View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Generation of Free Fatty Acids from Phospholipids Regulates Stratum Corneum Acidification and Integrity

Joachim W. Fluhr, Jack Kao, Mahendra Jain,* Sung K. Ahn, Kenneth R. Feingold, and Peter M. Elias

Dermatology Service, Veterans Affairs Medical Center, San Francisco, Department of Dermatology, University of California, San Francisco, and *Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware, U.S.A.

There is evidence that the ''acid mantle'' of the stratum corneum is important for both permeability barrier formation and cutaneous antimicrobial defense. The origin of the acidic pH of the stratum corneum remains conjectural, however. Both passive (e.g., eccrine/sebaceous secretions, proteolytic) and active (e.g., proton pumps) mechanisms have been proposed. We assessed here whether the free fatty acid pool, which is derived from phospholipase-mediated hydrolysis of phospholipids during cornification, contributes to stratum corneum acidification and function. Topical applications of two chemically unrelated secretory phospholipase $sPLA_2$ inhibitors, bromphenacylbromide and 1-hexadecyl-3-trifluoroethylglycero-sn-2-phosphomethanol, for 3 d produced an increase in the pH of murine skin surface that was paralleled not only by a permeability barrier abnormality but also altered stratum corneum integrity (number of strippings required to break the barrier) and decreased stratum corneum cohesion (protein weight removed per stripping). Not only stratum corneum pH but also all of the functional abnormalities normalized when either palmitic, stearic, or linoleic acids were coapplied with the inhibitors. Moreover, exposure of intact murine stratum corneum to a neutral pH for as little as 3 h produced comparable abnormalities in stratum corneum integrity and cohesion, and further amplified the inhibitor-induced functional alterations. Furthermore, short-term applications of an acidic pH buffer to inhibitor-treated skin also reversed the abnormalities in stratum corneum integrity and cohesion, despite the ongoing decrease in free fatty acid levels. Finally, the secretory-phospholipase-inhibitor-induced alterations in integrity/cohesion were in accordance with premature dissolution of desmosomes, demonstrated both by electron microscopy and by reduced desmoglein 1 levels in the stratum corneum (shown by immunofluorescence staining and vizualized by confocal microscopy). Together, these results demonstrate: (i) the importance of phospholipid-to-free-fatty-acid processing for normal stratum corneum acidification; and (ii) the potentially important role of this pathway not only for barrier homeostasis but also for the dual functions of stratum corneum integrity and cohesion. ***Key words: acidification/epidermis/fatty acids/free fatty acids/pH/phospholipases/phospholipids/secretory phospholipases/stratum corneum. J Invest Dermatol 117:44–51, 2001***

Although first recognized several decades ago (Schade and Marchionini, 1928; Blank, 1939; Draize, 1942; Beare *et al*, 1958; Baden and Pathak, 1967; Dikstein and Zlotogorski, 1994; Ohman and Vahlquist, 1994, 1998), the origin and function of the ''acid mantle'' of the stratum corneum (SC) remains conjectural. One function of SC acidification appears to be antimicrobial, because an acidic pH inhibits colonization by pathogenic bacteria such as *Staphylococcus aureus* (Aly *et al*, 1975; Puhvel *et al*, 1975). Conversely, alkalinization of the SC, as occurs in the urea-soaked skin of diaper dermatitis, is an important antecedent of bacterial and yeast infections (Leyden and Kligman, 1978; Brook, 1992). A second, key function of an acidic SC relates to the activation of lipid hydrolases, which process secreted polar lipids, a sequence required for normal barrier function (Elias and Menon, 1991). Barrier recovery after acute injuries is impeded at a neutral *versus* acidic pH (Mauro *et al*, 1998), apparently because one or more secreted hydrolases, e.g., β-glucocerebrosidase (Holleran *et al*, 1992; Takagi *et al*, 1999), display an acidic pH optimum. β-Glucocerebrosidase, with acid sphingomyelinase, represent two such critical processing enzymes, with acidic pH optimum, that generate a family of ceramides required for epidermal permeability barrier homeostasis (Holleran *et al*, 1993; Jensen *et al*, 1999; Schmuth *et al*, 2000).

Both active (energy-requiring) and passive (nonenergy-requiring) mechanisms have been proposed for the origin of the acid mantle of the SC. Passive exogenous mechanisms include: (i) free fatty acids (FFA), generated by lipases of bacterial and/or pilosebaceous origin (Puhvel *et al*, 1975); or (ii) eccrine-gland-derived products such as lactic acid (Ament *et al*, 1997). Passive processes, mediated by hydrolytic enzymes, include bulk proteo-

Manuscript received January 26, 2001; revised March 8, 2001; accepted for publication March 15, 2001.

Reprint requests to: Dr. Peter M. Elias, Dermatology Service (190), Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121. Email: eliaspm@itsa.ucsf.edu

Abbreviations: BPB, bromphenacylbromide; DSG-1, desmoglein 1; FFA, free fatty acids; MJ33, 1-hexadecyl-3-trifluoroethylglycero-sn-2-phosphomethanol; PA, palmitic acid; PL, phospholipids; SA, stearic acid; $sPLA_2$, secretory phospholipase; SC, stratum corneum; SG, stratum granulosum; TEWL, transepidermal water loss.

0022-202X/01/$15.00 · Copyright © 2001 by The Society for Investigative Dermatology, Inc.

lysis that accompanies catabolic processes late in terminal differentiation (Freinkel and Traczyk, 1983). Another passive mechanism, i.e., generation of *cis*-urocanic acid from histidine, was proposed recently to be the major, if not the sole, source of the acidification of the SC aqueous compartment (Krien and Kermici, 2000). This mechanism, however, may not be relevant either for hydrolytic processes that occur deep in the SC, or for those within the hydrophobic, extracellular compartments of the SC. It is in these domains that the processing events leading to the dual SC functions of barrier formation and desquamation largely occur (Elias and Menon, 1991). Moreover, to what extent this mechanism is operative under conditions of high humidity, when less histidine would be available as a precursor for *cis*-urocanic acid generation (Scott *et al*, 1982; Scott and Harding, 1986), is unknown.

A third, passive, enzymatic mechanism could come from the generation of proton-donating FFA, resulting from the bulk hydrolysis of epidermal phospholipids (PL) or other complex lipids late in terminal differentiation. PL disappear during cornification (Elias *et al*, 1988), and a family of nonessential FFA, required for normal barrier homeostasis, results from the bulk hydrolysis (processing) of PL within the SC interstices (Mao-Qiang *et al*, 1995). Although the responsible phospholipase(s) has (have) not been identified (Long and Yardley, 1972; Freinkel and Traczyk, 1980; Forster *et al*, 1983; 1985; Bergers *et al*, 1988; Andersen *et al*, 1994; Li-Stiles *et al*, 1998; Mazereeuw-Hautier *et al*, 2000), applications of inhibitors of the 14 kDa family of secretory phospholipases ($sPLA_2$) block the processing of PL, while inducing abnormalities in permeability barrier homeostasis (Mao-Qiang *et al*, 1995; 1996). We assessed here whether applications of these $sPLA_2$ inhibitors also induce abnormalities in SC acidity, as well as the potential functional consequences of such alterations. Our studies demonstrate that PL-to-FFA processing is an important source of SC acidification. Furthermore, we show that inhibition of FFA generation from PL results not only in altered barrier function, but also in abnormalities in SC integrity and cohesion.

