Stratum Corneum Acidification Is Impaired in Moderately Aged Human and Murine Skin

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Aged skin commonly is afflicted by inflammatory skin diseases or xerosis/eczema that could be triggered or exacerbated by impaired epidermal permeability barrier homeostasis. This defect is linked to reduced epidermal lipid synthesis in humans and in mice of advanced age (i.e., >75 years in human or >18–24 months in mice). We now report that barrier defects in moderately aged humans (50–80 years) or analogously aged mice (12–15 months) are linked instead to defective stratum corneum (SC) acidity. In moderately aged mouse epidermis, we find that abnormal acidification, in turn, is linked to decreased Na^+/H^+ antiporter (NHE1) expression. Decreased NHE1 levels lead to increased SC pH, which results in defective lipid processing and delayed maturation of lamellar membranes, due to suboptimal activation of the pH-sensitive essential, lipid-processing enzyme, β-glucocerebrosidase. Conversely, impaired SC integrity in moderately aged mice is due to increased pH-dependent activation of serine proteases, leading to premature degradation of corneodesmosomes. These abnormalities were normalized by exogenously acidifying the SC, suggesting a basis for the well-known acidification therapies that are widely used to treat the pathologic xerosis/eczema seen in moderately aged humans.

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INTRODUCTION

The epidermal permeability barrier is formed by two stratum corneum (SC) compartments: intact, protein-enriched corneocytes (the ''bricks'') interspersed within a lipid-enriched extracellular matrix that is organized into functional membrane bilayers (the ''mortar'' (Elias, 1983)). Formation of competent lipid bilayers requires the sequential: (1) epidermal lipid synthesis; (2) secretion of epidermal lamellar body (LB) lipids at the stratum granulosum (SG)–SC interface; followed by (3) the extracellular processing of the secreted polar lipid precursors into a hydrophobic mixture that forms functionally competent lamellar membranes. Although secretion is regulated by changes in extracellular Ca^{2+} and K^+ concentrations (Menon *et al.*, 1985; Mauro *et al.*, 1998a), lipid processing is controlled by the pH of the extracellular spaces (Mauro *et al.*, 1998b). Lipid processing requires two acidic-dependent lipid hydrolases: β-glucocerebrosidase (BGC) and acid sphingomyelinase (β-GlcCer'ase, aSM'ase), which are activated by extracellular acidity (Mauro *et al.*, 1998b; Hachem *et al.*, 2003, 2005a, b), but become inactive as the pH increases (Jensen *et al.*, 1999; Takagi *et al.*, 1999; Schmuth *et al.*, 2000), compromising permeability barrier homeostasis (Mauro *et al.*, 1998b; Behne *et al.*, 2002; Fluhr *et al.*, 2004a; Hachem *et al.*, 2003, 2005a, b). Conversely, increased pH activates a different family of enzymes, the serine proteases, which degrade corneodesmosomes (CDs), measured functionally as a decrease in SC integrity (Hachem *et al.*, 2003).

Although the SC acidic pH had been attributed to exogenous mechanisms (Korting *et al.*, 1987; Ohman and Vahlquist, 1998; Krien and Kermici, 2000; Patterson *et al.*, 2000), recent studies instead show that the SC is acidified largely by endogenous agents (Fluhr *et al.*, 2003, 2004a), including: (1) the Na^+/H^+ antiporter, NHE1 (Behne *et al.*, 2002, 2003b); and (2) one or more secretory phospholipase A2 (sPLA2) enzymes (Mao-Qiang *et al.*, 1996; Mazereeuw-Hautier *et al.*, 2000; Fluhr *et al.*, 2001, 2004a, b). Inhibiting either of these intrinsic mechanisms elevates SC pH, and inhibits lipid processing, resulting in altered epidermal permeability barrier homeostasis.

Xerosis, pruritus, and contact dermatitis increase progressively with human aging (Wilhelm *et al.*, 1991; Manuskiatti *et al.*, 1998). Both barrier recovery kinetics and SC integrity become highly abnormal with advanced age (Ghadially *et al.*, 1995; Reed *et al.*, 1997). These studies were performed in advanced aged humans and their murine counterparts (>75 years; 18–24 months), where decreased SC barrier function could be traced to a global decrease in SC lipids (Ghadially *et al.*, 1995, 1996; Zettersten *et al.*, 1997). We

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Abbreviations: BGC, β-glucocerebrosidase; cUCA, cis-urocanic acid; FLIM, fluorescence life-time imaging; SC, stratum corneum; SG, stratum granulosum

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report here that both moderately aged humans (50–80 years) and moderately aged mice (12–15 months) suffer from impaired SC acidification. Using mice as a model, we show that moderately aged mice also demonstrate defects in epidermal barrier recovery. Unlike advanced age mice, however, this barrier defect is due to impaired SC acidification leading to decreased lipid processing, not abnormal lipid synthesis. Moreover, moderately aged mice also display pH-dependent alterations in SC integrity, which could be further attributed to accelerated, pH-dependent degradation of CDs.

RESULTS

SC acidification is defective in moderately aged humans and mice

We first examined whether SC acidification is defective in moderately aged humans and mice. Using a flat surface electrode, we found that the surface pH of moderately aged SC increases progressively in aged humans beginning at about age 50 (Figure 1a) Although SC pH was slightly more acidic in males *versus* females of both age groups, SC acidity decreased in both groups as they aged (Figure 1b). SC acidification also was defective in a murine model, where surface pH in moderately aged mice (12–15 months) also was less acidic than in younger mice (8–12 weeks) (Figure 1c).

To assess the potential utility of moderately aged mice as a model for mechanistic studies (surface pH in mice), we next localized differences in SC pH, using fluorescence lifetime imaging (Behne *et al.*, 2002, 2003b). We found that pH is higher throughout the SC in moderately aged *versus* young mouse SC (Figure 2). The pH differences between young and moderately aged SC became apparent at the level where the outermost nucleated layers of epidermis transition into the lowest level of the SC (6–8 μm), and continue to diverge further towards the skin surface (Figure 2). These data demonstrate that SC acidification is impaired in moderately aged humans and mice. We next tested whether defective SC acidification leads to functional epidermal barrier defects.

