Accumulation of protein-bound epidermal glucosylceramides in β-glucocerebrosidase deficient type 2 Gaucher mice

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Abstract The epidermal permeability barrier for water is essentially maintained by extracellular lipid membranes within the interstices of the stratum corneum. Ceramides, the main components of these membranes, derive in large part from hydrolysis of glucosylceramides mediated by the lysosomal enzyme β-glucocerebrosidase. As analyzed in this work, the β-glucocerebrosidase deficiency in type 2 Gaucher mice (RecNci I) resulted in an accumulation of all epidermal glucosylceramide species accompanied with a decrease of the related ceramides. However, the levels of one ceramide subtype, which possesses an α-hydroxypalmitic acid, was not altered in RecNci I mice suggesting that the β-glucocerebrosidase pathway is not required for targeting of this lipid to interstices of the stratum corneum. Most importantly, ω-hydroxylated glucosylceramides which are protein-bound to the epidermal cornified cell envelope of the transgenic mice accumulated up to 35-fold whereas levels of related protein-bound ceramides and fatty acids were decreased to 10% of normal control. These data support the hypothesis that in wild-type epidermis ω-hydroxylated glucosylceramides are first transferred enzymatically from their linoleic esters to proteins of the epidermal cornified cell envelope and then catabolized to protein-bound ceramides and fatty acids, thus contributing at least in part to the formation of the lipid-bound envelope.

Key words: Glucocerebrosidase; Gaucher disease; Sphingolipid; Epidermal permeability barrier; Knockout mouse

1. Introduction

Terminal differentiation of epidermal keratinocytes results in the formation of the stratum corneum. In this outermost layer of the epidermis, corneocytes are embedded in a matrix of extracellular lipid membranes mainly consisting of ceramides (Cers), cholesterol, and fatty acids [1]. Moreover, corneocytes are coated by a monolayer of covalently protein-bound (i.e. ester-linked) ω-hydroxylated ceramides and fatty acids [2,3]. This lipid-bound envelope (LBE) is thought to be essential for the interaction of highly cross-linked proteins of the corneocyte (cornified cell envelope, CE) with the extracellular lipid matrix [4]. It is well documented that the function of the stratum corneum as a barrier to excess transepidermal water loss highly depends on the presence of extracellular lipid membranes. Ceramides, the main components, derive in large part from hydrolysis of glucosylceramides (GlcCers) of the lamellar bodies mediated by β-glucocerebrosidase (β-GlcCerase) [5–7].

Gaucher disease is a lysosomal storage disorder caused by mutations in the gene encoding the lysosomal enzyme β-GlcCerase. Among three distinct clinical types of Gaucher disease, type 2 (acute neuronopathic) is the most fatal form [10]. Complete loss of β-GlcCerase activity in some cases of type 2 Gaucher disease is fatal within a few days after birth and is accompanied with a severe ichthyosiform dermatosis (collodion baby phenotype) [8,9]. Recently, a mouse model for this disease has been created by introducing point mutations into the murine gene encoding β-GlcCerase resulting in an almost complete loss of enzyme activity [7]. These RecNci I point mutation mice exhibit severe ichthyosiform skin abnormalities presumably caused by accumulation of epidermal GlcCers together with decreased levels of Cers [7], as also shown for β-GlcCerase knockout (null allele) mice by Holleran et al. [6].

In contrast to the well documented precursor/product relationship between epidermal GlcCer and Cer, little is known about the metabolic relationship between free and protein bound lipids in the stratum corneum. Recently, we have identified a novel component of the lipid bound envelope, ω-hydroxylated GlcCer, suggesting that deglucosylation is not a required step for the covalent attachment of lipids to the cornified cell envelope [11]. In the present work we analyzed the levels of free and covalently bound glucosylceramides and their metabolic products in the epidermis of RecNci I mice with special regard to ω-hydroxylated glucosylceramides.

2. Materials and methods

2.1. Animals

Generation of RecNci I mice has been described previously [7]. Newborn RecNci I mice ($n=4$) as well as newborn wild-type littermates ($n=4$) were studied. Genotyping was performed by Southern blotting as described [7].

