E homogenizer (Kinematica AG, Luzernerstrasse, Luzern, Switzerland) at 12,500 rpm for 2 min on ice. Each homogenate was centrifuged at 600×*g* for 10 min and 4 °C, and the supernatant was considered a sample.

2.3. Metabolic response of GalCer in three-dimensional cultured skin epidermis homogenates

C12-NBD-GlcCer and C12-NBD-GalCer were dissolved in chloroform at a final concentration of 2.5 μM and dried under N_2 gas. The samples were redissolved in three-dimensional cultured skin epidermis homogenates and incubated for 24 or 72 h at 37 °C. Cell survival rates were measured using the alamarBlue assay. The reaction was stopped by adding chloroform:methanol (2:1 v/v). C12-NBD-GlcCer, C12-NBD-GalCer, C12-NBD-CER, and C12-NBD-FFA levels produced via the enzymatic reaction were measured using HPLC.

2.4. HPLC analysis of NBD lipids

HPLC was performed according to the method of Houben et al. [32]. HPLC was performed on Shimazu (Kyoto, Japan) LC20A system equipped with HPLC pump (LC-20AD), vacuum degasser for liquid chromatography (DGU-20AD), autosampler (SIL-20AC) and column oven (CTO-20AC). System parameters were controlled with system controller (CBM-20A). HPLC analysis of the NBD lipids was performed using a reverse-phase L-Column 2 ODS (5 μm, 2.1 × 150 mm; Chemicals Evaluation and Research Institute, Tokyo, Japan) (column oven temperature at 40 °C) and a fluorescence detector (RF-10XL fluorescence detector, Ex: 460 nm, Em: 534 nm). Mobile phases A (0.1% formic acid in acetonitrile) and B (0.1% formic acid in water) were used under binary linear gradient conditions. The gradient profile and run time were the same with each sample, the linear gradient from A/B = 30/70 to 100/0% was applied from 0 to 10 min. From 10 to 19.5 min, the mobile phase composition was constant A/B = 100/0%. From 19.5 to 23 min the mobile phase composition was change to A/B = 30/70%. From 23 to 30 min, the mobile phase composition was constant A/B = 30/70%. The flow rate was maintained at 0.4 mL/min throughout the assay. The sample injection volume was 10 μL. The calibration curve was prepared by analyzing under the same conditions at concentrations of NBD-GalCer, NBD-GlcCer, NBD-CER and NBD-FFA of 0, 0.25, 0.5, 0.75 and 1 μM, respectively.

2.5. Application of GalCer to the three-dimensional cultured human epidermis model

The three-dimensional cultured human epidermis was cultured at 37 °C in a 5% CO_2 atmosphere. The culture medium was obtained from Japan Tissue Engineering Co. Ltd. 1% GalCer solution (solvent composition: 73.9% water, 25.0% 1,3-butanediol, and 0.1% xanthan gum) and 1 mM CBE were applied to the SC side of the three-dimensional cultured human epidermis model followed by incubation at 37 °C for 2 or 14 days. Medium and 1% GalCer were changed every day. Twenty-four hours before lipid extraction, the medium was changed to L-ascorbic acid phosphate magnesium salt n-hydrate–containing medium to enhance keratinization. Transepidermal water loss (TEWL), as an indicator of skin barrier function, was measured using VAPO SCAN AS-VT100RS (Asch Japan Co., LTD., Tokyo, Japan). TEWL was measured after carefully removing the sample solution and allowing it to stand at

room temperature for 1 h.

