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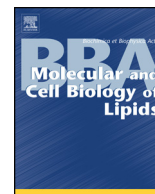
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Selectivity in cornified envelop binding of ceramides in human skin and the role of LXR inactivation on ceramide binding

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ABSTRACT

The cornified lipid envelope (CLE) is a lipid monolayer covalently bound to the outside of corneocytes and is part of the stratum corneum (SC). The CLE is suggested to act as a scaffold for the unbound SC lipids. By profiling the bound CLE ceramides, a new subclass was discovered and identified as an omega-hydroxylated dihydro-sphingosine (OdS) ceramide. Bound glucosylceramides were observed in superficial SC layers of healthy human skin. To investigate the relation between bound and unbound SC ceramides, the composition of both fractions was analyzed and compared. Selectivity in ceramide binding towards unsaturated ceramides and ceramides with a shorter chain length was observed. The selectivity in ceramide species bound to the cornified envelope is thought to have a physiological function in corneocyte flexibility. Next, it was examined if skin models exhibit an altered bound ceramide composition and if the composition was dependent on liver X-receptor (LXR) activation. The effects of an LXR agonist and antagonist on the bound ceramides composition of a full thickness model (FTM) were analyzed. In FTMs, a decreased amount of bound ceramides was observed compared to native human skin. Furthermore, FTMs had a bound ceramide fraction which consisted mostly of unsaturated and shorter ceramides. The LXR antagonist had a normalizing effect on the FTM bound ceramide composition. The agonist exhibited minimal effects. We show that ceramide binding is a selective process, yet, still is contingent on lipid synthesized.

1. Introduction

The outer skin layer, the stratum corneum (SC), consists mainly of terminally differentiated keratinocytes (corneocytes) embedded in an extracellular lipid matrix of ceramides, free fatty acids, and sterols. During the terminal differentiation process, the plasma membrane is replaced by a crosslinked protein structure called the cornified envelope (CE) [1]. Covalently bound to the CE is a monolayer of lipids referred to as the cornified lipid envelope (CLE). These lipids act as an interface between the hydrophilic corneocytes and the lipophilic extracellular lipids and are therefore an essential part of the skin barrier [2,3]. With a reduced CLE, mice exhibit severe skin barrier problems [4].

It has been reported that the CLE consists of ceramides and fatty

acids [5–7]. Ceramides are composed of two parts: a sphingoid base and an acyl chain. The molecular architecture of the ceramide its hydrophilic head group defines the ceramide subclass [8]. The bound ceramides comprise of a subset of the unbound subclasses [5,6], supplemental S1 depicts the structures of these subclasses. This subset has an omega-hydroxylated acyl chain (Ocers). The hydroxylated group at the end of the carbon tail facilitates an ester linkage to the CE proteins [9,10]. It also facilitates the binding of a linoleic acid. Both unbound SC ceramides (in the lipid matrix) and the bound SC ceramides (attached to cornified envelope) originate from the same pool of precursor lipids [11].

During the differentiation process the SC lipids are synthesized by keratinocytes [12]. In the upper part of the viable epidermis keratinocytes contain specific lipid vesicles called lamellar bodies. They contain

Abbreviations: SC, stratum corneum; CLE, cornified lipid envelope; OdS, omega-hydroxylated dihydro-sphingosine; LXR, liver X receptor; FTMs, full thickness models; CE, cornified envelope; Ocers, omega-hydroxylated ceramides; GlcCers, glycosylated ceramides; EOcers, linoleate esterified omega hydroxylated acyl chain ceramides; Rt, retention times; T, dihydroxy-dihydro-sphingosine; MuCer, ceramide with an unsaturated acyl chain; MCL, mean carbon chain length; PuCer, poly-unsaturated ceramides; pSAP^{-/-}, sphingolipid activator proteins precursor; GBA1, glucosyl cerebrosidase; UCGC, UDP-glucose ceramide glucosyltransferase

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the bound and unbound precursor lipids. At the interface between the viable epidermis and SC, ceramide precursors (glycosylated (GlcCers) or phosphocholinated (sphingomyelin) ceramides [13]) are released into the extracellular space together with the enzymes to convert them [14]. Simultaneously, formation of the CLE occurs [15,16]. Enzymes involved in ceramide binding to the CE have been colocalized at the viable epidermis SC interface [17]. Essential to ceramide binding is that ceramides have I) a linoleate esterified omega hydroxylated acyl chain (EOcers) [18–20] and II) are glycosylated. The esterified linoleate is removed, the GlcCers are bound, and thereafter, they are cleaved to ceramides [11]. Which ceramides of the pool of precursors are bound and if this is a selective process is not yet reported.

To study which ceramides are selected for CE binding, SC from *ex vivo* skin and from skin models was used. Comparing the composition of both bound and unbound lipid extracts of *ex vivo* SC could elucidate whether ceramide binding is a selective process. Human skin equivalent full thickness models (FTMs) were selected as skin model. These models mimic aspects of normal human skin including SC generation. Yet, in FTMs the SC lipids had an increased fraction of unsaturated ceramides and a reduced mean ceramide chain length [21,22]. It was hypothesized that the bound lipid fraction of FTMs was altered too. LXR is a nuclear receptor sensitive for sterols and responsible for multiple lipid processing pathways [23], one of which is regulation of Stearoyl coenzyme A desaturase 1 responsible for the desaturation of fatty acids also used in the epidermal ceramide biosynthesis [24]. By adding a liver X-receptor (LXR) agonist or antagonist, the lipid metabolism of FTMs can be manipulated [25] (paper is submitted to JLR). LXR signaling was hypothesized to affect the bound lipids in FTMs as well.