2.2. Preparation of epidermis

Whole skin was removed at autopsy from newborn mice. Epidermis was separated from dermis after floating the skin on Dispase (Boehringer Mannheim, grade II) diluted 1:1 in Hank's buffer at 4°C overnight.

2.3. Lipid analysis

Tissue samples were homogenized, lyophilyzed and weighed. Unbound lipids were extracted and separated as described previously [11]. For separation of glucosylceramides, the thin-layer silica gel 60 plates (Merck Darmstadt, Germany) were developed with chloroform-methanol-water (70:30:5, vol/vol/vol). Ceramides were resolved twice using chloroform-methanol-acetic acid (190:9:1, vol/vol/vol) as developing solvent. Bound lipids were recovered and analyzed as described previously [11]. Briefly, covalently bound lipids were released by incubation of preextracted samples with 1 M KOH in 95% methanol for

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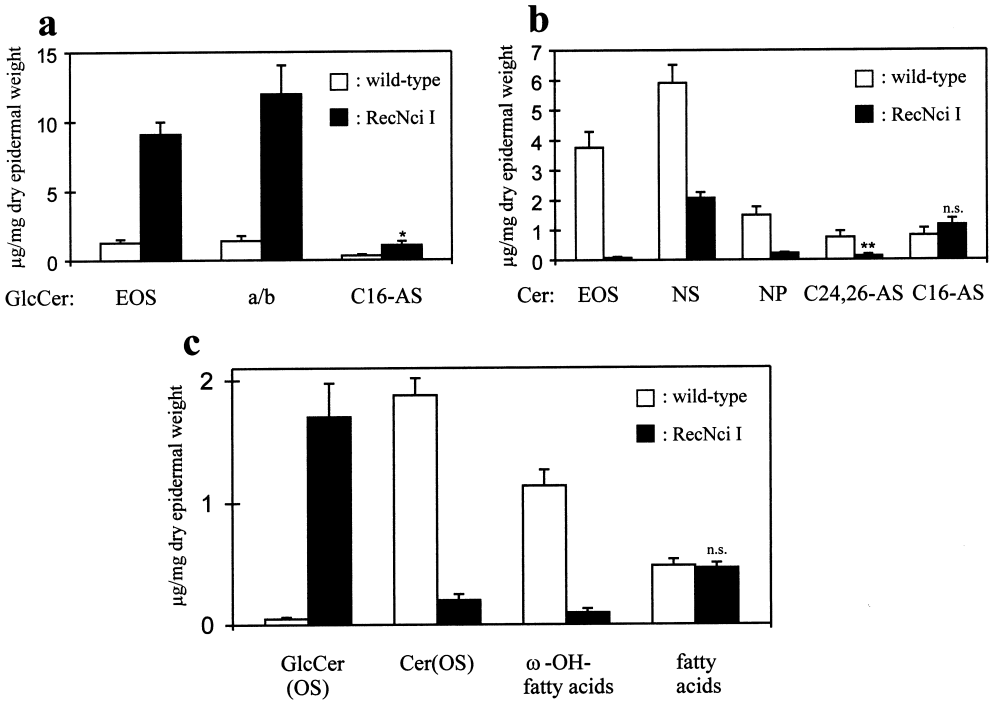

Fig. 1. Epidermal lipid composition of RecNci I mice compared to wild-type mice. After extraction of unbound lipids and separation by TLC GlcCers (panel a) and Cers (panel b) were quantified by photodensitometry. Bound lipids were released by base hydrolysis, separated by TLC and quantified (panel c). Data are presented as the mean ± S.E.M. ($n = 4$). Note the accumulation of all GlcCer fractions and, with the exception of Cer(C16-AS), the loss of Cers in RecNci I mice. $^{**}P \leq 0.001$ and $^{*}P < 0.05$; n.s., not significant; A, α-hydroxylated *N*-acyl fatty acid; E, esterified ω-position; S, sphingosine; O, ω-hydroxylated *N*-acyl fatty acid; N, non-hydroxylated *N*-acyl fatty acid; P, phytosphingosine.

