Extracellular pH Controls NHE1 Expression in Epidermis and Keratinocytes: Implications for Barrier Repair

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We have previously shown that the Na^+/H^+ antiporter (NHE1) is an essential endogenous pathway responsible for stratum corneum (SC) acidification. Since the epidermis must re-establish its epidermal barrier after acute barrier perturbations, we asked whether the NHE1 was, in turn, regulated by changes in barrier status. We found that *in vivo* epidermal NHE1 expression was upregulated within hours of barrier disruption. We next asked whether NHE1 was regulated by barrier status *per se*, or by the SC alkalinization that accompanies barrier perturbation. NHE1 was upregulated by alkalinizing SC pH, whereas this antiporter was downregulated by acidifying SC pH, independent of changes in barrier status. Moreover, acidifying SC pH overrode the effects of barrier break in regulating NHE1 expression, suggesting that SC alkalinization is the major stimulus for increased NHE1 expression. Finally, we confirmed that the keratinocyte NHE1 antiporter is regulated by extracellular pH independent of barrier status, by demonstrating that NHE1 was upregulated in cultured keratinocytes exposed to pH 8.3 medium and downregulated in cultured keratinocytes exposed to pH 6.3 medium. These data suggest that the keratinocyte NHE1 is regulated by extracellular pH. SC barrier break also upregulates NHE1 expression, but this response seems to be mediated by concomitant changes in SC pH.

Key words: barrier function/NHE1/polyhydroxyl acids/stratum corneum/superbase/transepidermal water loss
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The stratum corneum (SC), the uppermost epidermal layer, exhibits an acidic surface pH (Krapf *et al*, 1991), initially thought to primarily ensure anti-microbial defense (Schmid and Korting, 1995). SC acidification originally was thought to derive from exogenous sources, such as bacterial colonization or sebaceous gland lipid. More recent studies have however, demonstrated that these mechanisms, along with one endogenous mechanism, the histidase pathway (Krien and Kermici, 2000), are not essential in establishing SC acidity. Instead, two endogenous mechanisms, secretory phospholipase and one Na^+/H^+ antiporter, NHE1, seem to be the most important agents in producing an acidic SC (Fluhr *et al*, 2004). Fluorescence life-time imaging microscopy (FLIM) studies have shown that aqueous, acidic pockets within the lipid-rich extracellular matrix are also present at the stratum granulosum (SG)/SC interface (average pH = 6.0) (Hanson *et al*, 2002). NHE1 is essential for acidifying these acidic microdomains (Behne *et al*, 2002) and is required both for perinatal SC acidification and for postnatal barrier recovery (Behne *et al*, 2002, 2003). More recent biochemical and molecular biological studies demonstrated that the acidic pH of SC is also essential for adult epidermal permeability homeostasis (Mauro *et al*, 1998; Fluhr *et al*, 2001; Behne *et al*, 2003; Hachem *et al*, 2003) and SC integrity/cohesion (Fluhr *et al*, 2001; Hachem *et al*, 2003). An acidic environment is essential for barrier function by activating two key lipid-processing enzymes: β-glucocerebrosidase (β-Glc'er'ase) and acidic sphingomyelinase (aSMase) (Holleran *et al*, 1994; Jensen *et al*, 1999). If SC acidity is neutralized, using either buffer or superbase, barrier recovery is inhibited, because of decreased lipid-processing enzyme activity resulting in delayed lamellar membrane formation (Mauro *et al*, 1998; Hachem *et al*, 2003).

The NHE are among the major ion transporters involved in the regulation and maintenance of cell volume and the adjustment of intracellular pH (Noel and Pouyssegur, 1995). Six isoforms of the NHE, termed NHE1–6, have been characterized (Counillon and Pouyssegur, 1993; Bianchini and Poussegur, 1994; Noel and Pouyssegur, 1995). Whereas NHE1 and NHE6 are ubiquitously expressed, NHE2 to NHE5 isoforms remain restricted to specific tissues as they fulfil specialized functions (Ritter *et al*, 2001). Normal human skin (keratinocytes and melanocytes) as well as melanomas have been reported to express the NHE1 isoform (Sarangarajan *et al*, 2001). Within the lower SC and at the SG–SC interface, the NHE1 acidifies extracellular "microdomains" where lipid processing occurs (Behne *et al*, 2002, 2003). NHE1 knockout animals lack these acidic intercellular domains within the lower SC (Behne *et al*, 2002). Inhi-

Abbreviations: CHK, cultured human keratinocytes; LBA, lactobionic acid; NHE1, Na^+/H^+ antiporter; PBS, phosphate-buffered saline; SC, stratum corneum; SD, standard deviation; SG, stratum granulosum; Skh1/hr, hairless mice; TMG, 1,1,3,3-tetra-methylguanidine

bition of NHE1 activity partially blocks postnatal acidification of newborn rats (Fluhr *et al*, 2004). NHE1−/− mice and pharmacological NHE1 inhibition both impair barrier development or recovery (Mauro *et al*, 1998; Behne *et al*, 2002, 2003; Hachem *et al*, 2003).

