Functional Consequences of a Neutral pH in Neonatal Rat Stratum Corneum

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At birth, neonatal stratum corneum (SC) pH is close to neutral but acidifies with maturation, which can be ascribed, in part, to secretory phospholipase A_2 and sodium/hydrogen antiporter 1 (NHE1) activities. Here we assessed the functional consequences of a neutral SC pH in a newborn rat model. While basal transepidermal water loss rates are near normal, barrier recovery (BR) rates after acute barrier disruption were delayed in newborn animals. The abnormality in barrier homeostasis could be improved by topical applications of an acidic buffer, indicating that barrier abnormality is primarily due to high SC pH. The delay in BR correlated with incompletely processed lamellar membranes and decreased activity of beta-glucocerebrosidase. Inhibition of NHE1 delayed BR after acute barrier perturbation. SC integrity was abnormal in newborn animals. Electron microscopy demonstrated decreased corneodesmosomes (CD) in newborn animals with decreased expression of desmoglein 1 and corneodesmosin. Serine protease activation appears to be responsible for CD degradation in newborn animals, because serine protease activity is increased in the SC and it can be reduced by acidification of the SC. The delay in acidification of neonatal SC results in abnormalities in permeability barrier homeostasis and SC integrity and are likely due to pH-induced modulations in enzyme activity.

Key words: acidification/cohesion/desmoglein 1/integrity/neonatal Rat/pH/stratum corneum
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The initial observation that the skin has an acidic surface ("acid mantle") was made 70 y ago (Schade, 1928) and is noted repeatedly since then (Bernstein and Hermann, 1942; Beare *et al*, 1958; Jolly, 1960; Zlotogorski, 1987; Ohman and Vahlquist, 1994; Ohman and Vahlquist, 1998). Based upon a variety of indirect evidence, the major function of the acid mantle has been assumed to be primarily antimicrobial (Aly *et al*, 1975; Puhvel *et al*, 1975). Recent studies in adult mice, however, have demonstrated that acidification of the stratum corneum (SC) is important for both permeability barrier homeostasis and normal SC integrity and cohesion. The importance of pH for permeability barrier homeostasis was first suggested by the delay in barrier recovery that occurred when acutely disrupted skin sites were immersed in neutral pH buffers (Mauro *et al*, 1998). Moreover, blockade of endogenous acidifying mechanisms results not only in an increase in pH, but also in altered permeability barrier function and SC integrity/cohesion. For example, deletion or inhibition of the NHE1 antiporter, which selectively increases pH in the lower SC, produces a delay in barrier recovery kinetics following acute disruption, an effect that could be corrected by co-exposure of inhibitor-treated sites to an acidic buffer (Behne *et al*, 2002). Similarly, blockade of another endogenous acidification mechanism, the type 1 secretory phospholipases A_2 ($sPLA_2$), increases SC pH, while producing abnormalities both in permeability barrier homeostasis and in SC integrity/cohesion (Fluhr *et al*, 2001). Finally, direct applications of "superbases" to rodent skin also delay barrier recovery and alter SC integrity/cohesion (Hachem *et al*, 2003). All of the pH-induced abnormalities in barrier homeostasis are associated with a decrease in β-glucocerebrosidase (β-Gluc Cer'ase) activity in the SC that results in a lipid-processing defect with immature extracellular lamellar membranes on electron microscopy (Fluhr *et al*, 2001; Behne *et al*, 2003; Hachem *et al*, 2003). Neutralization of the SC, however, decreases β-Gluc Cer'ase activity resulting in impaired barrier homeostasis (Mauro *et al*, 1998; Hachem *et al*, 2003). Thus, it is likely that an increased SC pH adversely affects barrier homeostasis by decreasing the activity of β-Gluc Cer'ase and perhaps other enzymes with a preference for an acidic environment (e.g. acidic sphingomyelinase). Alternatively, it has been proposed that an acidic pH directly impacts lipid–lipid interactions in the SC extracellular

Abbreviations: AUC, area under the curve; BPB, bromophenacyl-bromide; CD, corneodesmosome; DSG 1, desmoglein 1; GA, glycolic acid; β-Gluc Cer'ase, β-glucocerebrosidase; PMSF, phenylmethylsulfonyl fluoride; SA, stearic acid; SC, stratum corneum; SG, stratum granulosum; SP, serine protease; $sPLA_2$, secretory phospholipase A_2; TEWL, transepidermal water loss; TMG, 1,1,3,3-tetramethyl-guanidine

lamellar bilayers (Bouwstra *et al*, 1998). An acidic SC pH also is essential for normal SC integrity and cohesion. Increasing the pH of adult SC by either exposure to neutral pH buffers, topical treatment with superbases, or $sPLA_2$ blockade, results in an enhanced tendency for the SC to be removed by tape stripping (decreased integrity), as well as increased amounts of protein removed per stripping (decreased cohesion) (Fluhr *et al*, 2001; Hachem *et al*, 2003).

Skin surface pH is neutral at birth both in humans and in various animal models (Behrendt and Green, 1971; Hardman *et al*, 1998; Visscher *et al*, 2000; Yosipovitch *et al*, 2000; Fluhr *et al*, 2004). In fact, human SC does not achieve adult levels of acidification for several weeks (Behrendt and Green, 1971). Yet, the mechanisms responsible for the delayed acidification of human SC remain unknown. Recently, we developed a neonatal rat model to study mechanisms of acidification in neonates. In newborn rats, the surface pH approaches neutrality at birth and acidification of SC attains normal adult levels over the first several days post-birth (Behne *et al*, 2003; Fluhr *et al*, 2004). Furthermore, the newborn rat model has a thick interfollicular epidermis with a competent SC at the time of birth under basal conditions. With a subsequent development of a fur, the interfollicular epidermis decreases to a few cell layers in thickness. Therefore, the epidermis in the newborn rodent, but not in the adult, manifests a model that is structurally and functionally similar to human stratum epidermis. When the NHE1 antiporter, which is expressed at birth (Behne *et al*, 2003) was inhibited, postnatal acidification of the SC is partially blocked (Fluhr *et al*, 2004). Additionally, $sPLA_2$ activity is low at birth, but increases progressively after birth over the first 4 d; again inhibition of $sPLA_2$ from days 1 to 5–6 delays postnatal SC acidification (Fluhr *et al*, 2004). Together, these results indicate that acidification of neonatal SC can be attributed, in part, to two endogenous mechanisms; namely, $sPLA_2$ and NHE1, which are known to be important for acidification of adult rodent SC.

In this paper, we determined whether the delay in acidification in neonatal rats resulted in functional consequences for neonatal skin: We demonstrate here that both permeability barrier homeostasis and SC integrity are defective in neonatal animals, and that both these defects are due specifically to delayed acidification, since they can be corrected by acidification of neonatal SC.

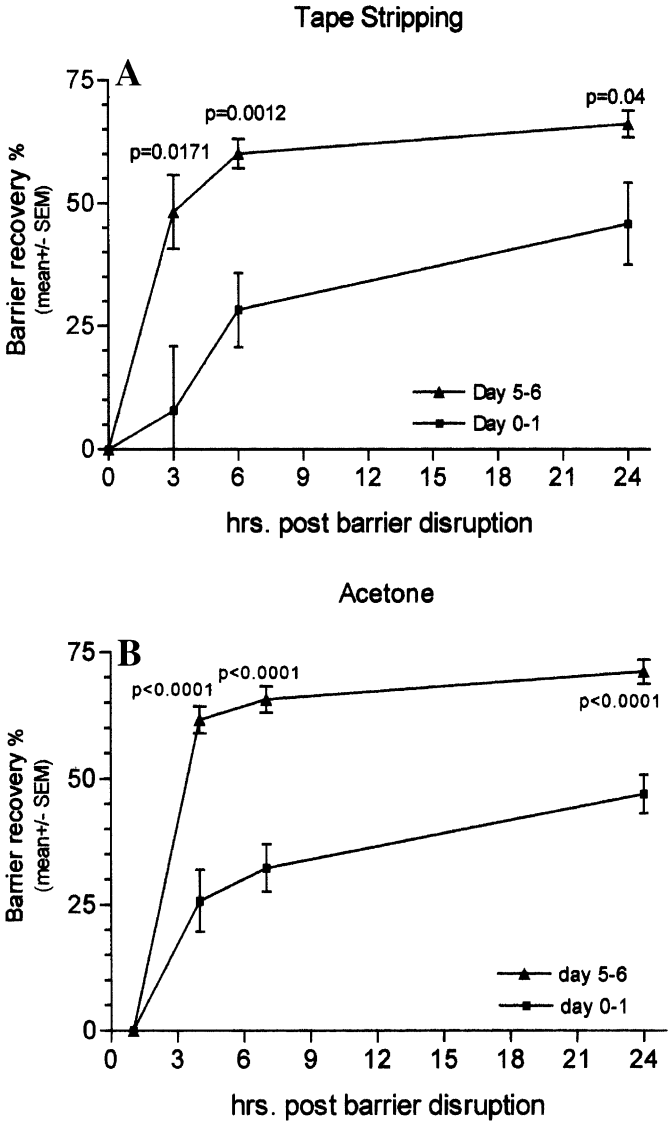

Figure 1
Barrier recovery is delayed at days 0–1 after acute barrier disruption induced by tape stripping and acetone treatment. (*A*) Acute disruption of the permeability barrier by repeated tape stripping on neonatal rats at days 0–1 ($n = 7$) and at days 5–6 ($n = 6$) reveals a significant delay in barrier recovery rate early after birth (days 0–1). The greatest difference occurs 6 h after barrier disruption ($p = 0.0012$). (*B*) Acute disruption of the permeability barrier by acetone-induced lipid extraction on neonatal rats at days 0–1 ($n = 10$) and at days 5–6 ($n = 9$) reveals a significant delay ($p < 0.0001$ at all time points) in barrier recovery rate early after birth (days 0–1).

