Extracellular processing of phospholipids is required for permeability barrier homeostasis

Man Mao-Qiang, Kenneth R. Feingold, Mahendra Jain, and Peter M. Elias

Dermatology and Medical (Metabolism) Services, Veterans Affairs Medical Center; Departments of Dermatology and Medicine, University of California, San Francisco, CA; and Department of Chemistry, University of Delaware, Newark DE

Abstract Three key lipid types, cholesterol, ceramides (CER), and free fatty acids (FA), form the stratum corneum (SC) lamellar bilayers that mediate barrier function. Prior studies have shown that barrier requirements regulate CER generation from glucosylCER, and that this catabolic step takes place within the SC interstices. Here, we addressed whether extracellular processing of phospholipids (PL), the FA precursor delivered to the SC interstices with glucosylCER and cholesterol during exocytosis of lamellar body contents, is also required for barrier homeostasis. We applied two chemically unrelated inhibitors of phospholipase A_2 (PLA_2-I), BPB (irreversible) and MJ33 (reversible), topically to hairless mice after barrier perturbation with acetone. Both inhibitors delayed barrier recovery at non-cytotoxic doses, while MJ45, an analogue known to inhibit PLA_2-II but not PLA_2-I, had no effect. Moreover, the delays in barrier recovery induced by BPB and MJ33 could be overridden by co-applications of palmitic acid, but not linoleic acid or lysolecithin. Furthermore, inhibitor-treatment resulted in accumulation of PL and depletion of FA in the SC by 4 h, as well as the persistence of "immature" lamellar body-derived membrane structures in the SC interstices. Finally, these changes in membrane structure were reversed when inhibitor-treated SC was incubated in vitro with palmitic acid. These studies show that: *1)* inhibition of PLA_2 results in abnormalities in lipid composition and SC structure that alter barrier homeostasis; and *2)* the functional defect can be attributed to a deficiency of nonessential FA within the SC. Thus, extracellular processing of PL into FA is required for normal barrier function.—**Mao-Qiang, M., K. R. Feingold, M. Jain, and P. M. Elias.** Extracellular processing of phospholipids is required for permeability barrier homeostasis. *J. Lipid Res.* 1995. **36:** 1925–1935.

Supplementary key words barrier function • epidermal ultrastructure • phospholipases • epidermal lipids • epidermal lipid metabolism

The central role of the three key stratum corneum lipids, cholesterol, fatty acid, and ceramides, in mediating mammalian permeability barrier function is now well established (1, 2). By either tape stripping or by selectively depleting the stratum corneum of lipids with topical acetone, we demonstrated previously that epidermal cholesterol, fatty acid, and sphingolipid synthesis are all regulated in response to barrier perturbation (2–4). Moreover, topical applications of inhibitors of epidermal HMG-CoA reductase and serine palmitoyl transferase, the rate-limiting enzymes in the cholesterol and sphingolipid biosynthetic pathways, respectively, delay barrier recovery after acute barrier abrogation (5, 6). Furthermore, selective inhibition of epidermal fatty acid synthesis also results in abnormal barrier recovery after barrier disruption (7). Finally, perturbations in barrier function also regulate other metabolic processes in the epidermis, including DNA synthesis (8), as well as the formation and secretion of epidermal lamellar body contents (9).

The epidermal lamellar body is enriched in glucosylsphingolipids, phospholipids, and cholesterol, which are secreted into the intercellular spaces at the stratum granulosum–stratum corneum junction (10). Biochemical studies have demonstrated that a decrease in both glucosylceramide and phospholipid contents is accompanied by an increase in ceramide and free fatty acid content during cornification (11–14). These changes suggest that extracellular processing of secreted lamellar body lipid precursors into more hydrophobic lipid products occurs during barrier formation. Moreover, the enzymes required for extracellular processing appear to derive, in large part, from secreted lamellar body contents, which are enriched in a number of hydrolytic enzymes, including proteases, glycosidases, acid phosphatase, and a family of lipases (phospholipase A, triacylglycerol hydrolase, and sphingomyelinase) (15, 16). These enzymes, including phospholipase A (type unspecified) have been shown biochemically to be concen-

---

Abbreviations: CER, ceramides; FA, fatty acids; SC, stratum corneum; PL, phospholipids; PLA_2, phospholipase A_2; BPD, bromphenacyl bromide; MJ33, 1-hexadecyl-3-trifluoro-ethylglycero-*sn*-2-phosphomethanol; MJ45, 1-hexadecyl-3-allyl-glycero-*sn*-2-phosphomethanol; TEWL, transepidermal water loss; PBS-CMF, phosphate-buffered saline, calcium- and magnesium-free.

Downloaded from www.jlr.org by guest, on June 18, 2012

trated in the outer epidermis (10, 17–19), where they have been localized to the intercellular domains of the stratum corneum (17, 19, 20). Thus, it appears that one or more phospholipases are delivered to the stratum corneum by lamellar body exocytosis. We have hypothesized further that activity of these enzymes is required for the lipid modifications that result in the sequential membrane structural modifications accompanying barrier formation (10). To address this hypothesis, we showed first that acute barrier disruption with topical acetone treatment increases the activity of β-glucocerebrosidase, the enzyme that hydrolyzes glucosylceramides to ceramides (21). In addition, either genetic deletion (22) or chemical inhibition (23) of epidermal β-glucocerebrosidase produces a barrier abnormality in intact skin in association with accumulation of glucosylceramides and structural evidence of impaired extracellular processing of secreted lamellar body contents into mature lamellar bilayer unit structures.

Phospholipase A_2 (EC3.1.1.4) is one of a group of phospholipid-hydrolyzing enzymes located in both cytosolic and extracellular sites that generate free fatty acids from phospholipids (24–28). As extracellular phospholipases appear to derive from the secretion of the contents of epidermal lamellar bodies (16, 17, 20), we hypothesized that inhibitors of extracellular phospholipase A_2 activity, such as either BPB (bromphenacyl bromide), MJ33 (1-hexadecyl-3-trifluoro-ethylglycero-*sn*-2-phosphomethanol), or MJ45 (1-hexadecyl-3-allyl-glycero-*sn*-2-phosphomethanol) should alter permeability barrier recovery after acetone treatment. Moreover, such inhibition should be attributable to either accumulated phospholipid and/or a failure to generate free fatty acids.

MATERIALS AND METHODS

Materials

Male hairless mice (Hr/Hr), 8–12 weeks old, were purchased from Simonsen Laboratories (Gilroy, CA), and fed Purina mouse diet and water ad libitum. Acetone and propylene glycol were from Fisher Scientific (Fairlane, NJ), and BPB, trypsin Type III, lysolecithin (*sn*-1), cholesterol, linoleic acid, and palmitic acid were from Sigma Chemical Co. (St. Louis, MO). MJ33 and MJ45 were synthesized as described previously (29). [^{14}C]acetate (60 mCi/mmol) was obtained from New England Nuclear (Boston, MA). High performance, silica-gel pre-coated, thin-layer chromatography plates were purchased from Brinkmann Instruments (Westbury, NY). Ruthenium tetroxide and osmium tetroxide were from Polysciences, Inc. (Warrington, PA).

Experimental protocols

Epidermal barrier function was disrupted by repeated gentle applications of absolute acetone, applied with cotton swabs, as described previously (30–32). This method removes stratum corneum intercellular lipids without producing morphological or biochemical evidence of toxicity in the underlying nucleated cell layers (9, 30, 32), and without altering the wet weight of the epidermis (30, 31, 33, 34). Barrier function was determined prior to and immediately after disruption by measurement of transepidermal water loss (TEWL), using an electrolytic water analyzer (MEECO, Warrington, PA). Either BPB (8 μg), MJ33, MJ45 (20 μg each), or either BPB or MJ33 with palmitic acid, linoleic acid, or lysolecithin (20 μg) was applied immediately after barrier perturbation in a propylene glycol–ethanol vehicle 7:3 (by vol) to a 5–6 cm^2 area (total volume applied: 8 or 20 μL). TEWL readings then were taken at different time points after drug (± lipid) or vehicle applications. The functional data are expressed as mean ± SEM of the percent change from initial reading.

