Skin Barrier Fine-Tuning through Low-Temperature Lipid Chain Transition

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ABBREVIATIONS: AFM, atomic force microscopy; FITC-inulin, fluorescein isothiocyanate-labeled inulin; HEX, hexagonal; OR, orthorhombic, SC, *stratum corneum*; TEWL, transepidermal water loss

ABSTRACT

The lipids in the mammalian *stratum corneum* (SC) adopt an unusually rigid arrangement to form a vital barrier preventing water loss and harmful environmental impacts. Just above the physiological temperature, a subset of barrier lipids undergoes a phase transition from a very tight orthorhombic to a looser hexagonal arrangement and vice versa. The purpose of this lipid transition in skin physiology is unknown. Permeability experiments on isolated human SC indicated that the transition affects the activation energy for a model compound that prefers lateral movement along lipid layers but not for water or a large polymer that would cross the SC via the pore pathway. The orthorhombic phase content of SC lipids, as determined by infrared spectroscopy, was also modulated by (de)hydration. Spontaneous rearrangement of human SC lipid monolayers into 10 nm higher multilamellar islets at 32 – 37°C, but not at room temperature, was revealed by atomic force microscopy. Our findings add to our knowledge of fundamental skin physiology suggesting a fine temperature- and hydration-controlled switch from fluid lipids (required for lipid barrier assembly) to rigid and tightly packed lipids in the mature SC (necessary for the water and permeability barriers).

INTRODUCTION

The lipid matrix of the uppermost skin layer, the *stratum corneum* (SC), plays an indispensable role in maintaining body fluid homeostasis while warding off harmful environmental noxae from xenobiotics to pathogens. Both the complex molecular composition and the intricate assembly of intercellular lipids are crucial for proper skin barrier function (Bouwstra and Ponec, 2006, Elias and Feingold, 2005, Madison, 2003, Suhonen et al., 1999).

At physiological conditions, the majority of the SC lipids occupy a tight orthorhombic (OR) packing (Figure 1), which is notably rigid and provides an efficient barrier (Damien and Boncheva, 2010). A portion of the lipids is found in a looser hexagonal (HEX) arrangement and a minority of the lipids are in a disordered liquid-crystalline phase (Boncheva et al., 2008, Mendelsohn et al., 2006). With rising temperature, the barrier lipids undergo endothermic phase transitions, leading to an increased proportion of HEX and liquid-crystalline phase lipids along with a decrease in barrier properties (Boncheva et al., 2008, Golden et al., 1986).

One of these transitions occurs at 35-40°C (Bouwstra et al., 1989, Cornwell et al., 1996, Gay et al., 1994, Golden et al., 1986, Groen et al., 2011, Silva et al., 2006, Van Duzee, 1975, Wilkes et al., 1973), where a subset of SC lipids is expected to undergo a transition from OR to HEX arrangement (Groen et al., 2011, Pensack et al., 2006). Since this transition is found close to the physiological skin surface temperature, which is approximately 30-35°C (Benedict et al., 1919, Lee et al., 2019), it appears to be physiologically relevant, raising the question of whether it has a purpose in skin barrier homeostasis.

In this study, we proposed and evaluated three hypotheses. First, loosening of the lipid chain packing at and above the transition could allow additional cooling by facilitating water loss during fever or inflammation. Second, the loosening of the lipid chains at temperatures at and above the transition could create space within the skin barrier lipids for the translocation of

larger, possibly signaling, molecules in heat stress or inflammation. Large hydrophilic molecules, such as antimicrobial peptides (Schröder and Harder, 2006), exist within SC (Breiden and Sandhoff, 2014, Elias, 2007, Feingold and Elias, 2014), but their movement is expected to be severely limited by the rigid lipid multilayers, and even the latest models of SC assembly (Narangifard et al., 2021) do not explain where these molecules reside in the SC.