MATERIALS AND METHODS

Materials Male hairless mice (Skh1/Hr), 8–12 wk old, were purchased form Charles River Laboratories (Wilmington, MA) and fed Purina mouse diet and water *ad libitum*. Propylene glycol, ethanol, NaOH, and HCl were from Fisher Scientific (Fairlane, NJ), whereas palmitic acid (PA), stearic acid, linoleic acid, HEPES buffer, and bromophenacyl bromide (BPB) were from Sigma Chemical (St. Louis, MO). 1-hexadecyl-3-trifluoroethylglycero-sn-2-phosphomethanol (MJ33) was synthesized as described previously (Jain *et al*, 1991). The stripping for the protein assay was performed with 22 mm D-Squame 100 tapes purchased from CuDerm (Dallas, TX). Bradford protein assay kits (Bio-Rad Protein Assay Dye), as well as lyophilized, bovine plasma gamma globulin were purchased from Bio-Rad (Hercules, CA).

Experimental procedures, pH determinations, and transepidermal water loss (TEWL) measurement Normal hairless mice were treated topically twice daily for 3 d with BPB (4 mg per ml) or MJ33 (4 mg per ml), both in propylene glycol:ethanol (7:3 vol/vol) vehicle, or the vehicle alone on an area of 5–6 cm^2 on the backs and flanks, as described previously (Mao-Qiang *et al*, 1996). The doses that we employed were shown previously to be nontoxic to murine skin (Mao-Qiang *et al*, 1995; 1996), and to inhibit secretory PLA_2 activity selectively in different tissues and cell types (Jain *et al*, 1991; Gelb *et al*, 1994). For the override experiments, animals received coapplications of either palmitic acid, stearic acid, or linoleic acid (10 mg per ml), at the same time that these sites were treated with the inhibitor in one solution. The acidification experiments were performed with 10 mM HEPES buffer, adjusted to either pH 5.5 or pH 7.4, as follows. One flank of the anesthetized mice was immersed on a mesh netting, as described previously (Lee *et al*, 1992). The mice were anesthetized with chloral hydrate (Morton Grove Pharmaceuticals, Morton Grove, IL).

After 3 h of immersion at 37°C, the mice were removed, and the remaining buffer was gently blotted off. After 15 min, barrier function was determined by measurement of TEWL with an electrolytic water analyzer (MEECO®; Warrington, PA). Surface pH was measured with a flat, glass surface electrode from Mettler-Toledo (Giessen, Germany), attached to a pH meter (Skin pH Meter PH 900; Courage & Khazaka, Cologne, Germany).

Immunofluorescence staining Hairless mouse skin was excised with a 6 mm punch biopsy, and the subcutaneous fat was removed. Tissue sections were incubated for 1 h in blocking buffer (1% bovine serum albumin, 0.1% cold water fish gelatin in phosphate-buffered saline, PBS) and were then incubated for 1 h further at room temperature with 1:500 dilution of polyclonal rabbit antimouse desmoglein 1 (DSG 1) antibody (gift of Dr. John Stanley, University of Pennsylvania) diluted in blocking buffer. The tissue was then washed with blocking buffer and incubated for 1 h at room temperature with fluorescein-labeled, isothiocyanate-conjugated, goat antirabbit IgG antibody (DAKO, Carpinteria, CA), diluted in blocking buffer. Either preincubation of DSG 1 antibody with DSG-1-recombinant protein (gift of Dr. Masayuki Amagai, Keio University, Tokyo, Japan), or omission of the DSG 1 primary antibody eliminated specific staining. Tissue sections were then washed with PBS and coverslipped before visualization under a confocal microscope (Leica TCS SP, Heidelberg, Germany) using fluorescein isothiocyanate at an excitation wavelength of 494 nm and an emission wavelength of 518 nm.

Protein assay on sequential tape strips The protein assay utilized the Bradford dye-binding procedure for quantification of total protein (Bradford, 1976). HEPES buffer and propylene glycol:ethanol, the two vehicles used in this study, are known to be compatible with this assay (Protein assay, Product Information, Bio-Rad, Hercules, CA). Before stripping the SC, the skin surface was cleaned with a single ethanol wipe. D-Squame tapes were then placed sequentially to the test areas for about 3 s each, removed with forceps, and stored in glass scintillation vials at 5°C.

The amount of protein removed per D-Squame was measured by a modification of the method of Dreher *et al* (1998). The microassay system was shown to be linear in the range 1–10 μg per ml, using human SC removed from a heel callosity. The calculated slope $R_f \pm$ SD is 0.0297 ± 0.00062; Spearman coefficient 0.999; $p < 0.0001$.

The protein content per stripping was determined with the Bio-Rad Protein Assay Kit. Lyophilized, bovine gamma globulin was used as the standard in all assays, because it correlated best with human SC. Each tape was incubated with 1 ml of 1 M NaOH for 1 h at 37°C in an incubator shaker at 80 rpm, and neutralized thereafter with 1 ml of 1 M HCl in the scintillation vials. Subsequently 0.2 ml of this solution was incubated in 0.6 ml distilled water plus 0.2 ml of the Bio-Rad protein dye for 5 min in borosilicate tubes. After incubations, the reagents were transferred to polysterene cuvettes, and absorption was measured with a Genesys 5 spectrophotometer (Spectronic, Rochester, NY) at 595 nm. An empty D-Squame tape, as well as distilled water incubated with the Bio-Rad dye, served as negative controls. The amount of calculated protein was then normalized to skin surface area (μg per cm^2). The amount of removed protein per D-Squame strip agreed with previous reports in untreated skin of hairless mice (i.e., range 2.5–4 μg per strip) (Weber *et al*, 1999).

Statistical analyses Statistical analyses were performed using Prism 2 (GraphPad Software, San Diego, CA). Normal distribution was tested before calculating the comparison. In the three group comparisons we calculated an ANOVA followed by a *posthoc* test (Bonferroni). Two groups were compaired with an unpaired *t* test.

RESULTS

Inhibition of PL-to-FFA processing increases the pH of the SC, while altering barrier homeostasis and SC integrity The surface pH of hairless mouse skin increased significantly after 1 d of BPB treatment, and continued to increase up to 3 d in comparison to vehicle-treated sites (**Fig 1*A***; $p < 0.01$). As previously reported (Mao-Qiang *et al*, 1995), daily topical applications of the group-1-specific $sPLA_2$ inhibitor BPB produce a concurrent, progressive abnormality in barrier function of normal skin, which becomes evident by day 2 (**Fig 1*B***). The integrity of the SC, quantitated as the number of tape strippings required to produce elevated TEWL levels, was also markedly abnormal after 3 d of BPB treatment. As seen in **Fig 1(*C*)**, a significant abnormality was already present at the first stripping, and SC integrity continued to deteriorate thereafter. These changes in integrity were paralleled by an increase in the amount of protein removed per D-Squame stripping (a measure of SC cohesion),