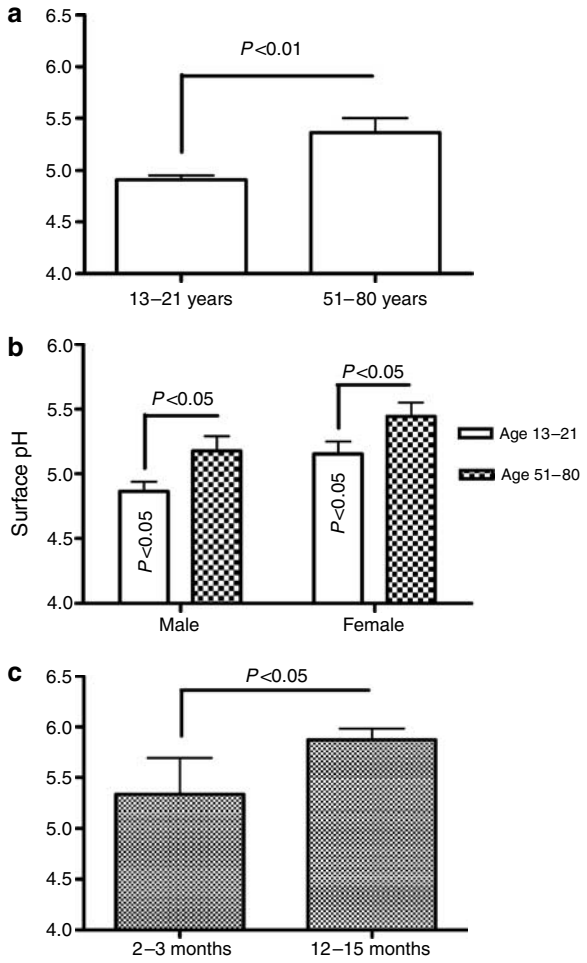

Figure 1. SC pH is less acidic in moderately aged humans and mice. (**a**) Surface pH was measured in unperturbed skin, using a flat electrode (see Materials and Methods) from the volar forearms of moderately aged humans (51–80 years, mean age 55.6 years, $n=55$) *versus* young (postpubertal) humans (13–21 years, mean age 20.2 years, $n=65$, $P<0.05$). Although SC pH was slightly more acidic in males *versus* females of both age groups, (**b**) SC acidification was less effective in both genders with aging. (**c**) SC surface pH measured from flanks also was less acidic in moderately aged (12–15 months) *versus* young (2–3 months) male mice ($n=4$–5 mice in each group, $P<0.05$). Data are presented as the mean $\pm$ SE.

Permeability barrier recovery is impaired in moderately aged epidermis

We next assessed whether permeability barrier recovery is impaired after acute insults in moderately aged mice. As shown in Figure 3, the kinetics of barrier recovery in moderately aged mice is delayed significantly at 6 hours after acute disruption in comparison to recovery rates in young mice of the same strain. To test our hypothesis that the pH abnormality is the primary defect that accounts for the barrier and integrity defects in moderately aged skin, we next assessed whether exogenous acidification would normalize rates of barrier recovery in moderately aged mice. We used lactobionic acid (LBA) because this agent has been shown to acidify the entire SC, without toxic effects on the SC or viable epidermis (Hachem *et al.*, 2005a). A single application of exogenous LBA (pKa 3.2; 10% in propylene glycol:ethanol 7:3 v/v, pH 2), which acidifies the surface pH to approximately pH 4.5 (Hachem *et al.*, 2005a), restored the kinetics of barrier recovery to rates comparable to young mice (Figure 3).

After acute barrier disruption by acetone, the surface pH increases in both young and moderately aged mice, largely returning toward normal in young mice by 12–18 hours (Hachem *et al.*, 2005a). Consistent with the impaired barrier recovery kinetics reported above, surface pH remained elevated at 12 hours: while young adult surface pH measured 5.97 ± 0.20 ($n=5$), moderately aged surface pH was still 6.28 ± 0.19 ($n=5$; $P<0.05$).

Abnormal lipid-processing accounts for the permeability barrier abnormality in moderately aged skin

We next assessed the basis for the permeability barrier abnormality in moderately aged skin. In contrast to advanced

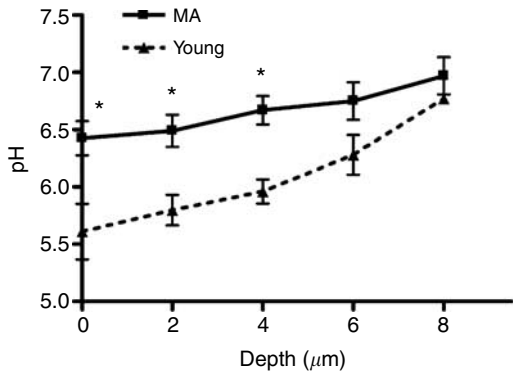

Figure 2. pH increases at all levels of moderately aged *versus* young SC. Skin biopsies were taken from three young (8–12 weeks) and three moderately aged (12–15 months) mice, and extracellular pH measured in unperturbed SC at increasing depths from the surface using two-photon microscopy and fluorescence lifetime imaging (see Materials and Methods). Data are presented as the mean ± SE. A 0 μm depth denotes SC surface, whereas 8 μm is the depth of the SC–SG interface. *$P<0.05$.