2 h at 60°C, recovered by extraction into chloroform and separated by TLC using twice chloroform-methanol-acetic acid (190:9:1, vol/vol/vol) as developing solvent. For quantitative analytical TLC determination, increasing amounts of standard lipids (*N*-stearoyl-sphingosine (kind gift of Beiersdorf AG, Hamburg, Germany), glucosylceramide (purified from Gaucher spleen in our laboratory)), and palmitic acid (Fluka Buchs, Switzerland) were applied. After development, plates were air-dried, sprayed with 8% (wt/vol) H_3PO_4 containing 10% (wt/vol) $CuSO_4$ charred at 180°C for 10 min.

2.4. MALDI mass spectrometry

Bound GlcCer(OS) was recovered and isolated from RecNci I and wild-type epidermis by TLC techniques as described above. The lipid was scraped from the plates and redissolved in chloroform-methanol 1:1 (vol/vol). Approximately 50 to 200 pmoles of lipid were mixed with 20 µg 2,5-dihydroxybenzoic acid (ICN Biochemicals, USA) directly on the target. Mass spectrometric analysis was performed on a TofSpec E (MicroMass, Manchester, UK) mass spectrometer operating at an acceleration voltage of 20 kV with a 337 nm nitrogen laser. External calibration was performed using PEG-1000 (Sigma, Deisenhofen, Germany).

3. Results and discussion

Although expression of β-GlcCerase is known to be required for epidermal function and homoeostasis [5–7], the consequences of β-GlcCerase deficiency for the formation of the lipid-bound envelope has not been addressed so far. Therefore, we analyzed the composition of the lipid-bound envelope in β-GlcCerase deficient Gaucher type 2 (RecNci I) mice. Unbound lipids were extracted from RecNci I and wild-type epidermis, separated by TLC and quantified (Fig. 1a, b). The structures of GlcCers and Cers are indicated according to the terminology proposed by Motta et al. [12] and modified by Robson et al. [13]. Briefly, the Cer structures are denoted by the composition of the sphingoid base (either sphingosine = S or phytosphingosine = P) and the *N*-acyl fatty acid by the presence of an α-hydroxy group = A, an ω-hydroxy group = O, or no hydroxy group = N, and whether the omega position is further acylated with linoleic acid (i.e. esterified = E). As expected, all three GlcCer species separated by TLC accumulated in RecNci I mice compared to wild-type levels, the most hydrophobic GlcCer(EOS) approximately 7-fold, the complex GlcCer a/b fraction 8.5-fold, and α-hydroxylated GlcCer(C16-AS) 3.5-fold over control (Fig. 1a). RecNci I mice mainly stored GlcCer(EOS) and GlcCer a/b in contrast to the previously described sphingolipid activator protein deficient pSAP knockout mice which mainly accumulated the rather hydrophobic GlcCer(EOS) within their epidermis [11]. Apparently its enzymatic hydrolysis is most dependent on the presence of SAP-C. In RecNci I point mutation mice the GlcCer accumulation was accompanied with an overall reduction of ceramides [7]. Here, we analyzed the levels of five individual ceramide subtypes in RecNci I mice in comparison to wild-type levels (Fig. 1b). Most importantly, Cer(EOS), the product of GlcCer(EOS) hydrolysis, was nearly absent in RecNci I mice. The levels of Cer(NS), (NP) and (C24,26-AS), which previously have been shown to comprise the lipid backbones of GlcCer a/b [11], also were reduced to less than 40% of normal control. Interestingly, Cer(C16-AS) levels remained unaffected indicating a direct de novo synthetic route not requiring subsequent glucosylation/deglucosylation steps for this most polar Cer species. It seems reasonable that transient glucosylation of the more hydrophobic ceramides is needed to allow intracellular transport to the

multilamellar bodies and after extrusion a proper insertion of the ceramides into the lamellar sheets within the interstices of the stratum corneum.

