ARTICLE IN PRESS

BBAMEM-81569; No. of pages: 12; 4C:

Biochimica et Biophysica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Effects of sphingomyelin/ceramide ratio on the permeability and microstructure of model stratum corneum lipid membranes

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ARTICLE INFO

Article history:
Received 4 February 2014
Received in revised form 10 April 2014
Accepted 3 May 2014
Available online xxxx

Keywords:
Sphingomyelin
Ceramide
Stratum corneum model
Lipid membrane
Permeability
X-ray diffraction

ABSTRACT

The conversion of sphingomyelin (SM) to a ceramide (Cer) by acid sphingomyelinase (aSMase) is an important event in skin barrier development. A deficiency in aSMase in diseases such as Niemann–Pick disease and atopic dermatitis coincides with impaired skin barrier recovery after disruption. We studied how an increased SM/Cer ratio influences the barrier function and microstructure of model stratum corneum (SC) lipid membranes. In the membranes composed of isolated human SC Cer (hCer)/cholesterol/free fatty acids/cholesteryl sulfate, partial or full replacement of hCer by SM increased water loss. Partial replacement of 25% and 50% of hCer by SM also increased the membrane permeability to theophylline and alternating electric current, while a higher SM content either did not alter or even decreased the membrane permeability. In contrast, in a simple membrane model with only one type of Cer (nonhydroxyacyl sphingosine, CerNS), an increased SM/Cer ratio provided a similar or better barrier against the permeation of various markers. X-ray powder diffraction revealed that the replacement of hCer by SM interferes with the formation of the long periodicity lamellar phase with a repeat distance of d = 12.7 nm. Our results suggest that SM-to-Cer processing in the human epidermis is essential for preventing excessive water loss, while the permeability barrier to exogenous compounds is less sensitive to the presence of sphingomyelin.

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1. Introduction

Lipids filling the intercellular space of the stratum corneum (SC) are essential for the function of the skin barrier in terrestrial mammals. The highly ordered skin barrier lipids include three main groups of hydrophobic compounds – ceramides (Cer), free fatty acids (FFA) and cholesterol (Chol) – in approximately equimolar fractions, with a minor amount of cholesteryl sulfate (CholS) [1]. Cer (*i.e.*, *N*-acylsphingosines) belong to the sphingolipids. There are at least 12 classes of Cer occurring in the human SC, including the very long acylCer (EO-class Cer), which contain 30–34C acyls with linoleic acid ester-linked to ω-hydroxyl [2,3]. AcylCer play a crucial role in the homeostasis of the permeability barrier [4–6]. The presence of acylCer is necessary for the formation of the so-called long periodicity lamellar phase (LPP, repeat distance of approximately 13 nm) that is typical for the human SC lipid matrix [7–9]. The lamellar structure of SC was at first visualized by freeze-fracture electron microscopy [10,11]. X-ray diffraction confirmed that the intercellular domains of murine SC form the lamellar phase with a repeat distance ~13 nm [12]. Since the 1980s, artificial lipid systems started to be systematically studied with the aim to propose the molecular arrangement of the lipid structures in the epidermal barrier [13].

In SC intercellular domains, hydrolytic enzymes release Cer from their polar precursors: glucosylceramides and sphingomyelins (SM). This processing is crucial for homeostasis of the epidermal barrier but is likely not complete; the total content of polar lipids was estimated to be between 2.3 and 5.2% (by weight) of healthy human SC lipids [14,15]. However, the amount of the individual precursors, glucosylceramides and SM, has not been reported.

In this work, we focused on the SM-to-Cer pathway. SM are converted to Cer (type NS or AS) and phosphocholine by acid sphingomyelinase (aSMase) (Fig. 1). These SM-derived Cer are indispensable for skin barrier function. For example, delayed recovery of the skin permeability barrier has been found in patients with Niemann–Pick disease, which is caused by a mutation in the SMPD1 gene that results in a severe decrease in aSMase activity [16]. Reduced enzymatic activity of aSMase

Abbreviations: aSMase, acid sphingomyelinase; acylCer, ω-O-acyl ceramide/s; Cer, ceramide/s; AD, atopic dermatitis; Chol, cholesterol from lanolin; FFA, free fatty acid/s; IND, indomethacin; HPTLC, high performance thin layer chromatography; hCer, ceramides of the human stratum corneum; LPP, long periodicity phase; PBS, phosphate-buffered saline; SPP, short periodicity phase; CholS, sodium cholesteryl sulfate; SM, sphingomyelin; eSM, sphingomyelin, chicken egg; bmSM, sphingomyelin, bovine milk; SEM, standard error of mean; SC, stratum corneum; TH, theophylline, anhydrous; TLC, thin layer chromatography

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http://dx.doi.org/10.1016/j.bbamem.2014.05.001
0005-2736/© 2014 Published by Elsevier B.V.