Results

Neonatal epidermis displays abnormal permeability barrier homeostasis As previously reported (Behne *et al*, 2003; Fluhr *et al*, 2004), at birth baseline permeability barrier function, assessed by measuring TEWL, was slightly, but not significantly higher in newborns than in adult rats (newborn 3.09 ± 0.22 g per m^2 per h SEM; $n = 9$ *vs* adult 1.7 ± 0.80 g per m^2 per h SEM; $n = 6$). During the 5 d following birth, baseline TEWL did not change significantly. Following acute barrier disruption by either tape stripping or acetone treatment, however, barrier recovery was markedly delayed in the newborn rats *versus* 5–6-d-old rats (Fig 1). To determine whether the delay in barrier recovery was pH dependent, we next determined whether application of an acidic solution (HEPES buffer at pH 5.5) would not only acidify the SC but also normalize barrier recovery kinetics. As shown in Fig 2*A*, the buffer acidified the surface pH of neonatal skin, and accelerated barrier recovery in comparison with a neutral pH solution (Fig 2*B*). These results show that the abnormality in barrier recovery in newborn rats is reversible with application of acidic pH buffer.

To determine the morphological basis for the defect in permeability barrier homeostasis, we next compared the ultrastructure of the extracellular lamellar membranes in newborn and 5–6-d-old rats, using ruthenium tetroxide post-fixation after barrier disruption (Fig 3). As previously

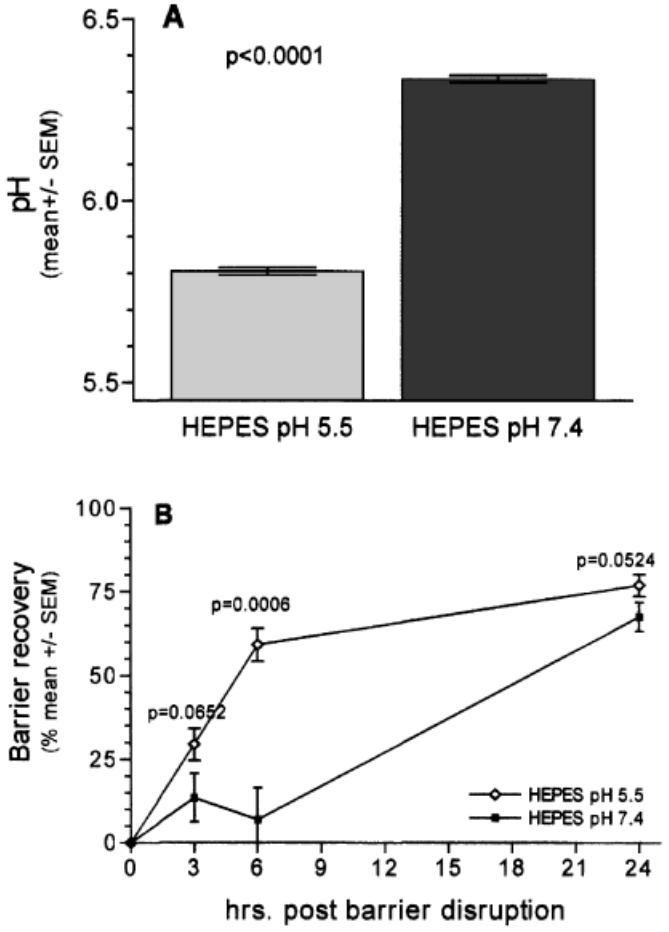

Figure 2
Neutral buffer increases stratum corneum (SC) pH and delays barrier recovery after acute barrier disruption. (*A*) Neutral HEPES buffer (10 mM) adjusted to a pH of 7.4 (n = 10) was compared with pH 5.5 (n = 11): A buffer-soaked gaze was placed over the newborns, changed every 30 min over a period of about 3 h. The neutral HEPES-buffer application increases significantly the SC pH after 3 d of treatment and 24 h after the last treatment ($p < 0.0001$). (*B*) The permeability barrier was disrupted after 3 d of treatment and 24 h after the last treatment by tape stripping (n = 4). The barrier recovery was recorded over the following 24 h. The animals treated with a neutral pH showed a delay in barrier recovery with the biggest delay 6 h after barrier disruption ($p = 0.0006$).

reported (Behne *et al*, 2003), small areas of incompletely processed extracellular lamellar membranes were observed in the SC of newborn rats, which disappeared by 3 d after birth (data not shown). Moreover, marked abnormalities in the extracellular lamellar membranes were observed in the newborn rats 3 and 6 h after barrier disruption (Fig 3*A* and *B*). Although large areas of incompletely processed lamellar membranes were present in newborn SC, the newly secreted membranes were almost completely processed by 3 and 6 h post-disruption in 5-d-old rats, as in adult rodents (Fig 3*C* and *D*). Thus, the defect in permeability barrier homeostasis is associated with a parallel delay in lipid processing.

Previous studies have shown that β-Gluc Cer'ase activity is reduced when SC pH is lowered resulting in immature extracellular lamellar membranes and abnormal barrier homeostasis. We again observed that β-Gluc Cer'ase activity is reduced in the SC of newborn rats as *versus* 5–6-d-old rats (data not shown; see Behne *et al*, 2003; Fluhr *et al*, 2004). Moreover, reduction in SC pH with topical buffers increased β-Gluc Cer'ase activity in newborn animals, an effect that is independent of enzyme expression, which is normal at birth (Behne *et al*, 2003 and data not shown). Together, these results demonstrate that β-Gluc Cer'ase protein is present in normal quantities in the SC of newborn rats, but the neutral SC pH results in suboptimal catalytic activity, leading to impaired processing of secreted lamellar membranes and abnormal barrier homeostasis.

Previous studies have shown that NHE1 protein expression is normal at birth (Behne *et al*, 2003) and that inhibition of NHE1 delays the acidification of the SC during neonatal development (Fluhr *et al*, 2004). To assess whether the reduction in pH from this mechanism is responsible for the barrier defect, we next examined if topical application of inhibitors of NHE1 would adversely affect permeability barrier homeostasis. Animals were treated for 3 d after birth with HOE694, a specific inhibitor of NHE1. As expected, SC pH increased in HOE694-treated animals in comparison with vehicle-treated controls (HOE694 6.61 ± 0.05 SEM *vs* control 6.35 ± 0.03 SEM; $p = 0.0002$, n = 8 and 11). As shown in Fig 4, inhibition of NHE1 activity delayed barrier recovery after acute disruption by tape stripping, further demonstrating the importance of SC acidification in regulating barrier homeostasis. These results show again that NHE1 contributes to postnatal acidification and that NHE1-induced acidification contributes to the postnatal normalization of permeability barrier function.

Neonatal SC displays abnormal integrity We next examined SC integrity, defined as the degree to which the SC can be abrogated, in neonatal *versus* days 5–6 rats. As shown in Fig 5, SC integrity was greatly impaired in newborn and 1-d-old rats in comparison with older (5–6 d) animals. To determine the role of pH in mediating the abnormalities in SC integrity, we next manipulated SC pH in neonatal rats using four different methods. First, we applied either an acidic HEPES buffer (pH 5.5) or a neutral (pH 7.4) HEPES buffer for 3 d to newborn rats, and then assessed SC integrity. As expected, SC pH was lower in animals treated with the acidic buffer (cf. Fig 2). More significantly, SC integrity improved in the animals treated with the acidic buffer compared with those treated with a neutral buffer (Fig 6*A*). As a second model, we treated neonatal animals with topical GA for the initial 1.5 d after birth. As expected, surface pH decreased in the GA- *versus* vehicle-treated animals (GA; 6.51 ± 0.04 *vs* control 6.87 ± 0.05; $p < 0.0001$; n = 9). Moreover, as shown in Fig 6*B*, SC integrity also improved in the animals treated with GA. As a third model, we compared treatment with the "superbase" TMG that would raise SC pH to treatment with a neutralized "superbase" that allows postnatal acidification to proceed normally. As expected, SC pH was lower in the animals treated with the neutralized base TMG *versus* animals treated with TMG (TMG 6.79 ± 0.15 *vs* neutralized TMG 5.99 ± 0.07; $p = 0.0143$; n = 4). As shown in Fig 6*C*, once again a higher SC pH resulted in an abnormal SC integrity whereas a lower SC pH was associated with a better SC integrity. Lastly, we treated animals with BPB, a drug previously shown to inhibit SC acidification during the postnatal period (BPB:

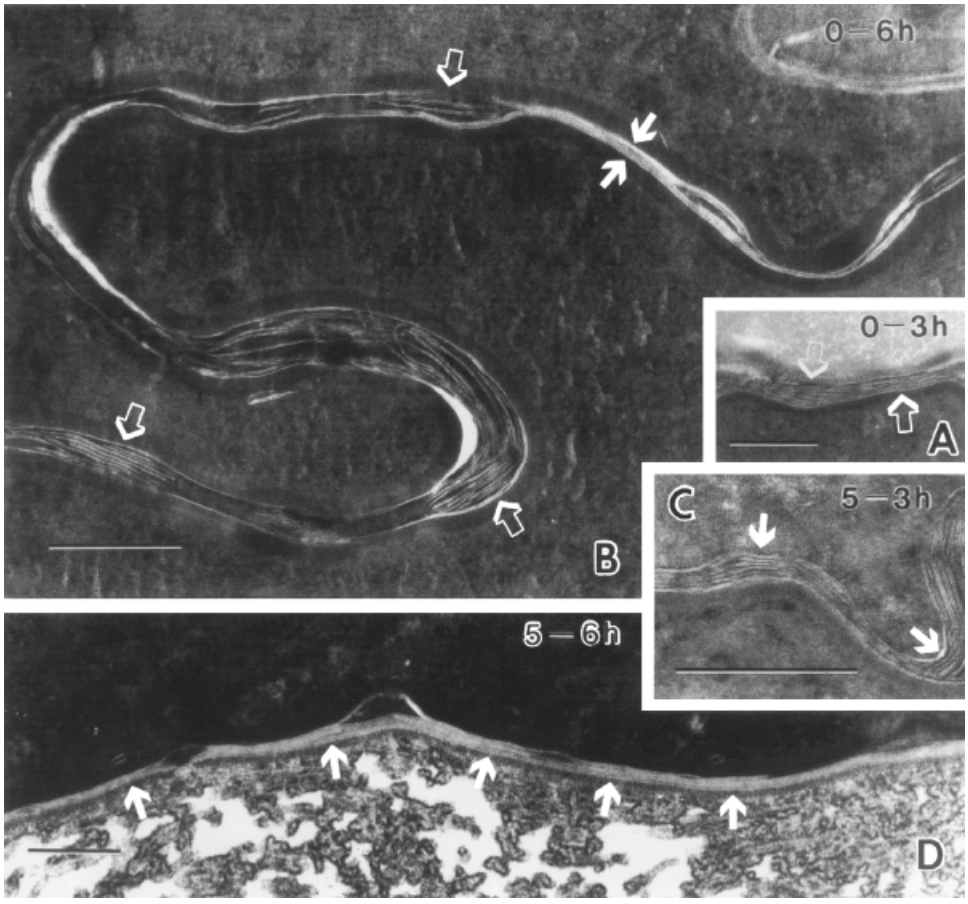

Figure 3
The delay in barrier recovery in 1 d rats correlates with incomplete lipid processing. (*A*, *B*) Secreted membrane structures in 1-d-old rat stratum corneum (SC) are incompletely processed, 3 h (*A*) and 6 h (*B*) after acute barrier disruption (*single arrows*, completely processed; *open arrows*, incompletely processed lamellae). (*C*, *D*) Secreted membrane structures in 5-d-old rat SC are almost-completely processed at 3 h (*C*, *arrows*), and fully processed by 6 h (*D*, *arrows*). (*A–D*), ruthenium tetroxide; *scale bars* = 0.25 μm.

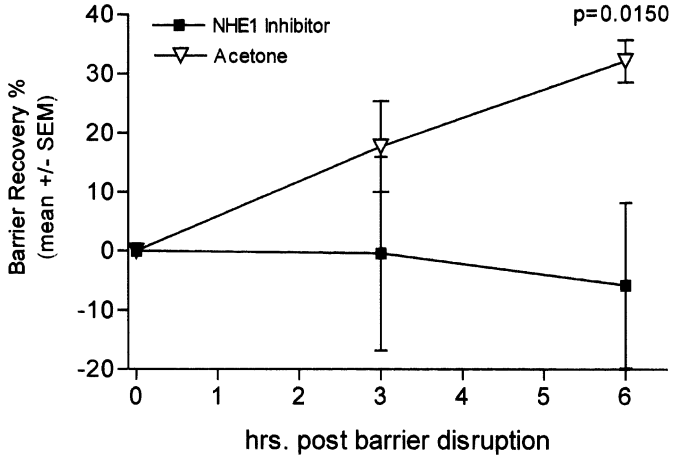

Figure 4
Inhibition of NHE1 antiporter delays barrier recovery in the postnatal period after acute barrier disruption. Blockade of the NHE1 antiporter by topical HOE694 for 3 d leads to a significant delay in barrier recovery of the neonatal permeability barrier in comparison with vehicle-treated animals (n = 6 in each group) (p = 0.0150).

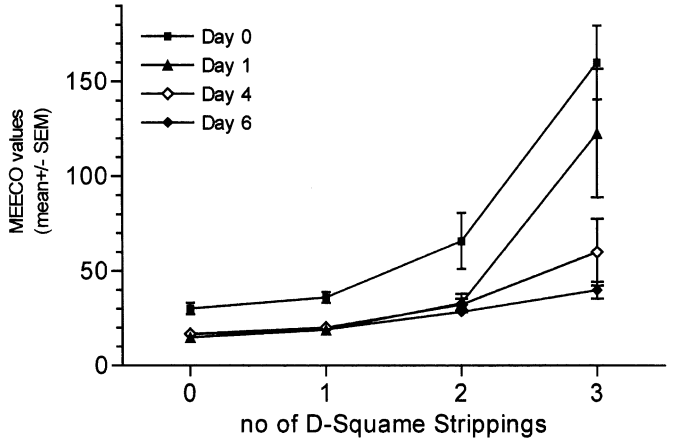

Figure 5
Stratum corneum (SC) integrity/cohesion improve after birth. Sequential tape stripping with D-Squame results in a larger increase in transepidermal water loss early after birth compared with later time points. A data reduction was performed calculating an area under the curve (AUC). ANOVA ($p = 0.0138$) and *post hoc* testing (Bonferroni adjusted) showed a significant difference of AUC from days 0–1 to days 1–2 in comparison with day x ($p < 0.05$) and day y ($p < 0.05$) (n = 7 in each group).

pH = 6.34 ± 0.02 SEM; n = 9 *vs* control: pH = 5.90 ± 0.02 SEM; n = 10; $p < 0.0001$). As shown in Fig 6*D*, BPB treatment resulted in decreased SC integrity compared with vehicle treatment, an abnormality that could be overridden by acidifying the SC with the enzyme product SA. Together, these experiments provide strong evidence that the neutral SC pH that occurs during the neonatal period adversely affects SC integrity.

We next determined the morphological basis for the abnormality in SC integrity in newborn animals. Newborn rats display a paucity of CD (Fig 7*A* and *C*) in comparison with 5-d-old rats (Fig 7*B* and *D*). Moreover, staining for DSG 1 and corneodesmosin, two major constituents of CD, also is decreased in newborn animals as compared with 5-d-old