Recently, a new LC-MS method to simultaneously profile and quantify the unbound SC ceramide fraction was developed and could be applied to discover new bound ceramides [26]. Quantification of the entire bound ceramide profile provides insight into subclass and chain length distribution and the degree of unsaturation. This method was applied to the bound ceramide fractions of *ex vivo* SC, tape stripped SC, and FTMs. We aimed to examine if ceramide binding is a selective process and if this process is affected in FTM by an LXR agonist or antagonist.

2. Materials and methods

2.1. Chemicals

For extraction and analysis of the lipids the following solvents were used: UPLC grade methanol, ethanol, isopropanol (Biosolve, Valkenswaard, the Netherlands), HPLC grade methanol, chloroform (Lab-Scan Gliwice, Poland), and n-heptane, and milli-Q water (18.2 Ω). KCl and NaOH (Merck KGaA, Darmstadt, Germany) were used in the extraction. Supplemental 1 contains a list of standards and the internal standard used for ceramide quantification.

2.2. Samples

All human skin was obtained after institutional approval and a written informed consent according the declaration of Helsinki.

2.2.1. *Ex vivo* SC samples

Skin from two mammoplasties and one abdominoplasty was dermatomed to a thickness of 0.6 mm within 24 h after surgery. The dermatomed skin was incubated overnight on filter paper containing 0.1% trypsin solution in PBS and thereafter digested for 1 h at 37 °C. SC sheets were peeled off, thoroughly washed with 0.1% trypsin-inhibitor followed by 2 \times milli-Q water, and were air dried before storage. For each donor, 3 SC samples of each \sim 3.5 mg SC dry weight were used for lipid extraction and subsequent bound lipid extraction.

2.2.2. *In vivo* tape-stripped SC samples

One site on the ventral forearm of the volunteer was tape-stripped using polyphenylene sulfide tape (Nichiban, Tokyo, Japan). Equal pressure was applied on the tapes using a 1 kg weight and a D-squame pressure instrument with additional foam pad and the removal direction alternated. The amount of material on the tapes was determined by detecting the absorption at 850 nm using a SquameScan (Heiland Electronic, Wetzlar, Germany), subtracting the absorbance of a blank tape. The first five tapes were discarded and four consecutive tapes were used for the bound lipid extraction. Each tape was extracted individually.

2.2.3. Full thickness models SC samples

FTMs were generated using isolated human keratinocytes obtained from three different skin donors. Keratinocytes were seeded on a collagen gel populated with fibroblasts and FTMs were generated as described by Thakoersing et al. [22]. LXR activation or inhibition was achieved by supplementation of the agonist T0901317 or antagonist GSK2033, respectively (Sigma Aldrich, Zwijndrecht, the Netherlands). The concentration of the agonist and antagonist in the medium was 500 nM (with an EC₅₀ of 50 nM and IC₅₀ of 32 nM); 0.05 vol% of a 1 mM stock in DMSO. For each donor FTMs were generated under four different conditions: FTM_{CONTROL} (normal medium), FTM_{DMSO} (medium + DMSO), FTM_{AGONIST} (Medium + Agonist), and FTM_{ANTAGONIST} (Medium + Antagonist). The FTMs SC was isolated as described above.

2.3. Lipid extractions

The SC samples were dried under vacuum at 50 °C, stored under N₂, and weighted using a microbalance ME5 (Sartorius, Bradford, MA, USA). To obtain the unbound lipids, SC sheets and tape-strips were first extracted using a modified four step Bligh and Dyer procedure [26]. MilliQ and 0.04 vol% of aqueous 0.25 M KCl was added to the combined extracts of the four steps. After phase separation over night at 4 °C the organic layer was collected. Thereafter, the hydrophilic layer was washed with chloroform and the organic layers were combined, filtered (0.20 μ M PTFE filter), evaporated under N₂, and reconstituted in chloroform:methanol (2:1, v:v), 0.4 ml per mg SC dry weight.

After extraction of the unbound lipids, the SC sheets were washed with chloroform and dried under vacuum. A solution of 10% 1 M aqueous NaOH and 90% methanol (v:v) was added to the dried samples to detach the bound lipids. For saponification the samples were shaken for 1 h with 120 rpm at 60 °C. To stop the reaction, the pH of the samples was decreased to a final pH between 4 and 7 using 1 M and 0.1 M HCl. After saponification tape-strips and SC sheets were further extracted at 40 °C by using subsequently: chloroform:methanol (1:1, v:v), chloroform:methanol (2:1, v:v), and heptane:isopropanol (1:1, v:v). Thereafter, the combined bound lipid extracts were treated as described above for the unbound lipids. The extraction efficiency and recovery of the bound lipid extraction are described in supplemental S2 and were both above 90%.

