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COMMUNICATION

Mass Spectrometric Confirmation of γ-Linolenic Acid Ester-Linked Ceramide 1 in the Epidermis of Borage Oil Fed Guinea Pigs

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Abstract Ceramide 1 (Cer1), a Cer species with eicosasphingenine (d20:1) amide-linked to two different ω-hydroxy fatty acids (C30wh:0:C32wh:1), which are, in turn, ester-linked to linoleic acid (LNA; 18:2n-6), plays a critical role in maintaining the structural integrity of the epidermal barrier. Prompted by the recovery of a disrupted epidermal barrier with dietary borage oil [BO: 36.5 % LNA and 23.5 % γ-linolenic acid (GLA; 18:3n-6)], in essential fatty acid (EFA)-deficient guinea pigs, we further investigated the effects of BO on the substitution of ester-linked GLA for LNA in these two epidermal Cer1 species by LC–MS in positive and negative modes. Dietary supplementation of BO for 2 weeks in EFA-deficient guinea pigs increased LNA ester-linked to C32wh:1/d20:1 and C30wh:0/d20:1 of Cer1. Moreover, GLA ester-linked to C32wh:1/d20:1, but not to C30wh:0/d20:1, of Cer1 was detected, which was further confirmed by the product ions of *m/z* 277.2 for ester-linked GLA and *m/z* 802.3 for the deprotonated C32wh:1/d20:1. C20-Metabolized fatty acids of LNA or GLA were not ester-linked to these Cer1 species. Dietary BO induced GLA ester-linked to C32wh:1/d20:1 of epidermal Cer1.

Keywords γ-Linolenic acid ester-linked ceramide 1 · γ-Linolenic acid · Ceramide 1 · LC–MS · Borage oil · Epidermis · Guinea pigs

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Abbreviations

EFA	Essential fatty acid
BO	Borage oil
Cer1	Ceramide 1
GLA	γ-Linolenic acid (18:3n-6)
FA	Fatty acid
HCO	Hydrogenated coconut oil
LNA	Linoleic acid (18:2n-6)
LC	Liquid chromatography
LC–ESI–MS/MS	Liquid chromatography coupled with electrospray tandem mass spectrometry
m/z	Mass/charge
MS	Mass spectrometry

Introduction

Ceramide (Cer) bears the structural moieties of ester-linked fatty acids (FA) and amide-linked non-hydroxy, α-hydroxy or ω-hydroxy FA on sphingoid bases, of which distinct compositions provide the diversity of heterogeneous Cer species (Cer1-9) in the epidermis [1]. Of various Cer species, Cer1, an acylCer with sphingosine (or sphingenine) amide-linked to long chain ω-hydroxy FA, which is, in turn, ester-linked to FA, plays a dominant role in maintaining the lamellar integrity of the epidermal barrier [2]. The marked depletion of Cer1 is thought to be an etiological factor for barrier disruption in abnormal skin conditions such as atopic dermatitis (AD) and essential fatty acid (EFA) deficiency [1, 3]. Moreover, the barrier disruption of EFA deficiency involves the substitution of non EFA for EFA ester-linked to ω-hydroxy FA of Cer1 [4].

In view of the functional importance of FA in maintaining the lamellar integrity of the epidermal barrier, dietary supplementation of ω-6 EFA is of particular interest. Specially,

γ-linolenic acid (GLA; 18:3n-6) has been reported to recover the disrupted barrier, and improve the clinical manifestation of AD and EFA deficiency [5, 6]. The oxidative metabolites of GLA have been reported to exert greater anti-inflammatory or anti-proliferative activities [7] than those of linoleic acid (LNA; 18:2n-6), the most abundant ω-6 EFA in human skin [7]; therefore borage oil (BO) (*Borago officinalis*), which contains a high concentration of GLA, has become popular for skin health [5, 7]. However, in contrast to the extensive reports on the functional importance of the LNA moiety of Cer1 in maintaining epidermal barrier [2, 4], there is only limited information available on the mechanism of GLA for recovering the disrupted epidermal barrier in abnormal skin conditions. In this regard, we previously reported that dietary supplementation of BO increases Cer synthesis and induces the incorporation of dihomo-γ-linolenic acid (DGLA; 20:3n-6), the C20 metabolized FA of GLA, into total Cer in the epidermis of EFA-deficient guinea pigs [6]. To further determine whether GLA or DGLA are ester-linked to Cer1, EFA-deficient guinea pigs were fed a BO diet in this study. The confirmation of ester-linked LNA, GLA, DGLA or arachidonic acid (ARA; 20:4n-6), the C20 metabolized FA of LNA, in epidermal Cer1, was performed by liquid chromatography coupled with electrospray tandem mass spectrometry (LC–ESI–MS/MS).