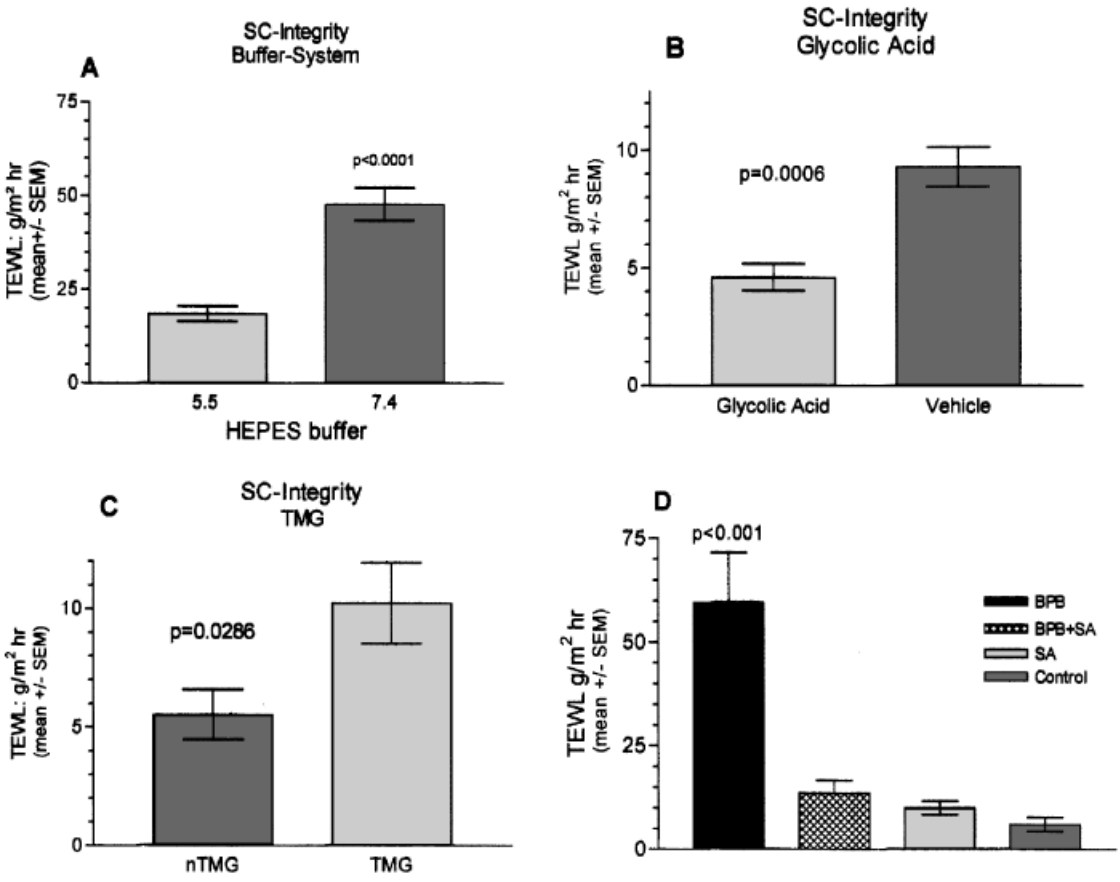

Figure 6
Alkalization of the stratum corneum (SC) impairs SC integrity within four independent models. (*A*) Repeated topical application (over 3 d, 3 h per d) of 10 mM HEPES buffer solution at a neutral pH (7.4) impairs the SC integrity in comparison with a group treated with acid HEPES buffer solution (pH 5.5) ($n = 8$ in both groups) significantly ($p < 0.0001$). The integrity was challenged by sequential stripping with three D-Squame tapes. (*B*) The application of topical glycol acids (1, 5 d) significantly improves the impaired SC integrity compared with vehicle ($n = 8$ in each group) ($p = 0.0006$). The integrity was challenged by sequential stripping with four D-Squame tapes. (*C*) Repeated exposure to 1% 1,1,3,3-tetramethylguanidine (TMG) (superbase) decreased the SC integrity compared with neutralized TMG ($n = 4$) ($p = 0.0286$). The integrity was challenged by sequential stripping with four D-Squame tapes. (*D*) SC integrity is impaired by topical secretory phospholipase A_2 ($sPLA_2$)-inhibitor (bromophenacyl bromide, BPB) application ($n = 6$) compared with control ($n = 7$) and stearic acid (SA) ($n = 8$) after three D-Squame strippings. Co-application of SA ($n = 6$) partially reverses the negative impact of the inhibited $sPLA_2$ pathway. ANOVA: $p < 0.0001$ and Bonferroni-adjusted *post hoc* comparison: $p < 0.001$ for BPB compared with all other three groups.

animals (Figs 8 and 9). These results show that a decrease in both CD, and its constituent proteins accounts for the defective SC integrity in newborns.

We next assessed whether the diminished SC integrity and CD density results from a developmental delay, or pH-dependent upregulation of SP activity. As shown in Fig 10, SP activity was markedly increased in newborn *versus* days 5–6. Moreover, decreasing SC pH in the newborn animals, by treatment with acidic HEPES buffer (see above), decreased SP activity both *in vivo* and *in vitro* (Fig 11), indicating that an elevated SC pH primarily accounts for the increase in SP activity in newborn rats. Finally, inhibiting SP activity in newborn animals increased CD density (Fig 12). Together these results suggest that the increase in SP activity induced by an elevated SC pH enhances the degradation of CD adversely effecting SC integrity.

Discussion

That the skin has an acid surface has been recognized for many years (Heuss, 1892; Schade, 1928; Marchionini and Hausknecht, 1938; Blank, 1939; Bernstein and Hermann, 1942; Draize, 1942; Arbenz, 1952; Jolly, 1960). Surprisingly, skin surface pH is neutral at birth in both humans and various animals but with maturation the SC acidifies (Behrendt and Green, 1958; Visscher *et al*, 2000; Yosipovitch *et al*, 2000; Behne *et al*, 2003; Fluhr *et al*, 2004). In rodents this acidification process occurs within 5–7 d, whereas in humans the SC does not achieve an adult level of acidification for several weeks. Recent studies by our laboratory have shown that both $sPLA_2$ and NHE1 contribute to the acidification of the SC during the neonatal period (Fluhr *et al*, 2004). $SPLA_2$ hydrolyzes phospholipids in the SC releasing free fatty acids that are acidic, whereas NHE1 in the apical plasma membranes of the outermost stratum granulosum cells secretes $H+$ into the SC intercellular spaces. Although $sPLA_2$ and NHE1 partially account for the decrease in SC pH that occurs with neonatal maturation (Fluhr *et al*, 2004), it is clear that other, yet to be defined pathways, also play a role in SC acidification.

In this study, we elucidated the pathologic consequences of an elevated SC pH in newborns. Both permeability barrier homeostasis and SC integrity were

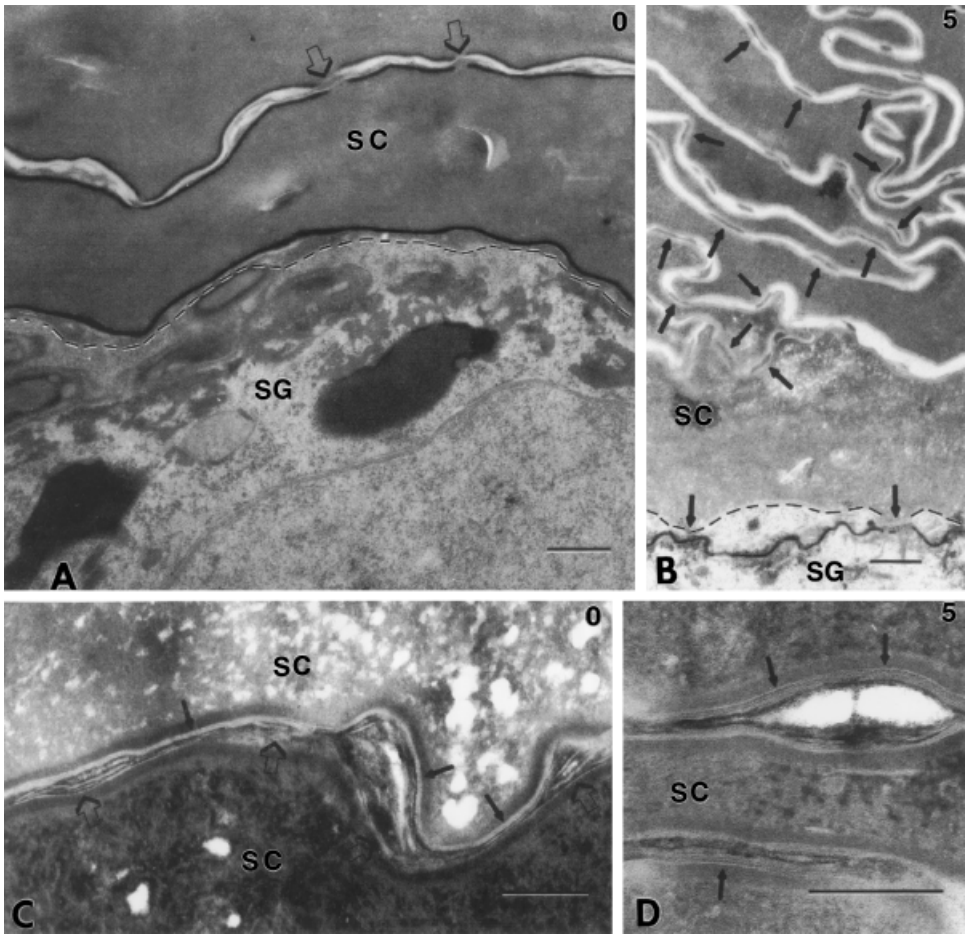

Figure 7
Lamellar membranes and corneodesmosomes are incompletely developed in 1-d-old rat epidermis. (*A*, *B*) A decrease in the number of corneodesmosomes is seen in 1-d-old (*A*, *open arrows*) *versus* 5-d-old (*B*, *single arrows*) stratum corneum (SC). Also, less secreted material is present at stratum granulosum (SG)–SC interface in 1-d-old epidermis (*dashed line*). (*C*, *D*) Secreted lamellae are incompletely processed at comparable levels in 1-d-old (*C*) *versus* 5-d-old (*D*) SC. Single arrows indicate mature lamellae, whereas open arrows indicate incompletely processed lamellae. (*A*, *B*) osmium tetroxide; *scale bars* = 0.5 μm. (*C*, *D*) ruthenium tetroxide; *scale bars* = 0.25 μm.

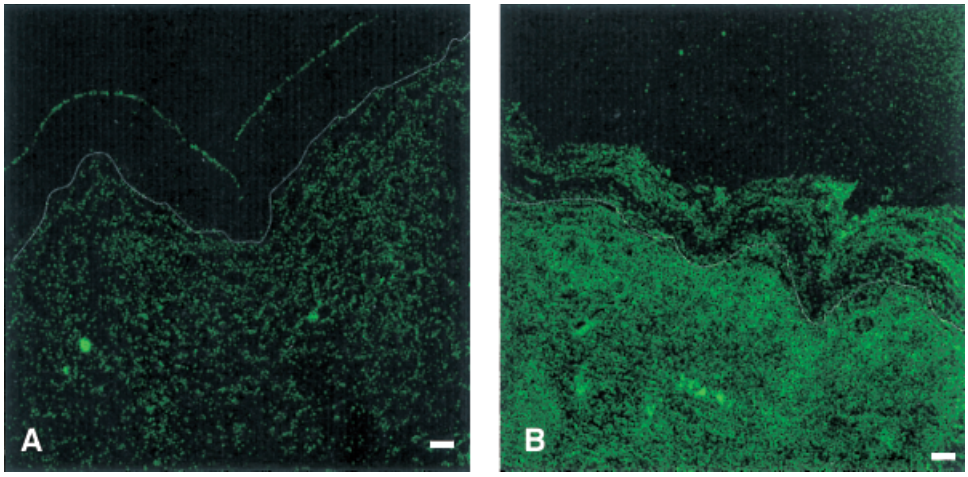

Figure 8
Desmoglein 1 (DSG 1) expression increases in the postnatal period in the stratum corneum (SC), stratum granulosum (SG)/SC interface and SG. (*A*) At days 0–1 a reduction in DSG 1 expression can be shown by the decrease of DSG 1 immunolabeling on laser-confocal microscopy. (*B*) In contrast DSG I staining at days 5–6 is markedly increased in the SC, SG/SC interface and the SG. Dotted line shows the SG/SC interface; *scale bars* = 10 μm.

abnormal. Basal permeability barrier, as measured by TEWL, was slightly increased compared with adult animals. More impressively, the recovery kinetics of permeability barrier function were markedly delayed after acute disruption of the barrier by either tape stripping or acetone treatment. Importantly, the defect in barrier homeostasis could be corrected by acidification of the SC, indicating that the abnormal permeability barrier was due to failure to acidify the SC and not to other developmental defects. The abnormal permeability barrier homeostasis was associated with incompletely processed extracellular lamellar membranes. In the basal state small areas of incompletely processed lamellar membranes were observed, which probably accounts for the very minor difference in permeability barrier function in newborns compared with adult animals (see also Behne *et al*, 2003) In contrast, following barrier disruption extensive areas of incompletely processed, lamellar membranes were observed, which would explain the major defect in barrier function observed following acute barrier disruption.