According to our third hypothesis, the transition could be a part of the mechanisms of lipid self-assembly during the formation and maturation of the skin barrier. Mature SC lipids are remarkably rigid, which is incompatible with the need for their remodeling during barrier formation. A higher flexibility of the loosely arranged lipids at temperatures at and above the OR-HEX transition, possibly accessible in the inner SC layers could (partly) explain how the barrier lipids attain their multilayer barrier structure without significant packing defects. The subsequent decrease in temperature and hydration in mature SC layers may provide a mechanism for chain stiffening to ensure proper barrier function (Figure 1). Indeed, there are examples of how phase transitions sculpt tissues, recently reviewed in (Lenne and Trivedi, 2022).

To test our first two hypotheses, we examined the permeability of human SC at temperatures between 26°C and 50°C to water (measured as transepidermal water loss, TEWL), a model lipophilic permeant, indomethacin, and a model large hydrophilic molecule inulin. Langmuir monolayer experiments and atomic force microscopy (AFM) of extracted human SC lipids were used to assess the effect of the transition on lipid assembly to investigate the third hypothesis. Simultaneously, infrared spectroscopy was employed to obtain an insight into the effects of temperature and hydration on the amount of OR lipid phase in isolated human SC lipids.

RESULTS AND DISCUSSION

The OR-HEX transition changes the activation energy for the SC permeability to lipophilic indomethacin, but not to water and inulin

To test our first hypothesis, the permeability of human SC to water, expressed as TEWL, was examined at 26 - 50°C (Figure 2a-b; one experiment also included 10°C) covering the temperature range of the transition (Bouwstra et al., 1989, Cornwell et al., 1996, Gay et al., 1994, Golden et al., 1986, Groen et al., 2011, Silva et al., 2006, Van Duzee, 1975, Wilkes et al., 1973). TEWL values increased with increasing temperature; however, Arrhenius plots showed that there was no change in the activation energy, i.e., the minimum energy required for this process, above the OR-HEX transition. Activation energy remained at 27-28 kJ/mol over the investigated temperature range. The first hypothesis was therefore rejected. The higher TEWL rates at higher temperatures were presumably due to the changing diffusivity according to Fick's laws (Mitragotri, 2003, Shahzad et al., 2015) while the OR-HEX transition played a minor role, if any. The likely explanation is that water molecules move through density fluctuations within the lipids at a higher rate than individual lipids within the lipid lamellae (Mitragotri, 2003).

The situation was different for indomethacin, a larger (358 g/mol) and lipophilic (logP over 4) model permeant. The indomethacin flux increased with temperature, and the Arrhenius plots showed a decrease of the activation energy above approximately 40°C to about half of the values observed at lower temperatures (Figure 2c-d, blue lines indicate region of decreased activation energy). To demonstrate that this change in activation energy for indomethacin permeation was not due to propylene glycol, which was added to the donor sample to increase indomethacin solubility, we tested the effect of propylene glycol on selected parameters of extracted human SC lipids by infrared spectroscopy. The relative OR phase content and lipid chain conformation (see below for details) did not differ between propylene

glycol-treated or hydrated human SC lipid samples from 20°C to 60°C (Supplementary Figure S1). This is in agreement with previous results that propylene glycol mainly affects SC protein structures rather than lipid chain order or packing. (Janůšová et al., 2013).

To further test our hypothesis that the OR-HEX transition may facilitate the translocation of larger molecules, we evaluated the SC permeability to fluorescein isothiocyanate labeled inulin, FITC-inulin (Figure 2e-f), a hydrophilic biopolymer with average molecular weight varying between 3,000 and 6,000 g/mol, which is a size comparable, for example, to some of the skin antimicrobial peptides (Schröder and Harder, 2006). The permeation of FITC-inulin was highly variable, but the Arrhenius plots showed similar activation energies over the investigated temperature range (122 kJ/mol and 125 kJ/mol for two independent experiments with SC from different donors). Thus, the simple increase in inulin diffusivity with rising temperature appears to dominate over the possible effect of the transition.