In this report, we ask whether a key acidifying mechanism, the NHE1 antiporter, is regulated by changes either in externally imposed pH changes or by alterations in barrier homeostasis. Although acidosis in other tissue stimulates NHE1 expression, we find the opposite direction of regulation: alkalinization stimulates, and acidification inhibits NHE1 expression. The unique response to extracellular pH allows keratinocytes to respond optimally to barrier perturbation.

Results

Acute barrier disruption alkalinizes surface pH and increases NHE1 expression We and others have shown previously that unperturbed SC contains a marked pH gradient, with a more neutral pH located at the base of the SC and a markedly more acidic pH found at the SC surface (Ohman and Vahlquist, 1994, 1998; Turner *et al*, 1998; Behne *et al*, 2003). We first tested whether acute perturbation of the epidermal barrier changes SC pH, or the kinetics of pH recovery. We found that barrier perturbation causes an immediate alkalinization of SC surface pH, which peaks between 0 and 5 h, returning gradually toward normal between 18 and 48 h (Fig 1). Because reacidification of SC is required for normal barrier recovery (Mauro *et al*, 1998; Hachem *et al*, 2003), we next asked whether the NHE1 antiporter is upregulated in response to the increase in SC pH and/or acute barrier perturbation. Immunohistochemistry revealed that NHE1 expression increased within 3 h after the epidermal barrier was perturbed (Fig 2), and protein expression, assessed by western immunoblotting, confirmed that this increase in NHE1 expression was sustained for at least 24 h after barrier disruption (Fig 3). These results demonstrate that acute barrier disruption causes a temporary increase in SC pH, which is paralleled by increased NHE1 protein levels.

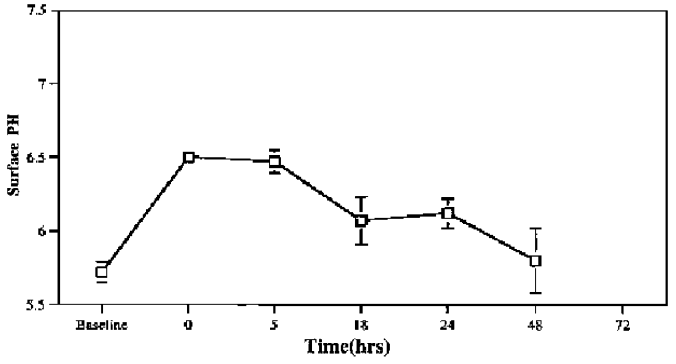

Figure 1
Acidity at the skin surface decreases after barrier disruption. Barrier disruption was induced by repetitive application of acetone-soaked cotton balls until transepidermal water loss reached values >4 mg per cm^2 per h. Surface pH was measured with a flat, glass surface electrode from Mettler-Toledo, attached to a pH meter (PH 900; Courage & Khazaka). Surface pH becomes more neutral immediately after barrier abrogation, and then gradually returns to baseline over the next 48 h. N = 4–5 mice/each time point. Results are shown average ± SD.

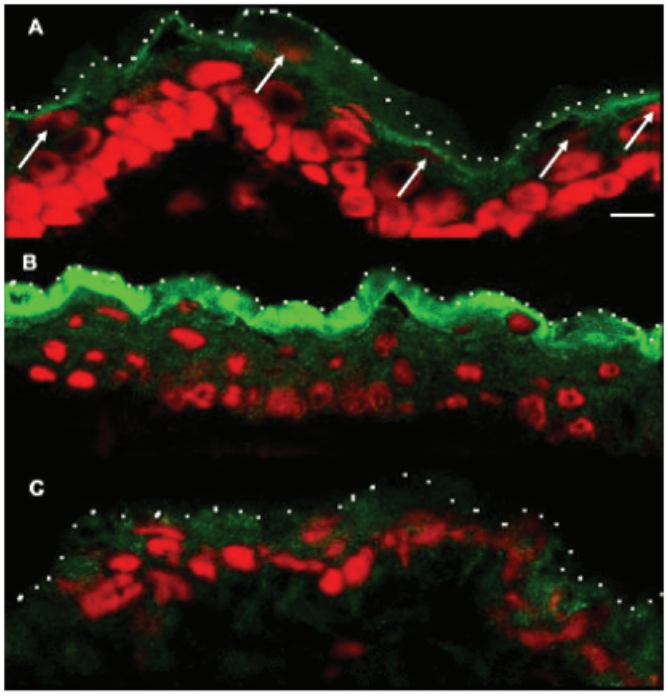

Figure 2
Acute barrier abrogation upregulates Na^+/H^+ antiporter (NHE1) expression. NHE1 expression was assessed using immunohistochemistry in frozen sections of mouse skin. Dotted lines indicate the stratum granulosum/stratum corneum (SG/SC) border. (*A*) NHE1 staining in normal unperturbed skin was localized primarily to the plasma membrane (arrows denote SG keratinocyte nuclei surrounded by plasma membranes that stain positively for NHE1) and was expressed most abundantly in the SG, with less staining in the basal layer and stratum spinosum, and very little staining in the SC. (*B*) Flanks of hairless mouse skin were treated with tape stripping, perturbing the epidermal permeability barrier to transepidermal water loss values >4 mg per cm^2 per h. Skin biopsies were obtained 3 h after barrier disruption. NHE1 expression was upregulated, especially in the SG, in sections in which the barrier was perturbed (*B*) *versus* normal control in which the barrier was left intact (*A*). Negative control staining in which the primary antibody was omitted demonstrated only background staining (*C*). Epidermal keratinocyte nuclei are counterstained with propidium iodide. *Scale bar*: 10 μm.