Previous studies have shown that the activity of β-Gluc Cer'ase, an enzyme that catalyzes the breakdown of glucosylceramide to ceramides, is required for the formation of mature lamellar membranes (Holleran *et al*, 1992,

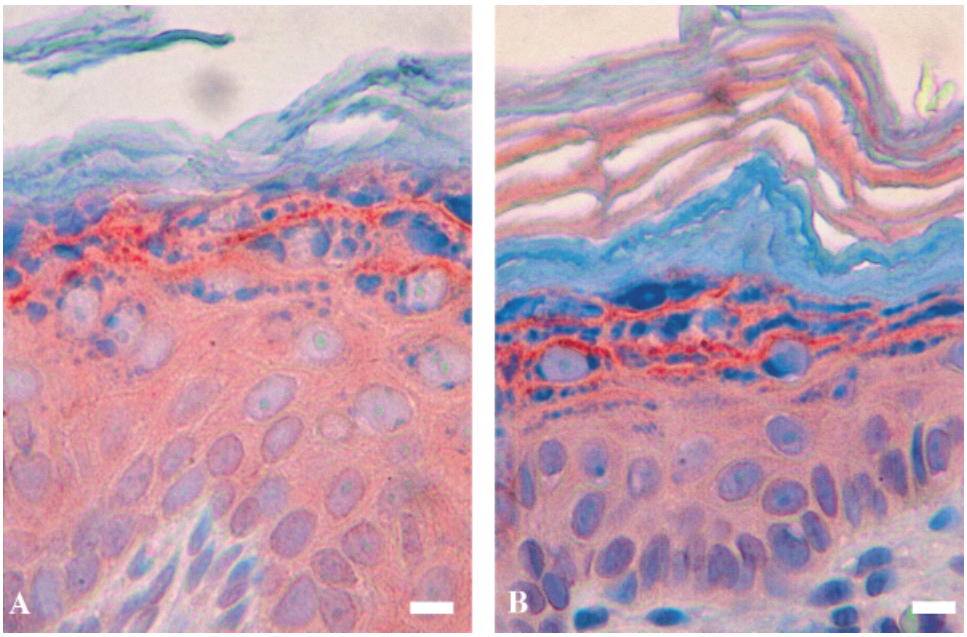

Figure 9
Corneodesmosin expression is detectable at birth at the stratum granulosum/stratum corneum (SG/SC) interface but increases in the postnatal period and moves outward through the SC. (*A*) Corneodesmosin expression visualized by immunohistochemistry of G36-39, a marker of persisting corneodesmosomes, was detectable at the SG/SC interface, but not in the SC at days 0–1 after birth. (*B*) Corneodesmosin expression increases markedly at days 5–6 after birth and moves outward through the SC; *scale bars* = 10 μm.

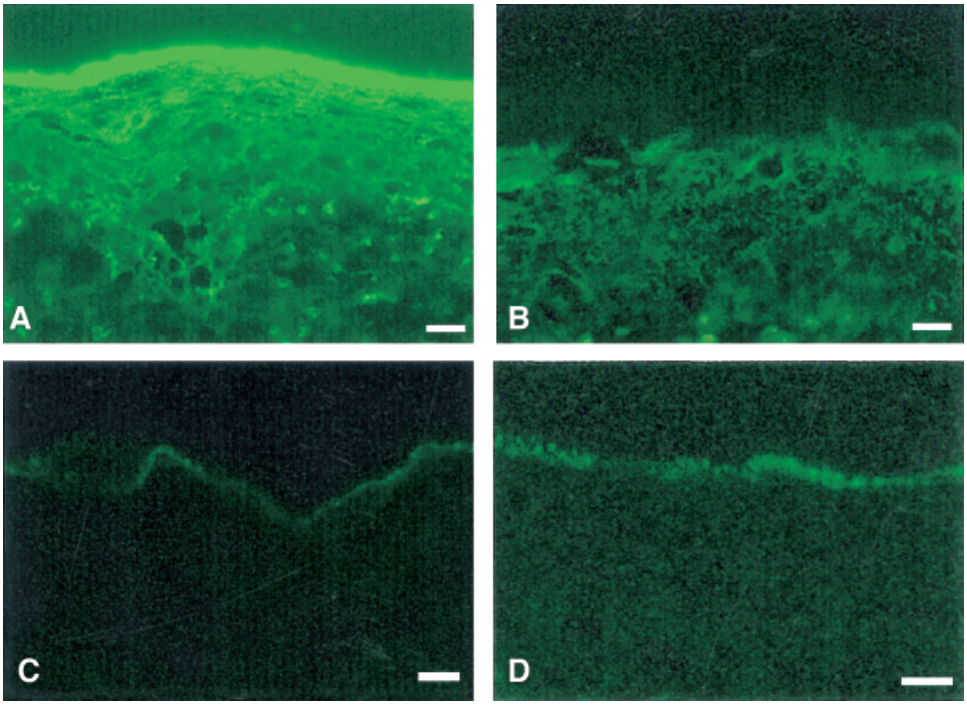

Figure 10
Serine protease (SP) activation declines in the postnatal period and can be inhibited by a SP inhibitor (phenylmethylsulfonyl fluoride, PMSF). (*A*) At days 0–1 a strong signal for protease activity in the stratum corneum (SC) and stratum granulosum (SG)/SC interface and less pronounced in the deeper part of the epidermis is detectable. (*B*) At days 5–6 after birth the protease activity is dramatically reduced and only is visible at the SG/SC interface. (*C*, *D*) Co-application of a serine protease (SP) inhibitor (PMSF) inhibits the protease activity almost completely indicating that the measured activity is mainly attributable to SP; *scale bars* = 10 μm.

1993, 1994; Doering *et al*, 1999; Takagi *et al*, 1999). Moreover, although enzyme activity is decreased in the SC of newborn rats (Fluhr *et al*, 2004), the mass of β-Gluc Cer'ase in the SC of newborns is not reduced (Behne *et al*, 2003) and we showed here that normal activity of β-Gluc Cer'ase could be restored by decreasing SC pH. The activity of β-Gluc Cer'ase is well known to be pH dependent, with enzyme activity optimal at pH 5.5, and therefore it is the increased SC pH of newborn SC that is responsible for the decrease in β-Gluc Cer'ase activity. It is also highly likely that the elevated SC pH and the decreased β-Gluc Cer'ase catalytic activity explain the failure to form mature lamellar membranes, thereby leading to disturbances in permeability barrier function. It should be noted, however, that β-Gluc Cer'ase activity, while decreased, is sufficient to allow for normal basal barrier function, but when the animals are stressed by barrier disruption the diminished β-Gluc Cer'ase activity results in major defects in permeability barrier homeostasis.

In addition to the abnormality in permeability barrier function, newborn animals also have abnormalities in SC integrity. This defect was demonstrated in newborn animals by a markedly greater rate of increase in TEWL with successive tape stripping compared with more mature animals (i.e., 5–6-d-old animals). That this functional defect is again due to the elevation in SC pH of newborns was demonstrated by the observation that acidifying the SC of newborn animals reversed the abnormal SC integrity. We showed

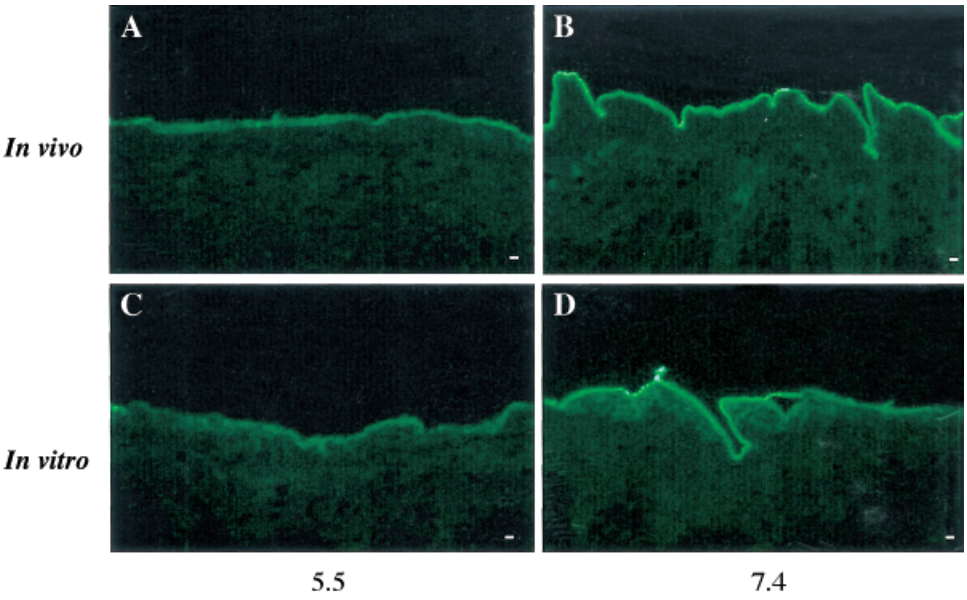

Figure 11
Acidic pH decreases serine protease (SP) activity both *in vivo* and *in vitro*. Stratum corneum (SC) acidification by applying HEPES buffer at a pH of 5.5 resulted in a decrease of SP activity *in vivo* (*A*) and *in vitro* (*C*) compared with HEPES buffer 7.4-treated skin *in vivo* (*B*) and *in vitro* (*D*). The increased SP activity at pH 7.4 is detectable throughout the whole epidermis whereas the lower activity at pH 5.5 is only detectable at the SC level.