Therefore, the OR-HEX transition seems to regulate the permeability barrier, but not for all compounds or permeation pathways. Lipophilic indomethacin would prefer the lateral movement pathway along lipid layers, whereas FITC-inulin is expected to translocate through the pore pathway (Mitragotri, 2003). Thus, the higher mobility of the chains of a certain subset of SC lipids caused by the transition seems to affect the lateral diffusion, as can be seen in the example of indomethacin. The permeation of other relatively large lipophilic drugs (such as topical corticosteroids) or, on the other hand, potentially hazardous chemicals (such as pesticides) would be expected to be similarly sensitive to the relative proportions of OR packed lipid chains. Of course, all substances will penetrate the SC lipids more rapidly at higher temperatures, but this effect will be more pronounced for relatively large and lipophilic substances that prefer lateral diffusion. Similar to our results, a significantly increased permeability of human epidermis to lidocaine (234 g/mol and logP 2.3) at 37-45°C compared to lower temperatures was reported (Wood et al., 2012). For benzoic acid (122 g/mol, logP 1.9),

the activation energy did not change during the transition (Groen et al., 2011); which is consistent with our findings, as benzoic acid is relatively small and would prefer the free diffusion permeation pathway, just like water.

Skin barrier lipids spontaneously arrange into multilayers at, but not below, the OR-HEX transition

Why does the OR-HEX transition only affect the lateral diffusion pathway? What if the main role of this transition in skin lipids is to alter lipid dynamics and therefore, we only see its effect on substances that prefer lateral diffusion? Thus, we explored our third hypothesis, that the OR-HEX transition (and thus the relative contribution of the OR phase) may act as a switch between more mobile chains (required to shape the lipid lamellae) and rigid chains (required to restrict permeability in mature SC).

To test this hypothesis, we investigated the lipid remodeling from monolayers to multilayers at various temperatures. First, we isolated and purified human SC lipids and characterized the relative proportions of OR-packed lipids at and after the studied transition using infrared spectroscopy (Figure 3). The OR packed lipids are so densely packed that intermolecular interactions can be detected by splitting of the scissoring and rocking methylene modes in infrared spectra (Figure 3a)(Lewis and McElhaney, 2010). The higher wavenumber component (at about 729 cm^{-1}) of the rocking mode, indicative of OR chains, decreased in intensity with temperatures above 28°C and disappeared at around 40°C. The OR-HEX transition was accompanied by an increase in the wavenumber of the methylene symmetric stretching vibration indicating less ordered lipid chains with temperature but still within the range of predominant all-*trans* chains (Figure 3b). Our data are consistent with previous reports on the OR-HEX transition in SC and extracted SC lipids using infrared spectroscopy (Golden

et al., 1986, Pensack et al., 2006, Sagrafena et al., 2022) and other methods (Bouwstra et al., 1989, Cornwell et al., 1996, Golden et al., 1986, Groen et al., 2011, Pilgram et al., 1999, Silva et al., 2006, Van Duzee, 1975, Wilkes et al., 1973). The thermotropic behavior of these (chromatographically purified) lipids clearly confirms that the OR-HEX transition is an intrinsic property of barrier lipids (Gay et al., 1994) and not of sebaceous lipids, as suggested by some (Cornwell et al., 1996, Golden et al., 1986, Shahzad et al., 2015).

The OR-HEX transition is also modulated by (de)hydration (Bouwstra et al., 2003, Golden et al., 1986, Van Duzee, 1975), which is relevant for the skin barrier lipids, as the water content decreases toward the skin surface. To characterize the dehydration-induced changes in OR phase content in the human SC lipid samples at 32°C and 37°C, which roughly correspond to the skin surface temperature and the temperature of the inner skin layers, respectively, we evaluated the infrared spectroscopic features in fully hydrated lipid films over 6 h (11 min steps) while the samples were allowed to freely lose moisture at 10-15% relative humidity (Figure 3c-d). Hydration of SC lipids at 100% relative humidity (even though these lipids have minimal water uptake) reduced the amount of OR phase similarly to the increase in temperature from 32°C to 37°C. For lipid chain conformation, the changes induced by hydration were even greater than those induced by a 5°C increase in temperature, based on methylene symmetric stretching frequency shifts (Supplementary Figure S2). These values are similar to those observed on human SC (Groen et al., 2011, Pensack et al., 2006). With dehydration, OR lipid phase proportion increased (higher relative intensity of the 729 cm^{-1} component of the CH_2 rocking mode) up to values approaching those of the dry samples at the same temperature (which had stable OR phase content over time; Figure 3). No significant changes in the lipid chain order were observed during dehydration in this experiment suggesting a longer time scale for this change (Supplementary Figure S2).