Before analysis, the concentration of extracts of unbound and bound lipids from SC sheets was determined by the gravimetric difference of a specific volume before and after drying. For LC-MS analysis, unbound lipid samples were prepared at 0.3 mg/ml of extract and tape-strip extract at 20 tapes/ml. Bound lipid samples were prepared at 0.1 mg/ml and tape-strip extracts at 40 tapes/ml. All samples were dissolved in heptane:chloroform:methanol (95:2.5:2.5, v:v:v) with internal standard added at 0.6 μ M.

2.4. LC-MS and LC-MS/MS analysis

All samples were analyzed and quantified using a Waters Acquity UPLC H-class (Waters, Milford, MA, USA) connected to an XEVO TQ-S mass spectrometer (Waters, Milford, MA, USA). Of the samples, 5 μ l was

injected and separated on a pva-silica column (5 μM particles, 100×2.1 mm i.d.) (YMC, Kyoto, Japan). Quantitative analysis was performed as described previously [26]. For identification, LC-MS/MS data were obtained with Scanwave daughter scan mode on the m/z values of interest with fragmentation energies of 40 or 50 eV. Supplement Table 2 shows the m/z ratios analyzed to identify the omega-hydroxylated dihydrosphingosine (OdS) ceramide.

2.5. Quantification

All ceramides that were identified in the bound and unbound fraction of the *ex vivo* SC samples were quantified to pmol and ng injected into the LC. Because the bound lipid extracts contained other materials than lipids (explained below), it was essential to normalize it to SC dry weight. The total amount of material extracted from the SC was calculated by multiplying the concentration of the extract with the volume in which it was dissolved. Either 1.5 μg (unbound sample) or 0.5 μg (bound sample) of this material was injected into the LC. The total amount of ceramides detected in this injected fraction of the sample was used to calculate the amount of ceramides in the complete extract (nmol or μg). The total amount of ceramides in the extract (μg) was normalized to the SC dry weight in mg. To determine which fraction of ceramides was bound, molar amounts per mg of SC of the unbound and bound ceramides were compared.

As a control for the gravimetric approach to determine the bound lipid amount extracted, three blank saponified extractions were performed. Supplemental S3 shows the results and discussion of these experiments. It was concluded that in saponified extracts excess non-lipid materials (most probably salts) were present.

2.6. Software and statistics

LC-MS and LC-MS/MS data analysis was performed using MassLynx and TargetLynx V4.1 SCN 843 (Water Inc. Milford, MA, USA). Statistical analyses were performed using linear mixed modeling in SPSS version 24 (IBM Corp.). For a detailed description of the models used see supplemental 4, 5, and 9.

3. Results

3.1. Profiling and quantification

To examine which bound ceramides could be detected by our method, a profile was made by LC-MS. Fig. 1A depicts an ion-map profile of a 0.5 μg *ex vivo* SC bound ceramide extract. Previously observed ceramide subclasses OS, OP, and OH were detected, however, additional compounds were detected as well. The retention times (Rts) of two groups of compounds corresponded to the Rts of GlcCers and had masses corresponding to Glc-OS and Glc-OH subclasses. A part of the GlcCers loses their glucose group during ionization. This was used to confirm the presence of bound GlcCers by detecting OS at the same Rt as Glc-OS (Supplemental S6). Bound GlcCers were also detected in a tape-strip sample, yet, they are not observed in the corresponding unbound fraction.

One group of compounds had a shorter Rt than that of subclass OS and a mass of 2 amu higher. To confirm that this was the suspected subclass OdS, fragmentation of 3 saturated, 2 unsaturated, and 1 odd number chain length of these compounds was performed (Supplementary Table S2). Fig. 1B depicts the fragmentation spectrum of OdS C52:0. The characteristic fragments of dS ceramides were observed (294.3 and 322.3 amu), depicted in Fig. 1B. As comparison a fragmentation spectrum of OS C52:0 is shown in Fig. 1C, depicting a sphingoid fragment 2 amu lower (292.3 and 320.3 amu). Finally, a group of compounds was observed with the mass of an omega-hydroxyl acyl chain with a dihydroxy-dihydrosphingosine (T). The latter was described by t'Kind et al. [27]. Combined with their long Rt we assumed

this is ceramide subclass OT. Only including the subclasses OdS, OS, OP and OH, a total of 103 different bound ceramides were detected in *ex vivo* SC bound lipid extracts.

Thereafter, the profiled bound ceramides were quantified. Next to *ex vivo* SC, a tape strip extract was examined to determine if this method was applicable to this sample type. Fig. 1D and E show the quantitative profiles of a representative *ex vivo* SC sample and a tape-strip sample, expressed as the molar ceramide amount per mg SC or the amount per amount SC stripped (expressed as absorbance at 850 nm), respectively. The profile showed that: 1) It is possible to isolate the bound ceramides from tape-stripped SC after extraction of the unbound ceramides; and 2) the bound ceramide profile of a tape-strip sample was similar to that of an *ex vivo* SC sample.

To compare the bound and unbound fractions, the total amount of unbound ceramides in the *ex vivo* SC was quantified. Unbound and bound ceramide amounts are provided in Fig. 2. Fig. 2A depicts the total unbound and bound ceramide amounts in μg per mg SC, whereas Fig. 2B depicts the molar percentage of ceramides that were bound. It was observed that: I) there were no large differences between the bound ceramide amounts of the three donors; II) 2.5–4 w% of the SC weight was ceramides; and III) about 20 mol% of the ceramides in the SC was bound. Table 1 depicts the relative molar amount (%) of each bound ceramide subclass to the total bound ceramides amount. In the next section, the bound ceramide fraction and how it relates to the unbound fraction will be examined in more detail.