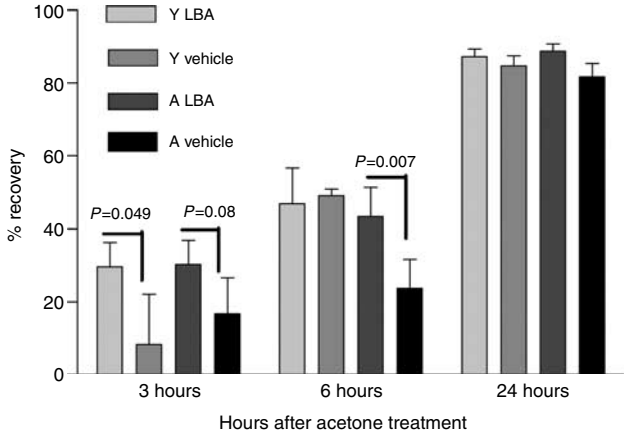

Figure 3. Barrier recovery is delayed in moderately aged epidermis, but normalized by reacidification. (**a**) Moderately aged (12–15 months) and young (Y) (8–12 weeks) hairless mice were treated with acetone to perturb the epidermal permeability barrier (transepidermal water loss >4 mg (cm^2/hour)); then LBA was applied to selected young (YLBA) or aged (ALBA) animals to acidify the SC. Vehicle-treated mice served as controls. Barrier recovery consistently lagged in moderately aged mice at 6 hours ($P=0.007$) after barrier perturbation. Three hours after barrier perturbation, LBA improved barrier recovery in both aged (ALBA) and young (YLBA) SC compared to vehicle controls (Y and A vehicle) confirming that neither group of animals can acidify its SC effectively at this time point (Hachem *et al.*, 2005a). There was no difference in barrier recovery at 3 hours in aged *versus* young vehicle-treated mice. In contrast, exogenous acidity improved barrier recovery in aged SC at 6 hours, implying that moderately aged SC cannot effectively reacidify its SC by this time point. Thus, exogenous acidification normalizes permeability barrier homeostasis in moderately aged SC. Because both young and aged SCs recover by 24 hours, it is likely that the barrier defect in moderately aged SC can be attributed to a delay in, rather than an absence of, SC acidification. Data are presented as the mean ± SE.

age skin, epidermal lipid synthesis in moderately aged (MA) murine epidermis was comparable to lipid synthetic rates in young (Y) epidermis, as assessed by morphologic and biochemical methods. First, the density of LBs in the cytosol

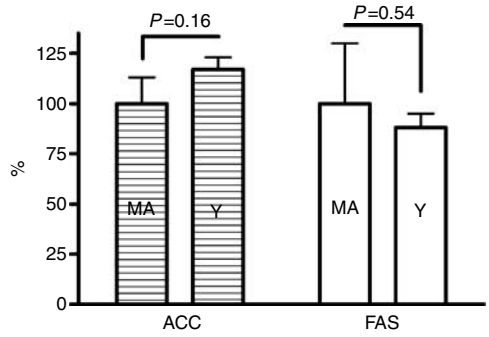

Figure 4. Epidermal lipid synthesis is normal in moderately aged mice. RNA was isolated from untreated epidermis from flanks of three moderately aged and three young mice. ACC and FAS levels were measured by quantitative PCR. Data are presented as the mean ± SE. No statistically significant difference was found between ACC or FAS synthesis in moderately aged *versus* young mice.

of granular cells, as assessed by electron microscopy, was unimpaired in moderately aged epidermis. Quantitative results of LB density (mean ± SE) were 13.92 ± 5.61(MA) *versus* 15.13 ± 4.76(Y) ($P=0.23$), and LB secretion were 2.0 ± 0.81(MA) *versus* 2.31 ± 0.83(Y) ($P=0.13$) ($n=6$). The volume densities of LB were measured by counting the LB in the cytosol of the uppermost two layers of the SG and expressed as average number per unit area of cytosol (Choi *et al.*, 2005) Secondly, we compared the levels of the key enzymes that control cholesterol and fatty acid synthesis in the skin, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Harris *et al.*, 1997). We found that RNA levels of both enzymes were comparable in moderately aged *versus* young skin (Figure 4), demonstrating that, unlike advanced aged skin (Ghadially *et al.*, 1995, 1996; Zettersten *et al.*, 1997), lipid synthesis is not impaired in moderately aged SC.

Hence, we next assessed whether defective lipid processing, particularly during re-establishment of the barrier after acute perturbation, accounts for the permeability barrier abnormality in moderately aged SC. An ultrastructural analysis of ruthenium tetroxide postfixed tissues, taken 6 hours after barrier perturbation, revealed delayed formation of ''mature'' (=fully processed) lamellar membranes in moderately aged epidermis treated with vehicle (Figure 5b). In contrast, in the SC of epidermis-treated LBA, an exogenous acidification, mature lamellar membranes begin to form within the SG–SC interface, and predominate by the first and second SC interspaces (Figure 5c). To ascertain whether the extracellular pH abnormality specifically impacts lipid-processing enzymes, we next examined whether BGC activity is diminished in moderately aged SC. Consistent with the structural and functional abnormalities described above, BGC activity, assessed by *in situ* zymography, is decreased in moderately aged SC (Figure 6). Because BGC activity normalized in moderately aged SC after exogenous acidification (Figure 6), pH-dependent changes in enzyme activity, rather than decreased BGC amount, appear to account for the diminished barrier function in aged SC.

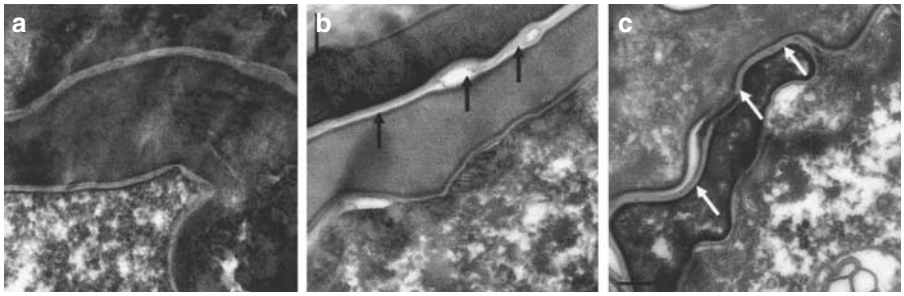

Figure 5. Abnormal lipid processing in moderately aged SC is reversed by reacidification. Moderately aged hairless mice were treated with acetone to perturb their epidermal barrier. Biopsies were obtained 6 hours after treatment, and analyzed with electron microscopy, using ruthenium tetroxide postfixation to visualize processing of lipid into bilayers. (**a**) Untreated normal skin of young mice shows normal appearance of SC intercellular lamellar structures. (**b**) Control moderately aged animals treated with vehicle display abnormal, poorly processed lipid (black arrows). (**c**) LBA acidification treatment, however, normalizes lipid processing into competent lipid bilayers (white arrows) in moderately aged SC. Bar = 0.2 μm.