To investigate the lipid reorganization into multilayers, we used Langmuir monolayer experiments at air-buffer interface at 23–46°C followed by deposition of the SC lipids on mica and AFM (Figure 4). At a physiologically relevant surface pressure of 30 mN/m, the area per lipid increased by approximately 23% at 32°C compared to 23°C, and remained at this value (approximately 27 $Å^2$) up to 46°C. The same trend was observed for the onset of the isotherms, i.e., when lipids begin to interact and the surface pressure increases to non-zero values. The maximum surface pressure that the SC lipid monolayer could withstand before collapsing decreased almost linearly from 59 mN/m to 44 mN/m as it moved from 23°C to 46°C (although the isotherms and the areas per lipid suggest some monolayer restructuring and possible lipid squeeze-out before these limiting pressures; Supplementary Figure S3a). The maximum compressibility moduli indicated liquid condensed phase, consistent with (Nováčková et al., 2021). The moduli were about 120 mN/m in monolayers prepared at 23 and 32°C, rose significantly to almost 150 mN/m at 37°C (indicating reduced elasticity) and decreased to 108 mN/m at 46°C (Supplementary Figure S3b-c).

Next, lipid monolayer was deposited on a solid mica support at 30 mN/m surface pressure using the Langmuir-Blodgett method and scanned by AFM (Figure 4d-e). At 23°C the samples formed a lipid monolayer with surface deviations of up to 2.5 nm, presumably caused by large variability in lipid chain lengths, and with frequent appearance of formations 4 to 6 nm high from the underlying monolayer. In samples prepared at 32°C and 37°C, additional formations 10–11 nm higher than the monolayer were found. These typically emerged from the 4-6 nm high structures, had a flat surface and phasing opposite to the underlying 4-6 nm high formations, which might be caused by the loss of substrate effect in the top layer of a horizontally stacked multilayer structure. Our findings are consistent with 10–11 nm high structures in human SC lipids at 32°C (Nováčková et al., 2021) and no such multilayers at 21°C (Norlén et al., 2007).

Since the mean area per lipid also increased with increasing temperature, we further tested whether multilayer formation depended primarily on temperature or on the mean molecular area per lipid. Two additional sets of samples were deposited at 27 $Å^2$/23°C and 22 $Å^2$/32°C. AFM scans (Supplementary Figure S4) confirmed that the multilayers formed at the higher temperature (and consequent higher chain mobility) rather than on the mean molecular area. Samples prepared at 27 $Å^2$/23°C showed a topography comparable to those prepared at 22 $Å^2$/23°C. Samples prepared at 22 $Å^2$/32°C showed formations peaking cca 10-11 nm above the base (although the monolayer was close to collapse) similar to those at 27 $Å^2$/32°C.

The step height of 10–11 nm (to which we must add the underlying monolayer height) is similar to the 11–13 nm repeat distance of the long periodicity lamellar phase of SC lipids (Bouwstra et al., 1991, Narangifard et al., 2021, White et al., 1988). Thus, a self-assembly of the SC barrier lipids into multilayers requires acidic pH (Nováčková et al., 2021) and temperatures at or above the OR-HEX phase transition. Below the transition, the energy barrier for the chain flip and multilayer formation is likely too high. Consistent with this result, a change in the SC mechanical properties has been observed at about 40°C (Wilkes and Wildnauer, 1973).