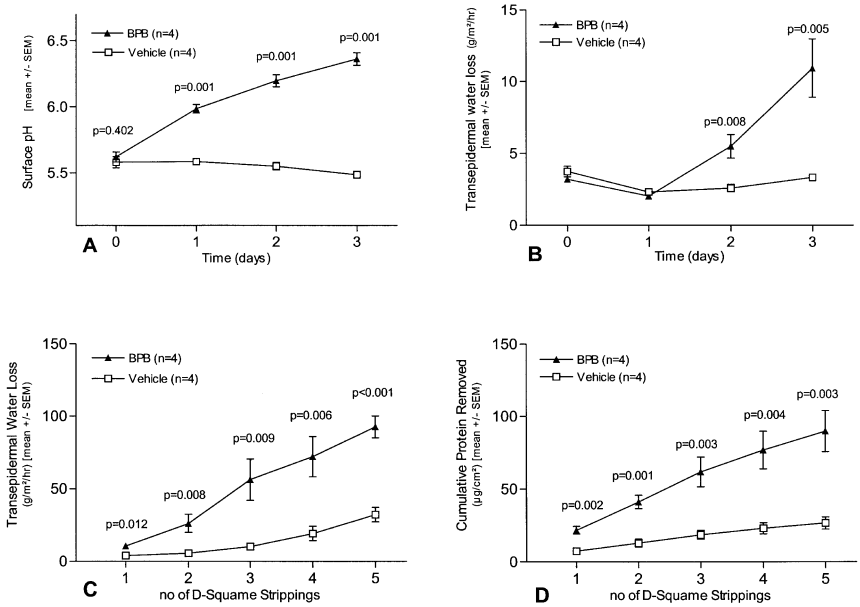

Figure 1. Alteration of stratum corneum pH with an $sPLA_2$ inhibitor correlates with altered barrier function, integrity, and cohesion. (*A*) Three days of topical BPB to intact skin results in a significant increase in SC surface pH. (*B*) Barrier function, assessed as TEWL, increases with 2 d of BPB applications. (*C*) SC integrity, assessed as number of tape strippings in relation to barrier function (TEWL), declines significantly (data shown are for 3 d of BPB). (*D*) SC cohesion, assessed as protein removed per D-Squame strip, also declines significantly (data shown are for 3 d of BPB).

which again was significant by the first strip, and continued to diverge from vehicle-treated sites with further stripping (**Fig 1*D***). These results show that repeated applications of the $sPLA_2$ inhibitor BPB result in an increased skin surface pH, a change that is accompanied not only by altered barrier function, but also by altered SC integrity and cohesion in comparison to the vehicle-treated control group.

Because BPB is an alkylating agent, which could nonspecifically affect other cellular processes, we performed additional studies with a chemically unrelated, competitive inhibitor of $sPLA_2$, MJ33, which is a highly specific, competitive inhibitor of group 1 $sPLA_2$. MJ33 treatment also produced a modest increase in SC surface pH *versus* vehicle (5.87 ± 0.06 *vs* 5.60 ± 0.05; $p = 0.0023$). Moreover, as seen previously (Mao-Qiang *et al*, 1995; 1996), repeated applications of MJ33 produced a progressive barrier abnormality, with a 2–3-fold increase in TEWL levels by day 3 (5.19 ± 0.81 *vs* 2.97 ± 0.16; $p < 0.001$, for MJ33 *versus* vehicle-treated animals). As with BPB treatments, 3 d of MJ33 applications also progressively altered SC integrity (**Fig 2*A***), reflected by a parallel change in SC cohesion (**Fig 2*B***). These results show that a second, chemically unrelated, $sPLA_2$ inhibitor produces comparable changes in skin surface pH, in parallel with altered SC barrier function, integrity, and cohesion.

The acidification and cohesion abnormalities are due to reduced generation of FFA Prior studies have shown that the barrier abnormality induced by both of the topical $sPLA_2$ inhibitors can be overridden by coapplications of certain end-products of PL hydrolysis, i.e., nonessential FFA (Mao-Qiang *et al*, 1995; 1996). We confirmed here, again, that coapplications of either PA (C16:0) or stearic acid (18:0), with either BPB or MJ33, prevented emergence of the barrier abnormality (data not shown). Coapplications of PA, stearic acid, and to a lesser extent linoleic acid prevented the BPB-induced increase in the pH of the SC (PA, 5.40 ± 0.14 *vs* 5.92 ± 0.05; stearic acid, 5.73 ± 0.09 *vs* 6.17 ± 0.08; linoleic acid, 5.77 ± 0.10 *vs* 6.28 ± 0.10). Furthermore, coapplications of either stearic acid, PA, and to a lesser extent linoleic acid also prevented emergence of the $sPLA_2$-inhibitor-induced abnormalities in SC integrity and cohesion (**Fig 3*A*–*D***; linoleic acid data not shown). Likewise, coapplications of PA with MJ33 prevented emergence of the MJ33-induced abnormality in SC integrity (**Fig 2*A***, ***B***). These results show that the $sPLA_2$-inhibitor-induced abnormalities in integrity and cohesion are linked to increased pH, resulting from decreased generation of FFA, the end-products of PL hydrolysis by $sPLA_2$.

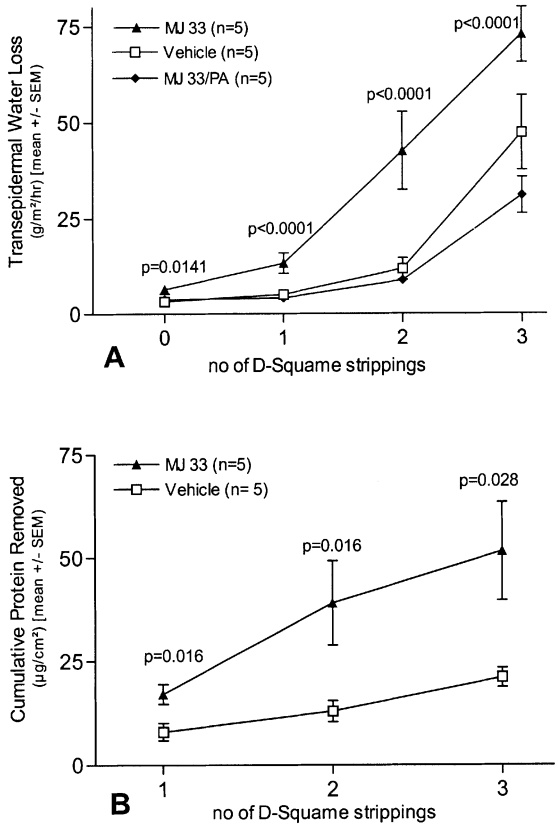

Figure 2. A chemically unrelated $sPLA_2$ inhibitor produces similar changes in SC integrity and cohesion. (*A*) Three days of topical MJ33 to intact skin results in a significant increase in TEWL, but coapplications of FFA (PA) could override this effect; indicated p-values were determined by ANOVA; the *posthoc* (Bonferroni) test revealed $p < 0.05$ for MJ33 *versus* vehicle at all data points, and $p < 0.01$ MJ33 *versus* MJ33/PA at all data points except at zero D-Squame strippings (which was not significant). (*B*) Integrity and cohesion of SC after 3 d of MJ33 applications to intact skin were significantly reduced; p-values determined by *t* test.