Lipid synthesis

One hour after barrier disruption, skin samples were incubated at 37°C in 2 ml of 10 mM EDTA in Dulbecco's phosphate-buffered saline, calcium- and magnesium-free (PBS-CMF), containing 40 μCi [^{14}C]acetate for 2 h, as described previously (33–35). After stopping the reaction by placing samples on ice, the epidermis was separated manually as an intact sheet from the dermis. The incorporation of [^{14}C]acetate into both cholesterol and fatty acids was determined after saponification, extraction of lipids with petroleum ether, and thin-layer chromatography, as described in detail previously (33–35). Results are expressed as nanomoles of acetate incorporated into lipid species per hour per gram initial wet weight of epidermis.

Stratum corneum isolation and lipid incubations

Four hours after acetone treatment with or without BPB, MJ33, or vehicle alone, epidermal sheets were separated from the dermis by heat separation (60°C, 60 sec), as described previously (30–32, 34, 35). The epidermal sheets then were incubated for 2 h at 37°C in 0.5% trypsin solution in PBS-CMF. Trypsin from this source was shown to have very low levels of PLA_2-I activity (<2 ng/100 μg trypsin). After incubations, the samples were vortexed gently to remove residual nucleated cells and the remaining intact stratum corneum sheets were washed with distilled water four times. Sample weights were obtained after the stratum corneum sheets were blotted dry with absorbent tissue.

In some studies, BPB was applied to acetone-treated sites for 2 h, followed by heat separation and trypsiniza-

Downloaded from www.jlr.org by guest, on June 18, 2012

tion to remove the nucleated layers, as described above. The isolated stratum corneum sheets then were spread and 200 μg of either palmitic acid, cholesterol, or vehicle was applied to the outer surface (original area: 5–6 cm^2), and the sheets were incubated in PBS for 2 h at 37°C. All samples then were fixed and processed for electron microscopy, as described below.

Lipid extraction and chromatography

Total lipids were extracted from the stratum corneum, and then fractionated and quantitated by high-performance thin-layer chromatography (HPTLC) followed by scanning densitometry, as described previously (23). Briefly, 2 μg of lipid was applied to plates for neutral lipid analysis, while either 20 or 100 μg was utilized for polar lipid analysis. To generate standard curves, and to identify the major species, 0.2–1.0 μg of a polar lipid standard and 0.12–1.0 μg of a neutral lipid standard were applied to each side of the plates. Neutral lipids were fractionated by developing the plates in petroleum ether–diethyl ether–acetic acid 80:20:1 (by vol), as described previously. Polar lipids were developed to 35 and 55 mm in chloroform–ethyl acetate–ethyl methylketone–2-propanol–ethanol–methanol–glacial acetic acid–hexyl acetate 34:4:4:6:20:28:4:1 (by vol), and then to 70 mm in chloroform–ethyl acetate–2-propanol–ethanol–methanol–H_2O 46:4:4:6:6:28:6 (by vol), and then to the top of the plate in chloroform–methanol–acetone 80:10:10 (by vol). The plates were painted with a charring solution, charred, scanned densitometrically, and the lipid fractions then were quantitated by using CATS II software, as described in detail previously (23).

Electron microscopy

Two hours after applications of either vehicle or one of the phospholipase inhibitors (± lipid), samples were taken from treated areas, minced to 0.5 mm^3, fixed in modified Karnovsky's fixative overnight, washed in 0.1 M cacodylate buffer, and post-fixed in 0.2% ruthenium tetroxide (RuO_4) or 1% osmium tetroxide, containing 0.5% potassium ferrocyanide (36). Ultrathin sections were examined in a Zeiss 10A electron microscope.

Statistical significance was determined using a two-tailed Student's t test.

RESULTS

Effect of phospholipase A_2 inhibitors on barrier homeostasis

Although numerous agents have been shown to alter phospholipase A_2 activity, BPB, MJ33, and MJ45 appear to have certain desirable features, i.e., putative activity against specific isoforms (24, 25, 28, 37, 38). Therefore, we first determined whether a single topical application of either of the PLA_2-I inhibitors would interfere with barrier recovery in the acetone-treated, acute barrier recovery model. Normal rates of barrier recovery, similar to those previously reported for air exposure, occur 2 and 4 h after vehicle treatment (20% and 30–35%, respectively) (**Table 1**, line 1). In contrast, a single application of BPB, an irreversible alkylator of PLA_2-I, delays barrier recovery at both 2 and 4 h (by 50% and 66%, respectively) (Table 1, line 2). Likewise, MJ33, a reversible, competitive inhibitor of PLA_2-I, also significantly delays barrier recovery at 2 and 4 h (by 100% and 66%, respectively) (Table 1, line 6).

To determine whether the delay in barrier recovery can be ascribed to a specific class of secretory PLA_2, namely the type I form (24, 25), we next applied MJ45, a chemically related inhibitor to MJ33, that affects the Type II, rather than the Type I isoform of PLA_2 (28, 29). As seen in Table 1 (line 10), in contrast to MJ33, MJ45

TABLE 1. Transepidermal water loss in inhibitor-treated mice (mean ± SEM)

Treatment	2 Hours (% of time 0)	P Value[a]	4 Hours (% of time 0)	P Value[a]
1. Acetone (A) + vehicle (n = 22)	80.7 ± 3.4		68.6 ± 5.2	
2. A + BPB (n = 20)	90.6 ± 4.6	< 0.01	91.6 ± 5.6	< 0.01
3. A + PA (n = 10)	105.1 ± 7.2	< 0.02	67.3 ± 5.8	NS
4. A + BPB + PA (n = 10)	77.1 ± 6.1	NS	64.0 ± 6.9	NS
5. A + BPB + LA (n = 10)	109.3 ± 9.6	< 0.01	88.8 ± 8.0	< 0.05
6. A + MJ33 (n = 10)	101.9 ± 8.5	< 0.05	91.3 ± 6.6	< 0.02
7. A + MJ33 + PA (n = 11)	93.2 ± 7.3	NS	61.1 ± 5.8	NS
8. A + MJ33 + LA (n = 12)	106.4 ± 8.8	< 0.02	83.8 ± 5.2	< 0.05
9. A + MJ33 + LPC (n = 10)	134.6 ± 10.8	< 0.005	91.4 ± 10.9	< 0.01
10. A + MJ45 (n = 15)	85.6 ± 4.7	NS	55.4 ± 4.0	< 0.1

Groups of hairless mice (n) were treated with acetone until transepidermal water loss levels reached > 4.0 mg/cm^2 per h. The various inhibitors (± lipid) or the vehicle alone were added immediately in a propylene glycol–ethanol vehicle to a 5–6 cm^2 area, and barrier recovery was assessed at 2 and 4 h. BPB, bromophenacyl bromide; PA, palmitic acid; LA, linoleic acid; LPC, lysolecithin (see Methods for amounts, volume, and concentration applied). Data in line 3 are from ref. 39.

[a]Statistical significance versus vehicle.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

does not significantly delay barrier recovery at either 2 or 4 h. As two chemically unrelated PLA_2-I inhibitors produced similar functional alterations, these results show that the likely target for the reversible inhibitors is the type I PLA_2. Moreover, as MJ45, a chemically related inhibitor of another PLA_2 isoform, had no effect, this suggests that the MJ33-induced delay in barrier recovery is not due to nonspecific or toxic effects.