2.6. RNA extraction and quantitative real-time PCR

1% GalCer was applied to the SC side of the three-dimensional cultured human epidermis model followed by 48 h incubation at 37 °C. Total RNA was then extracted using RNAiso Plus. cDNA was synthesized from total RNA by reverse transcription using a Prime Script™ reagent kit. Real-time PCR was performed using SYBR Premix Ex Taq™ and the Step One Plus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). mRNA expression was measured as relative values versus the control group (application of vehicle for 24 h). The following primers (Thermo Fisher Scientific) were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5′-GAAGGTGAAGGTCGGAGT-3′ (forward) and 5′-GAAGATGGTGATGGGATTTC-3′ (reverse); serine palmitoyltransferase 1 (SPT1), 5′-AGGGTTCTATGGCACATTTGATG-3′ (forward) and 5′-TGGCTTCTTCGGTCTTCATAAAC-3′ (reverse); CER synthase 3 (CerS3), 5′-ACATTCCACAAGGCAACCATTG-3′ (forward) and 5′-CTCTTGATTCCGCCGACTCC-3′ (reverse); GlcCer synthase (GCS), 5′-GCGTGTTATCCATGTGATGCTTG-3′ (forward) and 5′-TGGGCTGGCTCAGTAAGATGAA-3′ (reverse); β-GCase, 5′-GCTAGGCTCCTGGGATCGAG-3′ (forward) and 5′-GTTCAGGGCAAGGTTCCAGTC-3′ (reverse); SM synthase 1 (SMS1), 5′-GCCAGGACTTGATCAACCTAACC-3′ (forward) and 5′-CCATTGGCATGGCCGTTCTTG-3′ (reverse); SMS2, 5′-CACCCAGTGGCTGTTTCTGA-3′ (forward) and 5′-TGCATTCCAGGCACAGGTAGA-3′ (reverse); aSMase, 5′-ACTTTGATAACTGCTCCTCTGAC-3′ (forward) and 5′-TTCGTGTCCAGCAGAGTACC-3′ (reverse); peroxisome proliferator-activated receptor γ (PPARγ), 5′-ATTCTGGCCCACCAACTTTG-3′ (forward) and 5′-TCCATTACGGAGAGATCCACG-3′ (reverse); sterol regulatory element binding protein 1α (SREBP1α), 5′-GGTACCTGCAGCTGCTGA-3′ (forward) and 5′-TCAGGCTGGCTGGACCA-3′ (reverse); acetyl-CoA carboxylase (ACC), 5′-CCACTTGGCTGAGCGATT-3′ (forward) and 5′-CCAGGTCCTCCAGCAGAA-3′ (reverse); fatty acid synthase, 5′-CCGAGGAACTCCCCTCAT-3′ (forward) and 5′-GCCAGCGTCTTCCACACT-3′ (reverse); and carnitine palmitoyl-CoA transferase I (CPT-1), 5′-AAAAGCAGACCTGCGAGA-3′ (forward) and 5′-TCGCGGATGTGGTTTCCA-3′ (reverse). The mRNA expression level of each target gene was normalized to that of GAPDH mRNA and calculated using the ΔΔCt method.

2.7. Lipid extraction

Lipids in the three-dimensional cultured human epidermis model were extracted according to the methods of Bligh and Dyer [33]. Briefly, the three-dimensional cultured human epidermis model was dissolved in chloroform:methanol (2:1 v/v) and sonicated using a probe-type sonicator (Advanced Sonifier Model 250A, Branson, Danbury, CT, USA; 50 W, 10 min) on ice. The extracted lipid solution was dried under N_2 gas and dissolved in chloroform:methanol (2:1 v/v). The presence of the various CER species was determined via high-performance TLC (HPTLC).