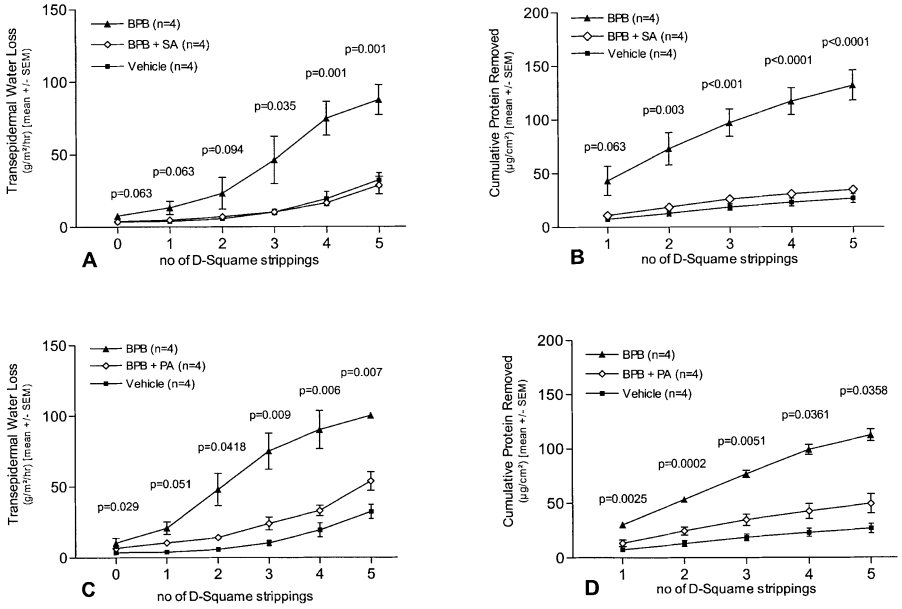

Figure 3. Coapplication of FFA (stearic acid, palmitic acid) with $sPLA_2$ inhibitor normalizes SC integrity and cohesion. (*A*, *B*) Integrity and cohesion data, respectively, after 3 d of BPB ± stearic acid application to intact skin. (*C*, *D*) Similar data on integrity and cohesion after 3 d of BPB ± PA application to intact skin. Indicated p-values for (*A*)–(*D*) were derived by ANOVA. (*A*) *Posthoc* (Bonferroni) test revealed $p < 0.01$ for BPB *versus* both groups for four and five D-Squame strippings, (*B*) $p < 0.01$ for BPB *versus* both groups for two, three, four, and five D-Squame strippings, and $p < 0.05$ for BPB *versus* vehicle for one D-Squame stripping. (*C*) Bonferroni test: $p < 0.01$ for BPB *versus* vehicle at all data points and $p < 0.05$ for BPB *versus* BPB/PA for three, four, and five D-Squame strippings. (*D*) Bonferroni test: $p < 0.01$ for BPB *versus* both groups for all data points.

Exposure of normal skin to a neutral pH also produces abnormalities in pH, barrier function, and integrity/cohesion To ascertain more directly whether the $sPLA_2$-inhibitor-induced functional alterations could be due to the associated pH changes, we next assessed SC integrity after short-term exposure of normal skin to a neutral *versus* acidic pH. After 3 h of exposure to a neutral pH buffer (HEPES), the surface pH of the SC rose from 5.86 ± 0.21 to 6.41 ± 0.20 (**Fig 4*A***, $p = 0.0257$). Whereas exposure to both buffers increased basal TEWL levels, a slightly greater increase in TEWL occurred following exposure to the neutral pH buffer for 3 h (all changes within normal range; data not shown). Even with such short-term exposures, SC integrity and cohesion declined significantly after 3 h of treatment with the neutral pH buffer (**Fig 4*B***, ***C***). These studies show that short-term exposure to a neutral pH buffer alone begin to produce functional abnormalities that mimic those induced by the $sPLA_2$ inhibitors.

Exposure to a neutral pH buffer amplifies the $sPLA_2$-inhibitor-induced alterations in SC integrity As a further test of the importance of PL-derived FFA for SC functions, we next assessed whether exposure of BPB-treated skin to a neutral or acidic buffer could amplify or override the functional abnormalities produced by the inhibitor. Exposure of skin sites to BPB for 3 d provoked an increase in SC pH, which was amplified further by exposure of the BPB-treated sites to a neutral pH buffer for 3 h. Exposure to an acidic buffer lowered the surface pH from 5.95 ± 0.05 to 5.72 ± 0.06 (**Fig 5*A***). In parallel with the increase in pH, exposure to the neutral pH buffer also accentuated the BPB-induced abnormality in SC integrity. In contrast, integrity remained unchanged in BPB-treated sites exposed to an acidic pH buffer (**Fig 5*B***). These studies demonstrate further that the abnormalities in SC integrity/cohesion induced by $sPLA_2$ blockage can be attributed largely to the acidification abnormality produced by inhibitor treatment.

Premature desmosomal dissolution accounts for the abnormality in SC integrity/cohesion We next assessed the basis for the abnormalities in SC integrity and cohesion that result from application of $sPLA_2$ inhibitors to murine skin. Both inhibitors provoked a dramatic decrease in the density of desmosomes in the lower SC and at the stratum granulosum (SG)–SC interface (**Figs 6**, **7**). Moreover, coapplication of free fatty acids with the inhibitors reversed the decrease in desmosome density in the lower SC (**Figs 6**, **7**). **Figure 8** shows the reduction of corneodesmosomes after 3 d of MJ33 treatment, an effect that could be prevented by coapplication of FFA (PA). BPB treatment resulted in a comparable reduction of corneodesmosomes (data not shown). A similar reduction in desmosomes was also shown by immunohistochemical assessment of DSG-1-positive structures in the lower SC of BPB *versus* vehicle-treated SC. On laser confocal microscopy, the density of DSG-1-positive clusters, which are presumed to correspond to intact desmosomes, declined dramatically in BPB-treated SC, whereas DSG-1-positive staining in vehicle-treated SC was comparable to control (**Fig 6**). These results show that the inhibitor-induced decline in SC integrity and cohesion can be attributed to a premature dissolution of desmosomes in the lower SC.

DISCUSSION

The acidic pH of the SC could be derived from one or more of several potential sources (Ohman and Vahlquist, 1998). Until recently, extrinsic sources were most commonly invoked, e.g., secreted eccrine/sebaceous gland products or metabolic byproducts of resident, cutaneous microflora (Aly *et al*, 1975; Puhvel *et al*, 1975). Among potential intrinsic (endogenous) sources, both passive (catabolic enzymes) and active (proton pumps or exchangers) sources have been postulated. Recently, Krien and Kermici (2000) proposed that *cis*-urocanic acid generation, the end-product of the filaggrin-to-histidine-to-urocanic acid proteolytic pathway, could alone explain the acidic pH of the SC. Their assay method, however, employed an externally applied aqueous receptor, which probably samples neither the lower SC nor hydrophobic microdomains within the SC interstices. It should also be noted that filaggrin hydrolysis is regulated by changes in external humidity (Scott *et al*, 1982; 1986), which, in turn, could influence the amount of histidine that would be available for *cis*-urocanic acid generation. Accordingly, we showed here that an alternative enzymatic mechanism, i.e., generation of FFA from $sPLA_2$-mediated catabolism of PL, regulates SC acidification. Topical applications of two chemically unrelated $sPLA_2$ inhibitors increased SC pH. As the inhibitor-induced increases, however, did not achieve a neutral pH, it is likely either that $sPLA_2$ blockade is incomplete or that other acidification mechanisms compensate, in part, for $sPLA_2$ blockade. Yet, it is also important to note that such potential, compensatory acidifying mechanisms, if operative, did not normalize pH during these experiments. We then demonstrated the specificity of this reaction through the ability of