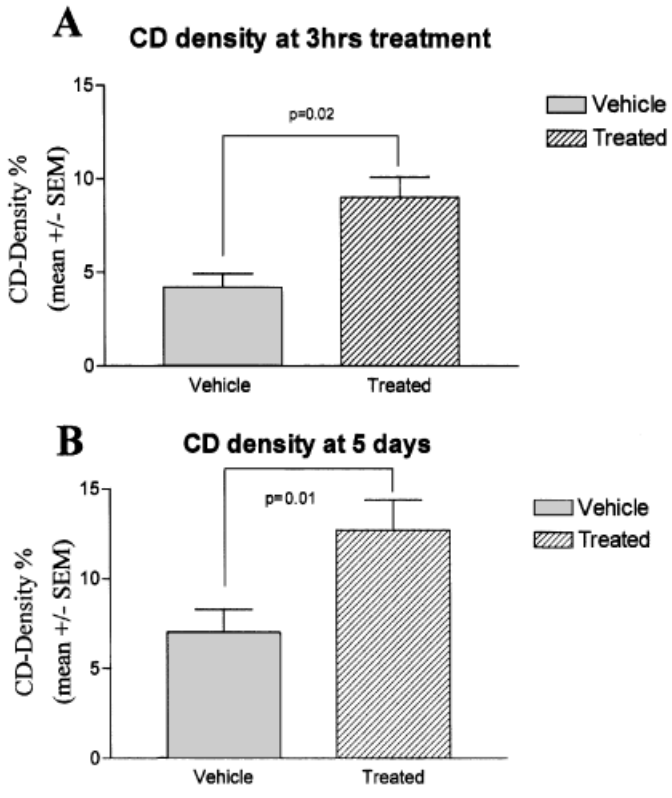

Figure 12
Corneodesmosome density is increased by topical protease inhibitor application *in vivo*. Newborn rats on their first day of life were treated topically with a single application of phenylmethylsulfonyl fluoride (PMSF) 10 mM in propylene glycol:ethanol (7:3 vol/vol) or vehicle alone on both flanks. The 5-d-old pups received three applications of PMSF or vehicle alone over 24 h. To quantify corneodesmosomes (CD) density, five pictures were taken from one block from one animal in each experiment at $\times$ 12,000 magnification. The ratio of the total length of intact CD to the total length of cornified envelopes in the first and second cell layers of the lower stratum corneum was determined using a planimeter ($n = 5$ for each group). (*A*) Represents the data in newborns after 3 h of treatment. (*B*) Results at day 5 after birth (three treatments).

further that the abnormality in SC integrity is associated with a reduction in CD, and at least two of its constituent proteins, DSG 1 and corneodesmosin. In addition, the decrease in CD and its constituent proteins was not developmental, but rather due to a pH-dependent increase in SP activity in the SC that is reversed by lowering the pH of the SC. It is well recognized that both chymotryptic and tryptic SP (SCCE and SCTE) in the SC mediate CD degradation leading to increased desquamation (i.e., decreased SC integrity) (Lundstrom and Egelrud, 1991; Lundström *et al*, 1994; Sondell *et al*, 1994; Haftek *et al*, 1997; Rogers *et al*, 1998; Brattsand and Egelrud, 1999; Horikoshi *et al*, 1999; Ekholm *et al*, 2000). The activity of two of SP involved in desquamation; i.e., SCCE and SCTE, is optimal at a neutral-alkaline pH (Lundstrom and Egelrud, 1991; Horikoshi *et al*, 1999). Therefore, the activity of these enzymes is increased in newborn SC. As the pH decreases during postnatal SC maturation, however, SP activities decline and CD number and SC integrity become similar to adult animals. The newborn rat has specific, glucocorticoid-inducible surface film at birth, the so-called periderm that is lost over the early postnatal period (Hoath *et al*, 1993). Even though this structure might be of biological relevance for barrier-related functions, we did not assess this structure in detail. It is likely that the pH-dependent abnormalities in two key epidermal functions; i.e., permeability barrier homeostasis and SC integrity, would have adverse clinical consequences for neonatal humans. The combination of a decrease in SC integrity, which would decrease frictional resistance, coupled with delayed barrier repair, translates into an increased susceptibility to external mechanical or chemical trauma. Although this propensity is well recognized among clinical neonatologists and pediatric dermatologists, the origin for barrier perturbation and SC dysfunction have not yet been methodologically studied in humans. Moreover, it is well recognized that injuries to the SC lead to the rapid release of cytokines that induce inflammation in adult skin (Nickoloff and Naidu, 1994; Wood *et al*, 1994; Elias *et al*, 1996, 1999), and thus an increased

susceptibility to injury and a deficient repair response might accentuate the risk for and/or severity of skin inflammation. In fact, the concept of "baby skin" conjures up an image of fragility and sensitivity, and newborn exposure to urine and/or feces readily injures the SC resulting in skin inflammation (diaper dermatitis), and these rashes often resolve slowly. Fecal enzymes and urine have been identified as key players in raising SC pH in diapered skin (Berg *et al*, 1986; Andersen *et al*, 1994; Berg *et al*, 1994), and these studies verify the increased susceptibility of neonatal skin to these and other irritants. Fecal enzymes also compromise epidermal barrier function (Buckingham and Berg, 1986), and the pathogens that colonize diapered neonatal skin elaborate abundant proteases. Furthermore, these pathogens grow better at a neutral than at an acidic pH (Korting *et al*, 1987, 1990). Recent data support the role of pathogen colonization in aggravating the irritative symptoms and delayed resolution of diaper dermatitis (Ferrazzini *et al*, 2003). Although propionibacteria grow well at pH 6.0–6.5, growth slows at pH 5.5 (Korting *et al*, 1987, 1990). In contrast, *Staphylococcus aureus* grow best at pH 7.5, but continue to proliferate slowly at pH 5.0–06.0 (Leyden *et al*, 1979; Korting *et al*, 1987, 1990). Thus, the acidic pH of the SC restricts colonization by pathogenic flora, and encourages persistence of normal microbial flora. Pertinently, both intertriginous and inflamed skin display an increased skin pH (Beare *et al*, 1958; Thune *et al*, 1988; Brook, 1992; Yosipovitch *et al*, 1993; Berg *et al*, 1994), as does newborn human SC, which only gradually develops an acidic pH over 1–3 mo of life (Behrendt and Green, 1958; Beare *et al*, 1960; Visscher *et al*, 2000; Giusti *et al*, 2001). Hence, the increased propensity for colonization by normal flora and reduced resistance to pathogens that characterize these settings.

Maternal deprivation, even for a short time, might have an impact on neonatal skin since standardized tactile stimuli leads to an increase in serum lactate persisting for 30 min at birth but only has slight effects on 7-d-old animals (Alasmi *et al*, 1997). In our studies, both vehicle-treated control and inhibitor-treated animals were treated identically so it is unlikely that tactile stimulation could account for our results.

But the developmental delay in acidification in neonates often is complicated by increased hydration and urea/fecal contamination, which can further increase SC pH, with a subsequent increased risk of infection and diaper dermatitis (Aly *et al*, 1978; Brook, 1992; Berg *et al*, 1994; Visscher *et al*, 2000). Thus, SC pH is closely linked to microbial colonization and possible diaper pathogenesis. A logical treatment for diaper dermatitis and/or a protective approach for newborn babies therefore might be to lower SC pH to improve barrier homeostasis and SC integrity. The relationship between pH of SC and barrier function has implications for the care of neonatal skin. In fact, several diaper brands already have acidic agents to lower SC pH.

In summary, this study demonstrates that the elevation in SC pH results in abnormalities in both permeability barrier homeostasis and SC integrity in newborn animals. These abnormalities are likely due to pH-induced modulations in enzyme activity with a decrease in β-Gluc Cer'ase activity, resulting in impaired barrier homeostasis, as well as an increase in SP activity leading to abnormal SC integrity. The regulation of SC pH has a similar local importance in the epidermal barrier as the exquisitely tight regulation of systemic pH does in internal medicine. It is likely that these functional abnormalities have adverse clinical consequences for the newborn.