Corneocytes are coated by a monolayer of covalently (i.e. ester linked) ω-hydroxylated Cers (Cer(OS)) and fatty acids [2,3,11]. Although little is known about the attachment of lipids to proteins of the epidermal cornified cell envelope, it was assumed that free Cer(EOS) are the direct precursors of bound Cer(OS). To test this hypothesis, we analyzed the covalently, i.e. ester linked epidermal lipids in RecNci I mice (Figs. 1c and 2). To ascertain that the isolated lipids were indeed covalently attached, each epidermal sample was preextracted until no additional lipids could be recovered (Fig. 2, lanes 1 and 3). Then the samples devoid of free lipids were subjected to alkaline hydrolysis and reextracted [11]. In wild-type mice, the predominant constituents of the lipid bound envelope were ω-OH fatty acids and Cer(OS). However, in RecNci I mice levels of both, bound Cer(OS) and ω-OH fatty acids were lowered down to 10% of normal control (Fig. 1c), whereas the predominant bound lipid in the mutant stratum corneum was GlcCer(OS) which was hardly detectable in wild-type epidermis (Figs. 1c and 2). MALDI mass spectrometrical analysis of GlcCer(OS) from RecNci I and wild-type mice revealed a fatty acid chain length distribution of mainly C32:1, C32:0 and C34:1 (Fig. 3), as previously found in Cer(OS) and ω-OH fatty acids [11]. In accordance with previous findings [3,11] these data support the view that free ω-hydroxylated glucosylceramides esterified to linoleic acid are transferred by a transesterification reaction to carboxylated side chains of surface proteins (e.g. involucrin, envoplakin and periplakin) of the corneocytes, a reaction in which the linoleoyl residue serves as leaving group. Then the protein linked glucosylceramides are hydrolyzed by the sequential action of glucocerebrosidase and acid ceramidase which need sphingolipid activator proteins (SAPs C and D) to enhance their activity against these rather hydrophobic substrates [11,14,15]. These catabolic steps are severely affected in RecNci I and in pSAP knock out mice, both of which suffer from a severely affected lipid bound envelope and an abnormality in lamellar membrane maturation within the interstices of the stratum corneum. Therefore, the formation of ω-hydroxylated glucosylceramides, presumably covalently linked through their ω-hydroxyacyl residues to glutamate residues of cell surface proteins such as involucrin seems to be a key step in the formation of the lipid bound envelope and the water permeability barrier in the stratum corneum of the skin of land living animals.

Since both, Cer(EOS) and GlcCer(EOS), now are putative precursors of the lipid bound envelope, it will be of importance for future work to identify the enzyme that attaches the lipid to the protein (i.e. a transacylase or transglutaminase) and to elucidate its substrate specificity.

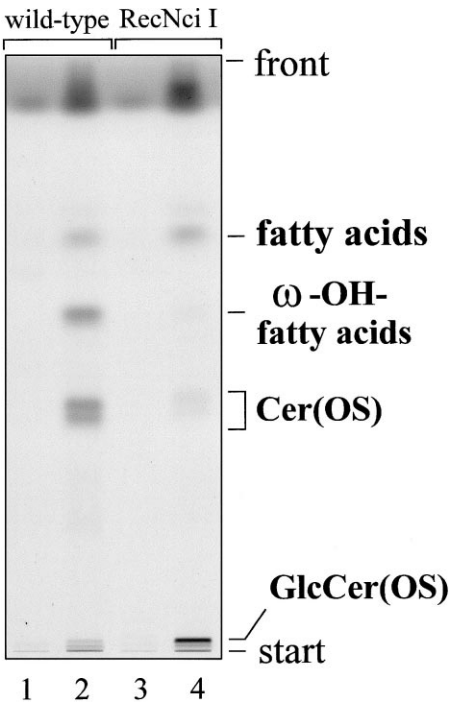

Fig. 2. Separation of covalently bound lipids of RecNci I and wild-type mice. Bound epidermal lipids were released by base hydrolysis and analyzed by TLC. A representative thin-layer chromatogram of recovered lipids from wild-type (lane 2) and RecNci I mice (lane 4) is shown. Note that the samples were devoid of unbound lipids before undergoing base hydrolysis (lanes 1 and 3). O, ω-hydroxylated *N*-acyl fatty acid; S, sphingosine.