SC pH modulates NHE1 expression independent of permeability barrier We next asked whether it is the epidermal barrier insult or the increases in pH that accounts for NHE1 upregulation. In previous studies, we demonstrated that transient perturbations in SC pH, induced by topical applications of either superbases (Hachem *et al*, 2003) or polyhydoxyl acids (Berardesca *et al*, 1997), do not alter basal permeability barrier function, as assessed by transepidermal water loss (TEWL) (see also Ritter *et al*, 2001). Moreover, prior studies have shown that treatment with agents such as 1,1,3,3-tetramethylguanidine (TMG) (a superbase) also does not produce cell toxicity, as assessed by light or electron microscopy (Berardesca *et al*, 1997; Hachem *et al*, 2003). To determine whether SC acidification/neutralization regulates NHE1 expression independent of barrier status, either TMG (a superbase) or lactobionic acid (LBA) (a polyhydroxyl acid) was applied to the flanks of

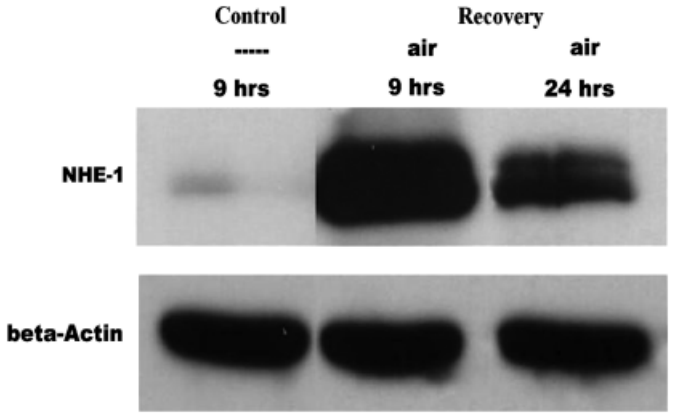

Figure 3
Epidermal Na^+/H^+ antiporter (NHE1) protein increases after barrier disruption. The epidermal barrier was perturbed as in Fig 2, above, and skin samples obtained after 6, 9, and 24 h. Analysis was performed (see Materials and Methods) using the same rabbit anti-murine polyclonal antibody used for immunohistochemistry in Fig 2, above. This antibody yielded a single band of 91 kDa, identical to the calculated molecular mass of NHE1 (Putney *et al*, 2002). Possibly because the entire epidermis was analyzed, NHE1 upregulation was not evident at time points earlier than 9 h (data not shown). NHE1 was however, strongly upregulated at 9 h, and NHE1 upregulation persisted for at least 24 h.

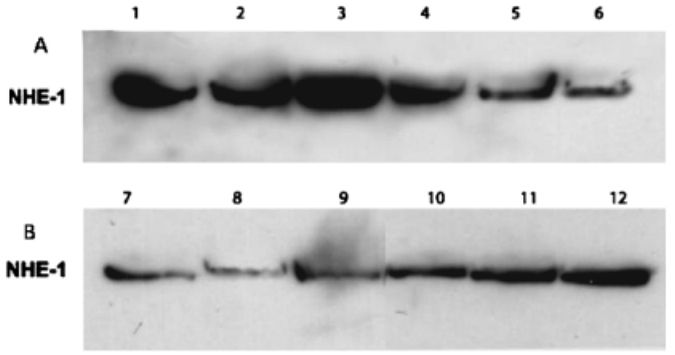

Figure 5
Stratum corneum (SC) acidity reduces, Whereas SC alkalinity enhances Na^+/H^+ antiporter (NHE1) expression. (*A*) After mice were treated topically with neutralized lactobionic acid (LBA) (*lanes 1–3*) or LBA (*lanes 4–6*) for 3 h, protein extracts (n = 3 each) from whole epidermis for NHE1 were loaded onto Tris-glycine polyacrylamide gel electrophoresis gels, and after transfer onto nitrocellulose membrane, incubated further with NHE1 antibody at 4°C overnight (*A*), identifying a single band of 91 kDa. Blots show decreased NHE1 expression in SC acidified using LBA (*lanes 4–6*), in comparison with control nLBA (*lanes 1–3*) (*B*). (*B*) Epidermis was treated, harvested, and processed as in (*A*), above, after alkalinization with tetramethylguanidine (TMG) for 3 h. Western blotting revealed that alkalinization with TMG led to an upregulation in NHE1 expression (*lanes 10–12*), compared with skin treated with control neutralized TMG (*lanes 7–9*).

hairless mouse skin to provoke SC alkalinization and acidification, respectively. Neutralized TMG or LBA, containing the same components, but adjusted to the pH of the propylene glycol:ethanol vehicle (pH = 7.0), were used as controls that both lack buffering capacity, and had previously been shown not to influence SC pH following topical application (Fluhr *et al*, 1999; Hachem *et al*, 2003). TMG or LBA applications alkalinized or acidified, respectively, all levels of the SC for ⩾24 h (Fig 4, TEWL data not shown). NHE1 immunoblotting demonstrated that NHE1 protein increased as a result of SC alkalinization, but decreased following SC acidification (Fig 5), results that were further confirmed by immunohistochemistry (Figs 6 and 7).