Materials and Methods

Materials and animal procedures Timed-pregnant Sprague–Dawley rats were obtained from Simonson Laboratories (Gilroy, California) and fed Purina mouse diet and water *ad libitum*. Daily measurements were performed from days 0 to 6 post-birth on animals of both sexes. Barrier function was determined by measurement of transepidermal water loss (TEWL) with an electrolytic water analyzer (MEECO, Warrington, Pennsylvania). 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). Propylene glycol, ethanol, NaOH, and HCl were from Fisher Scientific (Fairlane, New Jersey), whereas glycolic acid (GA), stearic acid (SA), HEPES-buffer, and bromophenacyl bromide (BPB) were from Sigma Chemical Co. (St Louis, Missouri). 1,1,3,3-tetramethylguanidine (TMG) was purchased from Aldrich (Sigma-Aldrich, Milwaukee, Wisconsin). EnzCheck Protease Assay Kit was purchased from Molecular Probes (Eugene, Oregon). The strippings were performed with 22 mm D-Squame 100 tapes, purchased from CuDerm (Dallas, Texas). The animal procedures were approved by the Animal Studies Committee of the San Francisco Veterans Affairs Medical Center and were performed in accordance with the guidelines.

Inhibitor treatment The newborn animals were treated topically twice daily for 3 d with $sPLA_2$ inhibitor BPB (4 mg per mL) in propylene glycol:ethanol (7:3 vol/vol) vehicle, or the vehicle alone on the backs and flanks of the newborn rats, as described previously for hairless mice (Mao-Qiang *et al*, 1996). The same protocol was used with the NHE1 inhibitor HOE694 at a concentration of 4 mg per mL in propylene glycol:ethanol (7:3 vol/vol) vehicle. In order to prevent the mothers from licking the applied substances off the newborn rats, we placed the pups in a plastic container in a 37°C incubator for 2–3 h. The inhibitor dose employed were shown previously to be non-toxic to murine skin (Mao-Qiang *et al*, 1995, 1996), and to inhibit $sPLA_2$ activity in different tissues and cell types (Jain *et al*, 1991; Gelb *et al*, 1994). For the override experiments, animals received co-applications of SA (10 mg per mL), dissolved in propylene glycol:ethanol (7:3 vol/vol) vehicle at the same time that these sites were treated with the $sPLA_2$ inhibitor. The NHE1 antiporter was blocked with the specific inhibitor, HOE 694 (gift from Aventis Pharma, Frankfurt, Germany). Even though the short-term maternal deprivation by placing the pups in an incubator has significant physiological effects e.g. increasing the serum lactate levels in neonatal rats after standardized tactile stimuli (Alasmi *et al*, 1997) this model was the only possible way to study the effects of the topical application of specific inhibitors. It should be noted that both the vehicle-treated controls and inhibitor-treated pups were separated from their mothers.

Neutralization and acidification models Despite the normal decrease in SC surface pH over time in neonatal rats (Fluhr *et al*, 2004), we used several different approaches to further modulate SC pH. For neutralization of the SC, 1% of the superbase TMG with a pK_a of 13.6 was dissolved in propylene glycol:ethanol 7:3 vol/vol (Hachem *et al*, 2003). At this dose TMG neutralizes SC pH without inducing signs of toxicity or inflammation (Hachem *et al*, 2003). Acidification/neutralization experiments were performed with 10 mM HEPES buffer, adjusted to either pH 5.5 and 7.4, as follows: A buffer-soaked gaze was placed over the newborns, changed every 30 min over a period of about 3 h. The newborns

were placed in the 37°C incubator, as described above. Acidification was performed using GA (1%), dissolved in water, and in an additional experiment, HEPES buffer, adjusted to either pH 5.5 or 7.4 (serine protease (SP) activity; *in vivo*). To assess SP activity *in vitro*, frozen sections were incubated with HEPES buffer adjusted to either pH 5.5 or 7.4, then assessed with *in situ* zymography (see below).

Quantification of corneodesmosomes (CD) Newborn rats on their first day of life were treated topically with a single application of phenylmethylsulfonyl fluoride (PMSF) 10 mM in propylene glycol:ethanol (7:3 vol/vol) on both flanks. Controls were treated with a single application of vehicle alone (propylene glycol:ethanol). The 5-d-old pups received three applications of PMSF or vehicle alone over 24 h.

SC integrity and barrier homeostasis SC integrity was assessed as the change in TEWL value after a predefined number of D-Squame tape strippings. To quantify epidermal permeability barrier function and barrier homeostasis, we measured TEWL with an electrolytic water analyzer (MEECO, Warrington, Pennsylvania). After acute barrier disruption by tape stripping or acetone treatment with a TEWL value of about 10–15 $\times$ over baseline, barrier recovery rates were calculated with the following formula: 1−(TEWL immediately after stripping−TEWL at different time points)/(TEWL immediately after stripping−baseline TEWL) $\times$ 100%.

Immunohistochemistry

Desmoglein 1 (DSG 1) expression DSG 1 expression was visualized as described previously (Fluhr *et al*, 2001). Paraffin tissue sections from newborn rat skin were incubated for 30 min in blocking buffer (1% bovine serum albumin, 0.1% cold-water fish gelatin in phosphate-buffered saline, PBS) and then incubated for 2 h at room temperature with 1:500 dilution of a primary, polyclonal, rabbit anti-mouse DSG 1 antibody (gift from Dr John Stanley, University of Pennsylvania), diluted in blocking buffer. The tissue then was washed with PBS and incubated for 1 h at room temperature with goat anti-rabbit IgG Alexa-labeled secondary antibodies (Molecular Probes), diluted in blocking buffer. Either pre-incubation 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. All sections were rinsed with PBS, counterstained with propidium iodide, mounted, and visualized directly in a confocal microscope (Leica TCS SP, Heidelberg, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 518 nm.

Immunolabeling for corneodesmosin Immunolabeling for corneodesmosin was performed as previously described (Haftek *et al*, 1998). After 1 h incubation with a murine monoclonal antibody to corneodesmosin (G36-19; Serre *et al*, 1991) diluted to 10 μg per mL in PBS, the sections were washed 3 $\times$ 5 min in PBS, and the antigen sites detected with the primary antibody were revealed using DAKO LSAB2 System peroxidase/AEC (DAKO Corp., Carpinteria, California), according to the manufacturer's protocol. Although the corneodesmosin epitope reactive with G36-19 antibody is lacking two amino acids in rodents, when compared with humans, the amplification system used in this study has allowed to compensate for a significantly lower affinity in the rat skin.

***In situ* zymography for SP activity** The *In situ* zymography for SP activity was assessed as described previously (Hachem *et al*, 2003). Briefly, biopsies were taken from newborn rats at different postnatal time points and the subcutaneous fat was removed by scraping with a #10 Bard-Parker blade. Frozen sections (5 μm thickness) were rinsed with a washing solution (2% Tween 20 in deionized water) and incubated at 37°C for 2 h with 250 μL of BODIPY-FI-casein in deionized water (2 μL per mL). All sections were rinsed with the washing solution, counterstained with propidium iodide, mounted, and visualized directly in a confocal microscopy (Leica TCS SP) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The control sections were treated with the SP inhibitor, PMSF.

Electron Microscopy Samples for electron microscopy were minced to 1 mm^3 cubes, placed in modified Karnovsky's fixative, and post-fixed in either reduced 1% osmium or buffered 0.2% ruthenium tetroxide with 0.5% ferrocyanide (Hou *et al*, 1991). Samples were then dehydrated in a graded ethanol series, embedded in Epon, and ultra-thin sectioned. Samples were examined in a Zeiss 10A electron microscope (Carl Zeiss, Thornwood, New York) operated at 60 kV. Pictures shown for both light and electron microscopy are representative of the changes observed in three or more samples obtained from at least two different litters, for each treatment.

To quantify CD density, five pictures were taken from one block from one animal in each experiment at $\times$ 12,000 magnification.

The ratio of the total length of intact CD to the total length of cornified envelopes in the first and second cell layers of the lower SC was determined using a planimeter (Morris, 2000).

Statistical analyses Statistical analyses were performed using Prism 3 (GraphPad Software Inc., San Diego, California). Normal distribution was tested before calculating the comparison with an unpaired *t* test. In experiments with more than three groups, an ANOVA was calculated followed by an α-corrected *post hoc* test (Bonferroni). Values are expressed as mean ± SEM. The significance level was set at $p < 0.05$.

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References

Alasmi MM, Pickens WL, Hoath SB: Effect of tactile stimulation on serum lactate in the newborn rat. Pediatr Res 41:857–861, 1997

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

Aly R, Shirley C, Cunico B, Maibach HI: Effect of prolonged occlusion on the microbial flora, pH, carbon dioxide and transepidermal water loss on human skin. J Invest Dermatol 71:378–381, 1978

Andersen PH, Bucher AP, Saeed I, Lee PC, Davis JA, Maibach HI: Faecal enzymes: *In vivo* human skin irritation. Contact Dermatitis 30:152–158, 1994

Arbenz H: Untersuchungen über die pH-Werte der normalen Hautoberfläche. Dermatologica 105:333–354, 1952

Beare J, Cheeseman E, Gailey A, Neill D, Merrett J: The effect of age on the pH of the skin surface in the first week of life. Br J Dermatol 72:62–66, 1960

Beare JT, Cheeseman EA, Gailey AAH, Neill DW: The pH of the skin surface of children with seborrhoeic dermatitis compared with unaffected children. Br J Dermatol 70:233–241, 1958

Behne MJ, Barry NP, Hanson KM, *et al*: Neonatal development of the stratum corneum pH gradient: Localization and mechanisms leading to emergence of optimal barrier function. J Invest Dermatol 120:998–1006, 2003

Behne MJ, Meyer J, Hanson KM, *et al*: NHE1 regulates the stratum corneum permeability barrier homeostasis: Microenvironment acidification assessed with FLIM. J Biol Chem 277:47399–47406, 2002

Behrendt H, Green M: Skin pH pattern in the newborn infant. J Dis Child 95:35–41, 1958