Relevance

During skin barrier formation, lipid precursors are delivered to the intercellular spaces at the *stratum granulosum*-SC interface and enzymatically processed (Menon et al., 2018, Norlén et al., 2022). Simultaneously, a progressive acidification and dehydration of the extracellular space occur (Behm et al., 2017, Caspers et al., 2001, Elias, 2017, Warner et al., 1988). Finally, in mature SC we find rigidly and intricately arranged lipid multilayers with little chain flexibility filling up the intercellular spaces (Boncheva, 2014). How do the lipids reach their position within the rigid lamellar architecture has not been satisfactorily explained so far. Our previous study indicated that, at some point, the lipids must be considerably fluid to assemble without significant packing defects (Sagrafena et al., 2022). Our

current results suggest that the low proportion of OR lipids is necessary for their assembly into multilayers *in vitro*. The fine-tuning of lipid chain mobility by the OR-HEX transition could form part of the explanation, because together with decreasing pH (Elias, 2017, Fluhr et al., 2004) and hydration (Behm et al., 2017, Boncheva, 2014, Caspers et al., 2001, Warner et al., 1988), the decrease in temperature to values below the transition is expected during the process of SC lipid barrier formation and maturation. Thus, the process of skin lipid barrier maturation, could possibly be co-regulated by the existence of the OR-HEX (or better HEX-OR) transition and by decreasing temperature and hydration across the inner skin-outer skin transition, consistent with the concept of tissue sculpting by phase transitions (Lenne and Trivedi, 2022). At lower temperatures and/or lower hydration levels, the proportion of OR lipids increases, lowering the activation energy for the translocation of a subset of compounds and lipids.

In diseases where the proportion of OR lipids is lower than in healthy skin, e.g. atopic dermatitis (Pilgram et al., 2001), lamellar ichthyosis (Pilgram et al., 2001), psoriasis (Uchino et al., 2023), or Netherton syndrome (Van Smeden et al., 2014), the permeation of lipophilic substances that move at least partially by lateral diffusion can be expected to be strongly increased compared to healthy skin. On the other hand, if SC lipids were predominantly OR at physiologically relevant temperatures (e.g., if metabolic selection of specific lipid structures shifted the OR-HEX transition to higher temperatures), they would probably be too rigid. Such rigid lipids would then be very reluctant to rearrange into multilamellar structures, defects would form, and they could hardly incorporate enzymes or peptides.

Our results suggest that the SC lipid matrix has fine-tuned its composition to take advantage of the presence of the OR-HEX transition at physiological temperatures. Thus, the low-temperature lipid packing transition associated with skin barrier lipids may act as a switch between lipid fluidity (required in lower SC layers for sculpting the lipids into their functional

arrangement) and rigidity (required in mature SC to resist water loss and penetration of exogenous compounds).

MATERIALS & METHODS

Material

Buffer components, gentamicin sulfate, solvents of HPLC grade, indomethacin, trypsin, dispase II, FITC-inulin (F3272, due to the polysaccharide nature of inulin it is supplied as a mixture of polymers of differing length where the average molecular weight varies between 3,000 and 6,000 g/mol including the FITC labelling - 0.001-0.015 mol FITC per mol monosaccharide), and polycarbonate filters (Magna Nylon 47 mm, 0.45µm pore size, Fisher Scientific EU) were purchased from Merck (Schnelldorf, Germany). All aqueous solutions were prepared using Millipore water (Milli-Q system, Millipore, Burlington, MA USA).

Human skin

Abdominal and breast skin was obtained from healthy female Caucasian donors, 44-57 years old, undergoing cosmetic or reparative surgery with their written, informed consent. The procedure was approved by the Ethics Committee of Sanus Sanatorium - First Private Surgical Centre, Hradec Králové, Czech Republic (No. 5/4/2018), and conducted according to the principles of the Helsinki Declaration. For permeability experiments abdominal skin was used immediately after surgery. For SC lipids extraction the subcutaneous tissue was removed, and the skin was stored at -20°C till the extraction.