3.2. Selectivity in ceramide binding

To examine if there is selectivity in ceramide binding, the differences in composition between the bound and unbound ceramide fraction of *ex vivo* SC were determined. It was observed that the bound ceramides contained large amounts of ceramides with a mono-unsaturated acyl chain (MuCers) and this was higher than the unbound fraction (Fig. 3A). Differences in MuCer % between the 3 donors were observed. Changes for each donor were consistent; if the unbound MuCer% increased, the bound MuCer% increased too. MS/MS fragmentation of the MuCers showed that the double bond was only present in the ceramides acyl chain (Supplemental S7).

To compare the bound and unbound ceramides originating from the same GlcCers, the unbound counterparts of the bound ceramides fraction were examined: EOcers and Ocers. The unbound ceramides with the highest MuCers content were the EO and O ceramide subclasses (Supplemental S8). Of the bound and unbound ceramides the MuCers percentage within each subclass was determined and plotted per subclass (Fig. 3B). Comparing these data with a linear mixed model showed that the bound Ocer fraction consisted of a significantly higher MuCers percentage than both the unbound EOcer and Ocer subclasses, except for subclass OdS (Supplemental S4 depicts the linear mixed model results). In the unbound fraction, subclasses OdS, OP, and OH contained a higher percentage MuCers than their corresponding EOcer subclasses. Comparing the bound and unbound ceramides degree of unsaturation indicates selective binding towards unsaturated ceramides.

Alongside the MuCers percentage, the bound and unbound fractions mean carbon chain lengths (MCL) were calculated. To compare the bound and unbound subclasses, the MCL of the Ocer and EOcer subclasses were determined. MuCers are depicted separately because they had a longer MCL than saturated ceramides (linear mixed model results Supplemental S5). To compare the EOcer to the Ocer, 18 carbons were subtracted from EOcers MCL to compensate for the esterified linoleates additional 18 carbons. Fig. 3C depicts the MCL for the bound Ocer, unbound Ocer, and unbound EOcer. The MCL of the bound ceramides differed significantly from that of the unbound ceramides. For both the saturated ceramides and MuCers, the MCL was shorter in the bound lipid fraction. The chain length of both unbound Ocers and the EO-C18 ceramides were comparable. These findings on MCL show that there was selectivity towards binding of shorter ceramides to the cornified

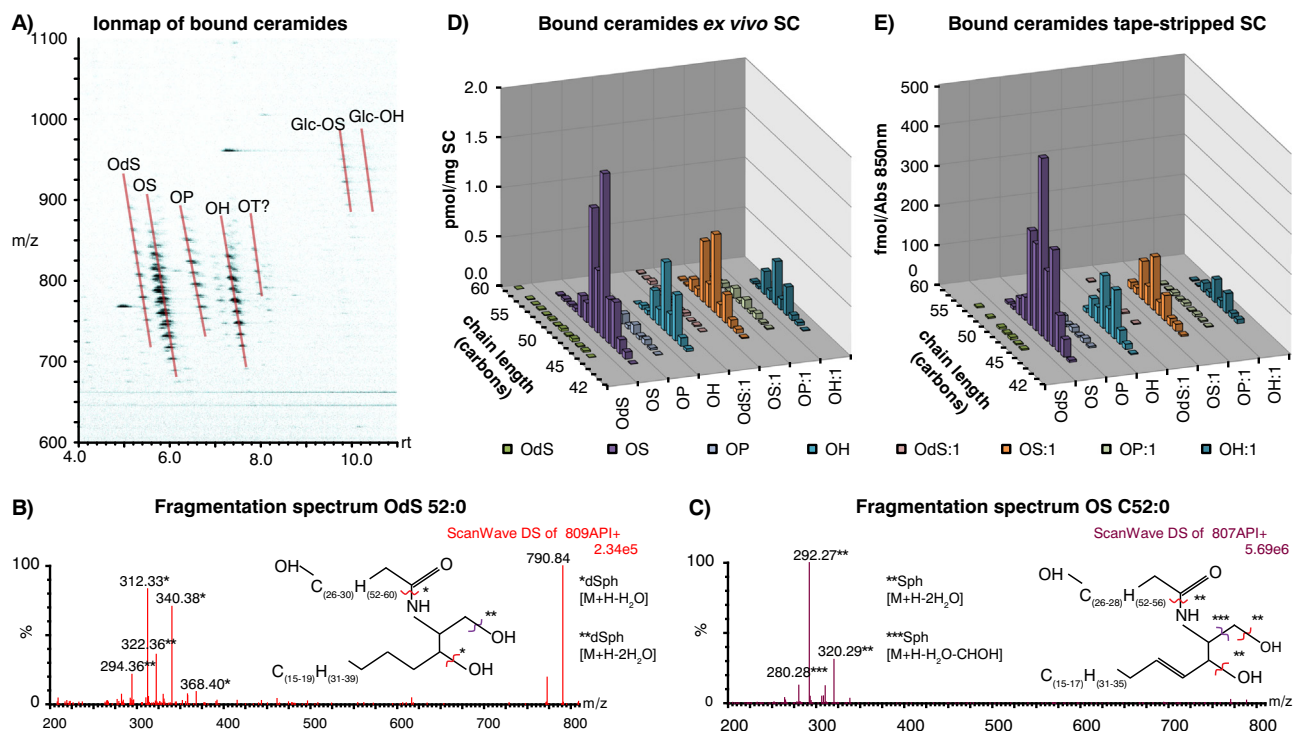


Fig. 1. Profiling of bound ceramide fraction.

