

Omega-Hydroxyceramides are Required for Corneocyte Lipid Envelope (CLE) Formation and Normal Epidermal Permeability Barrier Function

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Omega-hydroxyceramides (ω -OHCer) are the predominant lipid species of the corneocyte lipid envelope in the epidermis. Moreover, their ω -esterified-derivatives (acylCer) are major components of the stratum corneum extracellular lamellae, which regulate cutaneous permeability barrier function. Because epidermal ω -OHCer appear to be generated by a cytochrome P450-dependent process, we determined the effects of a mechanism-based inhibitor of ω -hydroxylation, aminobenzotriazole (ABT), on epidermal ω -OH Cer formation and barrier function. We first ascertained that ABT, but not hydroxybenzotriazole (OHBT), a chemical relative with no P450 inhibitory activity, inhibited the incorporation of [14 C]-acetate into the ω -OH-containing Cer species in cultured human keratinocytes ($68.1\% \pm 6.9\%$ inhibition *versus* vehicle-treated controls; $p < 0.001$), without altering the synthesis of other Cer and fatty acid species. In addition, ABT significantly inhibited the ω -hydroxylation of very long-chain fatty acids in cultured human keratinocytes. Topical application of ABT, but not OHBT, when applied to the skin of hairless mice following acute barrier disruption by tape-stripping, resulted in a significant

delay in barrier recovery (e.g., 38.3% delay at 6 h *versus* vehicle-treated animals), assessed as increased transepidermal water loss. The ABT-induced barrier abnormality was associated with: (i) a significant decrease in the quantities of ω -OHCer in both the unbound and the covalently bound Cer pools; (ii) marked alterations of lamellar body structure and contents; and (iii) abnormal stratum corneum extracellular lamellar membrane structures, with no signs of cellular toxicity. Furthermore, pyridine-extraction of ABT- *versus* vehicle-treated skin, which removes all of the extracellular lamellae, leaving the covalently attached lipids, showed numerous foci with absent corneocyte lipid envelope in ABT- *versus* vehicle-treated stratum corneum. These results provide the first direct evidence for the importance of ω -OHCer for epidermal permeability function, and suggest further that acylCer and/or corneocyte lipid envelope are required elements in permeability barrier homeostasis. **Key words:** acylceramides/ceramides/cytochrome p450/epidermal lipids/omega-hydroxylase/permeability barrier/stratum corneum. *J Invest Dermatol* 114:185-192, 2000

Epidermal permeability barrier function is mediated by an approximately equimolar mixture of nonessential free fatty acids (FFA), cholesterol, and ceramides (Cer), arranged as multilamellar membranes within the stratum corneum (SC) interstices (reviewed in Elias and Menon, 1991). Of these three key lipids, Cer comprise a family of at least seven subfractions (Wertz *et al*, 1985; Motta *et al*, 1993;

Robson *et al*, 1994), which account for up to 50% of SC lipid by weight (Elias and Menon, 1991). Not only because of their preponderance, but also because of their amphiphilic structure and extremely long-chain, constituent N-acyl fatty acids, Cer are presumed to be critical for barrier function. Indeed, blockade of either Cer synthesis or Cer formation from its immediate precursor, glucosylCer (GlcCer), results in abnormal permeability barrier homeostasis (Holleran *et al*, 1991b, 1993). Moreover, modulations in barrier function provoke changes in both mRNA levels and activities for the synthetic and GlcCer-processing enzymes that generate Cer (Holleran *et al*, 1991a, 1994b, 1995).

Although these studies demonstrate a broad requirement for the Cer family for barrier function, neither the function nor the requirement for specific species within the Cer family are known. Substantial indirect evidence, however, points to the importance for the barrier of two of these Cer fractions, the most nonpolar species, Cer 1 and Cer 4, which contain linoleic acid ω -esterified to an unusually long-chain, N-acyl fatty acid ($C \geq 30$; acylCer)

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Abbreviations: ABT, aminobenzotriazole; acylCer, acylceramides; Cer, ceramides; CHK, cultured human keratinocytes; CLE, corneocyte lipid envelope; FFA, free fatty acids; GlcCer, glucosylceramides; HPTLC, high-performance thin layer chromatography; OHBT, hydroxybenzotriazole; OsO₄, osmium tetroxide; RuO₄, ruthenium tetroxide; SC, stratum corneum; TEWL, transepidermal water loss; ω -OHCer, omega-hydroxyceramides.

(Wertz and Downing, 1983). For example, in essential fatty acid deficiency, oleate substitutes for linoleate as the predominant ω -esterified species in acylCer (Wertz *et al*, 1983), in association with a profound barrier abnormality (Elias and Brown, 1978; Elias *et al*, 1980). Moreover, only when acylCer are added to model lipid mixtures of cholesterol, FFA, and non- ω -esterified Cer, do membrane structures form *in vitro* with periodicities similar to those demonstrated by the SC extracellular lamellae (Bouwstra *et al*, 1998).

In addition to their putative role in extracellular lamellar membrane organization, a portion of the ω -OHCer pool is diverted to the external surface of the cornified envelope (CE), where it is attached covalently to involucrin and other constituent CE peptides (Swartzendruber *et al*, 1987; Wertz *et al*, 1989a; Marekov and Steinert, 1998), apparently through a unique transglutaminase 1 activity (Nemes *et al*, 1999). The resulting monolayer of ω -OHCer forms the corneocyte lipid envelope (CLE) (Wertz and Downing, 1986, 1987). Although the CLE itself possesses no intrinsic water barrier properties,¹ it is thought to be critical either for the normal deposition of the extracellular lamellae (scaffold function) (Downing, 1992), or for intercorneocyte cohesion (Wertz *et al*, 1989b), and/or as a semipermeable membrane.¹

To ascertain whether ω -OHCer are critical for the barrier, we assessed here whether an inhibitor of the cytochrome P450 isoform, which is responsible for ω -hydroxylation of the FFA prior to N-acylation of the sphingoid base, would alter epidermal structure and function. Because the type 4 subfamily of cytochrome P450 (CYP4) specifically performs this ω -hydroxylation step (Ortiz de Montellano, 1995), aminobenzotriazole (ABT), a suicide inhibitor of the CYP4 ω -hydroxylases (Ortiz de Montellano *et al*, 1992; Correia, 1995; Ortiz de Montellano and Correia, 1995; Dierks *et al*, 1998), was tested for its ability to alter permeability barrier function, SC membrane structure, and Cer composition. Our results show that inhibition of epidermal ω -hydroxylation results in abnormal permeability barrier homeostasis, attributable to a selective deletion of ω -OH-Cer, loss of CLE, altered lamellar body structure, and abnormal extracellular lamellar membrane structures. Together, these studies demonstrate the importance of ω -OHCer for normal cutaneous permeability barrier homeostasis.

MATERIALS AND METHODS

Materials Ethylenediaminetetraacetic acid (EDTA), 1-aminobenzotriazole (ABT), 1-hydroxybenzotriazole (OHBT), and Cer standards were from Sigma (St. Louis, MO). Bio-Rad protein reagent and bovine serum albumin were obtained from Bio-Rad Laboratories (Richmond, CA). High performance thin layer chromatography (HPTLC) plates (silica gel 60) were purchased from E. Merck (Darmstadt, Germany). All reagents were of analytical grade.