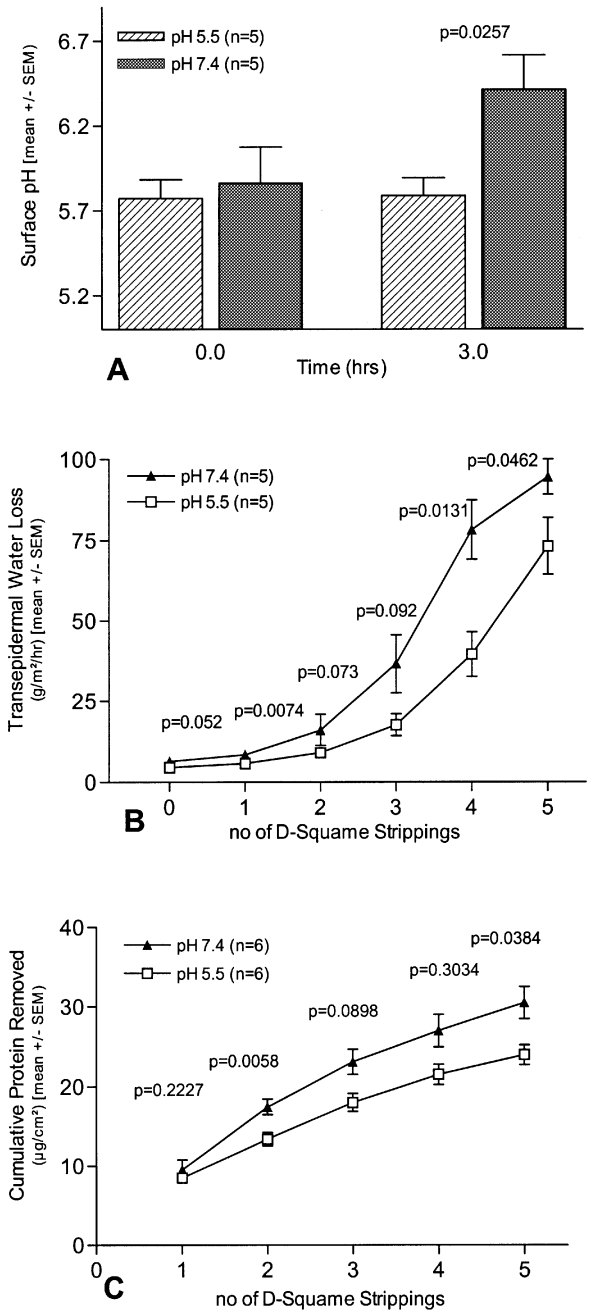

Figure 4. Exposure of intact skin to neutral pH buffer produces similar changes in SC pH, barrier function, and integrity. Anesthetized mice were immersed for 3 h in HEPES buffer, adjusted to pH 7.4 or pH 5.5. (*A*) After exposure to HEPES buffer pH 7.4 the pH of SC is significantly elevated in comparison to skin surfaces exposed to HEPES buffer pH 5.5. (*B*) An abnormality in SC integrity is also apparent. (*C*) The changes in SC cohesion after 3 h exposure to HEPES buffer, pH 7.4 *vs* pH 5.5, are also significant.

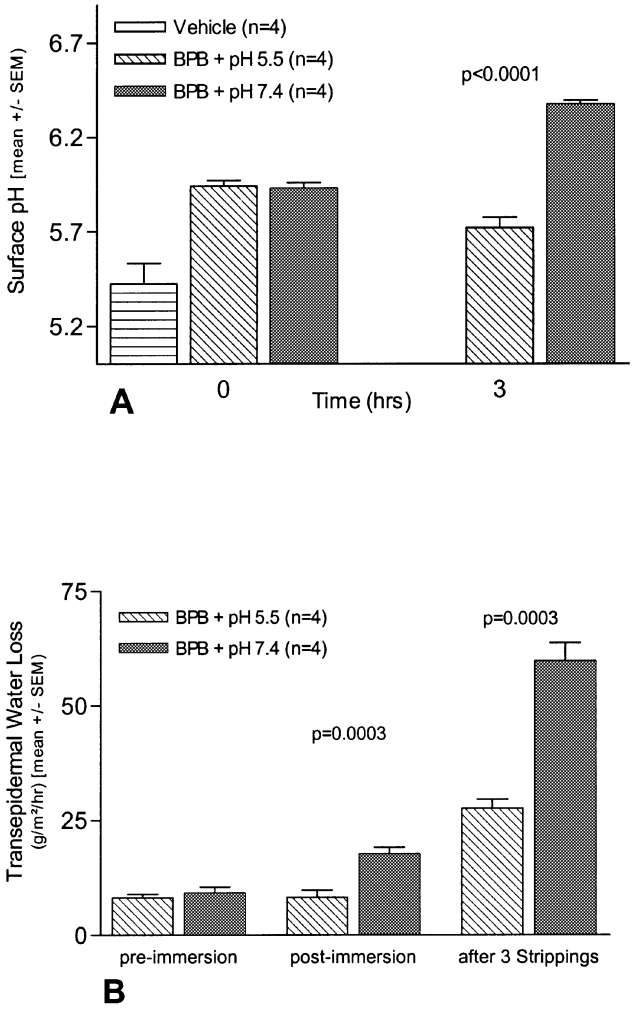

Figure 5. A neutral pH buffer amplifies the sPLA$_2$-inhibitor-induced alteration in pH, whereas an acidic buffer overrides inhibitor-induced alterations in barrier function and integrity. The hairless mice were first treated with the sPLA$_2$ inhibitor BPB for 3 d and then exposed to HEPES buffer, either pH 7.4 or pH 5.5, for 3 h. (*A*) Exposure to HEPES buffer pH 7.4 for 3 h amplifies the BPB-induced barrier abnormality, whereas exposure to HEPES buffer pH 5.5 prevents this change. The calculated ANOVA was not significant and thus a *posthoc* comparison was not performed. (*B*) After 3 d of BPB treatment, a significant barrier abnormality is present (cf. **Fig 2**). This abnormality is amplified by 3 h exposure to HEPES buffer at pH 7.4, but not at pH 5.5. (*C*) Three days of BPB treatment plus 3 h of exposure to HEPES buffer at either pH 7.4 or pH 5.5. Both treatments cause a further decline in SC integrity, but the defect is exacerbated significantly at pH 7.4 *vs* pH 5.5.

coapplications of either FFA or an acidic pH buffer to restore a normal acidic pH to inhibitor-treated SC. Thus, it can be assumed that sPLA$_2$-mediated catabolism of PL to FFA represents an important acidifying mechanism in normal SC.