A: An ion-map of the bound ceramide fraction of *ex vivo* SC. Each ceramide subclass is indicated by a red line. B: Fragmentations spectrum of the compound eluting before OS, m/z 808.81 and collision energy of 40 eV. C: Fragmentations spectrum of OS C52:0, m/z 806.80 and collision energy of 50 eV. D and E: The amount of all quantified bound ceramides shown per subclass and chain length of an *ex vivo* SC samples and a tape-stripped SC sample, respectively.

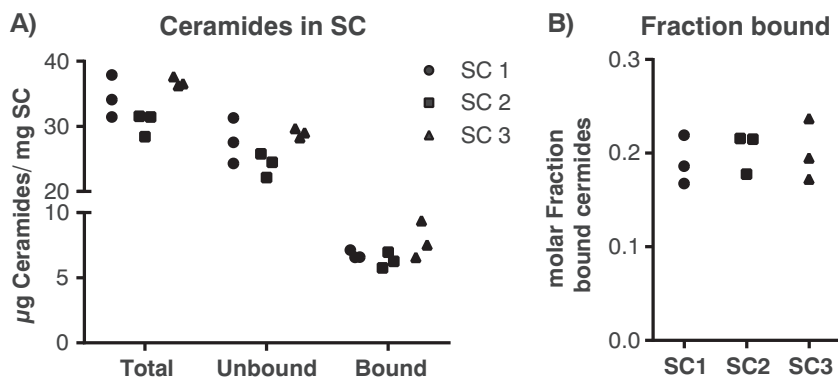


Fig. 2. Ceramide fraction of the SC.

A: The amount of ceramides in *ex vivo* SC as μg of ceramides per mg of SC. B: The percentage of ceramides which was bound for the three SC donors (SC1, 2 and 3).

Table 1

The relative molar amounts of the bound ceramides per subclass. Given as the mean and SD ($n = 3$). The Σ molar amount of all bound ceramides in a sample was set to 100%.

Subclass	SC 1		SC 2		SC 3	
	Molar %	SD	Molar %	SD	Molar %	SD
OdS	1.5	> 0.1	2.3	0.3	1.3	> 0.1
OS	51.1	0.5	48.6	0.7	43.9	1.3
OP	3.8	0.3	5.1	0.2	3.5	0.1
OH	20.6	0.2	25.3	0.7	17.7	1.0
OdS:1	0.8	0.1	0.9	0.2	1.4	0.3
OS:1	13.3	0.2	9.3	0.1	18.8	1.5
OP:1	2.1	> 0.1	2.1	0.2	3.1	0.2
OH:1	6.8	0.3	6.3	0.1	10.3	0.3

envelope.

3.3. The effect of LXR on the bound lipid fraction in FTMs

Previously, alterations in unbound SC ceramides of FTMs were observed. It was examined if similar changes were observed in the bound ceramide fraction and if the bound ceramides were influenced by the LXR. In Fig. 4A quantitative amounts of bound ceramides/mg SC are provided for four FTM conditions: FTM_{CONTROL}, FTM_{DMSO}, FTM_{AGONIST}, and FTM_{ANTAGONIST}. No differences in the total amount of bound ceramides were observed between the various FTM conditions. It was observed that there was a significantly lower quantity of bound ceramides in the FTMs compared to *ex vivo* SC samples. Supplemental S9 gives an overview of the statistical tests and output comparing the sample groups.

When examining the bound lipid fraction of the FTMs, it consisted predominantly of MuCers (Fig. 4B) and even polyunsaturated

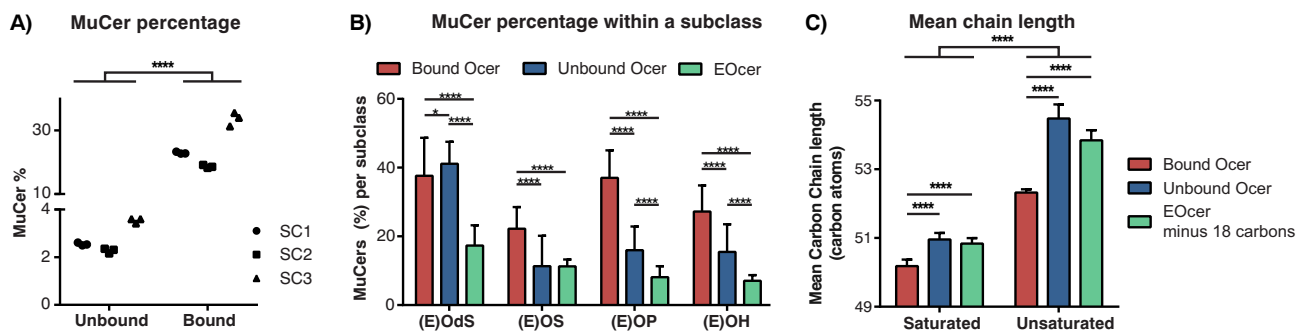


Fig. 3. Selectivity in ceramide binding for multiple parameters.