Since widely used topical therapeutic agents acidify the SC, we next addressed whether NHE1 downregulation would persist with more prolonged acidification of SC. NHE1 expression was assessed using immunohistochemistry at various times after topical applications of LBA (or nLBA) 3 or 9 h after a single dose, as well as 24 h after a double dose, applied at 12 h intervals. The surface pH after

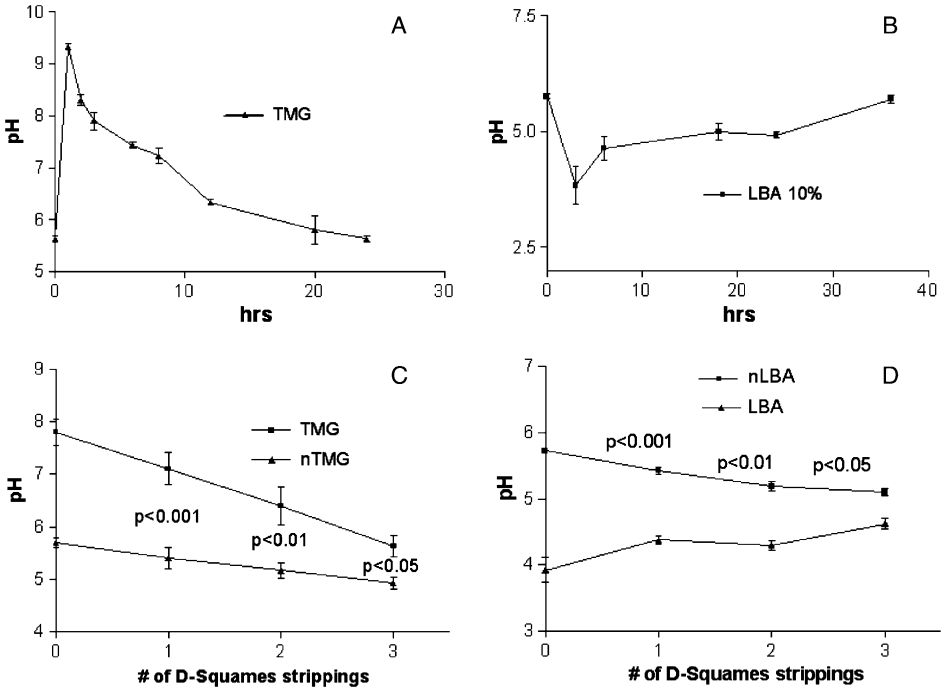

Figure 4
Tetramethylguanidine (TMG) and lactobionic acid (LBA) Modulate stratum corneum (SC) pH. (A) Application of TMG superbase (1/100, in propylene glycol: ethanol, 7:3 vol/vol) leads to a rapid and sustainable increase in SC pH. (*C*) The TMG-related increase in SC pH extends throughout the SC and is significantly more alkaline than SC treated with neutralized TMG (n-TMG). n-TMG application did not change SC pH (compared with n-LBA in *D*). N-TMG-treated sites did not differ significantly from untreated sites (historical data from Hachem *et al* (2003). (*B*) Application of LBA (10%, in propylene glycol: ethanol, 7:3 vol/vol) correspondingly decreased SC pH for a sustained period of time. (*D*) LBA likewise decreased SC pH throughout the SC, whereas neutralized LBA (n-LBA) did not change SC pH. pH was measured with a surface electrode. Results shown represent mean pH +/− SD (n = 4–6 animals in each group).

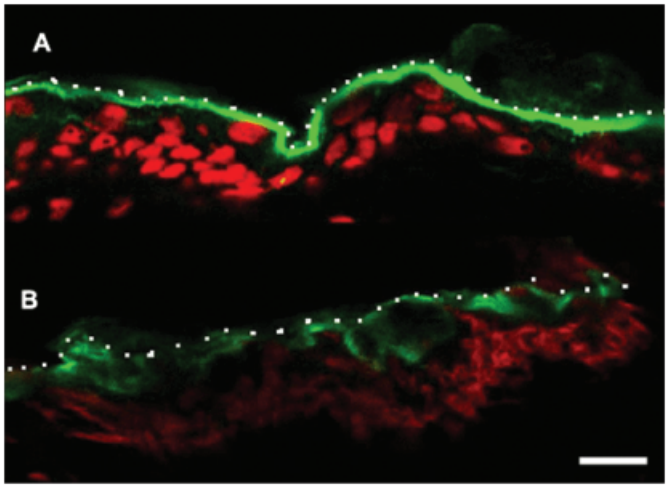

Figure 6
Stratum corneum (SC) alkalinization upregulates Na^+/H^+ antiporter (NHE1) expression in the stratum granulosum (SG). Tetramethylguanidine (TMG) (*A*) or neutralized TMG (*B*) was applied to skin flanks as described in Fig 4. Skin biopsies were taken after 3 h of treatment. Immunofluorescence staining was performed using rabbit anti-murine polyclonal antibody to identify NHE1 expression (green) in frozen sections. The epidermal keratinocyte nuclei are counterstained with propidium iodide (red). NHE1 expression is upregulated by alkalinization, as shown by the brighter signal in (*A*) *versus* (*B*). Dotted white line indicates SG/SC junction. *Scale bar*: 10 μm.