Cultured human keratinocytes (CHK) Human epidermis was isolated from newborn foreskins by incubation in Dispase, and a suspension of keratinocytes was obtained by incubation in EDTA and subsequent trypsinization (Rheinwald and Green, 1975). Second-passage cells were plated in serum-free keratinocyte growth medium (KGM; Cascade Biologics, Portland, OR), containing 0.07 mM calcium, and grown to 90%–100% confluence, as described previously (Pittelkow and Scott, 1986; Holleran *et al*, 1990). In order to further enhance ω -OHCer production (Madison and Howard, 1996), the medium was then switched to DMEM/Ham F-12 (2:1) supplemented with 10% FBS, 10 μ g insulin per ml, 0.4 μ g hydrocortisone per ml, 5 units penicillin per ml, and 5 μ g streptomycin per ml, for 5 d prior to harvesting.

Lipid synthesis To assess rates of lipid synthesis, second-passage CHK were incubated with [¹⁴C]-acetic acid (Amersham, Arlington Heights, IL) \pm one of the inhibitors (2.5–10 mM) or vehicle for 6 h, and harvested after rinsing with ice-cold phosphate-buffered saline (PBS). Total lipids were extracted as described below. Labeled Cer and GlcCer were separated

on HPTLC in the following solvent systems: for Cer, chloroform:methanol:acetic acid (94:1:4, vol/vol/vol); and for GlcCer, chloroform:methanol:water (40:10:1, vol/vol/vol). The incorporation of radioisotope into the N-(ω -OH)-acyl group was measured after the conversion to the corresponding fatty acid methyl esters (i.e., following methanolysis of total Cer and GlcCer fractions); fatty acid methyl esters were separated by HPTLC in n-hexane:diethylether:acetic acid (65:35:1, vol/vol/vol). Individual fractions were scraped, extracted into scintillation fluid, and counted by liquid scintillation spectrometry.

Animals and permeability barrier studies Male hairless mice (SKH1-hr) were purchased from Charles River Laboratories (Wilmington, MA), and fed Purina mouse diet and water *ad libitum*. All animals were 8–12 wk of age at the time of study. Transepidermal water loss (TEWL) was measured with an electrolytic water analyzer (Meeco, Warrington, PA), as described previously (Menon *et al*, 1985; Holleran *et al*, 1991b). By several consecutive strippings with adhesive tape (Tesa, Beiersdorf, Germany), parts of the SC and its constituent lipids were removed to induce an increase of TEWL over baseline (≥ 4 mg per cm² per h). This treatment results in approximately two residual corneocyte layers, focally distributed over the tape-stripped area. TEWL readings were obtained immediately after barrier disruption, and at 3, 6, and 24 h. Means of three individual measurements per treated animal and time point were combined as one statistical value.

Inhibitor studies The flanks (i.e., approximately 2.5 cm²) of hairless mice were treated topically 16 h before and immediately after tape stripping, with 100 μ l solution of ABT or OHBT over a range of molarities (3.7×10^{-6} – 3.7×10^{-1} M), in an ethanol/propylene glycol (3:7 vol/vol) vehicle. Approximately three-to-four times higher concentration of ABT was required to achieve comparable reductions in ω -OHCer production *in vivo* as *in vitro*, without evidence of toxicity. The vehicle alone was employed in the tape-stripped, control animals, or on the contralateral flank from inhibitor-treated animals.

Electron microscopy Freshly obtained biopsies of hairless mouse skin were either transferred to half-strength Karnovsky's fixative, or immersed first in absolute pyridine for 2 h (Elias *et al*, 1977) prior to aldehyde fixation. All samples were postfixed with 1% aqueous osmium tetroxide (OsO₄), containing 0.5% potassium ferrocyanide, and embedded in an Epon-epoxy mixture. For visualization of lamellar membrane structures, alternate samples were postfixed with ruthenium tetroxide (RuO₄) (Madison *et al*, 1987; Hou *et al*, 1991). Sections were cut on a Reichert Ultracut E microtome and counterstained with uranyl acetate and lead citrate. Sections were viewed in a Zeiss 10 CR electron microscope, operated at 60 kV.

Corneocyte lipid envelope quantitation For quantitation of the CLE, we evaluated randomly obtained and coded electron micrographs at a constant magnification from pyridine-extracted tissue samples. By planimetric measurement of the overall length of interdesmosomal corneocyte-surface *versus* the length of intact CLE within the same domains, ratios of CLE length *versus* length of cornified surface were obtained from ABT- *versus* vehicle- or OHBT-treated animals. Morphometric analysis was performed only on intercorneocyte domains in the first and second (most-proximal) cornified layers, to focus on nascent CLE.

Preparation of epidermal homogenates Epidermal sheets were obtained from normal and inhibitor-treated mice by first removing whole skin samples from euthanized animals, followed by submerging these samples in calcium- and magnesium-free Dulbecco's PBS containing 10 mM EDTA (pH 7.4) for 35–45 min (37°C), followed by gentle scraping with a scalpel blade (Holleran *et al*, 1991b). All subsequent steps were performed at 4°C, unless otherwise noted. Epidermal sheets were homogenized in 900 μ l of Dulbecco's PBS (pH 7.4, containing 0.250 M sucrose) with a Polytron PCU2 tissue homogenizer (35%; 2×15 s; 4°C), sonicated (35%; 3×10 s) using a Fisher sonic probe dismembrator (Artec, Farmingdale, NY), and cell debris was pelleted in a microfuge at $10\,000 \times g$ for 15 min (4°C).

Lipid extraction, fractionation, and quantitation Epidermal sheets were prepared as described above. Extractable lipids first were obtained with the method of Bligh and Dyer (1959), as previously modified (Wertz *et al*, 1989a; Holleran *et al*, 1991b). Covalently bound lipids were isolated by saponification of the exhaustively extracted samples (Wertz *et al*, 1989a). The following HPTLC procedure provided optimal separation and quantitation of Cer and GlcCer species by densitometry: Cer and GlcCer

¹Elias PM, Fartasch M, Uchida Y, Holleran WM: Observations on the structure, function, and origin of the lipid-bound envelope. *J Invest Dermatol* 112:542a, 1999

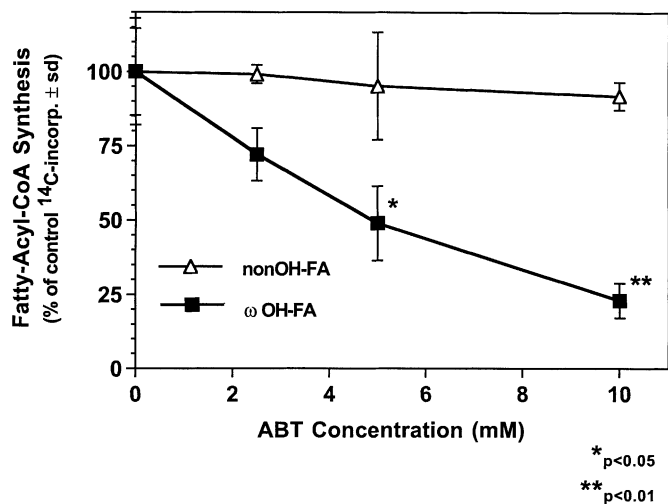


Figure 1. ABT in concentrations ≥ 5 mM selectively inhibits ω -OH-FA formation in CHK. CHK were treated with increasing concentrations of ABT (0–10 mM), and the incorporation of [14 C]-acetate was performed. The combined Cer fractions were acid-hydrolyzed to release acyl FA. The ω -OH and nonhydroxy fractions were separated by HPTLC, and incorporation into each fraction determined (see *Materials and Methods*). ABT, at concentrations of ≥ 5 mM significantly inhibited the incorporation into the ω -OH-FA (i.e., $p < 0.05$ and 0.01 at 5 and 10 mM, respectively), whereas incorporation into the non-OH-FA were unaffected. Values were normalized to vehicle controls (set to 100%) for each FA subfraction (i.e., ω -OH-FA and non-OH-FA), and represent the mean \pm SD; $n = 4$.

species were fractionated in the following solvent systems: (i) chloroform:methanol:water (40:10:1, vol/vol/vol) to 2 cm and 5 cm, successively; (ii) chloroform:methanol:acetic acid (94:4:1.5, vol/vol/vol) to the top; and (iii) n-hexane:diethylether:acetic acid (65:35:1, vol/vol/vol). After final development, the plates were dried, cooled, dipped in charring solution [1.5% cupric sulfate in acetic acid:sulfuric acid:orthophosphoric acid:water (50:10:10:30, by volume)] and charred at 160°C for 15 min. Plates were scanned with a variable wavelength scanning densitometer (Shimadzu, Kyoto, Japan) set at 684 nm. Lipid amounts were quantitated by cochromatography against known standards.