A: The percentage of MuCers in the bound and unbound ceramides of three SC donors. B: The percentage of MuCers within a subclass, comparing the bound O, unbound O, and EO ceramides with the same sphingoid base. In a sample, the sum of both the saturated and unsaturated ceramides of a subclass was set as a 100%. C: The MCL of the bound and unbound Ocers is shown for the saturated and unsaturated ceramides. From the EOcers MCL, 18 carbons were subtracted to compensate for the linoleate moiety. Bars are mean \pm SD of 9 samples, 3 per donor. Parameter estimates of the fixed effects are given in Supplemental S2 and S3.

ceramides (PuCer) were detected. Table 2 depicts the relative molar composition of the four different conditions. The percentage of bound MuCers in FTMs was significantly higher than in *ex vivo* SC. By adding an LXR antagonist a significant decrease in the percentage of MuCers was observed. No effect was observed from adding the LXR agonist.

For FTMs the bound ceramides MCL of saturated and unsaturated

bound ceramides was determined and compared to that of *ex vivo* SC (Fig. 4C). In FTMs, a significant decreased MuCers MCL was observed compared to *ex vivo* SC. The MCL was further decreased by the LXR agonist. However, using the LXR antagonist the MCL is increased to the MCL of *ex vivo* SC. Due to the lower percentages of saturated ceramides (see Table 2), the MCL of the saturated ceramides was predominantly

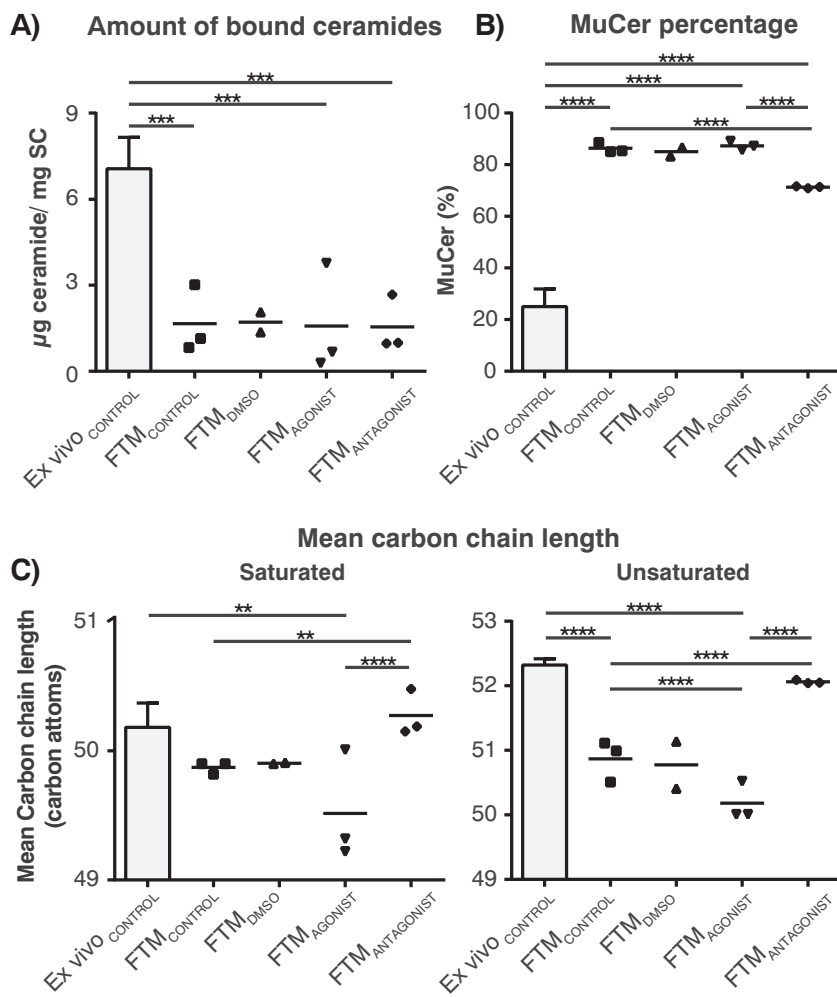


Fig. 4. Effects of LXR in FTMs on the bound lipids.

A: The amount of bound ceramides per mg SC. B: The percentage of the bound ceramides that had a monounsaturated acyl chain. C: The MCL of the saturated and unsaturated ceramides. All depict the *ex vivo* control as bar (mean \pm SD $n = 9$), FTMs: control, DMSO, AGONIST and ANTAGONIST, are shown as individual points and mean. Parameter estimates of the fixed effects are given in supplemental S9.

Table 2

The mean and SD (n = 3 except DMSO which had n = 2) of the bound lipids relative molar subclass amount in FTMs. For each sample the Σ molar amount of all bound ceramides was set to 100%.