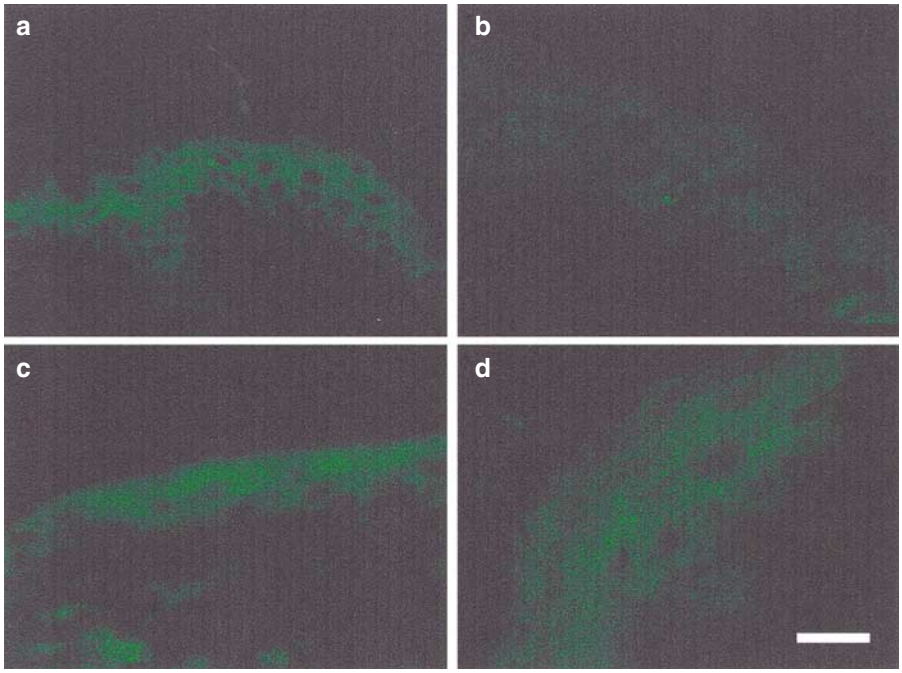

Figure 6. Decreased β-GlcCer'ase activity alone accounts for abnormal lipid processing in moderately aged epidermis. Unperturbed moderately aged and young mouse skin was assayed with enzyme zymography for β-GlcCer'ase (see Materials and Methods). (**a**) Young SC treated with vehicle (control) displayed robust β-GlcCer'ase activity (green staining), which was mildly increased after (**c**) exogenous acidification. In contrast, β-GlcCer'ase activity was almost absent in (**b**) control aged SC, but was increased after (**d**) LBA pretreatment, suggesting that β-GlcCer'ase amounts are not substantially decreased in aged SC, because comparable activity is seen after exogenous acidification. The same exposure time was used for both moderately aged and young skin samples, so that relative fluorescence intensity is a true measure of relative enzyme activity. Bar = 50 μm.

Abnormal SC integrity in moderately aged SC correlates with accelerated CD degradation by serine proteases

SC pH likewise controls the rate at which CDs are degraded, which in turn, is linked to SC integrity. We therefore compared SC integrity in moderately aged *versus* young mice. We found that SC integrity, assessed as the level of transepidermal water loss reached after five sequential tape strippings, is defective in moderately aged animals (Figure 7).

We next compared the density and structure of CDs in the SC of moderately aged *versus* young mice. As shown in Figure 8, the density of CDs decreases in moderately aged SC in comparison to young controls. Whereas CDs persist to the mid-SC in young SC (Figure 8a), CDs begin to disappear by the first intercellular space above the SG–SC interface in moderately aged SC (Figure 8b). Quantitative analysis for CDs showed a significant decrease in moderately aged mice *versus* young mice (mean $\pm$ SE = 26.89 $\pm$ 4.77% *vs* 33.22 $\pm$ 7.74%) ($n = 6$, $P = 0.033$). Thus, the age-related abnormality in SC integrity is linked to premature degradation of SC CDs.

SC CDs are degraded by several types of proteases. As the elevated SC pH of moderately aged skin would be expected to increase serine protease activity (Hachem *et al.*, 2003), we next compared serine protease activity in moderately aged *versus* young mice by *in situ* zymography. As seen in Figure 9, serine protease activity increases in moderately aged *versus* young SC. Because enzyme activity readily

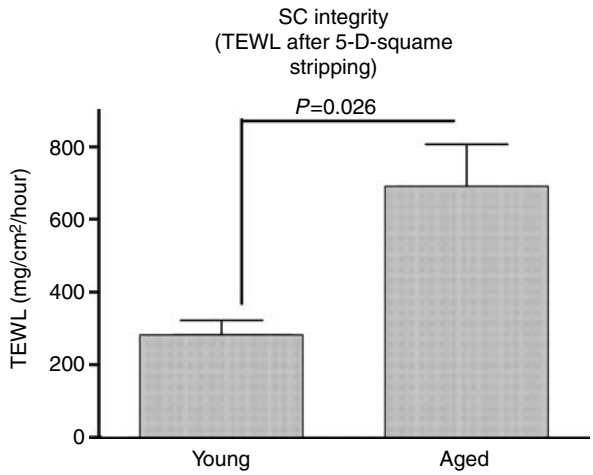

Figure 7. SC integrity is abnormal in moderately aged SC. Moderately aged (12–15 months) and young (8–12 weeks) hairless mice were treated with sequential D-squame strippings to perturb the SC, and transepidermal water loss was measured after each stripping as a measure of integrity. After five D-squame strippings, SC integrity was severely compromised in moderately aged SC, whereas damage to SC integrity in young SC was minimal. Data are presented as the mean ± SE.