application of 10% LBA twice was approximately 4.5 and NHE1 expression remained decreased in epidermis that was acidified for up to 9 h (Fig 7). Yet, when the decrease in surface SC pH was sustained for 24 h (Fig 4), NHE1 expression appeared to recover over time in comparison with the earlier time point (cf Fig 7). This transient response could reflect either homeostatic normalization of NHE1 expression, or recovery of NHE1 expression in parallel with initial normalization of SC pH at 24 h (see Fig 1). These results predict that long-term therapeutic SC acidification will not decrease NHE1 expression.

Alterations in NHE1 expression following acute barrier disruption are regulated by pH In order to assess further whether permeability barrier status or SC pH is more important in regulating NHE1 expression, we next tested whether acidifying the SC could override the upregulation of NHE1 that occurs after barrier disruption. To assess whether the barrier abrogation-dependent increase in pH accounts for NHE1 upregulation, we normalized SC pH with topical LBA immediately following barrier disruption. Normalization of SC pH blocked the expected barrier-induced increase in NHE1 expression, as shown by immunohistochemistry (Fig 8). These findings confirm that changes in SC pH predominate in directing NHE1 expression after barrier perturbation.

Extracellular pH directly regulates NHE1 expression in cultured keratinocytes Finally, to further confirm that the keratinocyte NHE1 is regulated by extracellular pH independent of barrier status, we compared NHE1 expression in cultured normal human keratinocytes, grown in keratinocyte growth medium (KGM) buffered to either pH = 6.3 or 8.3 *versus* basal control medium (pH = 7.3). Here again, NHE1

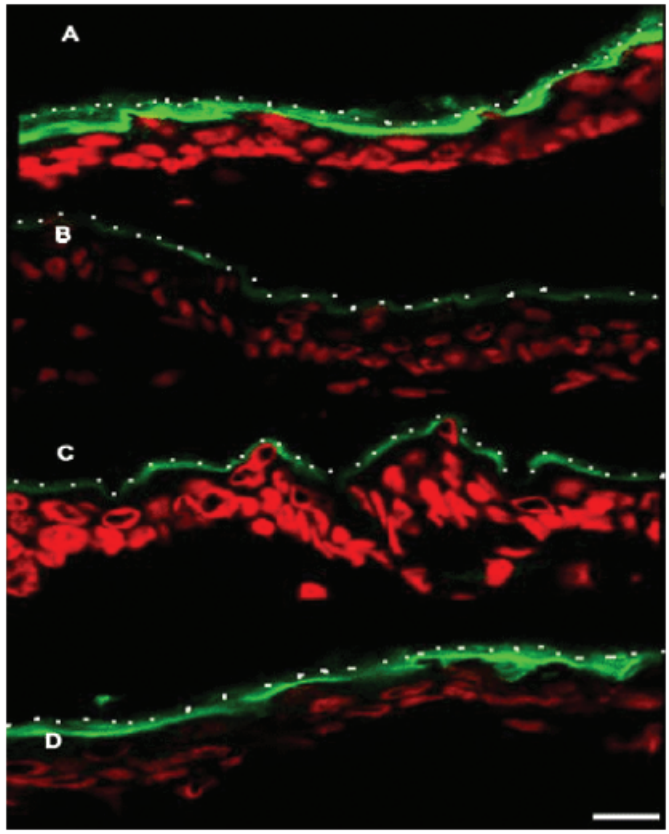

Figure 7
Na^+/H^+ antiporter (NHE1)-expression gradually recovers after startum corneum (SC) acidification. Immunofluorescence staining was performed to identify NHE1 expression in frozen sections from murine skin biopsies 3 h (*B*), 9 h (*C*) and 24 h (*D*) after application of lactobionic acid (LBA) and compared with control neutralized LBA (n-LBA)-treated skin sites (*A*). (*A*) Control n-LBA-treated sites expressed NHE1 in the same distribution as untreated epidermis (compare with *A* in Fig 2). (*B*) In agreement with our previous findings (see Fig 4, above), we observed a rapid and marked decrease in NHE1 expression, especially in the stratum granulosum (SG), 3 h after LBA application. (*C*, *D*) NHE1 expression gradually recovered to normal levels over 24 h, even with continued exposure to LBA. Dotted white lines indicate SG/SC junctions. *Scale bar*: 10 μm.

expression, as observed by western immunoblotting, was regulated by changes in medium pH, showing an increase in NHE1 protein expression when cells were grown at a basic pH, but downregulation of NHE1 at an acidic pH (Fig 9). These data confirm that NHE1 expression is regulated by extracellular pH, independent of SC barrier status.