To determine further whether the effects of either BPB or MJ33 on barrier recovery can be attributed either to a failure to generate free fatty acids or to nonspecific toxicity, we next co-applied either palmitic or linoleic acids, or lysolecithin, distal products of phospholipid breakdown, with either BPB or MJ33. As described previously, applications of palmitate alone to acetone-treated skin aggravated barrier recovery at 2 h (Table 1, line 3), (39). In contrast, the delay in barrier recovery induced by BPB was normalized by co-applications of palmitic acid with BPB at both 2 and 4 h after treatment (line 4). Likewise, when palmitic acid was applied simultaneously with MJ33, barrier recovery rates became comparable to those for vehicle treatment by 4 h after treatment (line 7). In contrast to palmitic acid, coapplications of linoleic acid with either BPB or MJ33 did not normalize barrier recovery (Table 1, lines 5 and 8). Finally, co-applications of lysolecithin also did not override inhibitor blockade (line 9). These data show, first, the requirement for nonessential free fatty acids for barrier recovery. Second, despite the fact that both BPB and MJ33 can have unrelated effects on other cell surface-associated enzymes, these studies demonstrate again that the barrier abnormalities induced by both BPB and MJ33 cannot be attributed to such nonspecific effects.

Effects of phospholipase A_2 inhibitors on stratum corneum lipid content

Several prior studies have shown that both BPB and MJ33 inhibit phospholipase A_2 activity, and that they decrease the generation of free fatty acids from phospholipids in vivo (24, 25, 37). We next assessed whether the topical applications of these inhibitors would either inhibit the production of free fatty acids and/or induce phospholipid accumulation in the stratum corneum. By 4 h after acetone plus MJ33 treatment, the free fatty acid content of stratum corneum was significantly decreased (26%; $P < 0.05$), and the total phospholipid content of stratum corneum was increased in comparison to vehicle-treated stratum corneum (37%; $P < 0.01$) (**Table 2**). The increase in total phospholipids can be attributed primarily to elevated phosphatidylcholine content, but other phospholipids, including lysolecithin, increased to a lesser extent. In contrast, the free sterol content of MJ33-treated stratum corneum remained comparable to that observed in vehicle-treated stratum corneum.

In order to confirm the above results with a chemically unrelated phospholipase A_2 inhibitor, we next applied BPB to acetone-treated skin. As with MJ33, both total phospholipid and phosphatidylcholine content increased and free fatty acid content decreased in BPB-treated stratum corneum by 4 h after treatment in comparison to vehicle-treated stratum corneum (data not shown). These results in epidermis are consistent with prior studies in lung that have shown that MJ33-induced inhibition of phospholipase A_2 activity leads to a reduction in free fatty acid production (38). Together, these results show that topical application of either of two chemically unrelated phospholipase A_2 inhibitors results in an accumulation of phospholipids and a reduction in the free fatty acid content of stratum corneum.

Effect of phospholipase A_2 inhibitors on epidermal lipid synthesis

As the abnormal barrier function induced by phospholipase A_2 inhibitors can be normalized by palmitic acid, and these inhibitors reduce the free fatty acid content in stratum corneum, we next determined

TABLE 2. Changes in lipid content in MJ33-treated stratum corneum (μg/mg wet weight of stratum corneum)

	Vehicle (n = 9)	P	MJ33 (n = 8)	% Change[a]
Neutral lipids				
FS	5.99 ± 0.62	NS	5.41 ± 0.49	
FFA	15.98 ± 1.22	< 0.05	11.81 ± 0.80	-26
Polar lipids				
LysoPC	0.33 ± 0.07	< 0.01	0.56 ± 0.09	+66
PC	1.61 ± 0.15	< 0.05	2.11 ± 0.14	+31
PI	0.31 ± 0.07	NS	0.40 ± 0.06	
PS	0.48 ± 0.09	NS	0.69 ± 0.09	
Total polar lipids	2.74 ± 0.29	< 0.01	3.76 ± 0.19	+37

Four hours after a single application of the inhibitor versus vehicle, the stratum corneum was isolated by heat treatment and trypsinization (see Methods). Total lipids were extracted by the Bligh-Dyer method, and both polar and neutral lipids were fractionated and quantitated by TLC-densitometry (see Methods). Data represent mean ± SEM. FS, free sterols; FFA, free fatty acids; LysoPC, lysolecithin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine.

[a]% Change, change in content versus vehicle.

Downloaded from www.jlr.org by guest, on June 18, 2012

whether the inhibitors of phospholipase A_2 inhibit fatty acid synthesis. As reported previously (31), barrier disruption followed by either vehicle-treatment or air exposure is accompanied by a significant increase in both epidermal fatty acid and cholesterol synthesis in comparison to normal untreated epidermis (**Fig. 1**). Moreover, a comparable increase in both cholesterol and fatty acid synthesis occurs after barrier disruption in mice treated with BPB (Fig. 1). Thus, at the doses used here, the BPB-induced decrease in fatty acid content of the stratum corneum can be attributed to inhibition of phospholipid hydrolysis rather than to inhibition of fatty acid synthesis.

Ultrastructural alterations in phospholipase A_2 inhibitor-treated epidermis (Figs. 2A–E)

To determine the structural basis for the abnormal barrier function caused by topical phospholipase A_2 inhibitors, we next examined the ultrastructure of inhibitor-treated epidermis. As reported previously (5–7, 30–32), acetone treatment removes all stainable lipids and ultrastructurally demonstrable lamellar bilayers from the stratum corneum without inducing cytotoxic effects on the nucleated layers of the epidermis. In acetone- and vehicle-treated animals, increased numbers of nascent lamellar bodies are present in the cytosol of outer stratum granulosum cells (Fig. 2B) and at the stratum granulosum–stratum corneum interface (not shown; see ref. 9) 2 h after acetone treatment. Moreover, by 2 h after vehicle treatment, normal-appearing membrane bilayer structures reappear in the lower to mid-stratum corneum (Fig. 2A and ref. 9). These membranes display a typical basic lamellar unit structure, and they appear in the first and/or second interspace above the stratum granulosum–stratum corneum interface both in untreated samples and in vehicle-treated controls (Fig. 2A).

In both BPB- and MJ33-treated epidermis, lamellar body structure appears comparable to vehicle-treated epidermis (Fig. 2D). However, in contrast to vehicle-treated samples, unprocessed, secreted lamellar body-derived membranes persist within the interstices for several layers above the stratum granulosum–stratum corneum interface in inhibitor-treated stratum corneum (Fig. 2C). Moreover, the initially secreted lamellar body sheets in inhibitor-treated samples do not form elongated membrane structures, an abnormality also seen in β-glucocerebrosidase-deficient stratum corneum (Fig. 2C, insert; **Fig. 3 A and B;** c.f., 22, 23). As a result, neither elongated sheets nor basic lamellar unit structures appear until corneocytes reach the mid- to outer stratum corneum. In addition, the absence of lamellar unit structures in BPB-treated samples results in a loose, irregular arrangement of "immature", unprocessed membrane material throughout the stratum corneum interstices (Figs. 2C, 3 A and B). Finally, co-applications of palmitic acid, but not linoleic acid, with BPB, normalize the appearance of lamellar bilayer unit structures in the stratum corneum interstices (Fig. 2E; linoleic acid data not shown). These results show that inhibition of phospholipase A_2, with increased phospholipids and decreased free fatty acids, results in abnormal extracellular processing of secreted lamellar body contents within the stratum corneum interstices.