FA are one of the three major lipid species of the SC, which together account for about 10% of SC by weight (Yardley and Summerly, 1981; Schurer and Elias, 1991). Cytochemical studies have shown that sPLA$_2$ activity (Freinkel and Traczyk, 1985) is codelivered to the SC interstices, along with PL (Grayson *et al*, 1985), through the secretion of epidermal lamellar body contents at the SG–SC interface (Elias *et al*, 1988). Moreover, there is also evidence that PL-to-FFA generation is an early catabolic event in a sequence of biochemical reactions that transforms secreted polar lipids into their nonpolar products (Long *et al*, 1985; Elias *et al*, 1988; Mao-Qiang *et al*, 1995; 1996). Although there are no other sources of FFA among the secreted, lamellar-body-derived polar lipids, in theory FFA could also be generated in the outer SC from the catabolism of nonlamellar-body-derived triglycerides by the abundant triacylglycerol lipase that is delivered to the SC interstices by lamellar body secretion (Grayson *et al*, 1985; Menon *et al*, 1986). Moreover, whereas only small quantities of triglycerides are present in SC (Hedberg *et al*, 1988; Schurer and Elias, 1991), large amounts of triglycerides are synthesized in both the nucleated layers of the epidermis (Monger *et al*, 1988) and sebaceous glands (Thody and Shuster, 1989; Stewart and Downing, 1991). Yet, although

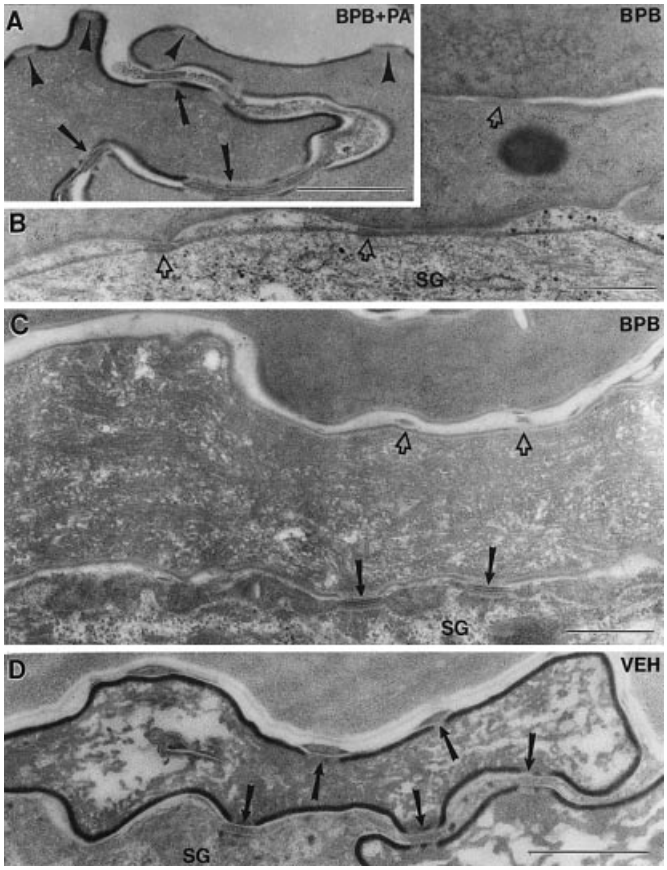

Figure 6. Treatment with two $sPLA_2$ inhibitors reduced desmosome density in the lower SC. (*B, C*) Hairless mouse skin treated with the $sPLA_2$ inhibitor BPB. Note the diminution of intact desmosomes (*solid arrow*) at the SG–SC interface with the presence of desmosomal remnants (*open arrows*). In contrast, coapplication of PA with BPB maintains normal desmosome numbers (*A*, *solid arrows* and *arrowheads*). (*D*) Vehicle (VEH)-treated SC – desmosome numbers in the SC appear to be normal (*D*, *solid arrows*). (*A*)–(*D*) OsO4 postfixation. *Magnification bars*, 0.5 μm.

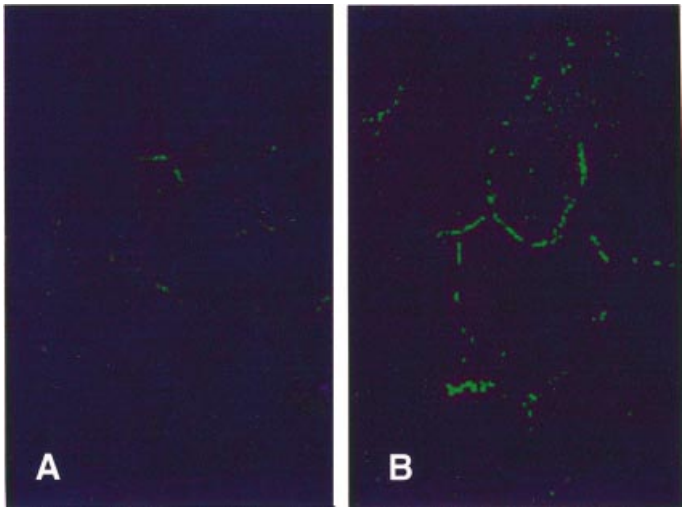

Figure 7. Treatment with the $sPLA_2$ inhibitor BPB reduces desmosoglein labeling in the lower SC. Reduction in desmosomes in inhibitor-treated skin is also shown by the decrease in DSG 1 immunolabeling in the lower SC of BPB *versus* vehicle-treated SC, on laser confocal microscopy (*A*). In contrast DSG 1 staining in vehicle-treated SC remained prominent (*B*).

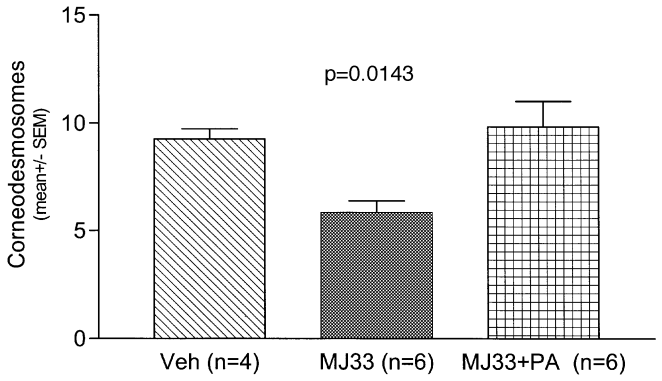

Figure 8. Treatment with the $sPLA_2$ inhibitor MJ33 reduces corneodesmosomes in the lower SC. Reduction of corneodesmosomes after MJ33 treatment, an effect that could be prevented by coapplication of the FFA PA. Indicated p-value was determined by ANOVA; the *posthoc* (Bonferroni) test revealed $p < 0.05$ for MJ33 *versus* MJ33 + PA and MJ33 *versus* vehicle.

some FFA could, in theory, derive from epidermal (nonlamellar body) and/or sebaceous gland sources, we found no changes in SC pH either in asebia (Asb-J) mice that lack sebaceous glands nor in animals treated with the topical lipase inhibitor orlistat (Elias PM, Mao-Qiang M, Kao J, Feingold KR, unpublished observations). Thus, a specific PL-derived pool of FFA accounts for the observed, FFA-induced changes in skin surface pH.

Regardless of the origin of FFA, these lipids are segregated within the interstices of normal SC. Within this compartment, they could regulate the pH selectively, resulting in modulation of pH-sensitive functions of the SC (**Fig 9**). Indeed, we showed here that certain key functions of the SC are regulated in parallel with the pH changes induced by a lack of PL-derived FFA. As shown previously, we showed again that inhibition of $sPLA_2$ results in a permeability barrier abnormality, and that coapplications of FFA normalize barrier function (Mao-Qiang *et al*, 1995, 1996). These prior studies showed that depletion of PL-derived FFA results in membrane structural abnormalities attributable to an altered ratio of the three key SC lipids function (Mao-Qiang *et al*, 1995, 1996). The question remains, however, whether an acidic pH alone could compensate for depletion of FFA in correcting the barrier. In fact, an alteration in pH alone could theoretically alter barrier homeostasis by at least two different mechanisms (**Fig 9**). First, barrier recovery after acute perturbations is delayed at a neutral pH, attributable to altered extracellular processing of secreted lamellar body polar lipids (Mauro *et al*, 1998). Moreover, both β-glucocerebrosidase and acid sphingomyelinase, enzymes that are required for the generation of ceramides from glucosylceramides and sphingomyelin, respectively, exhibit acidic pH optima (Holleran *et al*, 1992; Jensen *et al*, 1999; Takagi *et al*, 1999). Accordingly, an elevated SC pH could alter barrier function by blockade of ceramide generation from their two polar lipid precursors. Second, a neutral pH could directly impair the structural organization of lamellar membrane structures. Bouwstra *et al* (1998, 1999) have shown that the pKa resulting from an acidic pH allows FFA to assemble more efficiently into ceramide and cholesterol-containing membrane bilayers. Yet, whereas the BPB-induced barrier abnormality could be prevented by coapplied FFA, it could not be reversed by an acidic pH buffer alone: barrier recovery after acute perturbation is delayed comparably when BPB is applied in either a neutral or an acidic buffer (Fluhr JW, Feingold KR, Elias PM, unpublished observations). Thus, whereas the pH-related mechanism clearly is important for barrier homeostasis, it cannot substitute for PL-derived generation of FFA.