Statistical analysis Statistical analysis were performed using a paired or unpaired Student t test, as appropriate.

RESULTS

ABT selectively inhibits omega-hydroxylation in keratinocytes Prior to performing *in vivo* studies with the inhibitors, we assessed the optimal dose, time course, and specificity for inhibitor effects on the synthesis of ω -hydroxylated products in cultured human keratinocytes (CHK). To demonstrate the specificity of ABT for ω -OHCer synthesis, we first compared synthesis of ω -OH-fatty acids (FA) versus non-OH-containing FA with increasing concentrations of the inhibitor. ABT at concentrations of up to 10 mM did not inhibit the synthesis of non-OH-FA, but the inhibitor did reduce ω -OH-FA production in a progressive, concentration-dependent fashion (Fig 1). ABT also progressively inhibited the synthesis of ω -OHCer at concentrations from 2 to 10 mM, whereas the synthesis of other, non- ω -OH-containing Cer species was not significantly affected (data not shown; cf. Figure 1). Although the synthesis of α -hydroxy-containing fatty acids was slightly diminished at the highest ABT concentration tested (i.e., 10 mM), this difference did not achieve statistical significance (not shown).

As seen in Fig 2, ABT (10 mM) also did not inhibit the *in vitro* synthesis of either non- ω -OH-Cer or -GlcCer species, whereas the production of non- ω -OH Cer species increased with ABT treatment ($p < 0.01$ versus vehicle control). In contrast, the generation of both ω -esterified acylCer and acylGlcCer, end-products in the generation of ω -OHCers, was significantly

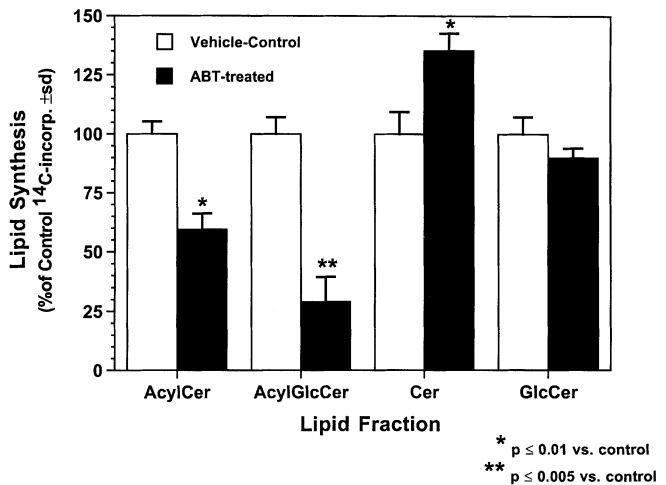


Figure 2. ABT selectively inhibits ω -hydroxy ceramide formation in CHK. CHK were treated with either vehicle control (light bars) or ABT (10 mM; solid bars) for 6 h, and the incorporation of [14 C]-acetate into extractable lipids was determined for this time period. ABT significantly decreased the formation of ω -OH-containing Cer fractions (i.e., acylCer and acylGlcCer; $p < 0.01$ and 0.005 , respectively), whereas the production of non- ω -OH-Cer were either increased (Cer) or unchanged (GlcCer). Values were normalized to vehicle controls for each fraction, and represent the mean \pm SD; $n = 4$.

inhibited under the same conditions ($p < 0.01$ and $p < 0.005$, respectively). These studies show that ABT specifically inhibits ω -OH FA and ω -OH Cer production in CHK, without producing nonspecific or toxic effects on either bulk sphingolipid or FA synthesis.

Inhibition of omega-hydroxylation interferes with normal permeability barrier homeostasis To ascertain whether ω -hydroxylation is important for barrier homeostasis, we next assessed the kinetics of barrier recovery in hairless mice treated twice with either ABT, or equal concentrations of the chemically related, inactive analog, hydroxybenzotriazole (OHBT). As seen in Fig 3(A), two topical applications of 37 mM ABT (i.e., 16 h before, and immediately after barrier disruption), but neither OHBT nor vehicle provoked a significant delay in barrier recovery at 3 and 6 h. By 24 h after acute disruption, barrier recovery rates were comparable in all groups. Whereas 10-fold higher and 10-fold lower concentrations produced comparable delays in barrier recovery, further dilutions of ABT ($< 370 \mu\text{M}$) did not alter barrier homeostasis significantly (and again OHBT had no effect) (Fig 3B; data for OHBT not shown). These results show that topical applications of an ω -hydroxylation inhibitor provoke abnormalities in permeability homeostasis.

Topical ABT also reduces omega-hydroxy ceramides in murine epidermis To ascertain the basis for the barrier abnormality, we next determined whether topical application of ABT selectively reduces the content of ω -OH-containing Cer in murine epidermis. Two applications of ABT to hairless mouse skin as above, produced a significant reduction in the content of ω -OH-containing Cer species in both the unbound and the covalently bound lipid fractions (Fig 4A). Likewise, the content of total unbound ω -OH-containing GlcCer species (i.e., acylGlcCer) was also reduced significantly by ABT treatment ($p < 0.02$) (Fig 4B). The quantities of bound ω -OH GlcCer were below detection levels. Conversely, ABT treatment did not alter the epidermal content of non- ω -OH-containing Cer (Fig 4C) and GlcCer (Fig 4B). Finally, OHBT showed no inhibitory effects on either epidermal glucosylated or nonglucosylated ω -OHCer species (Fig 4B, C). These studies show that ABT, at concentrations that alter barrier homeostasis, selectively deplete epidermal ω -OH Cer content, without altering other Cer and GlcCer.