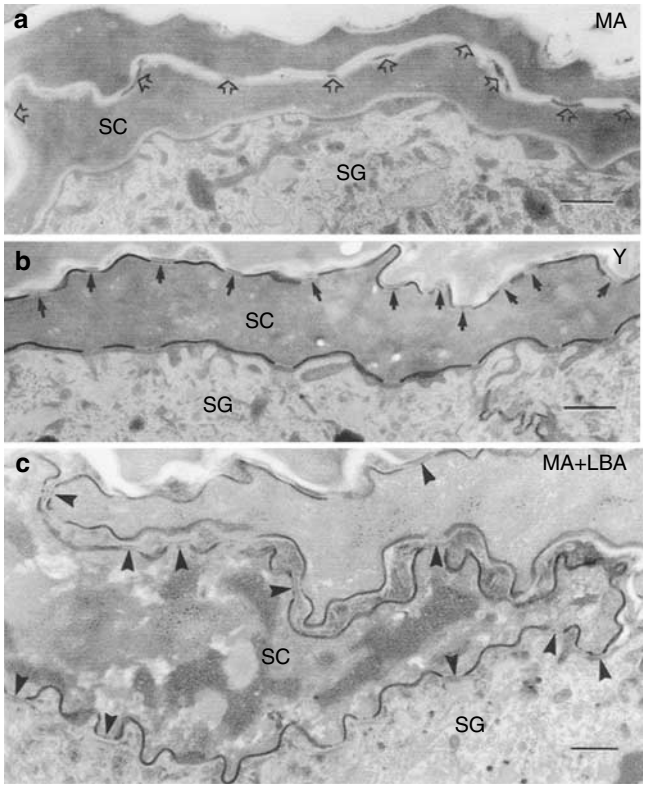

Figure 8. Premature degradation of CDs in moderately aged murine SC is reversed by reacidification. (**a** and **b**) Whereas CDs (solid arrows) are largely intact one layer up into SC in (**b**) young mouse epidermis, CDs appear to be disintegrating prematurely (open arrows) at the same level in (**a**) moderately aged mouse epidermis. After topical treatment with the polyhydroxyl acid, LBA and CDs (arrowheads) reappear in (**c**) SC of moderately aged mice. Osmium tetroxide post-fixation; bar = 0.5 μm.

normalizes with exogenous reacidification, the increased SP activity in moderately aged SC is likely due to changes in enzyme activity alone, rather than decreased amounts of this enzyme. This defect in SC integrity in moderately aged epidermis, associated with an increase in the activity of serine proteases, is consistent with the known activation of these enzymes at a neutral pH (Brattsand and Egelrud, 1999; Brattsand *et al.*, 2005). Together, these results suggest that accelerated CD degradation, due to increased serine protease activity, accounts for diminished SC integrity in moderately aged murine skin.

Decreased NHE1 accounts for the pH abnormality in moderately aged epidermis

Extracellular pH at the SG–SC interface and in the lower SC is largely regulated by the NHE1 (Behne *et al.*, 2002). Because this is the site where BGC and the serine proteases become active, we next investigated whether a developmental decrease in NHE1 expression occurs in moderately aged epidermis, accounting for the abnormal acidification of SC extracellular domains. As shown in Figure 10, immunostaining for NHE1 in the outer epidermis appears much lower in moderately aged than in young epidermis. This finding suggests that the pH abnormality in moderately aged epidermis can be ascribed, at least in part, to a decrease in NHE1 expression.

DISCUSSION

Although it has long been known that aged skin is at greater risk for symptomatic xerosis, severe pruritis, and an increased propensity to develop contact dermatitis, only recently has it been appreciated that abnormal permeability barrier function might underlie these skin conditions (Barland *et al.*, 2005). Initial studies focused on defects in markedly aged human skin (>75 years in humans (Ghadially *et al.*, 1995) or >18 months in mice (Barland *et al.*, 2004)). Although studies of mice near the end of their life expectancy (18–24 months) (Ghadially *et al.*, 1995) display decreased lipid synthesis and secretion as the primary defect (Ghadially *et al.*, 1995, 1996; Zettersten *et al.*, 1997), we found here that mice aged 12–15 months, and humans aged above 50, also suffer from an independent defect in SC acidification. Like their more elderly counterparts, these moderately aged mice also display a permeability barrier abnormality. But in contrast to the previously described mice of advanced age, these moderately aged mice display normal lipid synthesis and secretion. Instead, the basis for their barrier defect is a delay in lipid processing due to defective acidification of the SC. Because the acidification abnormality does not improve with further aging (Figure 1), advanced age epidermis (Ghadially *et al.*, 1995, 1996; Zettersten *et al.*, 1997; Choi *et al.*, 2002) is likely to display a combination of lipid synthetic and processing abnormalities, although lipid processing has not yet been studied in this age group.

In these mice, as is likely to be the case in moderately aged humans, we showed that delayed lipid processing results from impaired SC acidification. Abnormal SC acidification could provoke multiple defects in epidermal