A second functional consequence of FFA-mediated acidification relates to modulations of SC integrity/cohesion (**Fig 9**). Elevation

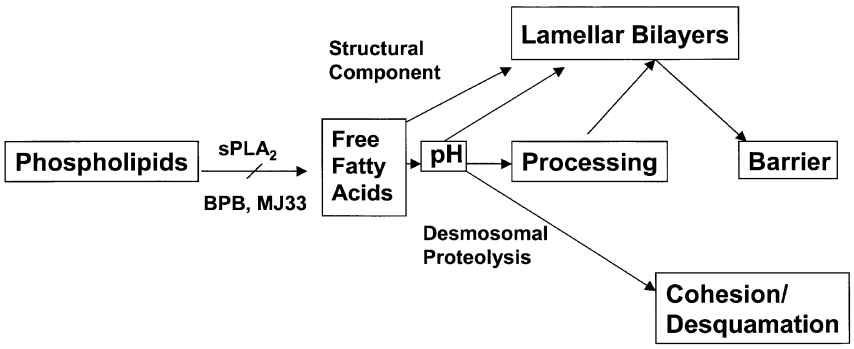

Figure 9. Structural and functional consequences of PL-to-FFA pathway. Lamellar body secretion delivers both PL and $sPLA_2$ to the SC interstices. Degradation of PL to FFA has distinct, separate downstream consequences for both permeability barrier function and SC integrity/cohesion.

of pH through inhibition of $sPLA_2$ compromised both SC integrity and SC cohesion, as evidenced by an $sPLA_2$-inhibitor-induced decrease in resistance to sequential strippings, and an increase in the quantity of protein removed per stripping, respectively. Again, the link between $sPLA_2$ inhibition and diminished SC integrity was shown by the normalization of SC integrity/cohesion that followed reacidification with either coapplied FFA or exposure to an acidic buffer. Conversely, integrity/cohesion deteriorated further when inhibitor-treated skin was exposed to a neutral pH buffer. We also showed that the basis for diminished SC cohesion with $sPLA_2$ blockade correlates with a premature dissolution of corneodesmosomes. Corneodesmosomes normally persist into the mid SC, but in $sPLA_2$-inhibitor-treated skin extensive degradation/dissolution was evident in the mid to lower SC, extending even to the SG–SC interface. Conversely, coapplication of FFA with the inhibitors restored normal corneodesmosome densities. Accordingly, desmoglein immunolabeling of corneocytes decreased abruptly above the SG–SC interface in inhibitor-treated skin. The accelerated dissolution of desmosomes in $sPLA_2$-inhibitor-treated skin could reflect increased activities of the SC chymotryptic and tryptic enzymes, which display neutral-to-alkaline pH optima (Brattsand and Egelrud, 1999; Egelrud, 2000). In contrast, the low desquamation rates (increased cohesion) that occur in intact skin could reflect a decrease in activity of one or both of these two enzymes. Further studies will be needed to ascertain the enzymatic basis for the $sPLA_2$-inhibitor-induced alterations in SC integrity and cohesion.

In summary, topical applications of two chemically unrelated $sPLA_2$ inhibitors produced an increase in the skin surface pH of murine SC that was paralleled by a permeability barrier abnormality, altered SC integrity, and decreased SC cohesion. Not only SC pH but also all of the functional abnormalities normalized when FFA were coapplied with the inhibitors. Short-term applications of a neutral pH buffer to inhibitor-treated skin further amplified the abnormalities in SC integrity and cohesion. Finally, the alterations in integrity/cohesion were linked to premature dissolution of corneodesmosomes.

Barbara E. Brown assisted greatly in the development of the protein assay, and Sue Allen superbly prepared and edited the manuscript. The authors thank Dr. Walter M. Holleran for numerous useful discussions regarding these experiments. This study has been supported by NIH grant HR 19098, Medical Research Service, VAMC, and the experiments regarding pH and barrier function by an unrestricted grant from Sebapharma AG, FRG.

REFERENCES

Aly R, Maibach HI, Rahman R, Shinefield HR, Mandel AD: Correlation of human *in vivo* and *in vitro* cutaneous antimicrobial factors. *J Infect Dis* 131:579–583, 1975

Ament W, Huizenga JR, Mook GA, Gips CH, Verkerke GJ: Lactate and ammonia concentration in blood and sweat during incremental cycle ergometer exercise. *Int J Sports Med* 18:35–39, 1997

Andersen S, Sjursen W, Laegreid A, Volden G, Johansen B: Elevated expression of human nonpancreatic phospholipase A2 in psoriatic tissue. *Inflammation* 18:1–12, 1994

Baden HP, Pathak MA: The metabolism and function of urocanic acid in skin. *J Invest Dermatol* 48:11–17, 1967

Beare JM, Cheeseman EA, Gailey AH, Neill DW: The pH of the skin surface of children with seborrheic dermatitis compared with unaffected children. *Br J Dermatol* 70:233, 1958

Bergers M, Verhagen DR, Jongerius M, van de Kerkhof PC, Mier PD: A unique phospholipase A2 in human epidermis: its physiologic function and its level in certain dermatoses. *J Invest Dermatol* 90:23–25, 1988

Blank IH: Measurement of pH of the skin surface. *J Invest Dermatol* 2:67, 1939

Bouwstra JA, Gooris GS, Dubbelaar FE, Weerheim AM, Ponec M: pH, cholesterol sulfate, and fatty acids affect the stratum corneum lipid organization. *J Invest Dermatol Symp Proc* 3:69–74, 1998

Bouwstra JA, Gooris GS, Dubbelaar FE, Ponec M: Cholesterol sulfate and calcium affect stratum corneum lipid organization over a wide temperature range. *J Lipid Res* 40:2303–2312, 1999

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976

Brattsand M, Egelrud T: Purification molecular cloning, and expression of a human stratum corneum trypsin-like serine protease with possible function in desquamation. *J Biol Chem* 274:30033–30040, 1999

Brook I: Microbiology of secondarily infected diaper dermatitis. *Int J Dermatol* 31:700–702, 1992

Dikstein S, Zlotogorski A: Measurement of skin pH. *Acta Derm Venereol (Suppl.)(Stockh)* 185:18–20, 1994

Draize JH: The determination of the pH of the skin of man and common laboratory animals. *J Invest Dermatol* 5:77, 1942

Dreher F, Arens A, Hostynek JJ, Mudumba S, Ademola J, Maibach HI: Colorimetric method for quantifying human stratum corneum removed by adhesive-tape stripping. *Acta Derm Venereol* 78:186–189, 1998