Subclass	FTM _{CONTROL}		FTM _{DMSO}		FTM _{AGONIST}		FTM _{ANTAGONIST}	
	Molar %	SD	Molar %	SD	Molar %	SD	Molar %	SD
OdS	n.d.*		0.4	n.a.**	0.6	n.a.**	0.6	0.4
OS	6.8	1.4	6.5	1.1	3.4	1.1	17.5	1.8
OP	0.6	n.a.**	0.8	n.a.**	0.5	0.2	0.9	0.6
OH	2.9	0.6	2.9	0.4	2.8	0.6	8.0	0.2
OdS:1	2.4	0.7	3.5	2.0	3.2	0.2	2.7	0.5
OS:1	53.4	1.2	52.2	3.4	51.6	4.3	42.8	1.2
OP:1	6.2	0.9	6.6	1.7	7.5	0.8	6.1	0.6
OH:1	24.4	3.4	22.7	2.6	25.1	4.3	19.7	2.0
OdS:2	0.3	n.a.**	0.9	n.a.**	0.9	0.0	0.2	n.a.**
OS:2	2.5	0.2	3.1	0.4	3.5	0.1	1.6	1.0
OP:2	0.3	n.a.**	0.2	n.a.**	0.5	n.a.**	0.1	n.a.**
OH:2	1.0	0.3	1.2	0.3	1.8	0.4	0.9	n.a.**

* n.d. not detected.

** n.a. not available (only detected in one of the replicates).

based on subclass OS. Nonetheless, the same trends were observed as in the MuCers. Concluding, FTMs had an altered bound lipid composition compared to *ex vivo* SC. An LXR antagonist had a normalizing effect on the FTMs bound ceramide composition, reducing the percentage of MuCers and increasing the MCL.

4. Discussion

Selectivity in ceramide cornified envelope binding was observed by comparing compositions of the unbound EOcer and Ocer subclasses to the bound SC ceramide fractions. In FTMs, the ceramide binding was reduced and the bound ceramide composition influenced by LXR. The new ceramide subclass OdS was identified [5–7]. Furthermore, a ceramide subclass with masses corresponding to an omega-hydroxy acyl dihydroxy-dihydrosphingosine (OT) was observed. It was hypothesized that dihydroxy-dihydrosphingosine exists as a bound ceramide, because these sphingoid bases have been observed attached to the various acyl chains of the unbound lipids (unpublished data).

Using the compositional differences between the bound and unbound ceramides, it was shown that bound ceramides had a higher degree of unsaturation and shorter carbon chains than their unbound counterparts. Both the bound and unbound ceramides are thought to originate from the same pool of precursor GlcCers [28]. Thus, during the binding process there was selectivity towards MuCers and shorter chain ceramides. Selectivity in ceramide binding conceivably has a physiological importance to provide corneocyte flexibility. Bound lipids form a template for the formation of the SC lipid organization and can align the lipid lamellae parallel to the cornified envelope [4,29,30]. Furthermore, the SC and within it the corneocytes, are not rigid structures but require flexibility [31–33]. Atomic force microscopy studies showed that the CLE is less rigid than the internal corneocyte structures [31,32]. With different hydration states the shape of corneocyte changes [33], requiring flexibility which should also be provided by the CLE. Flexibility of the CLE might be enhanced by a shorter MCL [34,35] and the presence of MuCers, although it is unknown if these are *cis* or *trans* configured either would impact the flexibility.

In FTMs, a lower amount of bound ceramides per mg dry SC was observed than in *ex vivo* SC. Previously published results showed no reduction [36]. It is likely that the observed reduction is due to the reduction of a precursor in the ceramide binding pathway (Fig. 5). In the unbound ceramides of FTMs a large fraction of EOcers contained an esterified oleic acid [37]. Previously, it has been shown that at the viable epidermis SC interface the linoleic acid is converted to an epoxide by 12R-LOX and subsequently to a hydroxy-epoxide by e-LOX3 [3,38]. In mice missing either of these enzymes, reduced amounts of bound

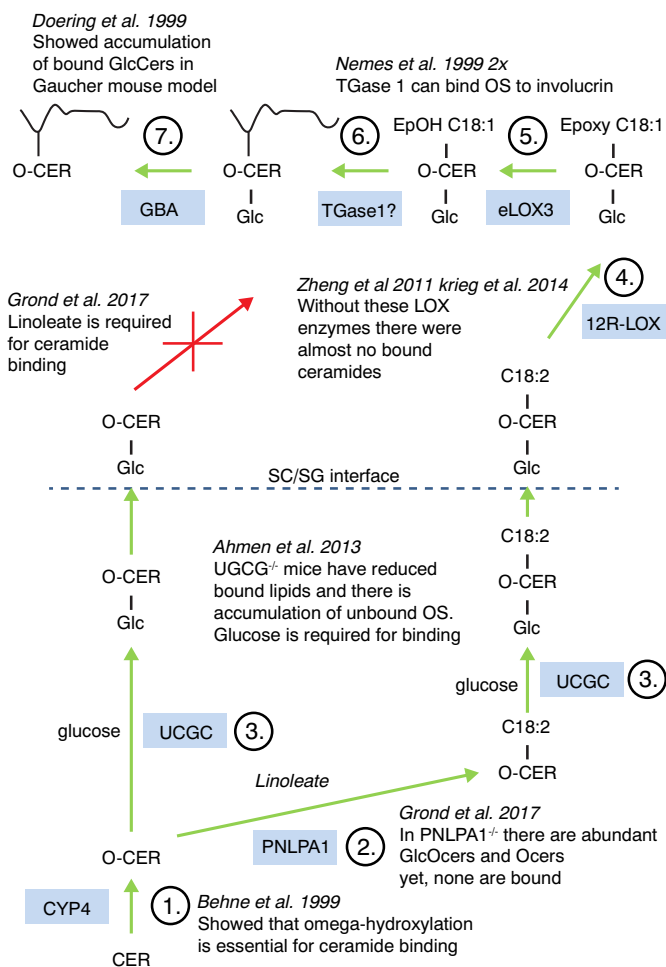


Fig. 5. The pathway of ceramide binding.