Table 1 FA composition of total lipids in the epidermis of groups (% of total FA)

FA	HCO-fed guinea pigs for 8 weeks[1]	Groups[1]	
		HCO	HCO + BO
16:0[2]	25.03 ± 1.508[3a]	20.97 ± 1.805[b]	15.32 ± 2.535[c]
18:0	14.38 ± 1.375[b]	20.29 ± 4.753[a]	15.10 ± 1.930[b]
18:1n-9	25.48 ± 0.654[a]	25.61 ± 4.608[a]	11.65 ± 3.480[b]
18:2n-6	15.32 ± 1.312[b]	9.29 ± 2.057[c]	25.59 ± 4.592[a]
18:3n-6	nd	nd	7.24 ± 0.692
20:3n-9	0.74 ± 0.116[b]	1.47 ± 0.300[a]	nd
20:3n-6	nd	nd	4.49 ± 0.894
20:4n-6	1.71 ± 0.457[c]	2.76 ± 0.723[b]	4.71 ± 0.567[a]

FA fatty acid, *nd* not detected

[1] Guinea pigs were fed a hydrogenated coconut oil (HCO) diet for 10 weeks (group HCO) or a HCO diet for 8 weeks followed by a borage oil (BO) diet for 2 weeks (group HCO + BO)

[2] Only the major fatty acids are listed

[3] Values are mean ± SD ($n = 6$)

[a,b,c] Different superscripts letters in the same row indicate significant differences ($p < 0.05$) using one way ANOVA and Tukey's honestly significant difference (HSD) *post hoc* test

Fig. 1 LC-ESI–MS/MS chromatograms of ceramide 1 (C32 wh:1-C(X)/d20:1) with C32 ω-hydroxy fatty acid with a double bond (C32wh:1) and C20-eicosasphingenine (d20:1). Guinea pigs were fed a hydrogenated coconut oil (HCO) diet for 10 weeks (group HCO) (**a**) or a HCO diet for 8 weeks followed by a borage oil (BO) diet for 2 weeks (group HCO + BO) (**b**). The epidermal ceramide 1 (Cer1) (C32wh:1-C(X)/d20:1) ester-linked with linoleic acid (LNA, 18:2n-6), γ-linoleic acid (GLA, 18:3n-6), dihomo γ-linoleic acid (DGLA, 20:3n-6) or arachidonic acid (ARA, 20:4n-6) was analyzed by liquid chromatography coupled with electrospray tandem mass spectrometry in the positive mode