2.8. HPTLC analysis of lipid levels

HPTLC was conducted to quantify CER levels using the method of Imokawa et al. [18], as described previously [34]. HPTLC to quantify FFA and CHOL levels was conducted using the method of Ohta and colleagues [35]. Lipid samples (10 μL) were dissolved in chloroform: methanol (2:1) and spotted onto the HPTLC plate (HPTLC Silica gel 60, Merck, Darmstadt, Germany). CER was developed in chloroform:methanol:acetic acid (190:9:1). SM was developed in chloroform:methanol: acetic acid:water (50:30:8:4). PA and CHOL were developed in hexane: diethyl ether:citric acid (80:20:1). The various lipids were visualized by treatment with a 10% $CuSO_4$ and 8% H_3PO_4 aqueous solution and heated to 180 °C for 10 min. The amount of each lipid present was quantitatively determined using a densitometer. Quantitative results for all lipids were related to the calibration curves of the standard substances CER [NS], CER [NP], CER [AS], CER [AP], SM, FFA and CHOL. The reference lipids were applied for absolute quantification in a concentration range from 0.5 to 5 μg/spot for CERs and in a concentration range from 0.5 to 10 μg/spot for FFA and CHOL. Stock solutions of all lipids were prepared in chloroform:methanol (1:1). The different concentrations of stock solution 10 μL were spotted on a HPTLC plate to obtain the concentrations 0.5, 1.0, 2.5, 5.0 for CERs and 0.5, 1.0, 2.5, 5.0, and 10 ng/spot for FFA and CHOL, respectively.

2.9. Data analysis

Analyses were performed using JMP® PRO 14 statistical software (SAS Institute, Cary, NC, USA). The indicated P-values were calculated using Tukey's post-hoc multiple comparison test.

3. Results

3.1. The metabolism of GalCer in the LabCyte EPI-MODEL homogenate

To evaluate the metabolism of GalCer in the epidermis, LabCyte EPI-MODEL homogenate and NBD-GalCer were mixed and incubated at 37 °C for 24 h. NBD-GlcCer was used as a positive control for metabolism. In total, 85.4 and 6.2% of the GlcCer input were metabolized to CER and FFA, respectively, whereas 8.4% of the input was not metabolized. Conversely, GalCer was slightly metabolized to FFA (7.5%), but most of its content remained unchanged (Fig. 1A). Next, the same examination was performed using a reaction time of 72 h. However, GalCer was hardly metabolized even with the extended reaction time (Fig. 1B).

3.2. Effects of GalCer on SC intercellular lipid synthesis and evaluation of barrier function in LabCyte EPI-MODEL

To investigate the effects of GalCer on lipogenesis, 1% GalCer was applied from the insert side of LabCyte EPI-MODEL. Fourteen days after the addition of GalCer, cell viability was determined using the alamarBlue assay. The viability of cells treated with GalCer (101.8%), CBE (99.1%), or both (105.8%) was not changed relative to the control (100.0%, Fig. 2).

Intracellular CER content in LabCyte EPI-MODEL were measured via HPTLC. GalCer treatment for 14 days increased CER [NS] and CER [APb] content compared with the effect of vehicle (Fig. 3A and E). In addition, the increase of CER [NS] levels following GalCer treatment

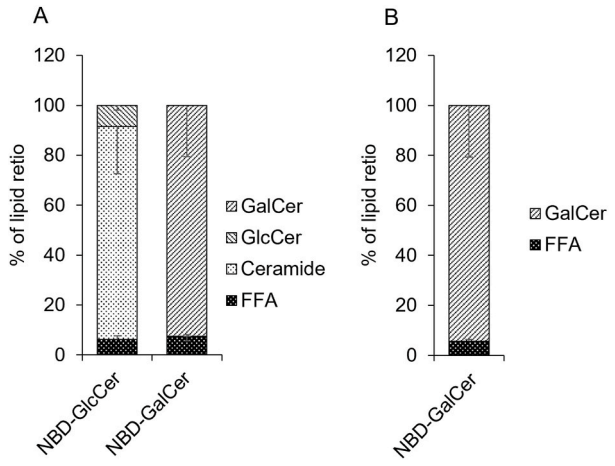

Fig. 1. Metabolism of C12-NBD-GalCer in the three-dimensional cultured human epidermis model. The metabolism of NBD-GlcCer and NBD-GalCer in the homogenate of three-dimensional cultured human epidermis was evaluated after 24 h (A) and 72 h (B). Values are presented as the mean ± SD (n = 5–8).