Please cite this article as: P. Pullmannová, et al., Effects of sphingomyelin/ceramide ratio on the permeability and microstructure of model stratum corneum lipid membranes, Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbamem.2014.05.001

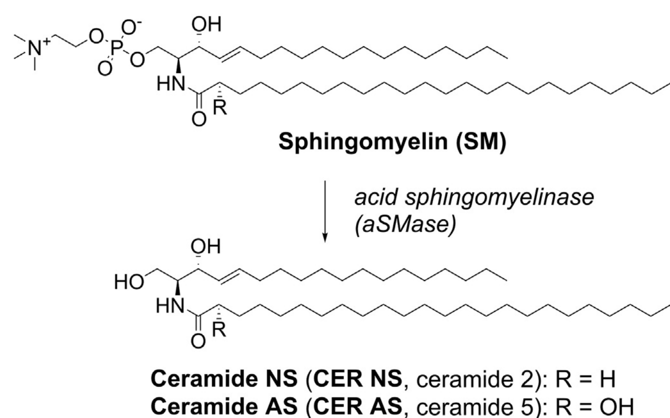

Fig. 1. Conversion of sphingomyelin (SM) to the ceramides (Cer) NS and AS — schematic representation.

has also been reported in atopic dermatitis (AD) patients and was proposed to be related to the decreased amount of Cer in SC lipids [17]. However, the role of aSMase in the development of skin barrier abnormalities remains unclear. In particular, there is also a report describing an increased amount of epidermal SMase in lesional human AD skin [18]. Evaluating the effect of decreased aSMase activity is also complicated by the abnormally expressed SM deacylase in atopic patients, which cleaves SM to FFA and sphingosylphosphorylcholine [19,20].

To provide greater mechanistic insight into the importance of this Cer-generating pathway, Schmuth et al. applied an aSMase inhibitor to the skin of hairless mice. The aSMase inhibition induced a 4-fold accumulation of SM in the murine SC after an acute barrier disruption (from 0.3% to 1.2% of the total SC lipid mass). The changed SM/Cer ratio was suggested to be responsible for the altered skin barrier function [16]. Similarly, the suppressed β-glucocerebrosidase enzymatic activity was followed by an accumulation of Cer precursors glucosylceramides in the SC [21]. However, a direct effect of SM on the permeability of SC has not been demonstrated.

The purpose of our work was to study how an increased SM/Cer ratio influences the permeability and biophysics of membranes composed of SM/Cer/FFA/Chol/CholS that model the SC intercellular lipid lamellae. We prepared lipid membranes containing either only one type of Cer — nonhydroxyacyl sphingosine (CerNS) or the full spectrum of isolated human SC Cer (hCer). To determine how SM accumulation affects the SC lipid barrier, various fractions of Cer (either CerNS or hCer) were replaced by equimolar SM. The barrier properties of these model membranes were studied in permeation experiments using four permeability markers, including relative water loss, the steady-state fluxes of theophylline and indomethacin and the electrical impedance. Furthermore, we used small- and wide-angle X-ray powder diffraction to provide insight into the microstructure of SC models with and without SM.