Materials and Methods

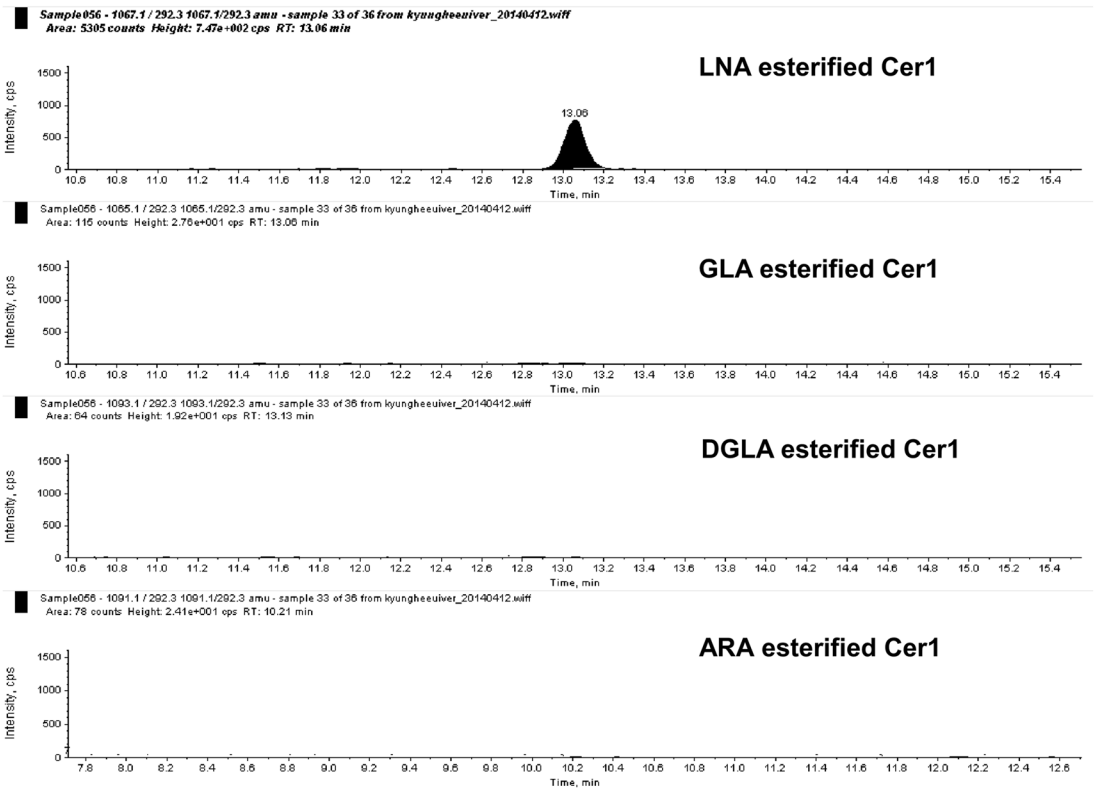
Guinea Pigs and Diet

After 1 week adaptation, fourteen-day-old male Hartley guinea pigs were initially fed a diet containing 40 g/kg hydrogenated coconut oil (HCO) (Dyets, Bethlehem, PA, USA) supplemented with 20 g/kg triolein (Sigma-Aldrich, St. Louis, MO, USA) for 8 weeks to induce EFA deficiency, as described previously [6]. At the end of 8 weeks, guinea pigs were divided into two groups. Group 1 was fed a diet containing 60 g/kg BO (Midlands Seed, Ashburton, New Zealand) (group HCO + BO: $n = 6$), and group 2, the EFA-deficient control, continued on the HCO diet (group HCO: $n = 6$) for 2 weeks. Details of the diet composition have been published elsewhere [6]. From FA analysis of the oils (% of total FA), BO contained 36.5 % LNA and 23.5 % GLA, and HCO contained saturated FA only. All aspects of animal handling and care procedures were approved by the Animal Care and Use Review Committee of Kyung Hee University (KHUASP-13-05). At the end of 10 weeks, all guinea pigs were sacrificed by cervical dislocation and epidermal strips were obtained, as described previously [6].