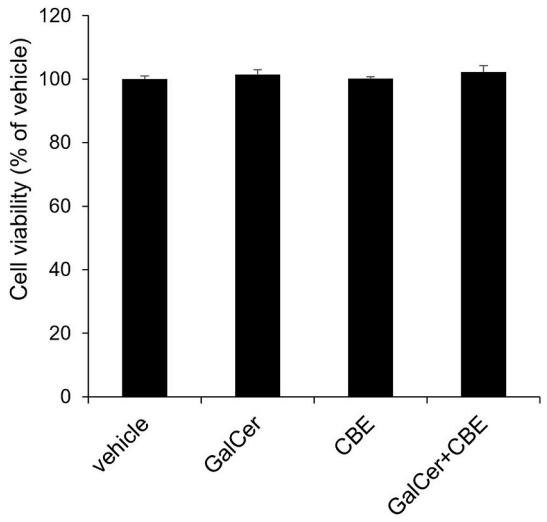

Fig. 2. Effect of GalCer on cell viability in the three-dimensional cultured human epidermis model. After 14 days of GalCer treatment, cell viability was evaluated using the alamarBlue assay. Values are presented as the mean ± SD (n = 3–6).

was suppressed by concurrent treatment with the β-GCase inhibitor CBE (Fig. 3A). Furthermore, FFA levels following GalCer treatment were significantly lower than those in the vehicle group (Fig. 3G). TEWL was measured on days 0, 7, and 14 of GalCer treatment. GalCer treatment for 14 days significant decreased TEWL (Fig. 4).

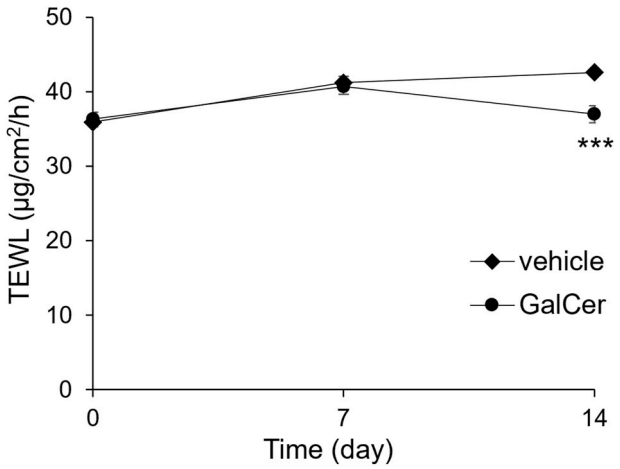

Fig. 4. Changes in TEWL after GalCer treatment in the three-dimensional cultured human epidermis model. TEWL was measured for 0, 7, and 14 days in the three-dimensional cultured human epidermis model. Values are presented as the mean ± SD (n = 4–8). ***$p < 0.001$, Student's *t*-test.

3.3. *mRNA expression of enzymes involved in the synthesis of CER and FFAs in the presence of GalCer*

GalCer treatment increased CER levels and decreased FFA levels, prompting us to examine the mechanism. The mRNA expression levels of CER (*SPT1*, *CerS3*, *GCS*, *β-GCase*, *SMS1*, *SMS2*, and *aSMase*) and FFA metabolism-related enzymes (*PPARγ*, *SREBP1α*, *ACC*, and *FAS*) were evaluated by real-time PCR. Relative to the effects of vehicle, the mRNA expression of *SPT1*, *CerS3*, *GCS*, and *β-GCase* was increased by 48 h of GalCer treatment (Fig. 5A–D). By contrast, the mRNA expression of

