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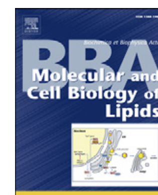

Effect of ceramide acyl chain length on skin permeability and thermotropic phase behavior of model stratum corneum lipid membranes

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ABSTRACT

Stratum corneum ceramides play an essential role in the barrier properties of skin. However, their structure–activity relationships are poorly understood. We investigated the effects of acyl chain length in the non-hydroxy acyl sphingosine type (NS) ceramides on the skin permeability and their thermotropic phase behavior. Neither the long- to medium-chain ceramides (8–24C) nor free sphingosine produced any changes of the skin barrier function. In contrast, the short-chain ceramides decreased skin electrical impedance and increased skin permeability for two marker drugs, theophylline and indomethacin, with maxima in the 4–6C acyl ceramides. The thermotropic phase behavior of pure ceramides and model stratum corneum lipid membranes composed of ceramide/lignoceric acid/cholesterol/cholesterol sulfate was studied by differential scanning calorimetry and infrared spectroscopy. Differences in thermotropic phase behavior of these lipids were found: those ceramides that had the greatest impact on the skin barrier properties displayed the lowest phase transitions and formed the least dense model stratum corneum lipid membranes at 32 °C. In conclusion, the long hydrophobic chains in the NS-type ceramides are essential for maintaining the skin barrier function. However, this ability is not shared by their short-chain counterparts despite their having the same polar head structure and hydrogen bonding ability.

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1. Introduction

The primary function of the skin is to protect the body from water loss and entrance of potential toxic compounds, allergens, irritants and microbes. The principal barrier to the penetration of most compounds resides in the uppermost skin layer, the stratum corneum (SC), which consists of several layers of corneocytes embedded in a lamellar lipid matrix. This lipid-based "mortar" is considered to be the main pathway for the diffusion of substances into the body. It consists of an approximately equimolar mixture of ceramides (Cer), cholesterol (Chol) and free fatty acids (FFA). In addition, minor amounts of cholesterol sulfate (CholS) are present. This unusual composition with high Cer content is essential for maintaining the skin barrier function. Moreover, lower Cer levels and altered Cer profiles are found in prominent skin diseases such as atopic dermatitis and psoriasis. For recent reviews on ceramides in the skin barrier physiology and pathology, see refs. [1–6].

Cer consist of a fatty acid bound to the amino group of sphingosine or another related long-chain amino alcohol. In human SC, 11 major Cer subclasses based on four sphingoid bases and three acyl chain types have been identified [7]. We have been interested in studying the structural requirements for Cer function in a competent skin barrier because little is known about the role of the individual Cer species and their structure–activity relationships (for a review on skin ceramide chemistry, see [8]). First, we hypothesized that the acyl chain length is important and that its shortening would decrease the skin diffusion resistance [9]. This assumption is based on the fact that the skin Cer possess exclusively very long acyl chains [7]. For example, the most abundant species is Cer NS, which contains sphingosine acylated mostly with 24C lignoceric acid (Supplementary data, Fig. S1). Although short-chain Cer have been used as Cer mimics with increased aqueous solubility in various experiments, recent studies showed substantial differences between the native long-chain Cer and their short-chain analogues in cell membranes [10,11] and the skin [12]. Another example relevant to our hypothesis is that the activity of amphiphilic transdermal permeation enhancers, which

Abbreviations: Cer, ceramide/s; Chol, cholesterol; CholS, cholesterol sulfate; DSC, differential scanning calorimetry; FFA, free fatty acid; FT-IR, Fourier transform infrared spectroscopy; IND, indomethacin; NS, non-hydroxyacyl sphingosine; SC, stratum corneum; TH, theophylline

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influence the permeability of the SC Cer membranes, is highly dependent on the chain length [13]. For example, a simple L-serine-based Cer analogue 14S24 with the same chain length as the natural Cer was able to repair damaged skin barrier [14,15], while its analogue with shorter chains behaved as a permeation enhancer [16].

In our preliminary study, we prepared a series of Cer analogues with the polar head identical to Cer NS, sphingosine truncated to 12 carbons, and acyl chain lengths of 2–12 carbons. No change in skin permeability was observed in Cer analogues with 2C and 12C acyl and Cer NS. However, Cer with 4–8C acyl chain increased the flux of two model permeants up to 10.8 times with a maximum effect at 6C acyl, suggesting that our hypothesis was basically correct [9]. Nevertheless, those Cer analogues contained not only shorter acyl but also shorter sphingosine (with 12-carbon backbone instead of the usual 18 carbons).

In this contribution, we report the effects of a series of Cer having natural 18C sphingosine and varying acyl chain length on the skin permeability, measured by two model permeants and electrical impedance. Apart from Cer with saturated acyl (Cer2-Cer24; the number indicates the acyl chain length), *N*-oleoylsphingosine (Cer18:1) containing a *cis*-double bond, and free sphingosine were included in this study (chemical structures and abbreviations used are given in Supplementary data, Fig. S1). Moreover, to gain some basic understanding of the observed differences between these sphingolipids, the thermotropic phase behavior of both pure Cer and the model SC lipid membranes composed of Cer/FFA/Chol/CholS was studied by differential scanning calorimetry and infrared spectroscopy.