Behrendt H, Green M: Pattern of Skin pH from birth through adolescence. Springfield, IL, USA: Charles C. Thomas, 1971

Berg RW, Buckingham KW, Stewart RL: Etiologic factors in diaper dermatitis: The role of urine. Pediatr Dermatol 3:102–106, 1986

Berg RW, Milligan MC, Sarbaugh FC: Association of skin wetness and pH with diaper dermatitis. Pediatr Dermatol 11:18–20, 1994

Bernstein ET, Hermann F: The acidity on the surface of the skin. NY State J Med 42:436–442, 1942

Blank IA: Measurement of pH of the skin surface. II. pH of the exposed surfaces of adults with no apparent skin lesions. J Invest Dermatol 2:75–79, 1939

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

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

Buckingham KW, Berg RW: Etiologic factors in diaper dermatitis: The role of feces. Pediatr Dermatol 3:107–112, 1986

Denda M, Sato J, Masuda Y, *et al*: Exposure to a dry environment enhances epidermal permeability barrier function. J Invest Dermatol 111:858–863, 1998

Denda M, Sato J, Tsuchiya T, Elias PM, Feingold KR: Low humidity stimulates epidermal DNA synthesis and amplifies the hyperproliferative response to barrier disruption: Implication for seasonal exacerbations of inflammatory dermatoses. J Invest Dermatol 111:873–878, 1998

Doering T, Proia RL, Sandhoff K: Accumulation of protein-bound epidermal glucosylceramides in beta-glucocerebrosidase deficient type 2 Gaucher mice. FEBS Lett 447:167–170, 1999

Draize JH: The determination of the pH of the skin of man and common laboratory animals. J Invest Dermatol 5:77–85, 1942

Ekholm IE, Brattsand M, Egelrud T: Stratum corneum tryptic enzyme in normal epidermis: A missing link in the desquamation process? J Invest Dermatol 114:56–63, 2000

Elias PM, Ansel JC, Woods LD, Feingold KR: Signaling networks in barrier homeostasis. The mystery widens. Arch Dermatol 132:1505–1506, 1996

Elias PM, Wood LC, Feingold KR: Epidermal pathogenesis of inflammatory dermatoses. Am J Contact Dermatol 10:119–126, 1999

Ferrazzini G, Kaiser RR, Hirsig Cheng SK, *et al*: Microbiological aspects of diaper dermatitis. Dermatology 206:136–141, 2003

Fluhr JW, Behne M, Brown BE, *et al*: Stratum corneum acidification in neonatal skin: Secretory phospholipase A2 and the NHE1 antiporter acidify neonatal rat stratum corneum. J Invest Dermatol, 2004

Fluhr JW, Kao J, Jain M, Ahn SK, Feingold KR, Elias PM: Generation of free fatty acids from phospholipids regulates stratum corneum acidification and integrity. J Invest Dermatol 117:44–51, 2001

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

Giusti F, Martella A, Bertoni L, Seidenari S: Skin barrier, hydration, and pH of the skin of infants under 2 years of age. Pediatr Dermatol 18:93–96, 2001

Hachem JP, Crumrine D, Fluhr J, Brown BE, Feingold KR, Elias PM: pH directly regulates epidermal permeability barrier homeostasis and stratum corneum integrity/cohesion. J Invest Dermatol 121:345–353, 2003

Haftek M, Simon M, Kanitakis J, Maréchal S, Claudy A, Serre G, Schmitt D: Expression of corneodesmosin in the granular layer and stratum corneum of normal and diseased epidermis. Br J Dermatol 137:864–873, 1997

Haftek M, Teillon MH, Schmitt D: Stratum corneum, corneodesmosomes and *ex vivo* percutaneous penetration. Microsc Res Technol 43:242–249, 1998

Hardman MJ, Sisi P, Banbury DN, Byrne C: Patterned acquisition of skin barrier function during development. Development 125:1541–1552, 1998

Heuss E: Die Reaktion des Scheisses beim gesunden Menschen. Monatsh Prakt Dermatol 14:343–359, 1892

Hoath SB, Tanaka R, Boyce ST: Rate of stratum corneum formation in the perinatal rat. J Invest Dermatol 100:400–406, 1993

Holleran WM, Ginns EI, Menon GK, *et al*: Consequences of beta-glucocerebrosidase deficiency in epidermis. Ultrastructure and permeability barrier alterations in Gaucher disease. J Clin Invest 93:1756–1764, 1994

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

Horikoshi T, Igarashi S, Uchiwa H, Brysk H, Brysk MM: Role of endogenous cathepsin D-like and chymotrypsin-like proteolysis in human epidermal desquamation. Br J Dermatol 141:453–459, 1999

Hou SY, Mitra AK, White SH, Menon GK, Ghadially R, Elias PM: Membrane structures in normal and essential fatty acid-deficient stratum corneum: Characterization by ruthenium tetroxide staining and x-ray diffraction. J Invest Dermatol 96:215–223, 1991

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

Jolly JHW: pH determinations of the skin: Readings under normal and abnormal conditions. J Invest Dermatol 305–308, 1960

Korting HC, Hubner K, Greiner K, Hamm G, Braun-Falco O: Differences in the skin surface pH and bacterial microflora due to the long-term application of synthetic detergent preparations of pH 5.5 and pH 7.0. Results of a crossover trial in healthy volunteers. Acta Derm Venereol 70:429–431, 1990

Korting HC, Kober M, Mueller M, Braun-Falco O: Influence of repeated washings with soap and synthetic detergents on pH and resident flora of the skin of forehead and forearm. Results of a cross-over trial in health probationers. Acta Derm Venereol 67:41–47, 1987

Leyden JJ, Stewart R, Kligman AM: Updated *in vivo* methods for evaluating topical antimicrobial agents on human skin. J Invest Dermatol 72:165–170, 1979

Lundstrom A, Egelrud T: Stratum corneum chymotryptic enzyme: A proteinase which may be generally present in the stratum corneum and with a possible involvement in desquamation. Acta Derm Venereol 71:471–474, 1991

Lundström A, Serre G, Haftek M, Egelrud T: Evidence for a role of corneodesmosin, a protein which may serve to modify desmosomes during cornification, in stratum corneum cell cohesion and desquamation. Arch Dermatol Res 286:369–375, 1994

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

Marchionini A, Hausknecht W: Säuremantel der Haut und Bakterienabwehr. Klin Wochenschr 17:663–666, 1938

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

Morris RE: The use of nonparametric statistics in quantitative electron microscopy. J Electron Microsc (Tokyo) 49:545–549, 2000

Nickoloff BJ, Naidu Y: Perturbation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. J Am Acad Dermatol 30:535–546, 1994

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 ichthyosi: A clue to the molecular origin of the "acid skin mantle"? J Invest Dermatol 111:674–677, 1998

Puhvel SM, Reisner RM, Sakamoto M: Analysis of lipid composition of isolated human sebaceous gland homogenates after incubation with cutaneous bacteria. Thin-layer chromatography. J Invest Dermatol 64:406–411, 1975

Rogers J, Watkinson A, Harding CR: Characterisation of the effects of protease inhibitors and lipids on human stratum corneum chymotrytic-like enzyme supports a role in desquamation. J Invest Dermatol 110:672, 1998

Sato J, Denda M, Ashida Y, Koyama J: Loss of water from the stratum corneum induces epidermal DNA synthesis in hairless mice. Arch Dermatol Res 290:634–637, 1998

Schade HMA: Zur physikalischen Chemie der Hautoberfläche. Arch Dermatol Syphil 154:690–716, 1928

Serre G, Mils V, Haftek M, *et al*: Identification of late differentiation antigens of human cornified epithelia, expressed in re-organized desmosomes and bound to cross-linked envelope. J Invest Dermatol 97:1061–1072, 1991

Sondell B, Thornell LE, Stigbrand T, Egelrud T: Immunolocalization of stratum corneum chymotryptic enzyme in human skin and oral epithelium with monoclonal antibodies: Evidence of a proteinase specifically expressed in keratinizing squamous epithelia. J Histochem Cytochem 42:459–465, 1994

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

Thune P, Nilsen T, Hanstad IK, Gustavsen T, Lovig Dahl H: The water barrier function of the skin in relation to the water content of stratum corneum, pH and skin lipids. The effect of alkaline soap and syndet on dry skin in elderly, non-atopic patients. Acta Derm Venereol 68:277–283, 1988

Visscher MO, Chatterjee R, Munson KA, Pickens WL, Hoath SB: Changes in diapered and nondiapered infant skin over the first month of life. Pediatr Dermatol 17:45–51, 2000

Williams ML, Elias PM: Ichthyosis. Genetic heterogeneity, genodermatoses, and genetic counseling. Arch Dermatol 122:529–531, 1986

Wood LC, Feingold KR, Sequeira-Martin SM, Elias PM, Grunfeld C: Barrier function coordinately regulates epidermal IL-1 and IL-1 receptor antagonist mRNA levels. Exp Dermatol 3:56–60, 1994

Yosipovitch G, Maayan-Metzger A, Merlob P, Sirota L: Skin barrier properties in different body areas in neonates. Pediatrics 106:105–108, 2000

Yosipovitch G, Tur E, Cohen O, Rusecki Y: Skin surface pH in intertriginous areas in NIDDM patients. Possible correlation to candidal intertrigo. Diabetes Care 16:560–563, 1993

Zlotogorski A: Distribution of skin surface pH on the forehead and cheek of adults. Arch Dermatol Res 279:398–401, 1987