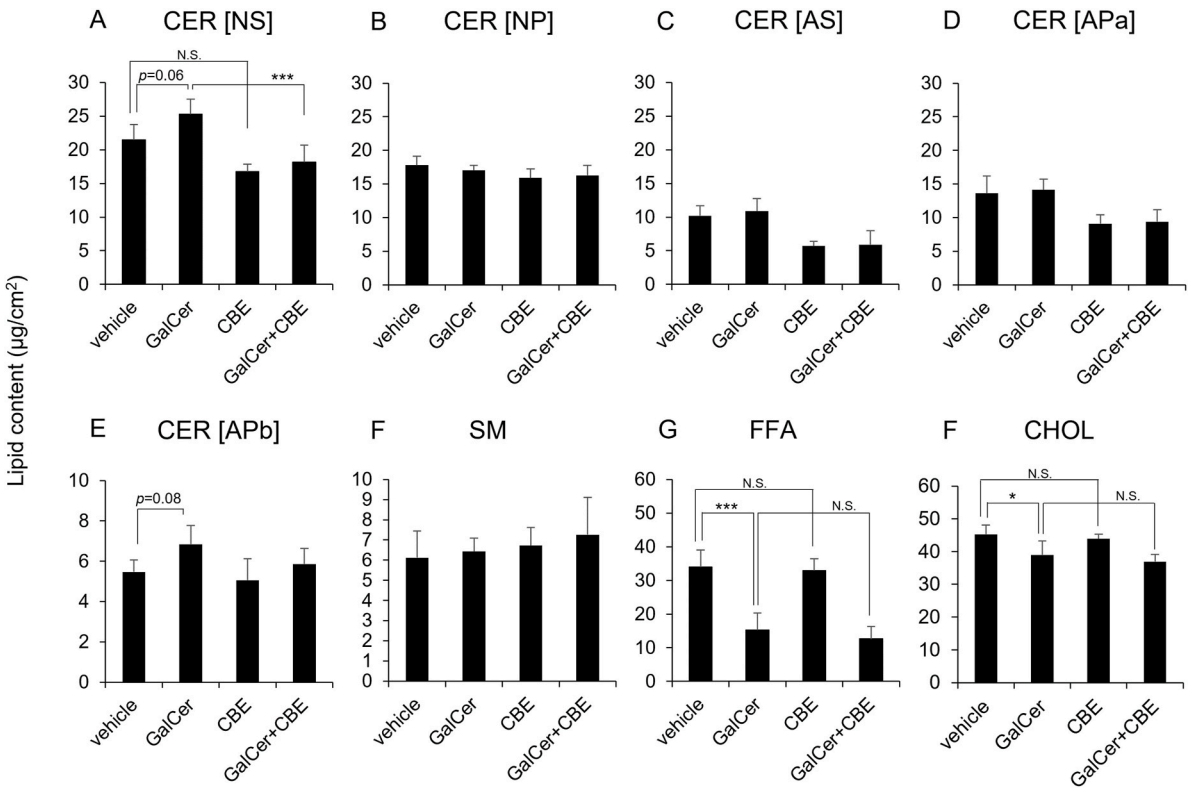

Fig. 3. Effect of 14 days of GalCer treatment on CER, FFA, and CHOL content in the 3-dimensional cultured human epidermis model. CER [NS] (A), CER [NP] (B), CER [AS] (C), CER [APa] (D), CER [APb] (E), SM (F), FFA (G), and CHOL levels (H) were evaluated in the three-dimensional cultured human epidermis model. Values are presented as the mean ± SD (n = 3–6). NS, not significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Tukey's *post hoc* multiple comparison test.

