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The role of ceramide chain length distribution on the barrier properties of the skin lipid membranes

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ABSTRACT

The skin barrier function is provided by the stratum corneum (SC). The lipids in the SC are composed of three lipid classes: ceramides (CERs), cholesterol (CHOL) and free fatty acids (FFAs) which form two crystalline lamellar structures. In the present study, we investigate the effect of CER chain length distribution on the barrier properties of model lipid membranes mimicking the lipid composition and organization of SC. The lipid membranes were prepared with either isolated pig CERs (PCERs) or synthetic CERs. While PCERs have a wide chain length distribution, the synthetic CERs are quite uniform in chain length. The barrier properties were examined by means of permeation studies using hydrocortisone as a model drug. Our studies revealed a reduced barrier in lipid membranes prepared with PCERs compared to synthetic CERs. Additional studies revealed that a wider chain length distribution of PCERs results in an enhanced hexagonal packing and increased conformational disordering of the lipid tails compared to synthetic CERs, while the lamellar phases did not change. This demonstrates that the chain length distribution affects the lipid barrier by reducing the lipid ordering and density within the lipid lamellae. In subsequent studies, the effect of increased levels of FFAs or CERs with a very long acyl chain in the PCERs membranes was also studied. These changes in lipid composition enhanced the level of orthorhombic packing, reduced the conformational disordering and increased the barrier properties of the lipid membranes. In conclusion, the CER chain length distribution is an important key factor for maintaining a proper barrier.

1. Introduction

The major role of the skin is to protect the body from the environment by providing an exceptionally efficient barrier. This physical barrier is located in the outer layer of the skin known as the stratum corneum (SC). The SC forms the rate limiting step for the penetration of drugs and other substances into the skin. Due to this role, it is important to understand the structure of the SC in relation to the skin barrier in more detail.

The composition of the SC is exclusive. It consists of dead flattened protein rich cells referred to as corneocytes being surrounded by intercellular lipids. These lipids greatly contribute to the barrier function of the skin. The main lipid classes in the SC are ceramides (CERs), cholesterol (CHOL) and free fatty acids (FFAs) in an approximately equimolar ratio [1–6]. In human and pig SC the lipids form two coexisting crystalline lamellar phases, referred to as the long periodicity phase (LPP) with a repeat distance of around 13 nm and the short periodicity phase (SPP) with a repeat distance of about 6 nm [7,8]. Apart from the lamellar phases, the arrangement of these lipids within the lipid lamellae, that is the compactness of the lipid chain packing, is also important for the skin barrier. This arrangement is referred to as the lateral packing. At skin temperature (~32 °C), the human SC lipids dominantly adopt a very dense orthorhombic packing. However, a small subpopulation of lipids is also present in a less dense hexagonal organization [7,9–12]. Both the lamellar phases and lateral packing are important and have been shown to play a role in the skin barrier function [13–16].

Further in-depth analysis of the SC lipid composition revealed different subclasses of CERs and the chain length distribution of CERs and FFAs in the SC. So far 12 different subclasses of CERs have been identified in the human SC, while in pig SC only 6 subclasses are present [1,4,5,17]. The CERs vary widely in their head group architecture and chain length distribution. Some of the CER classes have a very exceptional molecular architecture with a linoleic acid linked to a very long fatty acid chain. These CER subclasses are known as acyl CERs. The CERs are the key components in the skin barrier as they play a crucial role in maintaining this barrier [18]. In particular a reduction or complete abduction of the acyl CERs leads to an increased formation of the SPP and there are strong indications that this also leads to a reduced skin lipid barrier [13,19–21]. The FFAs present in native human SC are mainly saturated and range in chain length from C14 to C34 [20,22,23] with an average chain length between C20 and C22. It has been reported that the presence of FFAs enhances the formation of the orthorhombic lateral packing [24].

In our present study, we aimed to compare the barrier properties of lipid membranes prepared from isolated pig CERs (PCERs) with those

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prepared from synthetic CERs, in which the CER subclass composition mimics that of the PCER mixture. As each PCER subclass displays a multiple variation in chain length, in contrast to the synthetic CER composition having only 3 different chain lengths, a comparison between mixtures prepared with PCERs and synthetic CERs provides information on the role the chain length distribution plays in the skin lipid organization and barrier properties. In addition, the influence of increased levels of i) an acyl CER subclass, known as CER EOS (esterified -hydroxysphingosine, an acyl CER, see Fig. 1), and ii) FFAs on the barrier properties have also been examined. To accomplish our endeavors, we used small angle X-ray diffraction (SAXD) to elucidate the lamellar phases and Fourier transform infrared spectroscopy (FTIR) to examine the lateral packing, the conformational ordering and the mixing properties of the lipid mixtures. *In vitro* permeation studies were performed using a lipid model membrane casted on a porous support, referred to as the stratum corneum substitute (SCS) [25,26]. As model drug we used hydrocortisone.