Egelrud T: Desquamation in the stratum corneum. *Acta Derm Venereol Suppl* 208:44–45, 2000

Elias PM, Menon GK: Structural and lipid biochemical correlates of the epidermal permeability barrier. *Adv Lipid Res* 24:1–26, 1991

Elias PM, Menon GK, Grayson S, Brown BE: Membrane structural alterations in murine stratum corneum: relationship to the localization of polar lipids and phospholipases. *J Invest Dermatol* 91:3–10, 1988

Forster S, Ilderton E, Summerly R, Yardley HJ: Epidermal phospholipase A2 activity is raised in the uninvolved skin of psoriasis. *Br J Dermatol* 109:30–35, 1983

Forster S, Ilderton E, Summerly R, Yardley HJ: The level of phospholipase A2 activity is raised in the uninvolved epidermis of psoriasis. *Br J Dermatol* 108:103–105, 1983

Forster S, Ilderton E, Norris JF, Summerly R, Yardley HJ: Characterization and activity of phospholipase A2 in normal human epidermis and in lesion-free epidermis of patients with psoriasis or eczema. *Br J Dermatol* 112:135–147, 1985

Freinkel RK, Traczyk TN: The phospholipases A of epidermis. *J Invest Dermatol* 74:169–173, 1980

Freinkel RK, Traczyk TN: Acid hydrolases of the epidermis: subcellular localization and relationship to cornification. *J Invest Dermatol* 80:441–446, 1983

Freinkel RK, Traczyk TN: Lipid composition and acid hydrolase content of lamellar granules of fetal rat epidermis. *J Invest Dermatol* 85:295–298, 1985

Gelb MH, Jain MK, Berg OG: Inhibition of phospholipase A2. *Faseb J* 8:916–924, 1994

Grayson S, Johnson-Winegar AG, Wintroub BU, Isseroff 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

Hedberg CL, Wertz PW, Downing DT: The nonpolar lipids of pig epidermis. *J Invest Dermatol* 90:225–229, 1988

Holleran WM, Takagi Y, Imokawa G, Jackson S, Lee JM, Elias PM: Beta-glucocerebrosidase activity in murine epidermis: characterization and localization in relation to differentiation. *J Lipid Res* 33:1201–1209, 1992

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

Jain MK, Tao WJ, Rogers J, Arenson C, Eibl H, Yu BZ: Active-site-directed specific

competitive inhibitors of phospholipase A2: novel transition-state analogues. *Biochemistry* 30:10256–10268, 1991

Jensen JM, Schutze S, Forl M, Kronke M, Proksch E: Roles for tumor necrosis factor receptor p55 and sphingomyelinase in repairing the cutaneous permeability barrier. *J Clin Invest* 104:1761–1770, 1999

Krien PM, Kermici M: Evidence for the existence of a self-regulated enzymatic process within the human stratum corneum – an unexpected role for urocanic acid. *J Invest Dermatol* 115:414–420, 2000

Lee SH, Elias PM, Proksch E, Menon GK, Mao-Quiang M, Feingold KR: Calcium and potassium are important regulators of barrier homeostasis in murine epidermis. *J Clin Invest* 89:530–538, 1992

Leyden JJ, Kligman AM: The role of microorganisms in diaper dermatitis. *Arch Dermatol* 114:56–59, 1978

Li-Stiles B, Lo HH, Fischer SM: Identification and characterization of several forms of phospholipase A2 in mouse epidermal keratinocytes. *J Lipid Res* 39:569–582, 1998

Long SA, Wertz PW, Strauss JS, Downing DT: Human stratum corneum polar lipids and desquamation. *Arch Dermatol Res* 277:284–287, 1985

Long VJ, Yardley HJ: Phospholipase A activity in the epidermis. *J Invest Dermatol* 58:148–154, 1972

Mao-Qiang M, Feingold KR, Jain M, Elias PM: Extracellular processing of phospholipids is required for permeability barrier homeostasis. *J Lipid Res* 36:1925–1935, 1995

Mao-Qiang M, Jain M, Feingold KR, Elias PM: Secretory phospholipase A2 activity is required for permeability barrier homeostasis. *J Invest Dermatol* 106:57–63, 1996

Mauro T, Holleran WM, Grayson S, *et al*: Barrier recovery is impeded at neutral pH, independent of ionic effects: implications for extracellular lipid processing. *Arch Dermatol Res* 290:215–222, 1998

Mazereeuw-Hautier J, Redoules D, Tarroux R, et al: Identification of pancreatic type I secreted phospholipase A2 in human epidermis and its determination by tape stripping. *Br J Dermatol* 142:424–431, 2000

Menon GK, Grayson S, Elias PM Cytochemical and biochemical localization of lipase and sphingomyelinase activity in mammalian epidermis. *J Invest Dermatol* 86:591–597, 1986

Monger DJ, Williams ML, Feingold KR, Brown BE, Elias PM: Localization of sites of lipid biosynthesis in mammalian epidermis. *J Lipid Res* 29:603–612, 1988

Ohman H, Vahlquist A: *In vivo* studies concerning a pH gradient in human stratum corneum and upper epidermis. *Acta Derm Venereol* 74:375–379, 1994

Ohman H, Vahlquist A: The pH gradient over the stratum corneum differs in X-linked recessive and autosomal dominant ichthyosis: a clue to the molecular origin of the ''acid skin mantle''? *J Invest Dermatol* 111:674–677, 1998

Puhvel SM, Reisner RM, Amirian DA: Quantification of bacteria in isolated pilosebaceous follicles in normal skin. *J Invest Dermatol* 65:525–531, 1975

Schade H, Marchionini A: Der Säuremantel der Haut. *Klin Wochenschr* 7:12–14, 1928

Schmuth M, Man MQ, Weber F, *et al*: Permeability barrier disorder in Niemann–Pick disease: sphingomyelin-ceramide processing required for normal barrier homeostasis. *J Invest Dermatol* 115:459–466, 2000

Schurer NY, Elias PM: The biochemistry and function of stratum corneum lipids. *Adv Lipid Res* 24:27–56, 1991

Scott IR, Harding CR: Filaggrin breakdown to water binding compounds during development of the rat stratum corneum is controlled by the water activity of the environment. *Dev Biol* 115:84–92, 1986

Scott I, Harding C, Barrett J: Histidine-rich protein of the keratohyalin granules. Source of the free amino acids, urocanic acid and pyrrolidone carboxylic acid in the stratum corneum. *Biochimica Biophysica Acta* 719:110–117, 1982

Stewart ME, Downing DT: Chemistry and function of mammalian sebaceous lipids. *Adv Lipid Res* 24:263–301, 1991

Takagi Y, Kriehuber E, Imokawa G, Elias P, Holleran W: Beta-glucocerebrosidase activity in mammalian stratum corneum. *J Lipid Res* 40:861–869, 1999

Thody AJ, Shuster S: Control and function of sebaceous glands. *Physiol Rev* 69:383–416, 1989

Weber SU, Thiele JJ, Cross CE, Packer L: Vitamin C, uric acid, and glutathione gradients in murine stratum corneum and their susceptibility to ozone exposure. *J Invest Dermatol* 113:1128–1132, 1999

Yardley HJ, Summerly R: Lipid composition and metabolism in normal and diseased epidermis. *Pharmacol Ther* 13:357–383, 1981