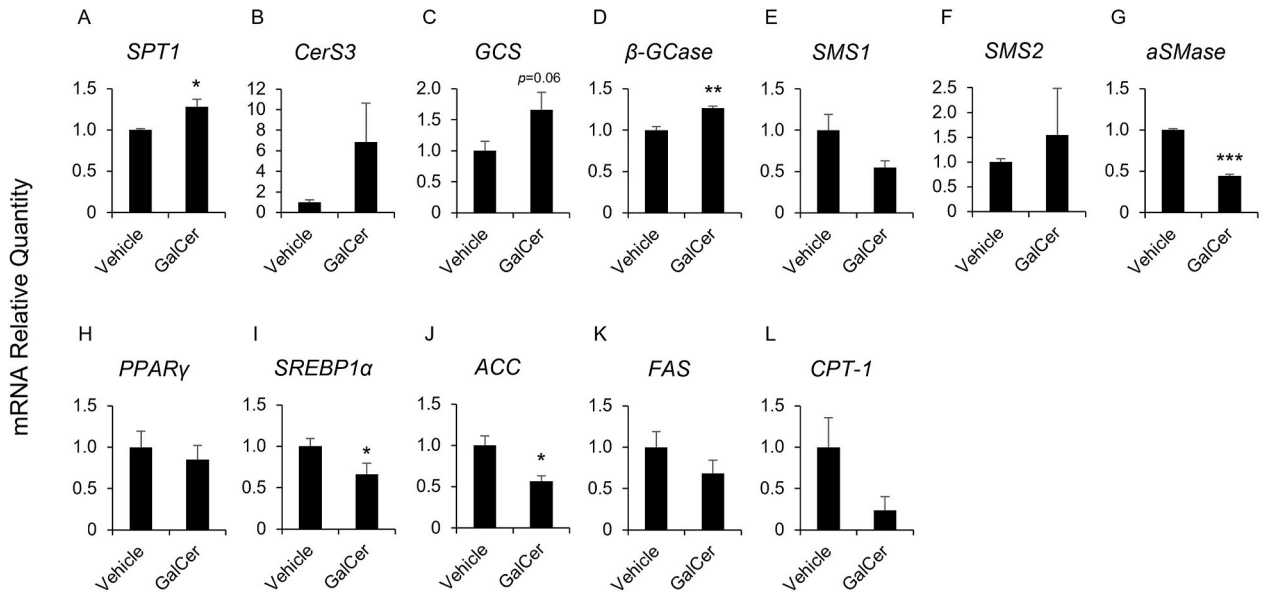

Fig. 5. Effect of GalCer treatment on the expression of enzymes involved in CER and FFA synthesis in the three-dimensional cultured human epidermis model. *SPT1* (A), *CerS3* (B), *GCS* (C), *β-GCase* (D), *SMS1* (E), *SMS2* (F), *aSMase* (G), *PPARγ* (H), *SREBP1α* (I), *ACC* (J), *FAS* (K) and *CPT-1* (L) mRNA levels were determined by quantitative real-time PCR. Values were normalized to those of GAPDH and compared with those in the vehicle group. Values are presented as the mean ± SD (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's *t*-test.

aSMase mRNA was significantly reduced by GalCer treatment (Fig. 5G). Meanwhile, the mRNA levels of *ACC* and *FAS* (involved in fatty acid synthesis) were decreased by GalCer treatment, whereas those of *CPT-1* (involved in fatty acid degradation) were decreased (Fig. 5J–L). In addition, the mRNA expression of *SREBP1α*, a transcription factor involved in fatty acid synthesis, was decreased by exposure to GalCer compared with the vehicle level (Fig. 5I).

4. Discussion

We investigated the effects of GalCer on the production of intercellular lipids in the SC using LabCyte EPI-MODEL 6D. The results suggested that GalCer promoted CER production via the β-GCase metabolic pathway and suppressed FFA production. Furthermore, it was suggested that GalCer was hardly metabolized, and it affected the production of these intercellular lipids in the SC. In the mature model of the three-dimensional cultured epidermis, the stratum corneum is formed, and it is considered that GalCer does not easily penetrate into the skin due to the skin barrier function. Therefore, the immature model was used in this study. In addition, since skin barrier function is impaired in atopic dermatitis and psoriasis, the effect of GalCer was evaluated using an immature model this manuscript.