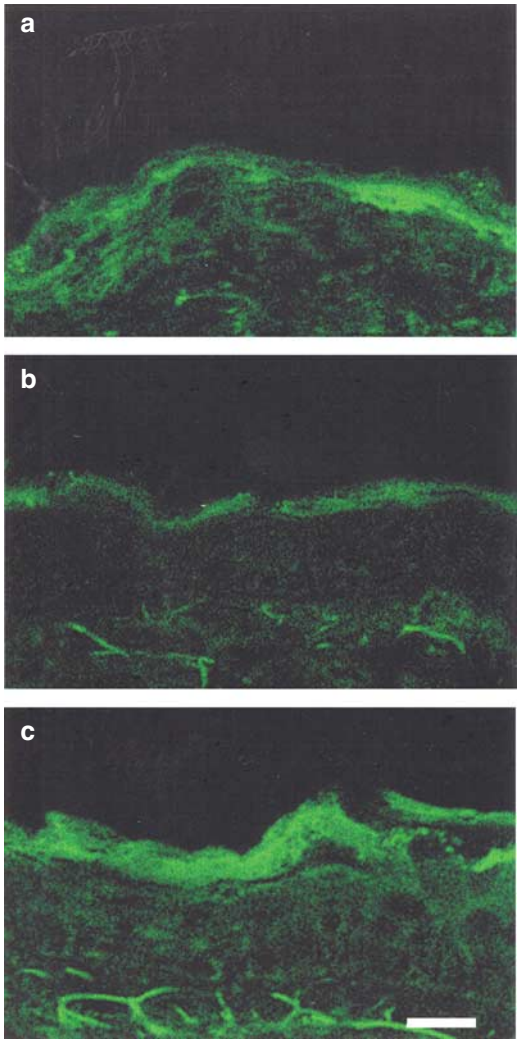

Figure 9. Serine protease activity increases in moderately aged murine epidermis. Serine protease (SP) activity was compared in unperturbed (**a**) moderately aged and (**b**) young SC by enzyme zymography. The exposure times are same in both samples. (**b**) Young SC, treated with vehicle alone, displayed little SP activity (green), compared with (**a**) moderately aged SC treated with vehicle alone. Increased SP activity in aged SC is likely the result of increased acidity, rather than altered enzyme protein levels, because activity in young SC increases to match aged SC, when the pH of young SC is raised to levels comparable to aged skin with a topical superbase; (**c**) tetramethylguanidine. Bar = 20 μm.

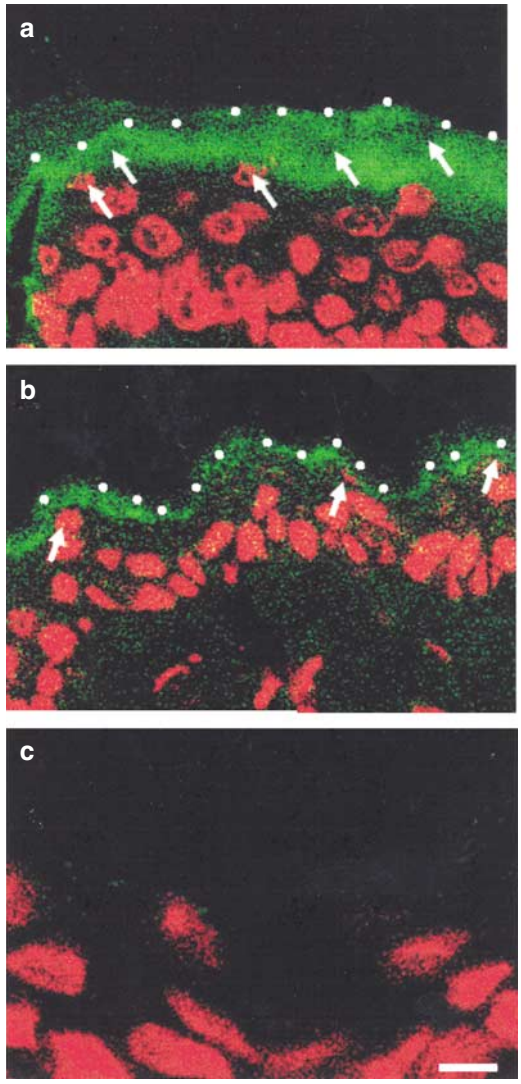

Figure 10. NHE1 expression is decreased in moderately aged *versus* young murine epidermis. NHE1 expression was assessed with immunohistochemistry, using an antibody specific for NHE1 (see Materials and Methods). Dotted lines indicate the SG/SC border. Arrows denote SG keratinocyte nuclei surrounded by plasma membranes that stain positively for NHE1. (**a**) NHE1 staining (green) in normal young skin was expressed most abundantly in the SG, with less staining in the basal layer and stratum spinosum. (**b**) Substantially less NHE1 protein is expressed in moderately aged epidermis. (**c**) Negative control staining in which the primary antibody was omitted demonstrated only background staining. Epidermal keratinocyte nuclei are counterstained with propidium idodide (red). Bar = 20 μm.

function, including delayed permeability barrier recovery after external perturbations and abnormal SC integrity, as confirmed here.

We show here first that the discrete and distinctive defect in lipid processing and lamellar membrane maturation in moderately aged mice is attributable to abnormal SC acidification. Abnormal SC acidification can provoke multiple defects in epidermal function, including delayed permeability barrier recovery after external perturbations, and abnormal SC integrity, as shown in young adult mice (Hachem *et al.*, 2003), and confirmed here for moderately aged mice. Moreover, our results show that decreased SC acidity downregulates pH-activated BGC enzyme activity, required for processing secreted lipids into functional lipid bilayers (Koone *et al.*, 1990; Holleran *et al.*, 1993). In addition, the increase in SC pH impairs SC integrity by upregulating the pH-sensitive serine proteases, which in turn, accelerate the degradation of SC CDs (Hachem *et al.*, 2003). We find further that each set of enzyme activity normalizes when SC pH is externally reacidified. Finally, and most importantly, we assessed directly whether the increases in SC pH are responsible for the functional abnormalities by testing whether these abnormalities normalize when SC pH is restored with exogenous acidification. Because acute

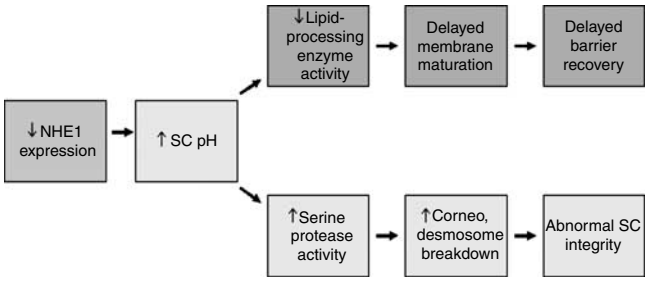

Figure 11. Proposed pathways leading to abnormal function in moderately aged epidermis.

restoration of a normally acidic pH (i.e., to levels found in younger mice) normalizes barrier recovery in these moderately aged mice, the defect in SC acidification clearly underlies the functional defects in these animals. The putative causes and consequences of decreased SC acidity in moderately aged SCs are summarized in Figure 11.