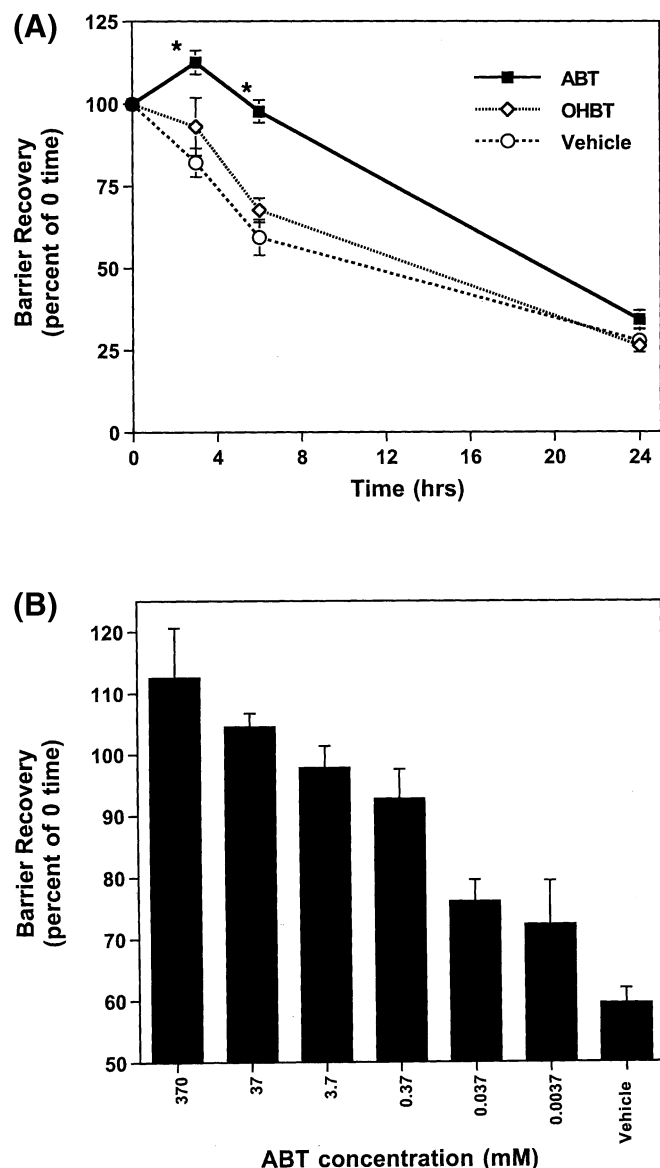


Figure 3. Permeability barrier recovery is delayed by inhibition of ω -hydroxylation with ABT. The flanks of hairless mice were treated twice with topical ABT (3.7 mM); i.e., 16 h prior to, and immediately following tape-stripping. Transepidermal water loss (TEWL) was measured at 3, 6, and 24 h following tape-stripping. (A) Barrier recovery of hairless mice is delayed by ABT at both 3 and 6 h following tape-stripping ($p < 0.0001$ for ABT- and vehicle-treated groups, respectively) but not by an equivalent concentration of the inactive congener OHBT ($n \geq 6$ mice with ≥ 3 TEWL measurements per mouse for each value). (B) Concentration-response for varying concentrations of ABT was performed at the time of maximum delay following acute barrier disruption [i.e., 6 h; see (A)]. All but the lowest ABT concentration applied (i.e., $> 3.7 \mu\text{M}$) significantly impeded barrier repair compared with that in the vehicle-treated control group ($n = 5-11$ animals for each treatment group; $n = 30$ for vehicle control group).

Inhibition of omega-hydroxylation results in depletion of CLE and abnormalities in extracellular lamellar membrane structure We next assessed two potential structural mechanisms that could account for the alterations in barrier homeostasis produced by inhibition of omega-hydroxylation. First, we assayed the morphology of the CLE ultrastructurally, including quantitative, stereologic methods [samples taken at times of maximal difference in rates of barrier recovery (i.e., 6 h; cf. Figure 3A)]. ABT-treated, but not vehicle-treated SC revealed lengthy regions of cross-sectioned CE with a reduction in

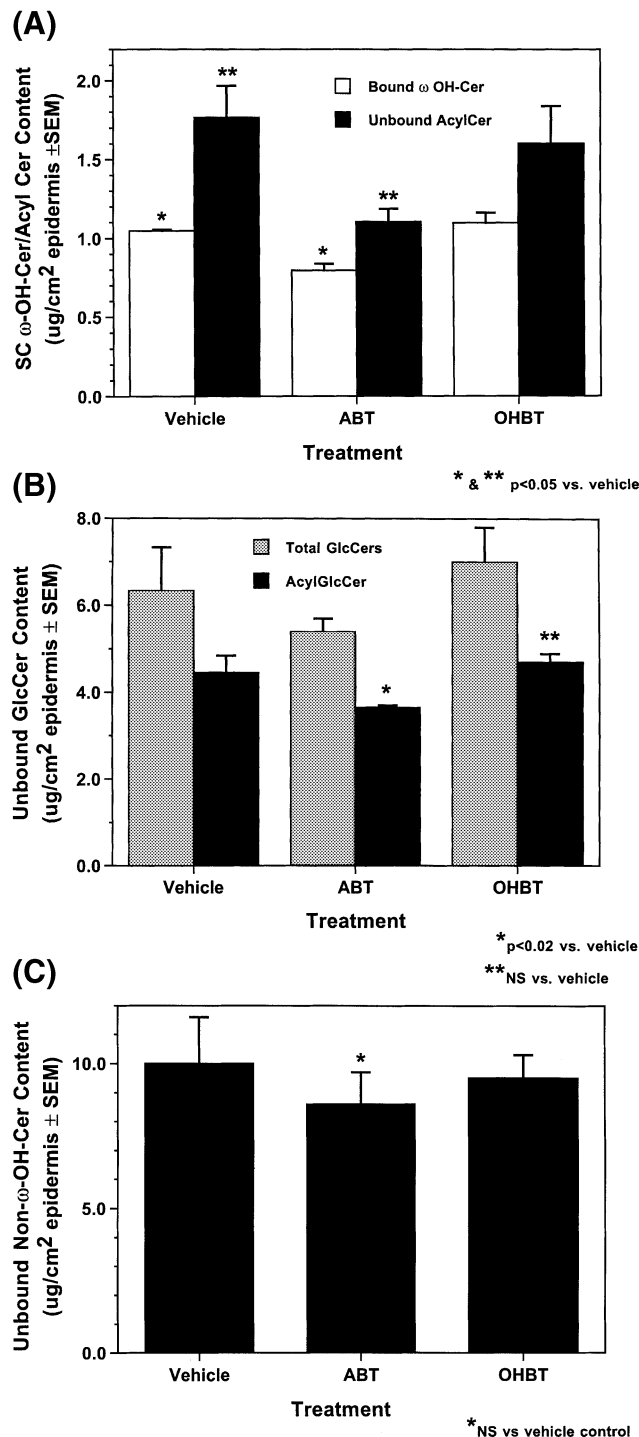


Figure 4. Topical ABT diminishes epidermal content of ω -OH-containing ceramides. The flanks of hairless mice were treated with ABT, OHBT, or vehicle (as in Fig 3, except $10\times$ higher concentrations of each compound were used to insure maximal effect(s); i.e., 37 mM). Both bound (i.e., CLE) and unbound lipids were extracted, separated by HPTLC, and the content of each lipid fraction determined (see Materials and Methods). Results are presented as the mean content (μg per cm^2 of epidermis) \pm SEM; $n = 3$ for each. (A) The content of both bound ω -OH-Cer and unbound acylCer were significantly diminished (i.e., $p < 0.05$ for each versus vehicle-control values) by ABT, whereas OHBT did not alter the content of these two Cer fractions. (B) The content of acylGlcCer also was decreased by ABT ($*p < 0.02$); the change in total GlcCer content was not statistically significant. Again, OHBT did not alter the content of either acylGlcCer or total GlcCer. (C) The content on non- ω -OH-Cer was not altered by either ABT or OHBT.

Figure 5. ABT treatment results in extensive deletion of CLE from corneocyte surface.

Electron micrographs of hairless mouse stratum corneum after two topical applications of ABT (37 mM; *A*) or vehicle (Con; *B*) (as in Fig 4). Two-headed arrows indicate cross-sections of cornified envelope without visible CLE. Open arrows indicate residual foci of CLE in ABT-treated samples (*A*), whereas solid arrows indicate intact CLE in controls (*B*). (*A, B*) Pyridine-treated, osmium tetroxide postfixation. Scale bar: 0.25 μ m.