Ultrastructural changes induced by lipid applications to isolated stratum corneum

To ascertain whether the defect in membrane structure in inhibitor-treated skin could be reversed by a direct effect of free fatty acids within the stratum corneum, we next treated BPB-treated stratum corneum with free fatty acids in vitro. As seen in Fig. 3A and B, BPB plus vehicle-treated sheets display abnormal-appearing, foreshortened intercellular lamellar structures, and extensive cleft formation, identical to those seen after in vivo application (c.f., Fig. 2C). In contrast, palmitic acid but not linoleic acid treatment restores normal-appearing lamellar structures to extensive domains within the intercellular spaces (Fig. 3C and insert; linoleic acid data not shown). Such normal structures do not reappear in stratum corneum sheets treated with equivalent concentrations of cholesterol (Fig. 3D). These studies provide further evidence that a deficiency of nonessential fatty acids accounts for the abnormal membrane structures in inhibitor-treated animals. Moreover, they show that applications of exogenous

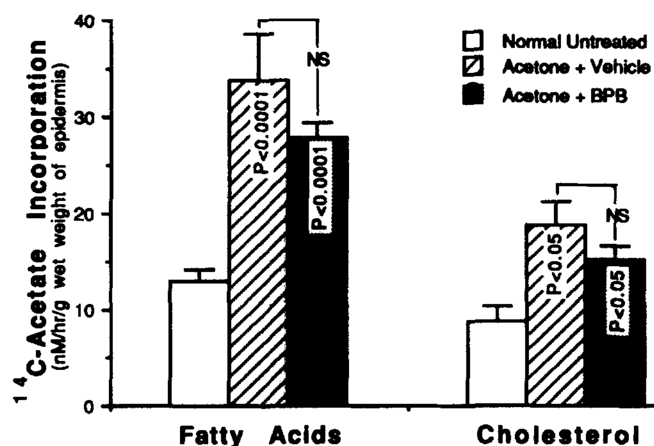
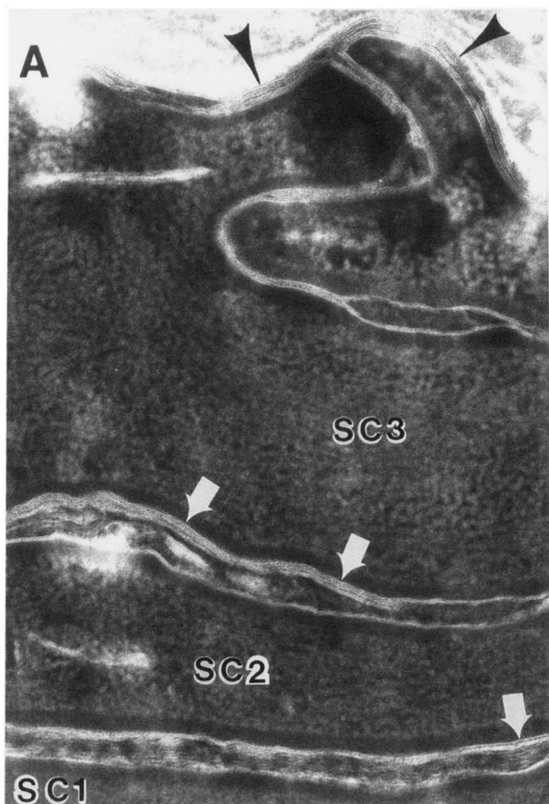
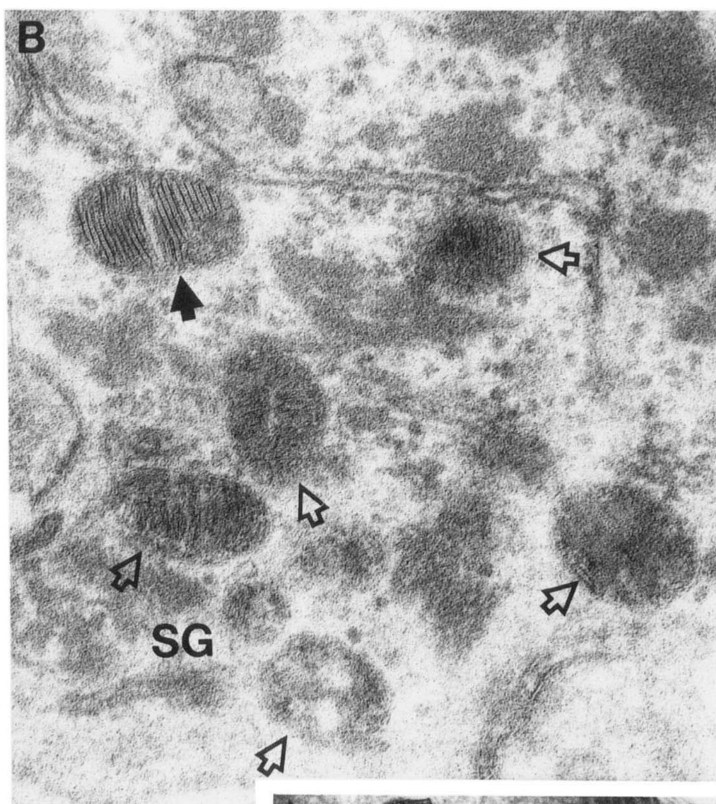


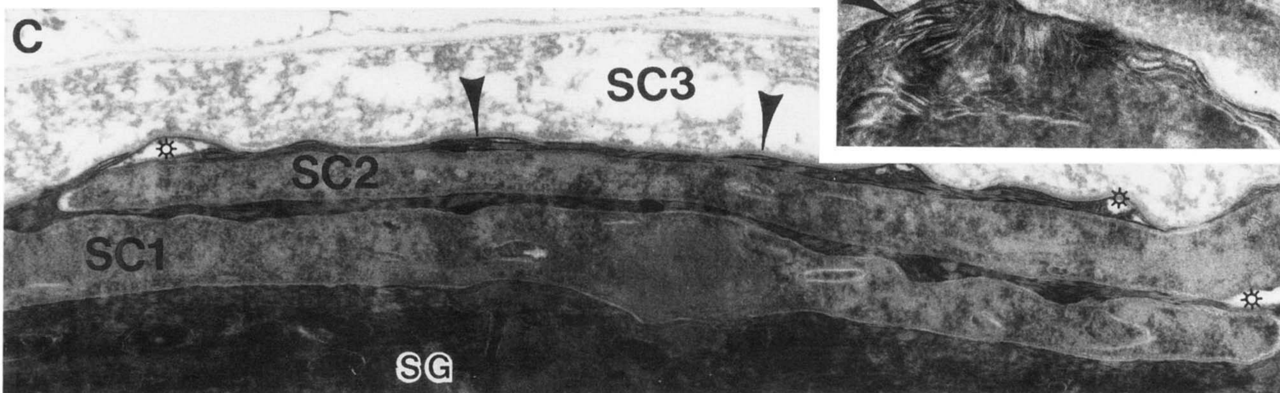
Fig. 1. Lipid synthesis in acetone plus inhibitor versus control epidermis. One hour after acetone ± inhibitor treatment, skin was isolated and incubated with [^{14}C]acetate. Two h later the epidermis was separated from the dermis, saponified, and the quantities of labeled fatty acids and cholesterol were determined as described in Methods. Results are expressed as nanomoles acetate incorporated/h per g wet weight. Data represent mean ± SEM; n = 5 from each group.