Discussion

In this paper, we focused on the mechanisms that regulate NHE1 expression in keratinocytes *in vivo* and *in vitro*. Whereas most cells other than keratinocytes increase NHE1 expression in response to acidification, NHE1 in murine epidermis and CHK was inversely regulated by changes in extracellular pH, namely: NHE1 was increased under alkalinizing conditions (i.e., TMG topical application, barrier abrogation, and alkaline KGM) and decreased by acidification (i.e., LBA topical application and acidic KGM).

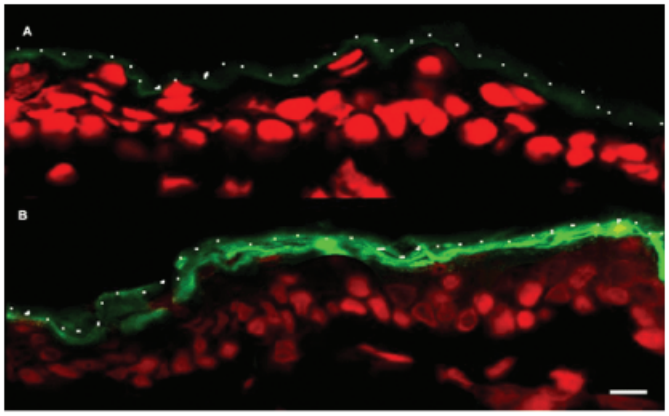

Figure 8
Simultaneous exogenous acidification overrides Na^+/H^+ antiporter (NHE1) upregulation caused by acute barrier abrogation. (*A*, *B*) Flanks of hairless mice were treated with tape stripping to abrogate the epidermal barrier to transepidermal water loss (TEWL) values >4 mg per cm^2 per h. (*A*) Lactobionic acid (LBA) was applied immediately and blocked NHE1 upregulation caused by barrier perturbation. (*B*) Control epidermis in which the barrier was perturbed in an identical fashion and treated with neutralized LBA (n-LBA) upregulated NHE1 expression, consistent with previous experiments (compare with Figs 2 and 7). Biopsies were taken from experimental and control sites 3 h after barrier break, and stained for NHE1 expression using immunohistochemistry on frozen mouse skin specimens. Dotted white lines indicate stratum granulosum/stratum corneum junction. *Scale bar*: 10 μm.

The NHE1 isoform is ubiquitously expressed on the plasma membrane of all cell lines (Noel and Pouyssegur, 1995). NHE1 regulates intracellular pH, and is involved in the preservation of cell volume under normal physiological conditions (Counillon and Pouyssegur, 1993; Bianchini and Poussegur, 1994; Noel and Pouyssegur, 1995). In many other cell types, an increase in NHE1 expression, both at the mRNA and protein level, follows sustained extracellular fluid acidification (Krapf *et al*, 1991; Quednau *et al*, 1994; Laghmani *et al*, 2001), protecting the cytosol by extruding excess H^+ while importing Na^+ ions. NHE1 was found to protect the esophageal mucosa from acute decreases in extracellular pH, and both protein and mRNA increased in patients with gastroesophageal reflux (Siddique and Khan, 2003). Similarly, chronic decreases in extracellular fluid pH cause an increase in NHE1 activity, protein synthesis, and mRNA abundance (Alpern *et al*, 1993). Primary myocytes cultures were shown to respond to low external pH by increasing NHE1 protein expression (Rehring *et al*, 1998). Likewise, cells derived from renal epithelia respond to extracellular acidity by increasing NHE1 levels (Moe *et al*, 1991; Gennari *et al*, 1992; Rutherford *et al*, 1997). But not all cells upregulate NHE1 in response to extracellular acidity. For example, acid pre-incubation of fibroblasts led to suppression of NHE1 activity and mRNA levels (Moe *et al*, 1991). This report adds keratinocytes to the very small group of cells that downregulate NHE1 expression in response to extracellular acidity.

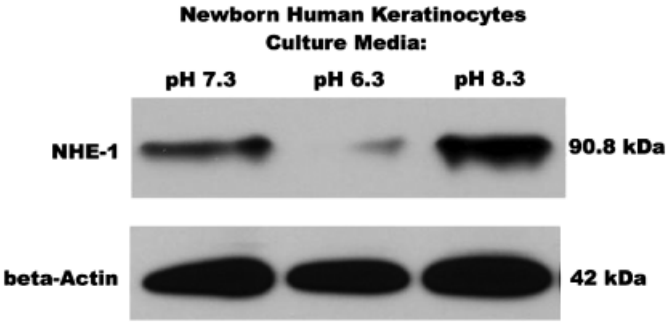

Figure 9
pH directly regulates Na^+/H^+ antiporter (NHE1) expression in cultured keratinocytes. Protein extracts from whole cultured human keratinocytes grown in cultured media buffered to acidic (pH = 6.3), alkaline (pH = 8.3), or neutral pH-buffered media (pH = 7.3) were loaded onto Tris-glycine polyacrylamide gel electrophoresis gels, and after transfer onto nitrocellulose membrane, incubated further with NHE1 antibody at 4°C overnight. Blots show a decrease in NHE1 expression when cells were incubated in acidic buffer (pH = 6.3) and an increase in NHE1 expression when cells were incubated in an alkaline buffer (pH = 8.3). Total protein levels did not change, as measured by β-actin levels, and cell numbers also remained constant (data not shown).