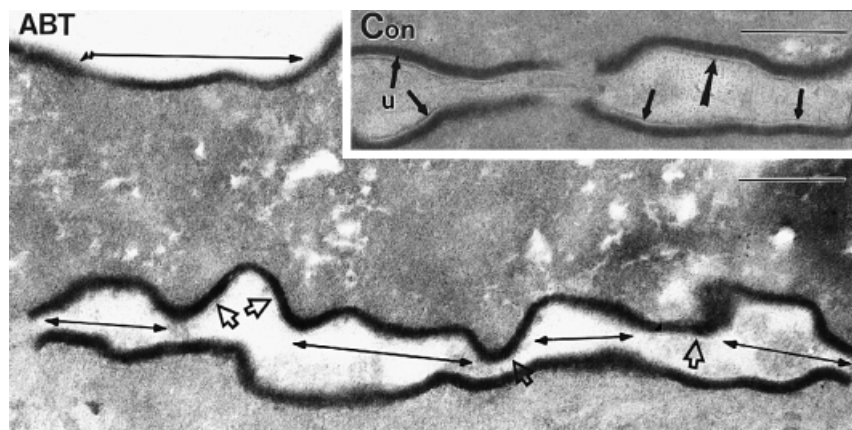
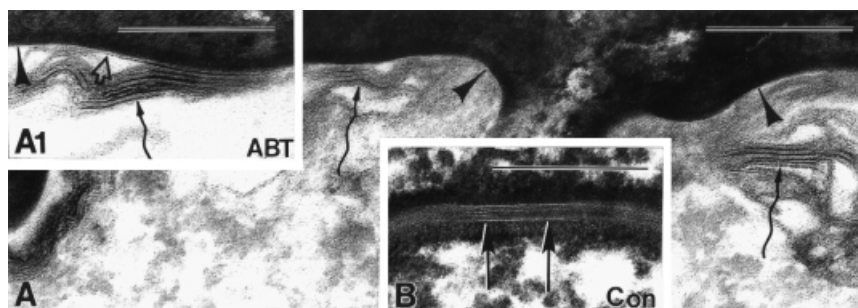


Figure 6. Secreted lamellar body contents fail to organize into mature lamellar membrane unit structures following ABT treatment. As in Figs 5 and 7. (*A*) Amorphous material and foreshortened lamellar stacks (waved arrow) predominate in SC interstices of ABT-treated skin. CLE is present focally (*A1*, open arrows), but absent from extensive regions of corneocyte surface (*A1*, arrowheads). (*B*) In contrast, normal bilamellar membrane arrays occur in vehicle-treated control samples (arrows). (*A, B*) Ruthenium tetroxide postfixation. Scale bar: 0.25 μ m.



contiguous CLE in the lower/nascent SC layer(s) (Figs 5, 6). These observations were confirmed by quantitative methods in randomly obtained, coded micrographs, assessed in an observer-blinded fashion. The extent of CLE was reduced by approximately 50% (Table I), which correlated well with the overall reduction in bound ω -OH Cer content (cf. Figure 4A). Focal areas in the suprajacent SC (i.e., mid-SC) displayed normal lamellar content and structures (not shown), likely representing residual, preformed SC prior to tape-stripping. Furthermore, the reduction in nascent CLE correlated with defects in the structure of the limiting membrane of lamellar bodies – organelles revealed either an apparent focal absence, complete deletion, or simple membranes rather than the normal, double membrane (Fig 7A). Such defective domains appear to be spliced into sites of fusion with the apical plasma membrane during lamellar body exocytosis at the SG–SC interface (Fig 7A2). Persistence of such abnormalities could result in the *in situ* formation of CLE with focal defects. These results show that ABT treatment, under conditions that inhibit both barrier permeability recovery and omega-hydroxylation, substantially decreases the CLE content of SC, and the structural mechanism that could account for both abnormal CLE formation and abnormal permeability barrier homeostasis in inhibitor-treated skin.

ABT also could alter barrier homeostasis by another or additional mechanism(s); i.e., disruption of extracellular lamellar membranes due to decreased production of acylCer. In fact, topical ABT significantly decreases the acylCer and acylGlcCer content of murine epidermis (Fig 4A, B), i.e., unbound acylCer in extracellular lamellae. Under these conditions, ABT-treated epidermis revealed abnormal lamellar body contents (Fig 7A1), depletion of secreted lamellar body contents at the SG–SC interface (Fig 7), as well as disruption of the architecture of the extracellular lamellae (Fig 6). Ruthenium tetroxide postfixation, ABT-treated samples revealed that nascent extracellular lamellae failed to transform into lamellar membrane unit structures in the lower SC (cf. Figure 6A vs 6B), despite extensive extracellular deposition of nonlamellar material (Fig 6A). These studies provide an alternate or additional structural mechanism whereby ABT could modulate barrier

Table I. Inhibition of omega-hydroxylation decreases CLE content

Treatment group	% CLE ^b (\pm SD)	n (fields)
Vehicle	74.7 \pm 20.4	14
Inhibitor (ABT)	32.5 \pm 16.4	26

^aThree days (topical).

^bPer cent of inter-desmosomal regions with CLE (planimetry of exhaustively-extracted SC).

homeostasis, i.e., by disruption of extracellular lamellar membrane structure due to decreased acylCer and acylGlcCer generation.

DISCUSSION

Abundant evidence indicates a critical role for bulk Cer for epidermal permeability barrier function. For example, inhibitors of either serine palmitoyltransferase or β -glucocerebrosidase, the key enzymes of Cer synthesis and Cer generation from GlcCer, respectively, alter barrier homeostasis (Holleran *et al*, 1991b, 1993). Moreover, Cer must be present in mixtures of physiologic lipids for permeability barrier recovery to occur at normal or accelerated rates after acute abrogations (Man *et al*, 1996). Yet, Cer comprise a heterogeneous family of at least seven subfractions (Wertz *et al*, 1985; Motta *et al*, 1993; Robson *et al*, 1994), whose specific functions remain unknown. Two of these species, Cer 1 and Cer 4 (acylCer) (Robson *et al*, 1994), which differ only in their sphingoid base, possess a linoleate moiety esterified via an ω -OH terminus to a very long-chain N-acyl group (Wertz *et al*, 1983). There is strong indirect evidence for the importance of these ω -esterified Cer for the permeability barrier, including their requirement to form lamellar membrane structures in model membranes that correspond to the dimensions of extracellular lamellae *in vivo* (Bouwstra *et al*, 1996, 1998), and their selective alteration in essential fatty acid