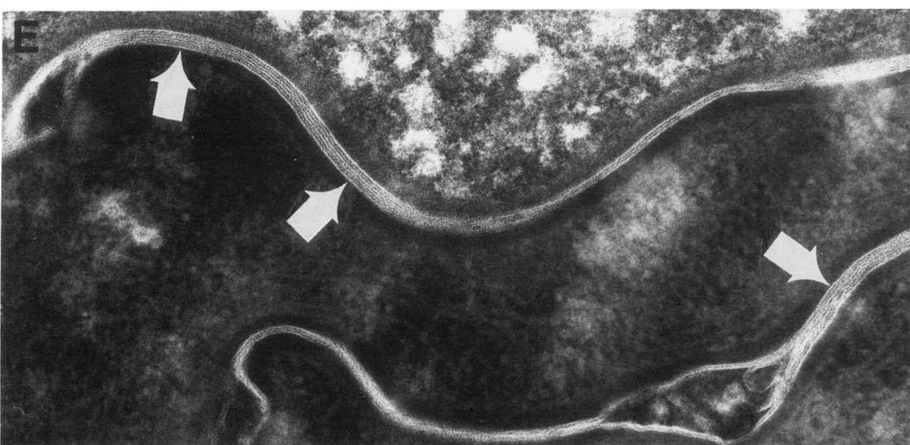
ASBMB JOURNAL OF LIPID RESEARCH JLR

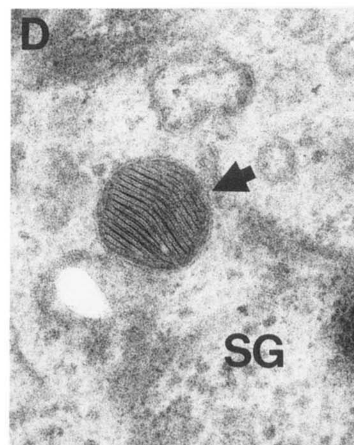
Downloaded from www.jlr.org by guest, on June 18, 2012











Downloaded from www.jlr.org by guest, on June 18, 2012

nonessential fatty acids lead to the formation of normal-appearing lamellar structures, without a prerequisite for intracellular processing through the lamellar body secretory system.

DISCUSSION

As its most important function, the epidermis generates a permeability barrier that prevents excessive transcutaneous water loss, allowing mammals to reside in a desiccating environment (2, 3, 10). A mixture of cholesterol, ceramides, and free fatty acids forms the mature membrane bilayers in the stratum corneum responsible for this permeability barrier function (1, 2). The epidermal lamellar body is believed to be the primary source of these lipids in the stratum corneum (10). But the majority of the lipids in both lamellar bodies and within the nucleated layers of epidermis consists of cholesterol, glycosphingolipids, and phospholipids; little ceramide and free fatty acids are present (11–14). Evidence from this and other laboratories suggests that the changes in lipid composition in the stratum corneum can be attributed to hydrolytic enzymes, co-localized with lipids within the lamellar body and the stratum corneum interstices (reviewed in ref. 10). To address the issue of post-secretion, extracellular processing within the stratum corneum, we initially studied β-glucocerebrosidase, which has been localized predominately to the outer epidermis and stratum corneum (19, 23). We used three approaches to demonstrate the extracellular processing of glucosylceramide precursors by co-localized β-glucocerebrosidase. First, we showed that inhibition of epidermal β-glucocerebrosidase by topical applications of a specific, irreversible inhibitor, bromoconduritol-B-epoxide, increased glucosylceramides in the stratum corneum, altered lamellar bilayer structure, and produced abnormal barrier function (23). Second, comparable changes in lipid content, structure, and function occurred in transgenic mice lacking the gene for β-glucocerebrosidase (22). Third, patients with neonatal Type II Gaucher's disease, who also display profound enzyme deficits, also demonstrate a similar abnormality in membrane structure (22).

Fig. 2. Ultrastructure of control versus inhibitor-treated epidermis. A: Two hours after acetone treatment + vehicle, normal lamellar unit structures (solid arrows) appear in the stratum corneum, between the first (SC1), second (SC2), and third (SC 3) cell layers. Above the third SC layer, the interstices appear filled with lamellar unit structures (arrowhead). B: Acetone treatment results in immediate secretion of lamellar body contents (not shown; 9), but by 2 h abundant nascent lamellar bodies (open arrows) appear again in the granular layer (SG) cytosol. C and insert: After acetone plus BPB treatment, the SC interstices contain lamellar body-derived membrane structures (arrowheads) that are only partially processed to mature lamellar unit structures. Moreover, these unprocessed structures persist several levels up into the SC, and are interspersed with non-lamellar domains (asterisks). D: Lamellar body structure is normal in BPB-treated epidermis (dark arrows). E: Two h after acetone + BPB + palmitate co-treatment, the maturation of secreted lamellar body contents into lamellar unit structures (white arrows) appear to be normal. A, × 72,000; B, × 112,500; C, × 65,000; C (insert), × 110,000; D, × 115,000; E, × 75,000.

Like β-glucocerebrosidase, phospholipase A_2 activity (type unspecified) has been localized to lamellar bodies (15, 16), the outer epidermis (17, 18), and more specifically to stratum corneum intercellular domains (17, 20). The intercellular pool of this enzyme(s) derives from lamellar body secretion, and both the formation and exocytosis of these organelles are accelerated by acetone treatment (9). Moreover, synthesis and delivery of lamellar body hydrolases appear to be augmented after acetone treatment, as shown specifically for β-glucocerebrosidase (21). Thus, it is likely that acetone treatment augments delivery of PLA_2 to the stratum corneum interstices. In this setting, we provide direct evidence that selective inhibition of type I phospholipase A_2 activity results in an abnormality in permeability barrier homeostasis, and that this functional alteration is associated with a reduction of free fatty acid content, with concurrent increases in the phospholipid content of treated stratum corneum. We showed first that topical applications of either of two chemically unrelated, type I phospholipase A_2 inhibitors delayed barrier recovery, while applications of a chemically related inhibitor of the type II isoform of secretory phospholipase A_2 had no effect. Moreover, the functional abnormalities produced by both BPB and MJ33 could be overridden with co-applications of palmitic acid, but not linoleic acid. Furthermore, this delay was associated with specific alterations in stratum corneum lipid content, i.e., increased phospholipids and decreased free fatty acids. Interestingly, the lysolecithin content of inhibitor-treated samples increased in parallel with other phospholipids. This finding could be explained if the inhibitors affected not only PLA_2-I, but also to a lesser extent, lysolecithinase. Finally, lamellar body-derived phospholipids could be degraded by an alternate pathway, i.e., PLA_1, followed by PLB, which, depending on rates, could explain monoacylglycerophospholipid accumulation in inhibitor-treated stratum corneum.

The biochemical alterations produced by the inhibitors are associated further with changes in stratum corneum lamellar bilayer structure, specifically, an apparent blockade in the end-to-end fusion of secreted lamellar body-derived membranes. Pertinently, these structural alterations could be overridden by co-applications of palmitic acid, but not linoleic acid, with either of the inhibitors, and reversed by incubations of iso-