Water loss and SC permeability at 26°C – 50°C

The effects of temperature on the permeability of isolated human SC were investigated using water (TEWL), indomethacin and FITC-inulin as model permeants. To isolate SC for permeation experiments, the skin was used immediately following the surgery. After mechanical removal of the subcutaneous tissues, the epidermis was separated enzymatically using dispase II (2 mg/ml) according to modified method of (Jian et al., 2020) to avoid any temperature-induced artefacts. SC was separated using trypsin (Kligman and Christophers,

1963) at temperatures not exceeding 37°C. Separated SC on supporting filter was fixed between Teflon holders in Franz-type diffusion cells with 1 cm^2 area available for diffusion, the lower SC layers facing the acceptor compartment. Each cell was filled with phosphate-buffered saline, pH 7.4 ± 0.01 with gentamicin (0.05 g/l). Acceptor volume of each cell was measured and considered in flux calculations. The cells were equilibrated at the studied temperature in dry bath for 12 h.

Temperature of SC was checked using infrared thermometer (Tecman TM900), the emissivity set to 0.98 (Bernard et al., 2013) and TEWL was measured with the upper part of the cell removed, using the Teflon holder opening (5 mm above the SC) with the Aquaflux probe (Aquaflux AF200 instrument, Biox Systems Ltd, UK) at 32-37 % relative air humidity.

Next, 150 µl of the donor sample (either indomethacin 2% in 60 % aqueous propylene glycol or FITC-inulin 1 mg/ml in water) was applied to the SC and occluded with a cover glass. Samples of acceptor phase were collected throughout 48 h and replaced by the same amount of fresh preheated acceptor solvent. From the concentrations (for quantification, see Supplementary Material), corrected for changes induced by sampling, a permeation profile was constructed, from which a steady state flux was determined for each cell as the slope of the linear portion of the profile.

Langmuir monolayers of extracted SC lipids

Extracted and chromatographically purified human SC lipids (Nováčková et al., 2021) pooled from SC samples from ≥10 donors were dissolved in chloroform/methanol 3:1 at 1 mg/ml. Hydrophobic PTFE trough (KSV NIMA, Espoo, Finland) was filled with isotonic acetate buffer (pH 5.5 ± 0.01). The subphase-air interphase was maintained at 23 ± 0.1°C, 32 ± 0.2°C, 37 ± 0.3°C or 46 ± 0.3°C. 10 µl of the lipid solution was spread over the pre-heated buffer-air interface. The solvent was left to evaporate for 15 min and then the interface area available

for lipids was decreased by side barriers at 10 mm/min. (i.e., 500 mm^2/min). The surface pressure was measured using a platinum Wilhelmy plate and plotted against the mean molecular area. The surface compressibility modulus, which reflects the lipid monolayer elasticity, was calculated (Vollhardt and Fainerman, 2006).

AFM of mica-deposited lipid layers

Monolayers were Langmuir-Blodgett deposited at 30 mN/m (Školová et al., 2013); see Supplementary Materials for details. AFM measurements were performed using NTEGRA (NT- MDT, Apeldoorn, Netherlands) equipped with NSG 10 tip (APPNANO, Mountain View, CA USA) in semicontact mode. For each sample overview scans of 10.0×10.0 μm and detailed scans of 2.0×2.0 μm were obtained.

Infrared spectroscopy of human SC lipids

The range of the OR-HEX transition in extracted SC lipids was verified using infrared spectroscopy. Isolated SC lipids were placed onto single-reflection MIRacle ATR ZnSe crystal (PIKE Technologies, Madison, WI USA) preheated to 20°C and infrared spectra were collected using Nicolet 6700 spectrometer (Thermo Scientific, Madison, WI USA) while gradually increasing the temperature in 2°C steps in 15 min intervals at a resolution of 2 cm^{-1}. The final spectra were generated by the co-addition of 256 scans.

To investigate the effects of hydration on the OR phase content, thin SC lipid films on a polycarbonate filter were prepared (Nováčková et al., 2021) and annealed in a sealed container at 100 % relative humidity at 70°C for 20 min, then left to cool down slowly (overnight) to 32°C or 37°C at 100 % relative humidity. Then, the samples were placed onto ATR ZnSe crystal and infrared spectra were recorded as above. During spectra collection (over 6 h), the lipids were maintained at 32°C or 37°C and were allowed to dehydrate at 10-15% relative humidity.