The specialized function of the epidermis as an interface between the internal neutral milieu and the external desiccating environment may explain the differential expression of NHE1 to extracellular pH stimuli. Stratified epidermal cells undergo a highly programmed form of cell death leading to the formation of a competent skin barrier (Chuong *et al*, 2002). Whereas simple epithelial cells remain in contact with the basement membrane throughout differentiation, stratified epidermal cells detach and continue to differentiate further (Green, 1977). Late in terminal differentiation, when the cornified envelope is formed, the keratinocytes degrade their organelles via the action of cellular proteases, resulting in cornification (Ashkenas and Werb, 1996) and the formation of the acid mantle. Thus, in response to acute alkalinization, i.e., barrier disruption, NHE1 upregulation would constitute a quick and highly localized response to restore specific skin functions, such as permeability barrier homeostasis.

Acute SC alkalinization has also been shown to produce negative effects on SC integrity and cohesion, mediated by the activation of SC serine protease (SP) (Hachem *et al*, 2003) (Egelrud and Lundstrom, 1991; Ekholm *et al*, 2000). Under normal physiologic conditions, these enzymes, with a known neutral pH optima (Egelrud and Lundstrom, 1991; Ekholm *et al*, 2000), are active at the lower levels of the SC where NHE1 acidification microdomains limit excessive SP activity, and ensure appropriate cohesion of SC (Egelrud and Lundstrom, 1991; Ekholm *et al*, 2000; Hachem *et al*, 2003). Therefore, the rapid increase in NHE1 activity following acute barrier abrogation or SC alkalization may support SC integrity/cohesion by limiting the increase in SP activity (Denda *et al*, 1997).

Rapid and massive secretion of lamellar bodies containing lipid precursors and processing enzymes is also observed immediately after barrier disruption and takes place at the SG/SC junction (Menon *et al*, 1992) paralleled by increased activities of β-Glc'er'ase and aSMase (Holleran *et al*, 1992; Uchida *et al*, 2000). Even though acute barrier disruption neutralizes SC surface pH, as observed after both tape stripping (Turner *et al*, 1998) and acetone application (see Results), barrier recovery occurs predictably over time (Taljebini *et al*, 1996). Thus, acidifying mechanisms within a deeper level of the SC may produce the acidic pH optima required to activate lipid-processing enzyme activity (Behne *et al*, 2002), while at the same time

restricting SP from exaggerated desquamation (Hachem *et al*, 2003).

In this paper, we have applied LBA, a polyhydroxyl acid, to acidify SC before and following barrier abrogation. In prior studies, α-hydroxy-acids, such as gluconolactone (GL), another polyhydroxyl acid, were shown to accelerate permeability barrier recovery and prevent skin irritation (Berardesca *et al*, 1997). This effect was not equal among all α-hydroxy-acids. Those characterized by antioxidant properties (i.e., LBA, GL) were superior to glycolic or lactic acid (Berardesca *et al*, 1997). Additionally, topical treatment with either glycolic and lactic acid appears to enhance UVB- and UVA-induced tanning (Tsai *et al*, 2000; Kaidbey *et al*, 2003), which does not seem to occur with GL (Green *et al*, 1999; Rona *et al*, 2004). SC acidification, however, can influence mechanisms taking place in the viable epidermis, at least at the SG level. For example, application of either lactic or glycolic acid to the SC reportedly increased the number and secretion of lamellar bodies and improved permeability barrier homeostasis (Kim *et al*, 2001).

Several signalling pathways could underlie the return of NHE1 protein levels to normal after chronic acidification. Normalization of NHE1 expression could be due either to changes in the molecular mechanisms that control NHE1 synthesis or degradation or adaptation of other cellular mechanisms that control intracellular pH, such as the Cl^-/HCO_3^- antiporter, or the H^+/K^+ ATPase. Regardless of the mechanism of normalization, the fact that NHE1 levels recover despite long-term exposure to exogenous acidification suggests that sustained use of popular acidifying agents, such as lactic acid, pose little or no risk to epidermal barrier function.

Finally, pH and barrier status may also be linked to NHE1 regulation through mechanisms that are linked to these agents, but not necessarily controlled by them. For example, NHE1 is known to be regulated by cell volume, which, coupled to Cl^-/HCO_3^- exchange, results in cellular uptake of NaCl and cell swelling consequent to the influx of osmotically obliged water (Lee *et al*, 2002; Cingolani *et al*, 2003; Kim *et al*, 2003). Although currently we have no direct evidence that this mechanism mediates keratinocyte NHE1 expression *in vivo* or *in vitro*, the possibility that NHE1 expression may be controlled by cell volume changes linked to extracellular pH induced merits mention.

In conclusion, keratinocytes feature a distinctive regulation of NHE1 expression in response to external changes in pH. Such observations could be explained by the functional consequences of NHE1 activation/inhibition on skin barrier homeostasis and the related formation of acidic microdomains within the SC. These in turn provide acidification deep within the SC interstices necessary for lipid processing and permeability barrier homeostasis.