2. Material and methods

2.1. Chemicals and skin

Cer and sphingosine were purchased from Avanti Polar Lipids (Alabaster, USA) and recrystallized from chloroform/methanol mixture. All other chemicals and solvents were from Sigma-Aldrich (Schnelldorf, Germany). Porcine ears were obtained from a local slaughterhouse. To ensure integrity of the skin barrier, ears were removed post-sacrifice before the carcass was exposed to the high-temperature cleaning procedure. Full-thickness dorsal skin was excised by blunt dissection and hairs were carefully trimmed. The skin grafts of 1000 μm thickness were cut with a dermatome (Aesculap, Tuttlingen, Germany). The skin fragments were immersed in 0.05% sodium azide solution in saline for 5 min for preservation and were stored at -20 °C.

2.2. Donor samples for permeation studies

Control donor samples were prepared as 5% (w/v) suspensions of theophylline (TH) or 2% (w/v) suspensions of indomethacin (IND) in propylene glycol/water 6:4 (v/v). Cer samples for co-application experiments were prepared by adding 1% (w/v) of the studied Cer to the aforementioned drug suspensions. The samples were stirred at 50 °C for 5 min and then allowed to equilibrate at 37 °C for 24 h. Before application to the skin, the samples were resuspended. All the samples were saturated with the pertinent model drug. To determine whether the added Cer had any effects on the solubility of the drugs in the donor solvent, the samples were prepared in triplicate as described above and allowed to equilibrate. After 24 h, the suspensions were centrifuged at 6700 × g for 5 min; the supernatant was withdrawn, diluted with the pertinent mobile phase and analyzed by HPLC.

Cer donor samples for pretreatment experiments containing 1% (w/v) Cer in propylene glycol/water 6:4 (v/v) without the model drug were prepared likewise.

2.3. Permeation experiments

The effects of the studied Cer on the skin permeability was evaluated using Franz diffusion cells with an available diffusion area of 1 cm^2 and an acceptor volume of approximately 17 ml. The skin fragments were slowly thawed immediately before use and carefully inspected for any visual damage. They were cut into squares ca. 2 × 2 cm, mounted into the diffusion cells dermal side down and sealed with silicone grease. The acceptor compartment of the cell was filled with phosphate-buffered saline (PBS, containing 10 mM phosphate buffer, 137 mM NaCl and 2.7 mM KCl) at pH 7.4 with 0.03% sodium azide as a preservative. The precise volume of the acceptor liquid was measured for each cell and included in the calculation. The Franz diffusion cells with mounted skin samples were placed in a water bath with a constant temperature of 32 °C equipped with a magnetic stirrer. After an equilibration period of 1 h, the skin integrity was checked by measuring the electrical impedance (see later).

The application protocol involved either co-application of the Cer and the model drugs or a pretreatment of the skin with the Cer followed by the permeation of the drugs. These approaches are complementary; both have their advantages and disadvantages. Pretreatment can eliminate any possible interactions between the Cer and the marker drug already in the donor sample, which may occur at co-application. However, in the in vitro pretreatment setup, the skin is exposed to Cer for a shorter time. Thus, the observed Cer effects may be more influenced by their penetration rates into the SC. Such differences in the penetration rates of fluorescent Cer with various chain lengths into the skin [12] and into other lipid membranes [17] were described previously.

For the co-application experiments, 150 μl (i.e., an infinite dose) of the donor sample, either with or without the studied Cer was applied to the SC side of the skin and covered with a glass slide. The acceptor phase was stirred at 32 °C throughout the experiment. Sink conditions were maintained for either drug. Samples of the acceptor phase (0.6 ml) were withdrawn at predetermined time intervals over 48 h and replaced with fresh PBS. In the pretreatment protocol, the skin first received 100 μl of the Cer samples in propylene glycol/water 6:4 without the drugs or 100 μl of the vehicle as the control. After 2 h, the donors were removed and the skin surface was briefly washed with 0.5 ml of 60% ethanol and 0.5 ml of PBS. The electrical impedance was recorded and, subsequently, the drug suspensions without Cer were applied on the skin. The cumulative amount of the drug penetrated across the skin, corrected for the acceptor phase replacement was plotted against time and the steady state flux was calculated from the linear region of the plot.

At the end of the permeation experiment (48 h), the diffusion cells were dismounted and the skin surface washed with 0.5 ml of ethanol and 0.5 ml of water. The exposed area of 1 cm^2 was punched out, blotted dry and weighed. The skin sample was then extracted with 5 ml of the appropriate mobile phase for 48 h and the concentration of the model drugs was determined by HPLC. The extraction efficacy was 98 ± 2% for TH and 101 ± 7% for IND [9], which is in accordance with the FDA Guidance for Industry for Bioanalytical Method Validation, 2001.

2.4. Electrical impedance measurement

The electrical impedance of the skin was recorded using an LCR meter 4080 (Conrad Electronic, Hirschau, Germany, measuring range 20 Ω to 10 MΩ, error at kΩ values <0.5%), operated in a parallel mode with an alternating frequency of 120 Hz, which yield the best sensitivity to small impedance changes [18]. The electrical impedance of skin reflects the condition of the SC. This value is the alternating current equivalent of the direct current resistance, and it is commonly used to non-invasively and rapidly evaluate skin integrity. The

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