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

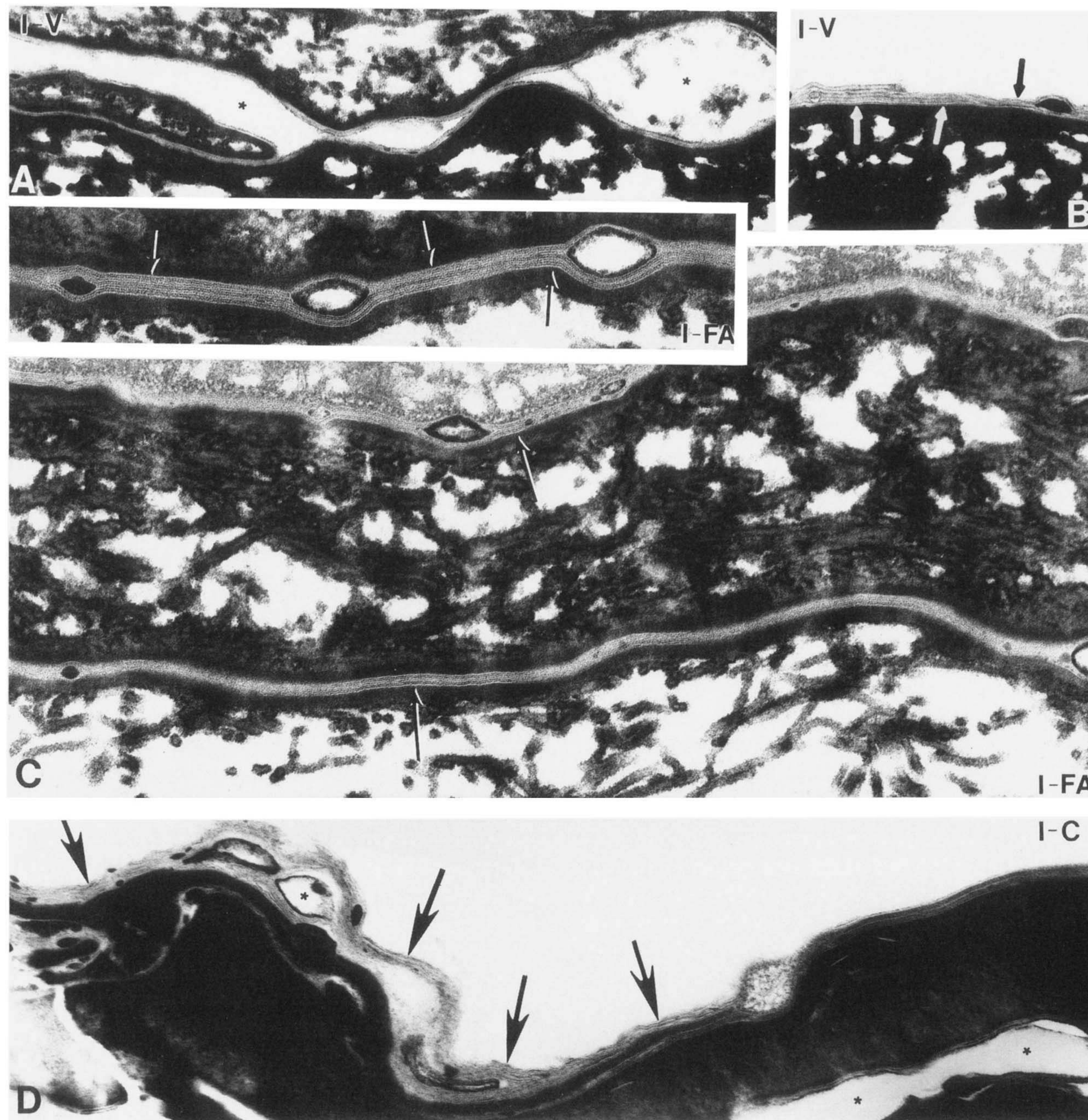


Fig. 3. Membrane structural alterations induced by incubation of stratum corneum (SC) with lipids. A + B: SC sheets isolated from acetone plus inhibitor (I, BPB)-treated skin, then incubated with vehicle (V) for 2 h. Note paucity of lamellar bilayers with formation of intercellular lacunae (A, asterisks). Those bilayers that are present are abnormal in substructure (B; c.f., C and insert, below), and greatly foreshortened (B, arrows). C and insert: SC sheets isolated from acetone plus inhibitor-treated skin, then incubated with a free fatty acid (FA, palmitic acid). Note reappearance of extensive domains with multilayered lamellar bilayers (C, arrows). D: SC sheets isolated from acetone plus inhibitor-treated skin, then incubated with cholesterol (C) for 2 h. In contrast to palmitate-treated samples, cholesterol-treated samples display abnormal, foreshortened lamellar bilayers (arrows) that are interspersed between or around numerous lacunar clefts (asterisks). A, × 70,000; B, × 95,000; C, × 75,000; C, insert, × 130,000; D, × 70,000.

lated, inhibitor-treated stratum corneum with palmitic acid, but again not with linoleic acid. The morphologic data are consistent with a site of maturational blockade proximal to the primary site affected in β-glucocerebrosidase-deficient epidermis (**Fig. 4;** 22, 23). An alternate explanation would be a direct effect of the inhibitors on stratum corneum intercellular membrane structures, which is less likely in light of the lipid override studies. Together, these results provide a biochemical and structural basis for the alterations in permeability

Downloaded from www.jlr.org by guest, on June 18, 2012

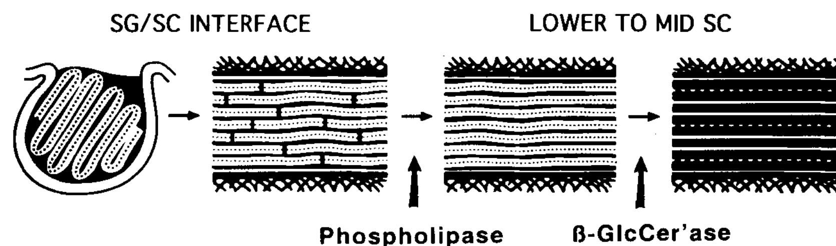


Fig. 4. Diagram of putative sites of extracellular processing of lamellar body-derived lipids. The studies described in this paper suggest that the phospholipase A family of enzymes may act at a more proximal site than β-glucocerebrosidase during the extracellular processing of lamellar body-derived lipid precursors.

barrier homeostasis in this model, which can be attributed to defective extracellular processing of one of the three key lipids required for barrier function, i.e., phospholipids to free fatty acids.

Our studies also address whether the biochemical basis for the barrier abnormality is attributable primarily to phospholipid accumulation or to free fatty acid depletion. Previous studies from this laboratory have demonstrated a requirement for free fatty acids for permeability barrier function (7). First, abrogations in barrier function result both in a burst in epidermal fatty acid synthesis (31), as well as increased activity of the rate-limiting enzymes for fatty acid synthesis, acetyl-CoA carboxylase and fatty acid synthase (40). Second, we demonstrated that topical applications of an incomplete lipid mixture of cholesterol and ceramides, which lack either essential or nonessential fatty acids, delay barrier recovery (39). Third, inhibition of epidermal fatty acid synthesis by applications of the acetyl-CoA carboxylase inhibitor, S-tetradecyloxyl-2-furancarboxylic acid (TOFA), induces abnormal lamellar body and membrane bilayer structure, as well as abnormal barrier function, which in turn could be normalized by topical coapplications of nonessential free fatty acids (7). In the studies described here, we have provided evidence that the abnormalities in both stratum corneum intercellular bilayer structure/maturation and barrier function, induced by either MJ33 or BPB, are due to a reduction of phospholipid-derived, nonessential free fatty acids, rather than to an accumulation of phospholipids because co-applications of palmitic acid, but not linoleic acid: *a)* override the inhibitory effects of the phospholipase inhibitors; and *b)* normalize stratum corneum membrane structure both in vivo and in isolated stratum corneum. Additionally, these findings show that nonessential free fatty acids are not only processed through the granular cell into nascent lamellar bodies, as shown previously (39), but also that they can correct the membrane structural abnormality directly within the stratum corneum interstices, i.e., prior processing through the lamellar body secretory system is not required. This result is consistent with X-ray diffraction data demonstrating the capacity of nonessential free fatty acids to form intercellular membrane structures in delipidized stratum corneum (41). This model, then, differs from the analogous prior studies using inhibitors of β-glucocerebrosidase, where the defect was not reversed by co-application of ceramides, suggesting that in that model, accumulation of glucosylceramides rather than a deficiency of ceramides, accounts for the barrier defect.