Recent studies assign most of the responsibility for SC acidification to two intrinsic mechanisms: (1) the NHE1, and (2) one or more isoforms of sPLA2 enzyme, which hydrolyzes membrane phospholipids, thereby generating acidifying free fatty acids (Behne *et al.*, 2002; Fluhr *et al.*, 2003, 2004a). We focused on NHE1 expression because: (1) the abnormality in SC pH occurs in the lower SC, where the NHE1 has been shown previously to acidify extracellular microdomains (Behne *et al.*, 2002); and (2) because NHE1 protein levels decrease after the first week of life (Fluhr *et al.*, 2003; Behne *et al.*, 2003a, b), whereas another acidification mechanism, $sPLA_2$, increases postnatally (Fluhr *et al.*, 2004a). In young adult epidermis, NHE1 localizes to the plasma membrane and is expressed most abundantly in keratinocytes of the SG, which abut extracellular domains in the lower SC where lipid processing is initiated (Behne *et al.*, 2003b). In contrast, NHE1 expression is markedly decreased in these sites in moderately aged mice. Although another endogenous mechanism, sPLA2, also contributes to SC acidification, in this report we concentrated on NHE1, because decreased NHE1 is sufficient to impair lipid processing and epidermal barrier homeostasis in both perinatal and young adult SC (Behne *et al.*, 2002, 2003a, b). However, further studies will be required to delineate whether altered sPLA2 activity also contributes to the functional abnormalities in moderately aged epidermis.

MATERIALS AND METHODS

Materials

Propylene glycol, ethanol, and HCl were from Fisher Scientific (Fairlane, NJ), whereas 1,1,3,3-tetramethylguanidine superbase and LBA polyhydroxyl acid were from Sigma Chemicals (St Louis, MO and Bornem, Belgium). 2′,7′-Bis-(2-carboxyethyl)-5(6)-carboxyfluorescein) was purchased from Molecular Probes (Eugene, OR). Stock (100 μM) was prepared by dissolving in phosphate-buffered saline (PBS). Primary rabbit anti-goat antibodies against mammalian NHE1 were purchased from Alpha Diagnostic International (San Antonio, TX). Goat anti-Rabbit Alexa®-labeled secondary antibodies were purchased from Molecular Probes (Leiden, The Netherlands). D-Squame-100 tapes of 22 mm were purchased from CuDerm (Dallas, TX). Bradford protein assay kits (Bio-Rad Protein Assay Dye), as well as lyophilized, bovine serum albumin were purchased from Bio-Rad (Hercules, CA and Nazareth Eke, Belgium). 4-Methylumbelliferone, 4-methylumbelliferyl-β-D-glucoside were obtained from Sigma Chemical Co. Bromoconduritol B epoxide was a gift from Dr G Legler (Köln University, FRG), whereas conduritol B epoxide was obtained from Toronto Research Chemicals (Ontario, Canada).

Humans

The human studies in Chinese subjects were carried out according to the Helsinki Declaration Principles and according to a protocol approved by the Scientific Research Committee of Dalian Skin Disease Hospital. Participants gave their written informed consent. Moderately aged humans (51–80 years, mean age 55.6 years, $n = 55$) were compared to young (postpubertal) humans (13–21 years, mean age 20.2 years, $n = 65$).

Animals

Male hairless mice (Skh1/Hr), 6 to 8-week old, were purchased from Charles River Laboratories (IFFA credo, Brussels, Belgium) and fed Purina mouse diet and water *ad libitum*. Hairless mice at 8–12 weeks or 12–15 months were used at the time of the experiments. Cages were changed 1 day before the experiment. All procedures were performed while mice were anesthetized with chloral hydrate under protocols approved by the San Francisco Veterans Affairs Medical Center and the University of California San Francisco Ethical Committees. Mouse and human analogous ages were extrapolated from optimal lifespans (approximately 120 years in humans and 21–24 months in mice).

Functional studies

SC integrity. The SC integrity was determined using sequential tape stripping with D-Squames (standard D-Squames, CUDERM, Dallas, TX). After cleaning the skin with ethanol, D-Squames were applied to the test areas for about 5 seconds, removed, and stored at 7°C. The amount of SC removed by sequential D-squame application was quantified using a protein assay (Bio-Rad Protein-Assay-Kit, Bio-Rad Laboratories, Munich, Germany). Briefly, D-Squames were first incubated in an incubation shaker at 120 r.p.m. with 1 M NaOH at 37°C. After 1 hour, the solution was neutralized with 10 ml 1 M HCl. Aliquots of 0.8 ml were then incubated with 0.2 ml of the Bio-Rad protein dye for 10 minutes and transferred in microcuvettes. Absorption at 595 nm was measured with a photospectrometer (Jasco V-530, Gross-Umstadt, Germany).

Barrier disruption. Barrier disruption was induced by either sequential cellophane or D-Squame tape stripping or by rubbing the skin surface with acetone-soaked cotton balls on normal hairless mice skin (transepidermal water loss rates $\geqslant 4$ mg/cm^2/hour). Immediately after disruption, hairless mice flanks were topically treated with a single application of LBA (10%) on a 5–6 cm^2 surface area. Control animals were prepared identically and treated with propylene glycol:ethanol.

Surface pH. SC surface pH was measured with a flat, glass surface-electrode from Mettler-Toledo (Giessen, FRG), attached to a pH meter (PH 900; Courage & Khazaka, Cologne, FRG). Surface

pH was measured from the volar forearm in all humans and from flanks in all mice.