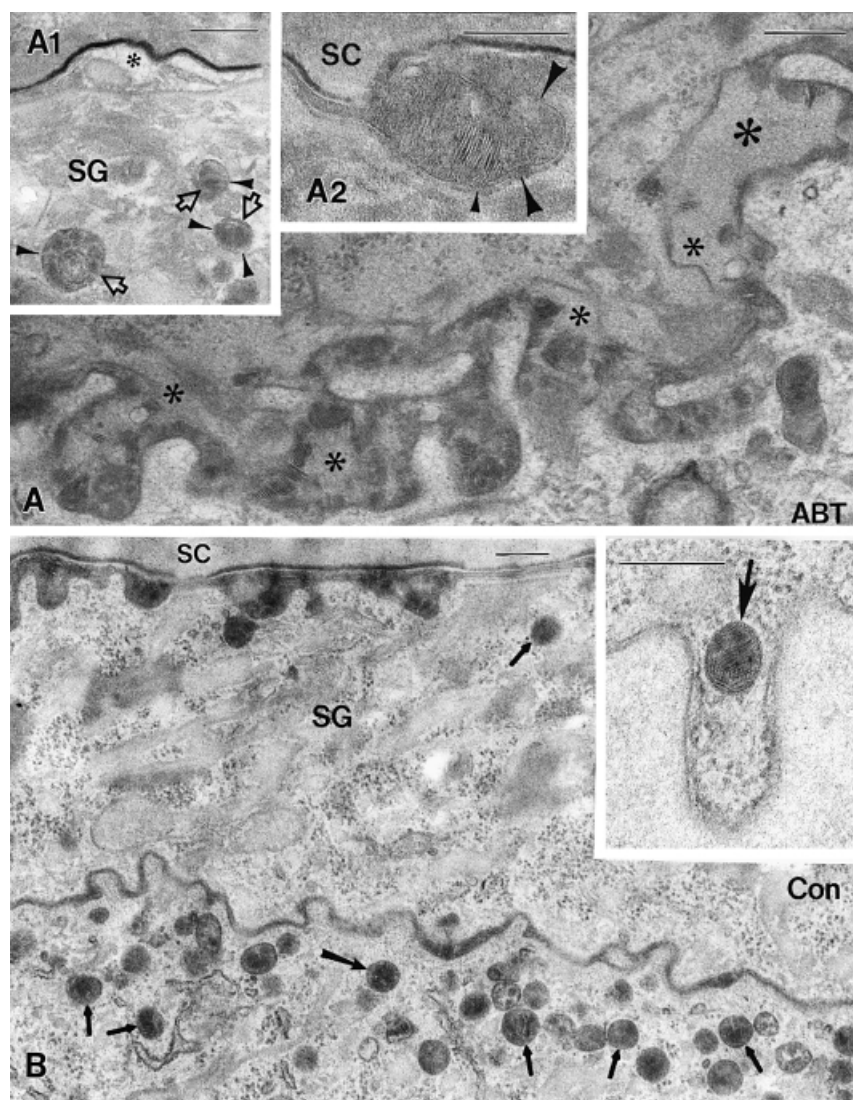


Figure 7. ABT treatment results in abnormalities in limiting membrane and contents of lamellar bodies. ABT-treated epidermis (as per Fig 5) reveals substitution of some amorphous material (*) for normal secreted LB contents, with reduction of secreted lamellar material at the stratum granulosum (SG)-stratum corneum (SC) interface (A, A1). Individual lamellar bodies display abnormal size and internal contents (A1, open arrows), as well as abnormalities in the organelle limiting membrane (A1, arrowheads). Abnormalities in the organelles' limiting membranes appear to correlate with focal deletion of apical plasma membrane of outermost SG cells at sites of lamellar body secretion (A2, arrowheads). (B, and inset) Vehicle-control epidermis displays numerous lamellar bodies (arrows) of uniform size in cytosol with partial-to-replete lamellar contents. (A, B) Osmium tetroxide postfixation.

deficiency (Wertz *et al*, 1983), which displays a profound barrier abnormality (Elias and Brown, 1978; Elias *et al*, 1980).

In this study, we sought direct evidence for the importance of ω -OHCer and/or their N-acyl esters for the barrier. Omega-OHCer are putative products of the type 4 cytochrome P450 enzyme subfamily, CYP4 (Ortiz de Montellano, 1995). We showed recently that CYP4A protein is present in epidermis, and concentrated in the cytosol of the stratum granulosum.² We also showed that ω -hydroxylation of the N-acylFA occurs prior to assembly of the N-acylFA with a sphingoid base to form Cer.³ Because lamellar body contents contain completely assembled acylGlcCer (Wertz *et al*, 1984), ω -hydroxylation must occur prior to packaging into this organelle (Fig 8). This conclusion is supported by our ultrastructural observations here of abnormal lamellar body contents in epidermis treated with the CYP4A suicide inhibitor, ABT. Moreover, we showed here that ABT inhibits ω -hydroxylation at concentrations that are inhibitory neither to FA nor to bulk Cer synthesis *in vitro* and *in vivo*. In contrast, the chemically related ABT analog, OHBT, which possesses no known enzyme-inhibitory properties (Su *et al*, 1998),

did not alter ω -OHCer production, further evidence both for the specificity of ABT for keratinocyte ω -hydroxylation and for its lack of toxicity at the concentrations employed here.

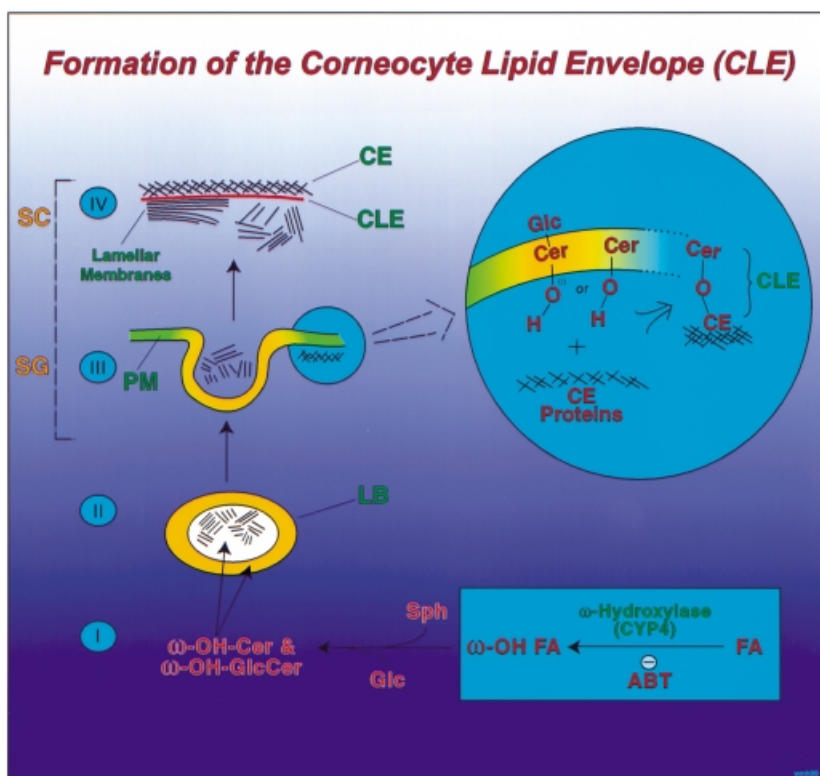
Omega-OHCer appear to be required for normal permeability barrier homeostasis, because barrier recovery was delayed following topical applications of nontoxic concentrations of ABT. The basis for the barrier abnormality is selective depletion of SC ω -OH-containing Cer content (by about 50% *versus* vehicle), resulting in abnormalities in SC membrane structure. In contrast, other epidermal synthetic activities are not altered under these conditions, nor is general toxicity visible by microscopy. Because the pool of preformed ω -OHCer in the SC would not be affected by short-term applications, as employed here, it is likely that more prolonged/frequent inhibitor applications would provoke a further decline in ω -OHCer (and a more-profound barrier abnormality), but at the potential cost of cumulative toxicity. Because our correlative data demonstrate selective inhibition of omega-hydroxylation under the same conditions that abrogate barrier recovery, these results indicate that ω -OHCer are required for permeability barrier homeostasis.