2. Materials and method

2.1. Materials

Five subclasses of synthetic CERs were used in our studies. These are an ester linked omega-hydroxyl acyl chain (30 carbons in the acyl chain (C30)) with a sphingosine chain (C18) referred to as CER EOS (C30), a non-hydroxy acyl chain (C24) linked to a sphingosine base (C18) referred to as CER NS (C24), a non-hydroxy acyl chain (C24 or C16) linked to a phytosphingosine base referred to as CER NP (C24) and CER NP (C16), respectively, an alpha-hydroxy acyl chain linked to a sphingosine base referred to as CER AS (C24), and an alpha-hydroxy acyl chain (C24) linked to a phytosphingosine base referred to as CER AP (C24). The number between parentheses indicates the number of carbon atoms present in the acyl chain of the CER subclass. The molecular structure of the various synthetic CERs is provided in Fig. 1. All the CERs were kindly provided by Evonik (Essen, Germany). The FFAs with a chain length of C16:0, C18:0, C20:0, C22:0, C23:0, C24:0 or C26:0 are obtained from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). CHOL, trypsin (type III, from bovine pancreas) and trypsin inhibitor (type II-S from soybean) were also purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). The perdeuterated FFAs with chain lengths of C16:0 and C22:0 and hydrocortisone were purchased from Larodan (Malmo, Sweden). The perdeuterated FFAs with chain lengths of C14:0, C18:0, and C20:0 were obtained from Cambridge Isotope Laboratories (Andover MA, USA). Nucleopore polycarbonate filter disks (pore size 50 nm) were obtained from Whatman (Kent, UK). All solvents were of analytical grade and supplied by Labscan (Dublin, Ireland). The water was of Millipore quality.

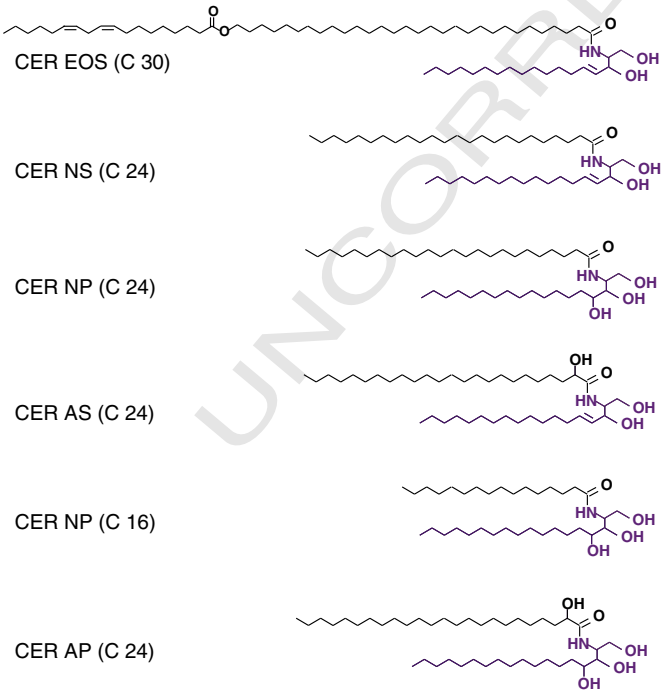

Fig. 1. Molecular structure of the synthetic CERs used in the present study. The CER consists of an acyl chain ranging from 24 to 30 carbons linked to a sphingoid base through an amide linkage.

2.2. Isolation and extraction of PCER

Fresh pig skin was obtained from the Central Veterinary Institute in Lelystad in the Netherlands. First, the hairs were removed with a razor blade. Then the pig skin was defatted and dermatomed at a thickness of 600 μm. The SC was isolated by trypsin digestion (0.1% in a phosphate-buffered saline solution) as described previously [27]. SC lipids were extracted using the method of Bligh and Dyer [28]. Briefly, first a series of chloroform:methanol extractions in various ratios (1:2, 1:1 and 2:1 v/v) was performed for 1 h at 37 °C. The extracts were then combined and treated with 2.5% KCl in demi-water to create an aqueous phase. The organic phase was collected, evaporated using a rotary evaporator and subsequently applied onto a silica gel 60 column after being redissolved in a suitable volume of chloroform:methanol (2:1). Then the various lipid classes were eluted sequentially using a series of solvent mixtures as published previously [27]. The lipid composition of the collected fractions was examined by one dimensional high performance thin layer chromatography (HPTLC), as described previously [27]. Then the fractions that contained only CERs were combined and the composition was analyzed. For quantification of the CER subclasses, HPTLC was performed: authentic standards (mixtures of 7 different CERs) and the isolated PCERs were run in parallel. To perform the quantification 12 different amounts of the CER standard solution and isolated PCER solution were applied on the plate. The quantification was performed after charring, using a photodensitometer with peak integration (Biorad, GS 800). The linear part of the calibration curves were used for calculating the relative levels of the PCER subclasses.

2.3. Mass spectrometry analysis

The PCERs were further analyzed by liquid chromatography coupled to mass spectrometry (LC/MS) in order to investigate the chain length distribution. A detailed description of the lipid analysis is given elsewhere [17]. In short, the PCER sample was redissolved in chloroform: methanol:heptane (2.5:2.5:95) to a final concentration of 1 mg/mL. 10 μL of sample was injected using an HPLC system (Waters, Thermo, Finnigan, San Jose, CA). The HPLC was coupled to a mass spectrometer (TSQ Quantum, Thermo, Finnigan, San Jose, CA) to analyze the PCER composition. The separation of the PCER was achieved by using a normal phase column (PVA-sil, YMC, Kyoto, Japan) while analyzing in positive ion mode. The software Xcalibur was used for data acquisition.

2.4. Compositions of stratum corneum substitute (SCS)

For the preparation of the SCS, synthetic CERs or PCER, CHOL and FFAs were used. The two compositions of the synthetic CERs with their corresponding molar ratios are provided in Table 1. One of these compositions is based on a CER subclass composition of pig SC reported earlier [27,29] and is referred to as $CER^{control}$. The other synthetic CER composition mimics the composition of the PCERs isolated in the present study and is referred to as CER^{spig}. The composition of the FFA mixture was comprised of seven different FFAs. The name, chain length and the molar ratios of the FFA mixture are provided in Table 1. This FFA composition is based on that reported for human SC [30]. In a few FTIR experiments, the protonated FFAs were replaced by deuterated FFAs

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