Two-photon fluorescence lifetime imaging microscopy

Skin biopsies were taken from three young (8–12 weeks) and three moderately aged (12–15 months) mice. Animals were anesthetized with chloral hydrate, and 20–30 μl of 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (50 μM made by adding 95% ethyl alcohol to stock) was applied to a small region ($\sim$0.25 cm^2) on the flank skin of the animal, four times at 10 minutes interval. The animal was then killed, and the 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein-incubated skin was harvested. Samples were viewed on a Zeiss LSM 510 (Carl Zeiss AG, Germany) inverted microscope equipped with Ti:Sapphire (Spectra-Physics, Mountain View, CA) laser as two-photon source. A single-photon counting module SPC830 (Becker Hikle GmbH, Germany) was used for fluorescence lifetime measurement. A long-pass dichroic blocking infrared light and a BG-39 interference filter (both supplied by Chroma Technologies) were added in the epi-fluorescence light path. The laser was operated mode-locked at 800 nm for two-photon excitation. Images were collected at 512 $\times$ 512 pixels. To obtain sufficient photons at each pixel for the exponential decay curve, an average of 20 scans were accumulated for each image. SC location was monitored both by measuring depth from the skin surface, and also by noting the characteristic morphology of nucleated epidermal cells, which appeared at 8–12 μm depth on average. Calibration was performed with powdered SC, suspended in aqueous solution at a range of pH. SC was obtained by incubation of full-thickness mouse skin in 0.5% trypsin (in H_2O) at 4°C overnight. Dermis and nucleated layers of epidermis came off sequentially; the remaining SC was then immersed in liquid nitrogen, and powdered and a drop of suspension at each pH was mounted on microscope slides and analyzed. The resulting calibration curve of fluorescence lifetime *versus* pH showed a nonlinear sigmoidal form. Image J (Freeware, Research Services Branch, National Institute of Health) was used for post-experimental data processing. Extracellular regions of interest were selected visually, and 3–5 regions were examined at each depth in each mouse. Data were measured in three moderated aged mice and three young mice, and were averaged to derive the mean lifetime $\pm$ SE.

Immunofluorescence

Hairless mouse skin was excised from treated animals, and the subcutaneous fat was removed. Tissue sections of 5 μm thickness were incubated for 30 minutes in blocking buffer (1% BSA, 0.1% cold water fish gelatin in PBS) and were then incubated for 2 hours at room temperature with 1:250 dilutions of primary antibodies NHE1 diluted in blocking buffer. The tissue was then washed with PBS and incubated for 1 hour at room temperature with goat anti-rabbit, diluted in blocking buffer. Tissue sections were then washed with PBS and counterstained with propidium iodide before mounting and visualization under a confocal microscope (Leica TCS SP, Heidelberg, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 518 nm.

In situ zymography

Briefly, freshly isolated murine skin was immediately placed into keratinocyte growth medium, and then snap-frozen in OCT (10.24% polyvinyl alcohol/4.26% polyethylene glycol; Miles Lab., Elkhart, IN) within 30 minutes of excision, sectioned (20 μm), and mounted onto poly-L-lysine-coated slides. Sections were fixed briefly (10 minutes; 22–25 μC) with 10% paraformaldehyde in Dulbecco's PBS (aldehyde fixation eliminated enzyme activity in this system), overlayered with substrate solution (0.5 mM 4-methylumbelliferyl-β-D-glucoside in McIlvaine citrate/phosphate buffer, pH 5.6) for 30 minutes, covered with glass coverslips and sealed. Additional sections were overlayered with substrate solutions containing 10 mM conduritol B epoxide (in citrate/phosphate buffer) for 30 minutes to inactivate GlcCer'ase. Sections then were incubated for 16 hours at 4°C (less signal diffusion occurs at 4°C), and viewed on an inverted Zeiss (Thornwood, NY) laser scanning confocal microscope (excitation wavelength, 360 nm; emission wavelength, 450 nm; objective $\times$ 40; aperture 1.2; brightness set to maximal and contrast adjusted to 329 arbitrary units). The same exposure time was used for both moderately aged and young skin samples, so that relative fluorescence intensity is a true measure of relative enzyme activity. Scans were subjected to signal analysis (representing relative rates of 4-methylumbelliferone release) using Zeiss imaging software. Controls included both inhibitor-treated samples (i.e., 10 mM conduritol B epoxide) and substrate-excluded samples. The pH of the buffer solutions was measured at the beginning and end of each incubation to ensure that the optimal pH for epidermal GlcCer'ase (i.e., approximately 5.6) was maintained.

Quantitative real-time PCR

Total RNA was extracted from mouse epidermis using TRIZOL reagent (Sigma). First-strand cDNA for PCR was synthesized using an Omniscript RT kit following the manufacturer's protocol (Qiagen Inc., Valencia, CA). Briefly, cDNA was synthesized from 2 μg of total RNA using Ommiscript Reverse Transcriptase with random hexamer primer at 37°C for 60 minutes. Relative mRNA levels of target genes (ACC and FAS) and an invariant transcript, 36B4, were determined using an Mx3000P™ Real-Time PCR System (110 V) with Notebook Computer (Stratagene, La Jolla, CA). This system employs SYBR Green chemistry for highly accurate quantification of mRNA levels. Individual PCR reactions were carried out in a mixture of 20 μl containing 40 ng cDNA, 450 nM forward or reverse primers (ACC: GAG CTG ACC TCC ATC CTC TCG TAG GCG ATG TAG CCT CT (AY451394.1); FAS: GCTGCGGAAACTTCAGGAAATAGAGACGT GTCACTCCTGGACTT (AF127033)), and 10 μl of 2 $\times$ 2SYBR Green Q-PCR Master Mix (Applied Biosystems, Foster City, CA). The reaction was performed at 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of amplification of melting at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds in Mx3000P 96-well plates (Stratagene). The PCR reaction was performed in triplicate. Gel electrophoresis and melting curve analyses were performed to confirm correct PCR product sizes and absence of nonspecific bands. The expression levels of each gene were normalized against 36B4 using the comparative C_T method according to the manufacturer's protocols.

Ultrastructural methods

Freshly obtained biopsies from mouse skin were fixed directly in modified Karnovsky's fixative, postfixed with reduced osmium tetroxide, and then embedded in an Epon-epoxy mixture. For visualization of lipid-enriched, lamellar bilayer structures, some

samples were postfixed with ruthenium tetroxide. Sections were cut on a Reichert Ultracut E microtome, counterstained with uranyl acetate and lead citrate, and viewed in a Zeiss 10 CR electron microscope, operated at 60 kV.

Statistical methods

Data are presented as the mean ± SE. Data are analyzed using an unpaired two-tailed Student's *t*-test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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