N-acylated and nonacylated ω -OHCer are constituents of two SC membrane structures (Fig 8), the CLE and extracellular lamellae, respectively, both of which were altered by ABT treatment. Thus, the functional abnormality resulting from inhibition of ω -OHCer production could be attributed to depletion or alterations in one or both of these structural constituents. ABT produced abnormalities in both lamellar body

²Behne M, Uchida Y, Seki T, Elias PM, Holleran WM: Omega-hydroxy ceramides in lipid-bound envelope (LBE) formation. *J Invest Dermatol* 112:605a, 1999 (abstr.)

³Uchida Y, Seki T, Elias PM, Holleran WM: Metabolic pathway for the formation of omega-hydroxy ceramides in mammalian epidermis. *J Invest Dermatol* 110:672a, 1998 (abstr.)

Figure 8. Formation, packaging, and organization of epidermal ω -OHCer proceed step-wise, and are disrupted by ABT. Formation of lamellar membranes in the interstices of the SC proceeds in a step-wise manner. Omega (ω)-hydroxylation of very long-chain fatty acids (FA) precedes condensation with sphingoid base (Sph) to form ω -OHCer species (Step I; in both lower and upper epidermal layers), including glucosylated and ω -acylated forms (not shown). This process is interrupted by the presence of ABT, which blocks the initial ω -hydroxylation reaction. The ω -OHCer species are then packaged, along with other barrier lipids, into both the limiting membranes and the central core of lamellar bodies (LB) (Step II; primarily in the mid-to-outer epidermal layers). Subsequent fusion of the LB limiting membrane with the apical plasma membrane (PM) of the outermost stratum granulosum (SG) cell delivers LB contents into the interstices between SG and cornified cells (Step III). This process also putatively enriches the apical plasma membrane with ω -OH-containing Cer species (i.e., from the LB limiting membrane), with subsequent covalent attachment of ω -OHCer to cornified envelope (CE) proteins to form the corneocyte lipid envelope (CLE) (inset). Finally, mature lamellar membrane unit structures form in the extracellular domains of the SC, the organization of which appears to depend upon the presence of an intact CLE (Step IV).



contents and their extracellular lamellar membrane products. Thus, abnormal extracellular lamellar membrane structure and contents could explain the barrier abnormality; however, our studies also demonstrate substantial deletion of the CLE following ABT treatment, which quantitatively paralleled the reduction in ω -OHCer content. Moreover, ABT treatment produced alterations in the limiting membranes of lamellar bodies that might also account for the CLE abnormality (Fig 8). These abnormalities suggest further that the CLE itself may result from splicing of the limiting membrane of the lamellar bodies into the plasma membrane of stratum granulosum cells. As previously suggested (Wertz, 1996), the limiting membrane of lamellar bodies must be ω -OHCer-enriched, and resistant to the hydrolytic digestion that degrades membrane phospholipids during cornification. One mechanism that could confer such resistance would be *in situ* transesterification of ω -OHCer from the acylCer to CE peptides, as recently suggested by Steinert and colleagues (Nemes *et al*, 1999). Although the CLE does not display intrinsic barrier properties,¹ it has been postulated to serve as a scaffold (Downing, 1992), critical for the deposition and organization of the extracellular lamellar membrane system. Whether the alterations in the extracellular lamellar membranes observed here result from a primary CLE abnormality leading to structural disorganization, and/or depletion of the acylCer component of the extracellular lamellae, could not be determined from this study. Nevertheless, these studies unequivocally demonstrate the requirement of ω -OHCer for normal epidermal permeability barrier homeostasis.

These results, together with other recently published work, have begun to reveal the sequence of biochemical events in the pathway of CLE formation and generation of mature lamellar membrane structures in the SC (Fig 8). First, as shown previously³ and again in this study (cf. Figure 1), ω -hydroxylation of very long-chain FA precedes condensation with sphingoid base moieties to form ω -OHCer species. Subsequent glucosylation and ω -acylation generates a mixture of primarily glucosylated, acylCer species (i.e., acylGlcCer), which then are packaged with other barrier lipids into both the central core and the limiting membranes of the LB (Wertz *et al*, 1984; Grayson *et al*, 1985; Wertz, 1996). Fusion of the

LB limiting membrane with the apical plasma membrane of the outermost SG cell delivers LB contents into the SG-SC interstices, and putatively enriches the apical plasma membrane with ω -OH-containing Cer species (i.e., from the LB limiting membrane). The subsequent covalent attachment of ω -OHCer to CE proteins to form the CLE may occur with either the free ω -OHCer (or acylCer species) (Fig 8, inset), or possibly with other Cer hydroxyl-residues;⁴ however, in the absence of β -glucocerebrosidase activity, a condition previously shown to significantly alter epidermal barrier structure and function (Holleran *et al*, 1993, 1994a), ω -OHGlcCer species also can be covalently linked to the CE (Doering *et al*, 1999a, b),⁵ suggesting that deglucosylation normally may occur following CLE formation. Finally, mature lamellar membrane unit structures form in the extracellular domains of the SC, the organization of which appears to depend upon the presence of an intact, deglucosylated CLE. This entire process is interrupted by the presence of ABT, which through an inhibition of the initial ω -hydroxylation reaction, disrupts both CLE and mature lamellar membrane formation, underscoring the key role for the unique ω -OHCer structures in epidermal permeability barrier homeostasis.

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⁴Downing DT, Stewart ME, Lazo N: Forty per cent of corneocyte envelope ω -hydroxyceramides are bound to protein through their ω -hydroxyl and 60 percent through their sphingosine 1-hydroxyl. *J Invest Dermatol* 112:575a, 1999 (abstr.)

⁵Uchida Y, Sidransky E, Ginns EI, Elias PM, Holleran WM: formation of the lipid-bound envelope (LBE): Insights from glucocerebrosidase-deficient Gaucher mouse epidermis. *J Invest Dermatol* 112:543a, 1999 (abstr.)