Materials and Methods

Materials Male hairless mice (Skh1/hr), 6–8-wk-old, were purchased form Charles River Laboratories (IFFA credo, Brussels, Belgium) and fed Purina mouse diet and water *ad libitum*. Propylene glycol, ethanol, and HCl were from Fisher Scientific (Fairlane, New Jersey), whereas TMG superbase and LBA polyhydoxyl acid were from Sigma Chemicals (St Louis, Missouri, USA and Bornem, Belgium). Primary rabbit anti-goat antibodies against mammalian NHE1 were purchased from Alpha Diagnostic International (San Antonio, Texas). Goat anti-Rabbit Alexa® -labelled secondary antibodies were purchased from Molecular Probes (Leiden, The Netherlands). Horseradish peroxidase, conjugated with anti-rabbit IgG, was purchased from Vector Labs (Burlingame, California). Twenty-two millimeter D-Squame-100 tapes were purchased from CuDerm (Dallas, Texas). Bradford protein assay kits (Bio-Rad Protein Assay Dye), as well as lyophilized, bovine albumin were purchased from Bio-Rad (Hercules, California and Nazareth Eke, Belgium). All procedures were performed and mice were anesthetized with chloral hydrate under protocols approved by the San Francisco Veterans Affairs Medical Center, University of California, San Francisco and the Vrije Universiteit Brussel Academische Ziekenhuis Ethical Committees. This study was conducted according to the Declaration of Helsinki Principles.

Experimental procedures

Acute SC alkalinization/acidification and pH recovery models Normal hairless mice were treated topically with a single application of either TMG (dose 1:100; vol/vol) or LBA (10%) in propylene glycol:ethanol (7:3 vol/vol) on 5–6 cm^2 areas on both flanks. Controls were treated with either HCl- or NaOH-neutralized TMG (nTMG) or LBA (nLBA) in the same propylene glycol: ethanol vehicle. For the 24 h acidification experiments, mice received two applications of LBA (10%) at 12 h intervals. The superbase alkalinization model has been previously described to be non-toxic on mouse skin in topical application (Hachem *et al*, 2003). In addition, LBA is used in cosmetic preparations (Berardesca *et al*, 1997).

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 (TEWL) rates $\geqslant$4 mg per cm^2 per h). Immediately following disruption, hairless, mice flanks remained either untreated or topically treated with a single application of LBA (10%) or TMG (1:100) on a 5–6 cm^2 surface area. Control animals were prepared identically and treated with nLBA or nTMG.

Surface pH SC surface pH was measured with a flat, glass surface electrode from Mettler-Toledo (Giessen, Germany), attached to a pH meter (PH 900; Courage & Khazaka, Cologne, Germany), immediately before and at 1, 2, 4, 6, 9, 12, 18, and 24 h after TMG and LBA applications.

Cultured human keratinocytes (CHK) Human epidermis was isolated from newborn human foreskins, obtained under a protocol approved by the San Francisco Veterans Affairs Medical Center and the University of California, San Francisco, by incubation in dispase, and a suspension of keratinocytes was obtained by incubation in 10 mm ethylenediamine tetraacetic acid and subsequent trypsinization. Second-passage cells were plated on to 100 mm plastic dishes in serum-free keratinocyte growth medium (Cascade Biologics, Portland, Oregon), containing 0.07 mM calcium, and grown to 90% confluence. The medium was then switched to pH buffered to either 6.3 or 8.3. A control group was left in the medium without changing pH (pH 7.3). After 24 h, cells were collected and NHE1 and actin levels were assessed using western blotting techniques.

Protein isolation

(a) *From whole epidermis*: Whole epidermis was isolated from treated flanks using the heat-split technique (Allen *et al*, 1997). Briefly, after excision skin flanks were placed dermis side downward in Petri dishes and exposed to heat (60°C). After 60 s, the epidermis was gently scraped off the dermis, and proteins were extracted by sonication (15 s, three times) in extraction buffer (Tris base 62 mM, 2% sodium dodecyl sulfate (SDS) containing a protease inhibitor cocktail).

(b) *From CHK*: Keratinocytes were solubilized with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, and protease inhibitors (Compete™, Roche Biochemical), and centrifuged to remove cell debris.

Immunofluorescence Hairless mouse skin was excised from treated animals, and the subcutaneous fat was removed. Five-micrometer thickness tissue sections were incubated for 30 min in blocking buffer (1% bovine serum albumin, 0.1% cold water fish gelatin in phosphate-buffered saline, PBS) and were then incubated for 2 h 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 h 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.

Western immunoblotting After protein isolation (see above), the protein content of CHK or whole epidermis extracts was determined using a Bradford protein assay kit as described by the manufacturer. Equal amounts of protein from each experimental group were loaded onto 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred from the slab gel onto nitrocellulose membranes and immunoblotted to detect either NHE1 or β-actin using the Western Lightening chemiluminescence kit (PerkinElmer Life Sciences, Boston, Massachusetts).

Statistical analysis Changes in SC pH were compared in paired samples (Fig 4) using a paired, two-sided Student's *t*-test.

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