The ability of palmitic acid, but not linoleic acid, to override both BPB and MJ33 inhibition of PLA_2 suggests that a substantial portion of the phospholipids in epidermal lamellar bodies may possess saturated acyl groups not only in the *sn*-1, but also in the *sn*-2 position. Indeed, phospholipids in the outer epidermis become progressively enriched in saturated fatty acids (1). However, the specific fatty acid composition of phospholipids residing within lamellar bodies is not known. Nevertheless, it seems plausible to speculate that the epidermal lamellar body, like the lamellar body of the lung type 2 cell, may also be enriched in nonessential fatty acids. Certainly, the free fatty acids resulting from phospholipid hydrolysis, which accumulate in the stratum corneum interstices, are enriched in very long-chain, saturated species, with reduced levels of linoleic acid (1, 11, 13). Linoleic acid, instead, is found almost exclusively ϖ-esterified to the N-acyl group of one type of ceramide (1, 11, 42). Whether lamellar body-derived phospholipids provide both nonessential and essential fatty acids for membrane assembly, or whether the essential fatty acid, linoleic acid, derives from a separate pool (? plasma membrane), remains to be determined. Both essential and nonessential fatty acids also could result from the hydrolysis of other esterified lipids, e.g., triglycerides or cholesteryl esters. However, neither of these lipids is present in lamellar bodies (15, 16). Moreover, though sebaceous lipids could deliver these two lipids to the skin surface, barrier function is not impacted by differences in sebaceous gland function (1). Thus, despite the theoretical possibility that some of the free fatty acids in the stratum corneum could arise from the hydrolysis of other esterified lipids, phospholipids provide the principal reservoir for a critical component of the free fatty acid pool.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH

Phospholipase A_2 hydrolyzes fatty acids esterified at the *sn*-2 position of glycerophospholipids. Much attention has focused on PLA_2 because of its central role in controlling the generation of arachidonic acid leading to eicosanoid generation and modulation of signal transduction (24-27). Two major types of PLA_2 (secreted and cytosolic) have been described in mammalian tissues (24-27), and the secreted PLA_2 have been grouped into at least three separate isoforms. The secreted and cytosolic PLA_2 vary according to molecular weight, preferential substrate, Ca^+ requirements, susceptibility to various inhibitors, and putative biological function (24-27). Whereas the type I secreted PLA_2 (PLA_2-I; pancreatic PLA_2) classically is associated with exocrine pancreatic secretions, it also appears to be generated by a variety of non-pancreatic cell types (24, 27). Because PLA_2-I is inhibited by both BPB and MJ33 (op. cit.), it is tempting to assume that PLA_2-I is the principal epidermal isoform responsible for the phenomena described in these studies. In contrast, the type II isoform of secreted PLA_2 also is a known target of BPB, but not of MJ33, and additionally it is inhibited by MJ45 (29, 43). Finally, while the type III isoform is sensitive to both BPB and MJ33, it is not known to be present in mammalian tissues. Although the sensitivity of the epidermal enzyme to MJ33 suggests a similarity to a newly described, acidic phospholipase A_2 in rat lung (38, 44-46), this isoform is not inhibited by BPB. Whether the interstices of the lower stratum corneum, where the relevant membrane transformations occur, is acidified remains to be determined. If the epidermal extracellular target enzyme exhibits an acidic pH optimum, as does the lung isoform (38), the epidermis could contain a unique form of secreted PLA_2. Although some types of phospholipase A_2 activity present in epidermis have been partially characterized and localized (18, 47-49), the inhibitor profile of these and other epidermal phospholipases has not been evaluated.

In summary, inhibition of epidermal phospholipase A_2 activity decreases free fatty acid content and increases phospholipid content in stratum corneum, resulting in abnormalities in both membrane bilayer structure and barrier function, changes that could be normalized by coapplications of free fatty acids. These results indicate that the extracellular processing of phospholipids into free fatty acids is required for barrier homeostasis.

This work was supported by National Institutes of Health grants AR 19098, AR 39639, AR 39448, SM 29703, and the Medical Research Service, Veterans Affairs Medical Service. Ms. Debbie Crumrine provided invaluable technical assistance, and Ms. Sally Michael, Angie Kho-Kim, and Michie Wong provided excellent secretarial assistance.

Manuscript received 24 January 1995 and in revised form 15 May 1995.

REFERENCES

1. Schurer, N. Y., and P. M. Elias. 1991. The biochemistry and function of stratum corneum lipids. *Adv. Lipid Res.* **24:** 27-56.
2. Elias, P. M., and K. R. Feingold. 1992. Lipids and the epidermal water barrier: metabolism, regulation, and pathophysiology. *Semin. Dermatol.* **11:** 176-182.
3. Feingold, K. R. 1991. The regulation and role of epidermal lipid synthesis. *Adv. Lipid Res.* **24:** 57-79.
4. Elias, P. M., W. M. Holleran, G. K. Menon, R. Ghadially, M. L. Williams, and K. R. Feingold. 1993. Normal mechanisms and pathophysiology of epidermal permeability barrier homeostasis. *Curr. Opin. Dermatol.* **1:** 231-237.
5. Feingold, K. R., M. Mao-Qiang, G. K. Menon, S. S. Cho, B. E. Brown, and P. M. Elias. 1990. Cholesterol synthesis is required for cutaneous barrier function in mice. *J. Clin. Invest.* **86:** 1738-1745.
6. Holleran, W. M., M. Mao-Qiang, W. N. Gao, G. K. Menon, P. M. Elias, and K. R. Feingold. 1991. Sphingolipids are required for mammalian barrier function: inhibition of sphingolipid synthesis delays barrier recovery after acute perturbation. *J. Clin. Invest.* **88:** 1338-1345.
7. Mao-Qiang, M., P. M. Elias, and K. R. Feingold. 1993. Fatty acids are required for epidermal permeability barrier homeostasis. *J. Clin. Invest.* **92:** 791-798.
8. Proksch, E., K. R. Feingold, M. Mao-Qiang, and P. M. Elias. 1991. Barrier function regulates epidermal DNA-synthesis. *J. Clin. Invest.* **87:** 1668-1673.
9. Menon, G. K., K. R. Feingold, and P. M. Elias. 1992. The lamellar body secretory response to barrier disruption. *J. Invest. Dermatol.* **98:** 279-289.
10. Elias, P. M., and G. K. Menon. 1991. Structural and lipid biochemical correlates of the epidermal permeability barrier. *Adv. Lipid. Res.* **24:** 1-26.
11. Elias, P. M., B. E. Brown, P. O. Fritsch, R. J. Goerke, G. M. Gray, and R. J. White. 1979. Localization and composition of lipids in neonatal mouse stratum granulosum and stratum corneum. *J. Invest. Dermatol.* **73:** 339-348.
12. Gray, G. M., and H. J. Yardley. 1975. Different populations of pig epidermal cells: isolation and lipid composition. *J. Lipid Res.* **16:** 441-447.
13. Lampe, M. A., M. L. Williams, and P. M. Elias. 1983. Human epidermal lipids: characterization and modulations during differentiation. *J. Lipid Res.* **24:** 131-140.
14. Cox, P., and C. A. Squier. 1986. Variations in lipids in different layers of porcine epidermis. *J. Invest. Dermatol.* **87:** 741-744.
15. Freinkel, R. K., and N. Traczyk. 1985. Lipid composition and acid hydrolase content of lamellar granules of fetal rat epidermis. *J. Invest. Dermatol.* **85:** 295-298.
16. Grayson, S., A. G. Johnson-Winegar, B. U. Wintroub, E. H. Epstein, Jr., and P. M. Elias. 1985. Lamellar body-enriched fractions from neonatal mice: preparative techniques and partial characterization. *J. Invest. Dermatol.* **85:** 289-295.
17. Elias, P. M., G. K. Menon, S. Grayson, and B. E. Brown. 1988. Membrane structural alterations in murine stratum corneum. Relationship to the localization of polar lipids and phospholipases. *J. Invest. Dermatol.* **91:** 3-10.
18. Bergers, M., D. R. Verhagen, M. Jongerius, P. C. van der Kerkhof, and P. D. Mier. 1988. A unique phospholipase A_2 in human epidermis: its physiologic function and its