2. Material and methods

2.1. Chemicals and material

N-tetracosanoyl-D-*erythro*-sphingosine (CerNS), *N*-(2′-(*R*)-hydroxytetracosanoyl)-D-*erythro*-sphingosine (CerAS), sphingomyelin from chicken egg (eSM) and sphingomyelin from bovine milk (bmSM) were purchased from Avanti Polar Lipids (Alabaster, USA). *N*-tetracosanoyl-D-*erytho*-phytosphingosine (CerNP) was synthesized according to the previously described method [22]. Cholesterol from lanolin (Chol), sodium cholesteryl sulfate (CholS), hexadecanoic acid, octadecanoic acid, eicosanoic acid, docosanoic acid, tetracosanoic acid, theophylline, anhydrous (TH), indomethacin (IND), gentamicin sulfate from Micromonospora purpurea, trypsin from porcine pancreas (1:250 powder, 1500 BAEE units/mg solid), sodium phosphate dibasic dodecahydrate, propylene glycol and solvents were purchased from Sigma-Aldrich Chemie Gmbh (Schnelldorf, Germany). All solvents were analytical or HPLC grade. *N*-(2-hydroxy octadecanoyl)-phytosphingosine 94.8% (CerAP) was purchased from Evonik Industries AG (Essen, Germany). Sodium hydroxide, sodium chloride, potassium chloride and sodium phosphate monobasic dihydrate were supplied from Lachema (Neratovice, Czech Republic). Hydrochloric acid was purchased from Penta (Prague, Czech Republic). The chemicals were analytical grade and used without further purification. The silica gel 60 (230–400 mesh) for column chromatography, HPTLC 20 × 10 cm glass plates (silica gel 60) and TLC plates (silica gel 60 F 254, aluminum back) were from Merck (Darmstadt, Germany). Nuclepore™ track-etched polycarbonate membranes of 0.015 μm pore size were from Whatman (Kent, Maidstone, United Kingdom). The aqueous solutions were prepared with Millipore water.

2.2. Skin

Human abdominal or breast skin from Caucasian female patients (n = 6), who had undergone plastic surgery, was provided by the University Hospital Hradec Králové, Clinics of Plastic Surgery. The procedure was approved by the Ethics Committee of the University Hospital Hradec Králové, Czech Republic (No. 200609 S09P) and was conducted according to the Declaration of Helsinki Principles. Within 6 h of the surgery, residual subcutaneous fat was removed from the skin by a scalpel. The skin was gently wiped with a tissue paper soaked in acetone to remove skin sebum and contaminating subcutaneous fat. The skin fragments were washed in phosphate-buffered saline at pH 7.4 (PBS solution, 10 mM buffer adjusted to 150 mM ionic strength containing 137 mM NaCl, 8 mM $Na_2HPO_4.12H_2O$, 2 mM $NaH_2PO_4.2H_2O$ and 2.7 mM KCl) with 50 mg/L gentamicin for preservation and were stored at −20 °C.

2.3. Isolation of human SC lipids

The SC was isolated using a modified procedure described by Kligman and Christophers [23]. Frozen human skin was slowly thawed and subsequently immersed in 60 °C Millipore water for 30 s. The epidermis was peeled off using tweezers. Then, the epidermis was incubated overnight with the basal layer down in a solution of 0.5% trypsin in PBS at pH 7.4 and 32 °C. The residual epidermal cells were gently removed from the SC using cotton swabs. The SC was washed several times in Millipore water and was washed once briefly in acetone. Afterward, the SC was dried in vacuum over P_4O_{10} and solid paraffin in a desiccator and stored in nitrogen environment at −20 °C.

The SC lipids were extracted from pooled SC of 6 objects by a modified method of Bligh and Dyer [24] with a series of chloroform:methanol mixtures (2:1, 1:1, and 1:2 v/v) for 2 h each. The solvent from the combined extracts was removed using a rotary vacuum evaporator.

2.4. Isolation of human SC Cer (hCer)

The obtained human SC lipids were redissolved in a suitable volume of chloroform–methanol (2:1 v/v) and applied to a silica gel column (Silicagel 60, Merck, Darmstadt, Germany). Similar separation of hCer by column chromatography was described previously by Bouwstra et al. [25]. We used a simpler mobile phase composition: the SC lipid classes were eluted sequentially using gradient elution with solvent mixtures (v/v) in the following sequence: chloroform–acetic acid 99:1 and then chloroform–methanol in ratios of 100:1, 50:1, 10:1, 3:1, 2:1, 1:1 and 1:2. The lipid composition of the individual fractions was established by one-dimensional thin layer chromatography that was run in parallel with standards. To ensure complete separation of the least polar Cer and FFA, the separation of these fractions was repeated.

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