1. Attachment of an omega hydroxyl group. 2. Binding of a linoleate. 3. Binding of glucose. 4. Converting the linoleate to an epoxide. 5. Adding a hydroxyl group to the epoxide. 6. Binding of the ceramides. 7. Cleavage of the glucose group from the ceramide.

ceramides were observed, indicating that this process is essential for ceramide binding [4]. Furthermore, in essential fatty acid deficient pigs and mice, linoleic acid of EOcers became substituted with oleic acid, resulted in skin barrier dysfunction [11,39]. Thus, in FTMs the

decreased amount of bound ceramides was likely due to the absence of sufficient amounts of linoleic acid esterified EO ceramides.

FTMs bound ceramides consisted predominately of MuCers and had a decreased chain length compared to those in *ex vivo* SC. Similar as to the bound ceramides, FTMs unbound ceramides also had an increased MuCer fraction and reduced chain length compared to *ex vivo* SC [25] (paper is submitted to JLR) [21,40]. Hence, changes in FTMs bound ceramide composition were comparable to changes in the free ceramide fraction. It was observed that the LXR could alter the unbound SC lipid composition of FTMs [25] (paper is submitted to JLR). Here, it was shown that the bound lipid composition improved upon LXR inhibition compared to the FTM_{control}, reducing the MuCers fraction and increasing the FTMs MCL. Contrastingly, an LXR agonist induced minor changes in the bound ceramide composition, expected as the endogenous substrate would be present in FTMs. Effects of LXR inhibition were comparable to the changes observed in the unbound lipid fraction upon LXR inhibition [25] (paper submitted to JID). This showed that LXR has down-stream effects and can change the CLE composition.

In this study, bound glycosylated ceramides were observed in human *ex vivo* SC and also in tape-strips samples. Other reported that mice with a deficiency in sphingolipid activator proteins precursor (pSAP^{-/-}) and that in a Gaucher disease mouse model, the glycosylated bound ceramides were increased compared to wild type mice [41,42]. Yet, to our knowledge bound GlcCers had never been observed in healthy human SC samples. The bound GlcCers in tape-stripped SC samples demonstrates that the bound GlcCers are also present in superficial layers of the SC, while GlcCers were not detected in the unbound lipids of tape-stripped samples. Free GlcCers primarily reside at the viable epidermis SC interface [14,43]. Whether the bound GlcCers escaped cleavage or that they have biological function is not known, yet, it does indicate that ceramide binding and glucosyl-cleavage are two separate processes. Previous studies showed that glycosylation was required for ceramide binding and free OS was not able to bind to the CE [18,44]. Furthermore, large amounts of bound glycosylated ceramides were observed when the glucosyl-cleavage capacity decreased [41,42]. Together with the present data, there is strong evidence that GlcCers are first bound to the CE followed by cleavage by glucosyl cerebrosidase (GBA1) to a bound ceramide [14].

Our data demonstrate selectivity in the binding process for ceramide linkage to the CE. Here, we propose a selective mechanism for ceramide binding based on our and previously published data (Fig. 5). We propose the following: 1) Ω -hydroxylation of the acyl chain creating Ocers [29]. 2) Mediated by PNLPA1 and with ABHD5 as a co-factor, a linoleate (C18:2) is bound to the Ocers resulting in EOcer [18–20,45]. 3) By UDP-glucose ceramide glucosyltransferase (UGGC), glucose is attached to the remaining Ocers and EOcers and both are stored in lamellar bodies. Glucose attachment is essential for ceramide binding [44]. GlcCers are released into the viable epidermis SC interface. During this event, enzymes essential for ceramide binding colocalize [17]. In the next two steps [4,5] 12R-LOX and eLOX3 convert the linoleate to a hydroxy-epoxide, yielding a ceramide with a hydroxy-epoxide and glucose attached. Literature reports that both modifications are necessary for ceramide binding, shown by PNLPA1 deficient mice which have abundant free OS and free Glc-OS in the SC, but virtually no bound ceramides [18]. 6) Transglutaminase 1 is hypothesized to bind the ceramides to the cornified envelope [46], but other mechanisms have not been excluded [47]. 7) GBA1 removes the glucose of the bound ceramide [14]. We show that this mechanism is selective towards MuCers and short chain ceramides, which further increases in FTMs. Yet, when the lipid synthesis is altered by LXR inhibition, compositional changes in the unbound ceramides were observed in the bound ceramides as well. This indicates that the ceramide binding is selective but still contingent on the lipid synthesis. Although it cannot be specified which part of the binding process is responsible for the selectivity, it can be deduced that it serves an important physiological function by providing flexibility to the corneocytes. Alterations in the binding and

composition of the bound lipids could affect the formation of a proper SC lipid barrier.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

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