Downloaded from www.jlr.org by guest, on June 18, 2012

level in certain dermatoses. *J. Invest. Dermatol.* **90:** 23–25.
19. Holleran, W. M., Y. Takagi, G. Imokawa, S. Jackson, J. M. Lee, and P. M. Elias. 1992. β-Glucocerebrosidase activity in murine epidermis: characterization and localization in relation to differentiation. *J. Lipid Res.* **33:** 1201–1209.
20. Menon, G. K., S. Grayson, and P. M. Elias. 1986. Cytochemical and biochemical localization of lipase and sphingomyelinase activity in mammalian epidermis. *J. Invest. Dermatol.* **86:** 591–597.
21. Holleran, W. M., Y. Takagi, G. K. Menon, S. M. Jackson, J. M. Lee, K. R. Feingold, and P. M. Elias. 1994. Permeability barrier requirements regulate epidermal β-glucocerebrosidase. *J. Lipid Res.* **35:** 905–912.
22. Holleran, W. M., E. Sidransky, G. K. Menon, M. Fartasch, J. U. Grundmann, E. I. Ginns, and P. M. Elias. 1994. Consequences of β-glucocerebrosidase deficiency in epidermis: ultrastructure and permeability barrier alterations in Gauchers disease. *J. Clin. Invest.* **93:** 1756–1764.
23. Holleran, W. M., Y. Takagi, K. R. Feingold, G. K. Menon, G. Legler, and P. M. Elias. 1993. Processing of epidermal glucosylceramides is required for optimal mammalian permeability barrier function. *J. Clin. Invest.* **91:** 1656–1664.
24. Kudo, I., M. Murakami, S. Hara, and K. Inoue. 1993. Mammalian non-pancreatic phospholipase A_2. *Biochem. Biophys. Acta.* **117:** 217–231.
25. Glaser, K. B., D. Mobilio, J. Y. Chang, and N. Senko. 1993. Phospholipase A_2 enzymes: regulation and inhibition. *TIPS Rev.* **14:** 92–98.
26. Mayer, R. J., and L. A. Marshall. 1993. New insights on mammalian phospholipase A_2(s); comparison of arachidonoyl-selective and non-selective enzymes. *FASEB J.* **7:** 339–348.
27. Dennis, E. A. 1994. Diversity of group types, regulation, and function of phospholipase A_2. *J. Biol. Chem.* **269:** 13057–13060.
28. Gelb, M. H., M. K. Jain, and O. G. Berg. 1994. Inhibition of phospholipase A_2. *FASEB J.* **8:** 916–924.
29. Jain, M. K., W. Tao, J. Rogers, C. Arenson, H. Eibl, and B-Z. Yu. 1991. Active-site-directed specific competitive inhibitors of phospholipase A_2: novel transition site analogues. *Biochemistry.* **30:** 10256–102568.
30. Menon, G. K., K. R. Feingold, A. H. Moser, B. E. Brown, and P. M. Elias. 1985. De novo sterologenesis in the skin. II. Regulation by cutaneous barrier requirements. *J. Lipid Res.* **26:** 418–427.
31. Grubauer, G., K. R. Feingold, and P. M. Elias. 1987. Relationship of epidermal lipogenesis to cutaneous barrier function. *J. Lipid Res.* **28:** 746–752.
32. Grubauer, G., P. M. Elias, and K. R. Feingold. 1989. Transepidermal water loss: the signal for recovery of barrier structure and function. *J. Lipid Res.* **30:** 323–333.
33. Monger, D. J., M. L. Williams, K. R. Feingold, B. E. Brown, and P. M. Elias. 1988. Localization of sites of lipid biosynthesis in mammalian epidermis. *J. Lipid Res.* **29:** 603–612.
34. Proksch, E., P. M. Elias, and K. R. Feingold. 1991. Localization and regulation of epidermal HMG-CoA reductase activity by barrier requirements. *Biochim. Biophys. Acta.* **1083:** 71–79.
35. Holleran, W. M., W. N. Gao, K. R. Feingold, and P. M. Elias. 1995. Localization of epidermal sphingolipid synthesis and serine palmitoyl transferase activity. *Arch. Dermatol. Res.* **287:** 254–258.
36. Hou, S. Y. E., A. K. Mitra, S. H. White, G. K. Menon, R. Ghadially, and P. M. Elias. 1991. 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.
37. Al-Mehdi, A. B., C. Dodia, M. K. Jain, and A. B. Fisher. 1993. A phospholipase A_2 inhibitor decreases generation of thiobarbituric acid reactive substance during lung ischemia-reperfusion. *Biochim. Biophys. Acta.* **1167:** 56–62.
38. Fisher, A. B., C. Dodia, A. Chander, and M. Jain. 1992. A competitive inhibitor of phospholipase A_2 decreases surfactant phosphatidylcholine degradation by the rat lung. *Biochem. J.* **288:** 407–411.
39. Mao-Qiang, M., K. R. Feingold, and P. M. Elias. 1993. Exogenous lipids influence permeability barrier recovery in acetone-treated murine skin. *Arch. Dermatol.* **129:** 728–738.
40. Ottey, K. A., L. C. Wood, C. Grunfelt, P. M. Elias, and K. R. Feingold. 1995. Cutaneous permeability barrier disruption increases fatty acid synthetic enzyme activity in the epidermis of hairless mice. *J. Invest. Dermatol.* **104:** 391–395.
41. Friberg, S. E., I. Kayali, W. Beckerman, L. D. Rhein, and A. Simion. 1990. Water permeation of reaggregated stratum corneum with model lipids. *J. Invest. Dermatol.* **94:** 377–380.
42. Bowser, P. A., D. H. Nugteren, R. J. White, U. M. T. Houtsmuller, and C. Prottey. 1985. Identification, isolation, and characterization of epidermal lipids containing linoleic acid. *Biochim. Biophys. Acta.* **834:** 419–428.
43. Bayburt, T., B-Z. Yu, H-K. Lin, J. Browning, M. K. Jain, and M. H. Gelb. 1993. Human nonpancreatic secreted phospholipase A_2: interfacial parameters, substrate specificities, and competitive inhibitors. *Biochemistry.* **32:** 573–582.
44. Heath, M. F., and W. Jacobson. 1976. Phospholipase A_1 and A_2 in lamellar inclusion bodies of the alveolar epithelium of rabbit lung. *Biochim. Biophys. Acta.* **441:** 443–452.
45. Hook, G. E. R., and L. B. Gilmore. 1982. Hydrolases of pulmonary lysosomes and lamellar bodies. *J. Biol. Chem.* **257:** 9211–9220.
46. Wang, R., C. R. Dodia, M. K. Jain, and A. B. Fisher. 1994. Purification and characterization of a calcium-independent acid phospholipase A_2 from rat lung. *Biochem. J.* **304:** 131–137.
47. Long, V. J. W., and H. J. Yardley. 1972. Phospholipase activity in the epidermis. *J. Invest. Dermatol.* **58:** 148–153.
48. Ziboh, V. A., and J. T. Lord. 1979. Phospholipase activity in the skin. *Biochem. J.* **184:** 283–290.
49. Freinkel, R. K., and T. N. Traczyk. 1980. The phospholipases of epidermis. *J. Invest. Dermatol.* **74:** 169–173.

Downloaded from www.jlr.org by guest, on June 18, 2012