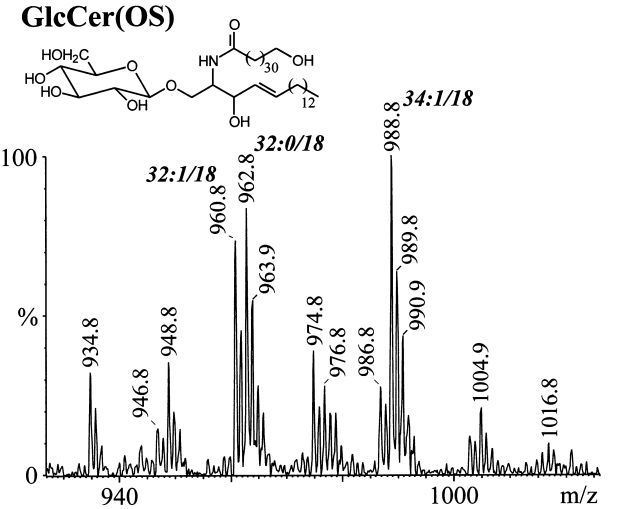

Fig. 3. MALDI mass spectrometry of GlcCer(OS). Bound GlcCer(OS) was released by base hydrolysis, isolated by TCL, and analyzed using a TofSpec E MALDI mass spectrometer operating in positive ion mode. Indicated molecular masses represent sodium adducts of intact molecules. Likely compositions of most prominent peaks are indicated. The fatty acids are indicated first followed by the chain length of the sphingoid bases. O, ω-hydroxylated *N*-acyl fatty acid; S, sphingosine.

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References

[1] Elias, P.M. and Menon, G.K. (1991) Adv. Lipid Res. 33, 301–313.
[2] Wertz, P.W. and Downing, D.T. (1987) Biochim. Biophys. Acta 917, 108–111.
[3] Marekov, L.N. and Steinert, P.M. (1998) J. Biol. Chem. 273, 17763–17770.
[4] Swartzendruber, D.C., Wertz, P.W., Madison, K.C. and Downing, D.T. (1987) J. Invest. Dermatol. 88, 710–713.
[5] Holleran, W.M., Takagi, Y., Menon, G.K., Legler, G., Feingold, K.R. and Elias, P.M. (1993) J. Clin. Invest. 91, 1656–1664.

[6] Holleran, W.M., Ginns, E.I., Menon, G.K., Grundmann, J.U., Fartasch, M., McKinney, C.E., Elias, P.M. and Sidransky, E. (1994) J. Clin. Invest. 93, 1756–1764.
[7] Liu, Y., Suzuki, K., Reed, J.D., Grinberg, A., Westphal, H., Hoffmann, A., Doering, T., Sandhoff, K. and Proia, R.L. (1998) Proc. Natl. Acad. Sci. USA 95, 2503–2508.
[8] Liu, K., Commens, C., Choong, R. and Jaworski, R. (1988) Arch. Dis. Child. 63, 854–856.
[9] Sidransky, E., Sherer, D.M. and Ginns, E.I. (1992) Pediatr. Res. 32, 494–498; 63, 854–856.
[10] Beutler, E. and Grabowski, G.A. (1995) in: C.R. Scriver, A.L. Beaudet, W.S. Sly and D. Valle (Eds.), The Metabolic and Molecular Basis of Inherited Disease, McGraw-Hill, New York, pp. 2641–2671.
[11] Doering, T., Holleran, W.M., Potratz, A., Vielhaber, G., Elias, P.M., Suzuki, K. and Sandhoff, K. (1999) J. Biol. Chem., in press.
[12] Motta, S., Monti, M., Sesana, S., Caputo, R., Carelli, S. and Ghidoni, R. (1993) Biochim. Biophys. Acta 1182, 147–151.
[13] Robson, K.J., Stewart, M.E., Michelsen, S., Lazo, N.D. and Downing, D.T. (1994) J. Lipid Res. 35, 2060–2068.
[14] Ho, M.W. and O'Brien, J.S. (1971) Proc. Natl. Acad. Sci. USA 68, 2810–2813.
[15] Klein, A., Henseler, M., Klein, C., Suzuki, K., Harzer, K. and Sandhoff, K. (1994) Biochem. Biophys. Res. Commun. 200, 1440–1448.