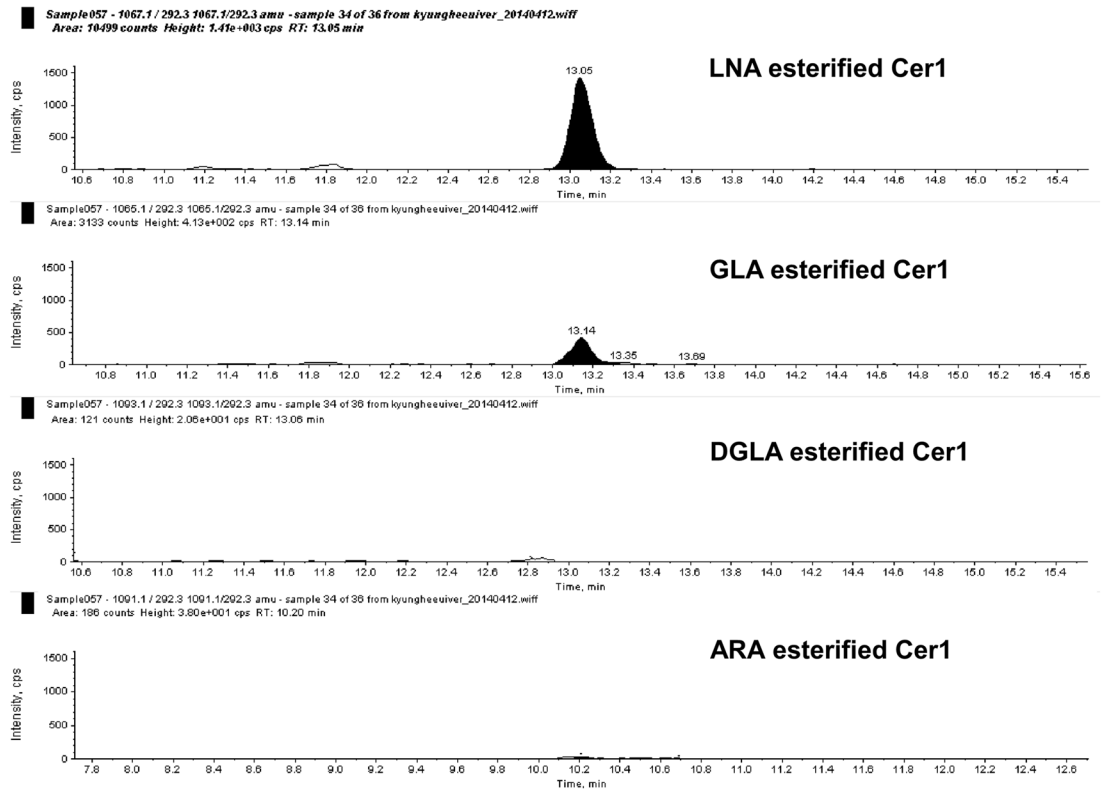
Confirmation of EFA Deficiency by FA Analysis

FA composition of epidermal total lipids was analyzed for an accumulation of mead acid (20:3n-9) and a ratio of mead acid to ARA, biomarkers of EFA deficiency [8]. Specifically, epidermal strips were homogenized, and total lipids were extracted with chloroform ($CHCl_3$) and methanol (MeOH) (2:1, by vol). Fatty acid composition was analyzed by gas chromatography (Shimadzu, Kyoto, Japan) after transmethylation with 6 % hydrochloride in MeOH, as described previously [6]. The separated FA were identified and quantitated with external standards of FA methyl ester mixtures (GLC-19A, GLC-91, GLC-455) and an internal standard of heptadecanoic acid (C17:0) (N-17-M) (Nu-Check Prep, Elysian, MN, USA).

a Group HCO



b Group HCO+BO



Epidermal Sample Preparation for LC–ESI–MS/MS Analysis

Epidermal strips (0.1 g wet weight) were homogenized in 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM $CaCl_2$ and 30 units proteinase K (Sigma-Aldrich, St. Louis, MO, USA). MeOH and $CHCl_3$ (2:1, by vol) were added and sonicated, followed by the addition of 100 pmol of Cer (C18:0/d17:1) as an internal standard [9]. After centrifugation, the lower phase was collected, dried down using a Speed Vacuum type concentrator (Vision, Seoul, Korea), and dissolved in 100 µL of MeOH.

Ceramide 1 Analysis by LC–ESI–MS/MS

Cer1 species were separated by reverse-phase high performance liquid chromatography (HPLC) (NANOSPACE SI-2 HPLC equipped with HTS autosampler Z, Shiseido, Tokyo, Japan) using a LUNA C18 column (2.1 × 150 mm, ID: 5 µm) (Phenomenex, St. Louis, MO, USA) as described previously [10]. The column was pre-equilibrated in solvent A (MeOH/0.05 % formic acid in H_2O, 95:5, by vol) and lipids were eluted with increasing percentages of solvent B (2-propanol/0.05 % formic acid in MeOH, 99:1, by vol) at a flow rate of 0.3 mL/min using a modified method by Merrill *et al*. [9]. Both solvent A and B also contained 1 mmol/L ammonium formate.

The HPLC column effluent was introduced onto an API 3200 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbo-ESI in the positive ionization mode for LC–MS/MS analysis. Because LNA ester-linked Cer1 is mainly composed of 20-carbon dihydroxysphingosine with one double bond (C20-eicosasphingenine: d20:1, *m/z* 292) [9] and two different amide-linked *ω*-hydroxy FA (C30 *ω*-hydroxy FA: C30wh:0, C32 *ω*-hydroxy FA with one double bond: C32wh:1) [11], the confirmation of ester-linked LNA, GLA, DGLA or ARA in Cer1 was performed by the Selected Reaction Monitoring (SRM) transitions (*m/z*) of these protonated Cer1 species to fragment C (loss of amide-linked *ω*-hydroxy FA) [10]. The levels of each FA ester-linked to Cer1 were quantitated using the ratio of peak area between each analyte and the internal standard, and expressed as the ratio between each analyte peak area and the internal standard area/g wet weight of epidermis.

