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.

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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.

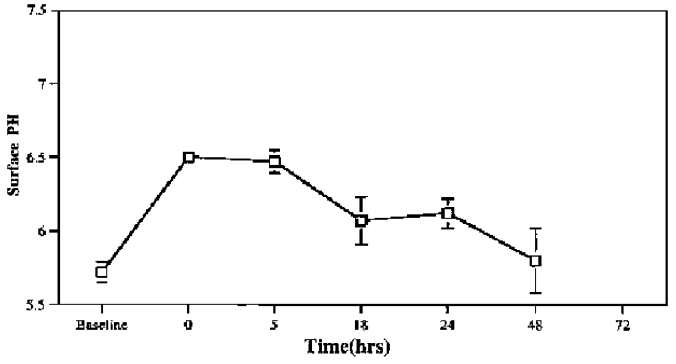

Figure 1
Acidity at the skin surface decreases after barrier disruption. Barrier disruption was induced by repetitive application of acetone-soaked cotton balls until transepidermal water loss reached values >4 mg per cm^2 per h. Surface pH was measured with a flat, glass surface electrode from Mettler-Toledo, 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.

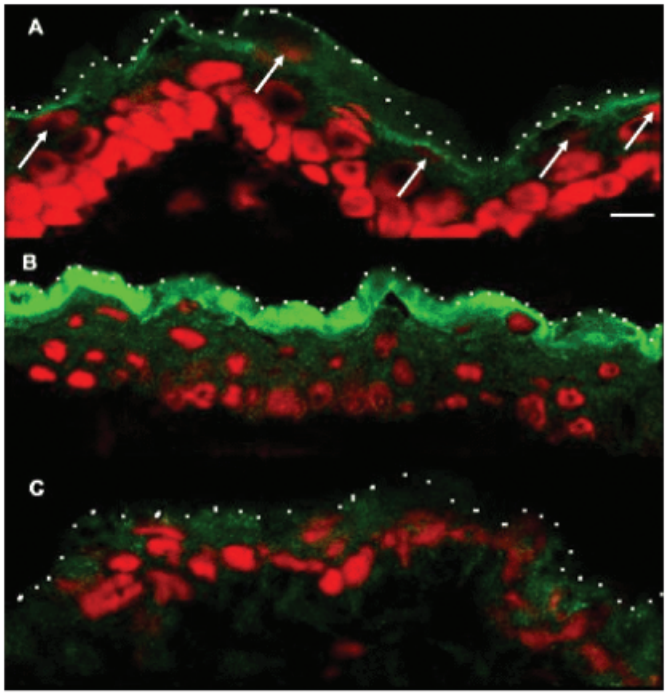

Figure 2
Acute barrier abrogation upregulates Na^+/H^+ antiporter (NHE1) expression. NHE1 expression was assessed using immunohistochemistry in frozen sections of mouse skin. Dotted lines indicate the stratum granulosum/stratum corneum (SG/SC) border. (*A*) NHE1 staining in normal unperturbed skin was 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