We first examined whether GalCer was directly metabolized by enzymes in LabCyte EPI-MODEL 6D. When LabCyte EPI-MODEL 6D homogenate was examined, C12-NBD-GlcCer was metabolized to CER, but C12-NBD-GalCer was hardly metabolized (Fig. 1). This suggested that GalCer promotes β-GCase activity [30,31], but it is not directly metabolized by enzymes such as β-GCase in LabCyte EPI-MODEL 6D homogenate. GlcCer, a precursor lipid for the synthesis of diverse glycolipids, is contained in all cells in mammals. Its synthesis is mainly initiated by glucose transferase (UDP-glucose ceramide glycosyltransferase, GCS) present on the cytoplasmic side of the Golgi membrane. By contrast, GalCer is metabolized by the action of UDP-galactose CER galactosyl transferase. In the skin, GlcCer is metabolized to CER by β-glucocerebrosidase. By contrast, GalCer, a major glycosphingolipid of myelin, is degraded to CER via degalactosylation by both GM1β-galactosidase and galactosylceramide β-galactosidase. In addition, psychosine, a cytotoxic lysolipid, can be synthesized from GalCer by the action of N-deacylase, and fatty acids are produced as a by-product. Sphingolipid CER N-deacylase (SCDase) acts on sphingolipids and SM to release fatty acids and produce their respective lysoforms [36]. However, this enzyme acts on gangliosides, including so-called acidic glycolipids such as GD1a, GM1, GM2, and GM3, but it does not act on GalCer and GlcCer. Furthermore, SCDase is bacterial enzyme and has not been identified in eukaryotes. In addition, CER, which hydrolyzes the bond between the sphingosine base and the fatty acid, cannot hydrolyze the bond between the sphingosine base and the fatty acid in the CER portion of the glycolipid. Therefore, GalCer is unlikely to be metabolized in the skin.

GalCer was added from the insert side of LabCyte EPI-MODEL 6D, and after 14 days of culture, lipid extraction was performed followed by HPTLC. The CER [NS] content was increased by GalCer treatment relative to the vehicle, and the increase was suppressed by the combined use of CBE (Fig. 3). It has already been reported that GalCer enhances β-GCase activity, and our results also proved that β-GCase is involved in the increased production of CER from GalCer. However, since the amount of CER [APb] did not change with the combined use of CBE, it is considered that CBE could not completely suppress β-GCase activity. In addition, GalCer treatment increased the expression of *GCS* and *β-GCase*, which are involved in GlcCer metabolism (Fig. 5). Conversely, *SMS* and *aSMase*, which are involved in SM metabolism, were downregulated, suggesting that the increased production of CER from GalCer may be caused by activation of the *de novo* synthesis pathway mediated by GlcCer. In addition, although the levels of FFA and CHOL were decreased by GalCer treatment, TEWL, which is an index of barrier function, was significantly decreased by 14 days of GalCer treatment (Figs. 3 and 4). This suggests that barrier function was not altered by GalCer treatment. There was no difference in TEWL on the 7 days after GalCer treatment, and a difference was obtained on the 14 days. This is probably because the promotion of ceramide production by the treatment of GalCer was almost unchanged in 7 days (data not shown) and was confirmed in the 14 days, so that the skin barrier function was formed by the production of CER. GalCer treatment reduced the expression of *ACC* and *FAS*, which are involved in fatty acid synthesis, and *CPT-1*, which is involved in fatty acid degradation (Fig. 5). Therefore, it was suggested that the decrease in fatty acid content following GalCer treatment was caused by the suppression of fatty acid synthesis. CER is metabolized to fatty acids and sphingosine by ceramidase. GalCer may affect the process of metabolism from CER to sphingosine and fatty

acids by reducing ceramidase activity. In the future, it will be important to conduct detailed research on the effects of GalCer on ceramidase activity. CHOL biosynthesis in the epidermis is regulated by barrier function. Thus, it is considered that CHOL levels also fluctuated to maintain the balance as CER and PA levels fluctuated.

GalCer is used in several cosmetics and medicines, and there are various reports on its efficacy and safety [37,38]. This study illustrated that GalCer may affect CER, PA, and CHOL levels in the epidermis when applied to the skin. Intercellular lipids in the SC such as CER are greatly involved in skin barrier function, and imbalances among their levels lead to skin barrier dysfunction. Elucidation of the detailed mechanisms in the future may be useful for treating skin diseases such as psoriasis and atopic dermatitis.

Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.

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