REFERENCES

- Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911-917, 1959
- Bouwstra JA, Cheng K, Gooris GS, Weerheim A, Ponc M: The role of ceramides 1 and 2 in the stratum corneum lipid organisation. *Biochim Biophys Acta* 1300:177-186, 1996
- Bouwstra JA, Gooris GS, Dubbelaar FE, Weerheim AM, Ijzerman AP, Ponc M: Role of ceramide 1 in the molecular organization of the stratum corneum lipids. *J Lipid Res* 39:186-196, 1998
- Correia MA. Rat and human cytochrome P450. substrate and inhibitor specificities and functional markers. In: Ortiz de Montellano P (ed.). *Cytochrome P450 Structure, Mechanism and Biochemistry*. New York: Plenum Press, 1995, pp. 607-621
- Dierks EA, Davis SC, Ortiz de Montellano PR: Glu-320 and Asp-323 are determinants of the CYP4A1 hydroxylation regioselectivity and resistance to inactivation by 1-aminobenzotriazole. *Biochemistry* 37:1839-1847, 1998
- Doering T, Holleran WM, Potratz A, Vielhaber G, Elias PM, Suzuki K, Sandhoff K: Sphingolipid activator proteins are required for epidermal permeability barrier formation. *J Biol Chem* 274:11038-11045, 1999a
- Doering T, Proia RL, Sandhoff K: Accumulation of protein-bound epidermal glucosylceramides in beta-glucocerebrosidase deficient type 2 Gaucher mice. *FEBS Lett* 447:167-170, 1999b
- Downing DT: Lipid and protein structures in the permeability barrier of mammalian epidermis. *J Lipid Res* 33:301-313, 1992
- Elias PM, Brown BE: The mammalian cutaneous permeability barrier: defective barrier function in essential fatty acid deficiency correlates with abnormal intercellular lipid deposition. *Lab Invest* 39:574-583, 1978
- Elias PM, Menon GK: Structural and lipid biochemical correlates of the epidermal permeability barrier. *Adv Lipid Res* 24:1-26, 1991
- Elias PM, Brown BE, Ziboh VA: The permeability barrier in essential fatty acid deficiency: evidence for a direct role for linoleic acid in barrier function. *J Invest Dermatol* 74:230-233, 1980
- Elias PM, Goerke J, Friend DS: Mammalian epidermal barrier layer lipids: composition and influence on structure. *J Invest Dermatol* 69:535-546, 1977
- Grayson S, Johnson-Winegar AG, Wintroub BU, Iseroff RR, Epstein EH Jr, Elias PM: Lamellar body-enriched fractions from neonatal mice: preparative techniques and partial characterization. *J Invest Dermatol* 85:289-294, 1985
- Holleran WM, Williams ML, Gao WN, Elias PM: Serine-palmitoyl transferase activity in cultured human keratinocytes. *J Lipid Res* 31:1655-1661, 1990
- Holleran WM, Feingold KR, Man MQ, Gao WN, Lee JM, Elias PM: Regulation of epidermal sphingolipid synthesis by permeability barrier function. *J Lipid Res* 32:1151-1158, 1991a
- Holleran WM, Man MQ, Gao WN, Menon GK, Elias PM, Feingold KR: Sphingolipids are required for mammalian epidermal barrier function. Inhibition of sphingolipid synthesis delays barrier recovery after acute perturbation. *J Clin Invest* 88:1338-1345, 1991b
- Holleran WM, Takagi Y, Menon GK, Legler G, Feingold KR, Elias PM: Processing of epidermal glucosylceramides is required for optimal mammalian cutaneous permeability barrier function. *J Clin Invest* 91:1656-1664, 1993
- Holleran WM, Ginns EI, Menon GK et al: Consequences of beta-glucocerebrosidase deficiency in epidermis. Ultrastructure and permeability barrier alterations in Gaucher disease. *J Clin Invest* 93:1756-1764, 1994a
- Holleran WM, Takagi Y, Menon GK, Jackson SM, Lee JM, Feingold KR, Elias PM: Permeability barrier requirements regulate epidermal beta-glucocerebrosidase. *J Lipid Res* 35:905-912, 1994b
- Holleran WM, Gao WN, Feingold KR, Elias PM: Localization of epidermal sphingolipid synthesis and serine palmitoyl transferase activity: alterations imposed by permeability barrier requirements. *Arch Dermatol Res* 287:254-258, 1995
- Hou SY, Mitra AK, White SH, Menon GK, Ghadially R, Elias PM: Membrane structures in normal and essential fatty acid-deficient stratum corneum: characterization by ruthenium tetroxide staining and x-ray diffraction. *J Invest Dermatol* 96:215-223, 1991
- Madison KC, Howard EJ: Ceramides are transported through the Golgi apparatus in human keratinocytes in vitro. *J Invest Dermatol* 106:1030-1035, 1996
- Madison KC, Swartzendruber DC, Wertz PW, Downing DT: Presence of intact intercellular lipid lamellae in the upper layers of the stratum corneum. *J Invest Dermatol* 88:714-718, 1987
- Man MM, Feingold KR, Thornfeldt CR, Elias PM: Optimization of physiological lipid mixtures for barrier repair. *J Invest Dermatol* 106:1096-1101, 1996
- Marekov LN, Steinert PM: Ceramides are bound to structural proteins of the human foreskin epidermal cornified cell envelope. *J Biol Chem* 273:17763-17770, 1998
- Menon GK, Feingold KR, Moser AH, Brown BE, Elias PM: De novo sterologenesis in the skin. II. Regulation by cutaneous barrier requirements. *J Lipid Res* 26:418-427, 1985
- Motta S, Monti M, Sesana S, Caputo R, Carelli S, Ghidoni R: Ceramide composition of the psoriatic scale. *Biochim Biophys Acta* 1182:147-151, 1993
- Nemes Z, Marekov LN, Fésüs L, Steinert PM: A novel function for transglutaminase 1: attachment of long-chain omega-hydroxyceramides to involucrin by ester bond formation. *Proc Natl Acad Sci USA* 96:8402-8407, 1999
- Ortiz de Montellano P: *Cytochrome P450. Structure, Mechanism, and Biochemistry*, 2nd edn. New York: Plenum Press, 1995
- Ortiz de Montellano PR, Chan WK, Tuck SF, Kaikau RM, Bass NM, Peterson JA: Mechanism-based probes of the topology and function of fatty acid hydroxylases. *FASEB J* 6:695-699, 1992
- Ortiz de Montellano PR, Correia MA: Inhibitors of cytochrome P450 enzymes. In: Ortiz de Montellano P (ed.). *Cytochrome P450 Structure, Mechanism and Biochemistry*. New York: Plenum Press, 1995, pp. 305-364
- Pittelkow MR, Scott RE: New techniques for the in vitro culture of human skin keratinocytes and perspectives on their use for grafting of patients with extensive burns. *Mayo Clinic Proceedings* 61:771-777, 1986
- Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331-343, 1975
- Robson KJ, Stewart ME, Michelsen S, Lazo ND, Downing DT: 6-Hydroxy-4-sphingene in human epidermal ceramides. *J Lipid Res* 35:2060-2068, 1994
- Su P, Kaushal KM, Kroetz DL: Inhibition of renal arachidonic acid omega-hydroxylase activity with ABT reduces blood pressure in the SHR. *Am J Physiol* 275:R426-R438, 1998
- Swartzendruber DC, Wertz PW, Madison KC, Downing DT: Evidence that the corneocyte has a chemically bound lipid envelope. *J Invest Dermatol* 88:709-713, 1987
- Wertz PW: The nature of the epidermal barrier: Biochemical aspects. *Adv Drug Del Rev* 18:283-294, 1996
- Wertz PW, Downing DT: Ceramides of pig epidermis: structure determination. *J Lipid Res* 24:759-765, 1983
- Wertz PW, Downing DT: Covalent attachment of omega-hydroxyacid derivatives to epidermal macromolecules: a preliminary characterization. *Biochem Biophys Res Commun* 137:992-997, 1986
- Wertz PW, Downing DT: Covalently bound omega-hydroxyacylsphingosine in the stratum corneum. *Biochim Biophys Acta* 917:108-111, 1987
- Wertz PW, Cho ES, Downing DT: Effect of essential fatty acid deficiency on the epidermal sphingolipids of the rat. *Biochim Biophys Acta* 753:350-355, 1983
- Wertz PW, Downing DT, Freinkel RK, Traczyk TN: Sphingolipids of the stratum corneum and lamellar granules of fetal rat epidermis. *J Invest Dermatol* 83:193-195, 1984
- Wertz PW, Miethke MC, Long SA, Strauss JS, Downing DT: The composition of the ceramides from human stratum corneum and from comedones. *J Invest Dermatol* 84:410-412, 1985
- Wertz PW, Madison KC, Downing DT: Covalently bound lipids of human stratum corneum. *J Invest Dermatol* 92:109-111, 1989a
- Wertz PW, Swartzendruber DC, Kitko DJ, Madison KC, Downing DT: The role of the corneocyte lipid envelopes in cohesion of the stratum corneum. *J Invest Dermatol* 93:169-172, 1989b