Further Confirmation of LNA or GLA Ester-Linked Cer1 by Ion Trap MS

LNA or GLA ester-linked to C32wh:1/d20:1 of epidermal Cer1 in the HPLC column effluent of group HCO + BO were further confirmed by direct infusion mass spectrometry (DIMS) using an LCQ FLEET ion trap mass spectrometer (Thermo, San Jose, CA, USA) with ESI in the negative mode, as described previously [10]. Data was collected by the isolation width (*m/z* 5) and fragmentation with 35 % relative collision energy.

Results and Discussion

Mead acid, an abnormal FA generated from oleic acid (18:1n-9) during the development of EFA deficiency [8],

Table 2 Comparative composition of PUFA ester-linked to ceramide 1 in the epidermis of groups[a]

Ester-linked PUFA of Cer1	Amide-linked FA and sphingoid base moiety of Cer1	Groups[b]	
		HCO	HCO + BO
18:2n-6	C30wh:0/d20:1	0.27 ± 0.031[c]	0.46 ± 0.068**
	C32wh:1/d20:1	0.49 ± 0.055	0.61 ± 0.100*
18:3n-6	C30wh:0/d20:1	nd	nd
	C32wh:1/d20:1	nd	0.19 ± 0.033
20:3n-6	C30wh:0/d20:1	nd	nd
	C32wh:1/d20:1	nd	nd
20:4n-6	C30wh:0/d20:1	nd	nd
	C32wh:1/d20:1	nd	nd

PUFA polyunsaturated fatty acids, *FA* fatty acid, *Cer1* ceramide 1, *nd* not detected

* $p < 0.05$, ** $p < 0.01$ *versus* group HCO in the same row by the student's *t* test

[a] 100 pmol of ceramide (C18:0/d17:1) was added as an internal standard in the epidermal sample preparation for ceramide 1 analysis by liquid chromatography coupled with electrospray tandem mass spectrometry

[b] Guinea pigs were fed a hydrogenated coconut oil (HCO) diet for 10 weeks (group HCO) or a HCO diet for 8 weeks followed by a borage oil (BO) diet for 2 weeks (group HCO + BO)

[c] Comparative values were determined as ratios of peak areas between each analyte and the internal standard[a]/g wet weight of epidermis. Values are means ± SD ($n = 6$)

was detected in the epidermis of guinea pigs fed a HCO diet for 8 weeks, and further accumulated in group HCO (Table 1). In parallel, the ratio of mead acid to ARA was increased from 0.43 to 0.53. However, in group HCO + BO, mead acid was not detected, and levels of LNA and ARA were higher than in group HCO. GLA and DGLA were also detected. These data, together with a ratio of mead acid to ARA of greater than 0.4 diagnosed as EFA deficiency [8], indicate that EFA deficiency was evident with a HCO diet for more than 8 weeks, and reversed with a BO diet for 2 weeks (group HCO + BO).

In LC–ESI–MS/MS analysis, the small peak of LNA ester-linked Cer1 was detected in group HCO (Fig. 1a). However, in group HCO + BO, LNA or GLA ester-linked Cer1 were detected as two distinct peaks with slight different retention times (13.05 min for LNA; 13.14 min for GLA; Fig. 1b), which were confirmed by their specific SRM transition condition, as summarized in Table 2. Although LNA was more preferentially ester-linked to C32wh:1/d20:1 than C30wh:0/d20:1 of Cer1 in all groups, these levels were higher in group HCO + BO than in group HCO, reflecting the LNA content of BO (36.5 % LNA in BO; no LNA in HCO). GLA was not ester-linked to C30wh:0/d20:1 of epidermal Cer1 in group HCO + BO. However, a small but significant level of ester-linked GLA was detected in Cer1 with C32wh:1/d20:1 in the epidermis of group HCO + BO. DGLA and ARA, C20-metabolized FA of GLA and LNA, were not detected in these two Cer1 species of any group.