Data treatment

Data are presented as mean and standard deviation. The number of replicates is indicated in each figure. Three or more values were compared using a one-way ANOVA with Tukey post hoc test; two values were compared using unpaired t-test (GraphPad Prism version 8.2.1, GraphPad Software, USA). $P \leq 0.05$ was considered significant.

DATA AVAILABILITY

Datasets related to this study can be found at https://doi.org/10.17632/vr37vj22s5.2, an open-source online data repository hosted at Mendeley Data.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: KV, PJ; Data Curation: PJ, MKo, MKu, KV; Funding Acquisition: KV, PJ; Investigation: PJ, MKo, MKu, LO, AK, IS; Methodology: PJ, MKo, MKu, LO, AK, KV; Resources: LO; Supervision: KV; Validation: PJ, MKo, MKu, AK; Visualization: PJ, MKu, KV; Writing - Original Draft Preparation: PJ, KV; Writing - Review and Editing: all authors

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/.

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FIGURE LEGENDS

Figure 1. Cartoon of SC structure and lateral arrangement of lipid chains. In the inner layers of the SC, where temperature and hydration are higher than at the skin surface, lipids are arranged into the structures necessary for proper permeability barrier function. The arrangement of lipids requires some flexibility, which is more compatible with HEX chain packing. In contrast, very tight and rigid OR packing of lipid chains is required for proper permeability barrier in mature SC layers.

Figure 2. Permeability barrier of human SC as a function of temperature around the OR-HEX transition. Three model markers were studied on isolated human SC in Franz diffusion cells: (**a**-**b**) water (measured as transepidermal water loss, TEWL), (**c**-**d**) indomethacin, and (**e**-**f**) FITC-labeled inulin. Panels (**a**, **c**, **e**) show the fluxes of the respective molecules as a function of temperature, and (**b**, **d**, **f**) show the Arrhenius curves (natural logarithm of the flux multiplied by the gas constant (R) versus the inverse of the absolute temperature) and the activation energy (slope of the linear fit). Symbols indicate the mean ± SD of individual experiments on SC isolated from different donors (SC1-SC4), n≥3 (2 for indomethacin at 44°C).

Figure 3. OR phase content in barrier lipids isolated from human SC as a function of temperature and hydration. (**a**) Shape evolution of the infrared rocking methylene vibration at 20-44°C. The band at ~730 cm^{-1} indicates OR lateral packing. Representative spectra. (**b**) Relative OR phase content (intensity ratio of ~730 cm^{-1} and ~720 cm^{-1} bands) and lipid chain conformation (methylene symmetric stretching vibration, $\nu_{sym(CH2)}$; lower values indicate more ordered all-*trans* chains) around the OR-HEX transition, approximately indicated by the gray rectangle. (**c**-**d**) Evolution of the relative OR phase content in SC lipids that were fully hydrated

and allowed to dehydrate freely at 10-15% relative humidity at 32°C (**c**) and 37°C (**d**). Mean ± SD, n≥3 (2 for $\nu_{sym(CH2)}$ at 30°C), * P < 0.05.

Figure 4. Skin barrier lipids spontaneously rearrange into multilayers at 32°C and 37°C, but not at 23°C, when the OR phase content is high. (**a**) Cartoon transition of lipids from monolayers to multilayers. (**b**) Representative isotherms of SC lipids at the air-buffer interface at 23-46°C. (**c**) Area per lipid at 30 mN/m versus temperature; mean±SD, n≥3, * P < 0.05. The blue area shows the relative content of the OR phase at these temperatures and the vertical lines delineate the approximate extent of the transition. (**d**) Representative atomic force micrographs of lipid samples prepared at 23-37°C (n=3-5, each scanned at least 5×), blue lines indicate analyzed height profiles. (**e**) Height profiles of lipid samples prepared at 23-37°C (representative of at least 10 measurements).