The HPLC effluent of Cer1 species in group HCO + BO was further analyzed by ion trap MS, and Fig. 2 represents the product ion spectrum obtained in negative ion mode. The most abundant signal observed at *m/z* 279.2 and *m/z* 277.2 represented ester-linked LNA (*m/z* 279.2) and GLA (*m/z* 277.2), respectively. The fragmentation at *m/z* 802.3 was from the deprotonated C32wh:1/d20:1 moiety of Cer1,

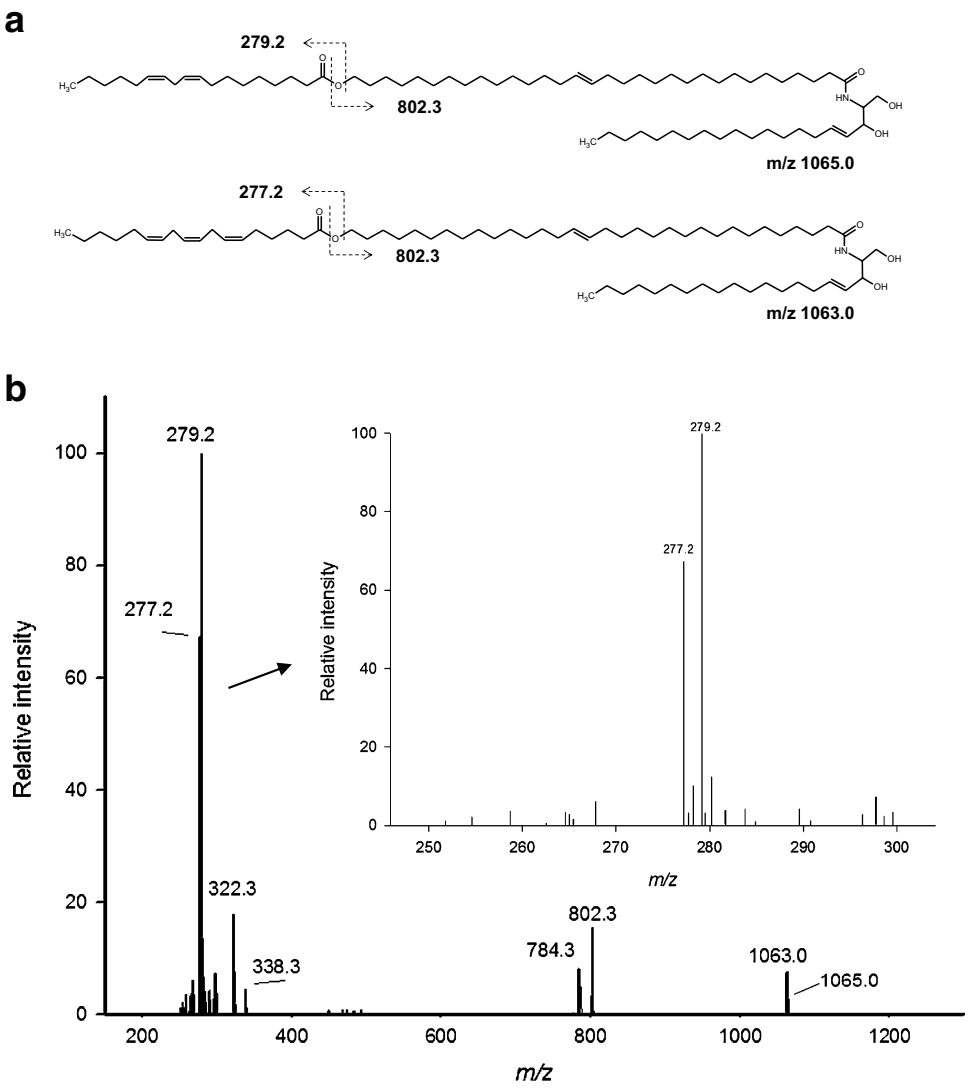


Fig. 2 Product ion spectrum of linoleic acid or γ-linoleic acid ester-linked to C32wh:1/d20:1 of ceramide 1 in group HCO + BO by ion trap MS. Guinea pigs were fed a hydrogenated coconut oil (HCO) diet for 8 weeks followed by a borage oil (BO) diet for 2 weeks in group HCO + BO. The liquid chromatography column effluent of ceramide 1 (Cer1) with C32 ω-hydroxy fatty acid with a double bond (C32wh:1) and C20-eicosasphingenine (d20:1) in group HCO + BO was analyzed by ion trap mass spectrometry (MS) in the negative ion mode. **a** Structure of linoleic acid (LNA, 18:2n-6) or γ-linoleic acid (GLA, 18:3n-6) ester-linked to C32wh:1/d20:1 moiety of Cer1 **b** LNA (*m/z* 279.2) and GLA (*m/z* 277.2) were presented along with the cleaved product ions of the deprotonated C32wh:1/d20:1 moiety (*m/z* 802.3) from LNA ester-linked Cer1 (C32wh:1-C18:2n-6/d20:1) (*m/z* 1065.0) or GLA ester-linked Cer1 (C32wh:1-C18:3n-6/d20:1) (*m/z* 1063.0). The inset shows an enlargement of the spectrum in *m/z* 250–300

and the subsequent loss of a water molecule resulted in a product ion at *m/z* 784.3. The combined ion signals of *m/z* 1065.0 and *m/z* 1063.0 for either the deprotonated LNA ester-linked Cer1 (C32wh:1-C18:2n-6/d20:1) or GLA ester-linked Cer1 (C32wh:1-C18:3n-6/d20:1) were also detected. Although several cleavage and rearrangement reactions resulted in unidentified signals such as *m/z* 322.3 and *m/z* 338.3, these data, along with the lack of GLA ester-linked to C30wh:0/d20:1 of Cer1 (Table 2), further confirmed that GLA is ester-linked to the C32wh:1/d20:1 moiety of Cer1. The ion trap MS analysis in the negative mode confirmed no DGLA or ARA ester-linked to Cer1.

Distinct from the fractionation of total Cer by high performance thin layer chromatography (HPTLC) in our previous study [6], further fractionation of Cer1 and species-specific product ion confirmation of LC–MS analysis were employed in the present study, which allowed a more specific analysis of ester-linked FA in Cer1. The GLA ester-linked to C32wh:1/d20:1 of Cer1 in group HCO + BO agrees with a previous study of EFA-deficient rats fed the same amount of LNA or columbinic acid (18:3n-6, $\Delta^{5t,9c,12c}$) esters for 10 days; similar levels of LNA or columbinic acid were incorporated into epidermal acylCer [12]. Furthermore, the lack of ester-linked DGLA and ARA in the epidermal Cer1 of groups HCO and HCO + BO also agrees with no incorporation of ARA into epidermal acylCer despite dietary supplementation of the same amount of either LNA or ARA in EFA-deficient rats [12], which supports again that C18 FA is preferentially ester-linked to Cer1 [4]. However, despite 36.5 % LNA and 23.5 % GLA in BO, the level of GLA ester-linked to C32wh:1 of Cer1 was approximately 31.1 % of LNA ester-linked to C32wh of Cer1. These data, coupled with no incorporation of α-linolenic acid (ALA) (18:3n-3) into acylCer in the epidermis of EFA deficient rats fed ALA esters for 13 weeks at 2 % of total energy levels [12], suggest that the level of C18 FA ester-linked to Cer1 does not simply reflect the C18 FA content of dietary oil, but is extremely specific.

In the human epidermis, there are two other acylCer species, Cer4 and Cer9 with 6-hydroxysphingosine (Cer4) or phytosphingosine (Cer9) amide-linked to ω-hydroxy FA, which is, in turn, ester-linked to FA [1]. Although a disrupted lamellar integrity is more related with the depletion of Cer1 than that of Cer4, and is not recovered despite the presence of Cer4 and Cer9 [3], LNA is highly ester-linked to the ω-hydroxy FA moiety of Cer4 and Cer9 [13] as similar with Cer1; further studies are required in depth to investigate the substitution of GLA for LA ester-linked to Cer4 or Cer9, and the significance of GLA ester-linked Cer1 in the recovery of a disrupted epidermal barrier. This study demonstrated, for the first time, that dietary supplementation of BO induces GLA ester-linked to C32wh:1 of epidermal Cer1 in EFA-deficient guinea pigs.

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