

## ORIGINAL PAPER

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## Barrier recovery is impeded at neutral pH, independent of ionic effects: implications for extracellular lipid processing

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**Abstract** Epidermal permeability barrier homeostasis requires the postsecretory processing of polar lipid precursors into nonpolar lipid products within the stratum corneum (SC) interstices by a family of lipid hydrolases. A specific requirement for  $\beta$ -glucocerebrosidase ( $\beta$ -GlcCer'ase), which exhibits a distinct acidic pH optimum, is particularly well documented. Therefore, we sought to determine whether the recovery of the barrier after acute insults requires acidification of the SC. We examined permeability barrier recovery by assessing changes in transepidermal water loss (TEWL), SC membrane ultrastructure utilizing ruthenium tetroxide ( $\text{RuO}_4$ ) postfixation, and  $\beta$ -GlcCer'ase activity by in situ zymography at an acidic vs neutral pH. Barrier recovery proceeded normally when acetone-treated skin was exposed to solutions buffered to an acidic pH. In contrast, the initiation of barrier recovery was slowed when treated skin was exposed to neutral or alkaline pH, regardless of buffer composition. In addition, enhancement of the alkaline buffer-induced delay in barrier recovery occurred with  $\text{Ca}^{2+}$  and  $\text{K}^+$  inclusion in the buffer. Moreover, the pH-dependent alteration in barrier recovery appeared to occur through a mechanism that was independent of  $\text{Ca}^{2+}$ - or  $\text{K}^+$ -controlled lamellar body secretion, since both the formation and secretion of lamellar bodies proceeded comparably at pH 5.5 and pH 7.4. In contrast, exposure to pH 7.4 (but not pH 5.5) resulted in both the persistence of immature, extracellular lamellar membrane structures, and a marked decrease in the in situ activity of  $\beta$ -GlcCer'ase. These results suggest first that an acidic extracellular pH

is necessary for the initiation of barrier recovery, and second that the delay in barrier recovery is a consequence of inhibition of postsecretory lipid processing.

**Key words** Barrier function · pH · Stratum corneum · Lamellar body · Lipid content · Ultrastructure

**Abbreviations**  $\beta$ -GlcCer'ase  $\beta$ -glucocerebrosidase · CBE conduritol B-epoxide · HEPES *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid · 4-MUG 4-methylumbelliferyl- $\beta$ -D-glucoside · PBS phosphate-buffered saline · PIPES piperazine-*N,N'*-bis(2-ethanesulfonic acid) · SC stratum corneum · TEWL transepidermal water loss · Tris tris(hydroxymethyl) aminomethane

### Introduction

Formation of the cutaneous permeability barrier requires both the secretion of the lipid and the hydrolytic enzyme contents of lamellar bodies and the subsequent postsecretory processing of polar lipids into their nonpolar lipid products. Whereas lamellar body secretion is regulated by changes in extracellular ions [16, 17], lipid processing appears to be mediated by a set of hydrolytic enzymes, which produce structural transformations within the stratum corneum (SC) interstices [4, 6], leading to barrier formation [4, 12, 21]. To date, two lipid processing enzymes have been shown to be required for the membrane transformations that result in barrier competence,  $\beta$ -glucocerebrosidase ( $\beta$ -GlcCer'ase) [10, 11], and an as-yet-uncharacterized secretory phospholipase  $A_2$  [4, 19]. Whereas the pH optimum of the SC secretory phospholipase  $A_2$  isoform is not known, epidermal  $\beta$ -GlcCer'ase exhibits a distinct acidic pH optimum [9, 10, 26]. Likewise, two other potential processing enzymes, steroid sulfatase and acid sphingomyelinase, also are concentrated in the SC interstices [5, 20], but their dependence on variations in the pH of the SC are not yet known.

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Whereas the epidermal surface has been known for more than a century to be acidic [3, 8, 28], the importance of the acidic pH of the SC for barrier homeostasis is suggested by: (a) the worsening of barrier function associated with alkalization of the skin [25]; (b) the exacerbation of experimentally induced contact dermatitis at alkaline pH [27]; and (c) the association of an alkaline skin pH with diaper dermatitis [1]. Since these observations suggest that the pH of the SC may influence barrier homeostasis, we tested the effects of pH on barrier recovery following acute barrier perturbations in hairless mice. We found that barrier recovery proceeds normally at an acidic pH, while recovery is delayed at neutral pH [7], independently of the ionic environment. Moreover, while lamellar body secretion appears to be unimpeded at neutral pH, postsecretory processing into mature lamellar membrane structures is impeded, and the *in situ* activity of  $\beta$ -GlcCer'ase is inhibited. Together, these results suggest that extracellular pH influences barrier homeostasis by modulating the processing of lipids in the SC interstices.

## Methods

### Materials

Conduritol-B-epoxide (CBE) was obtained from Toronto Chemical Co. (Toronto, Canada); 4-methyl umbelliferyl- $\beta$ -D-glucoside (4-MUG) was obtained from Fluka Chemical Co. (Buchs, Switzerland); ruthenium and osmium tetroxide were from Polysciences (Warrington, Pa.). All other chemicals were from Sigma (St. Louis, Mo.).

### Experimental protocols

To produce marked barrier perturbation, flanks of male hairless mice (hr/hr; Simonsen Laboratories, Gilroy, Calif.; <3 months of age), previously anesthetized with intraperitoneal injection of chloral hydrate, were treated with acetone-soaked cotton balls by gently rubbing the skin for several minutes, until the transepidermal water loss (TEWL) rates exceeded 5 mg/cm<sup>2</sup> per h (normal TEWL rates for unperturbed epidermal barrier are <0.1 mg/cm<sup>2</sup> per h). Each group comprised three or four animals. To produce moderate barrier perturbation, the same protocol was used to give a TEWL level of about 1 mg/cm<sup>2</sup> per h (single experiment, three animals).

Immediately after acetone treatment, the skin was warmed to approximately 35°C and the TEWL was measured using an electrolytic water analyzer (Meeco, Warrington, Pa.). The device's sampling cup, covered with Parafilm, was flushed with 99.9% pure nitrogen gas at 100 ml/min. After removal of the Parafilm, the cup was placed on the previously treated flank. TEWL was measured immediately after acetone treatment, and at various times after barrier disruption, up to 24 h, as shown. To minimize hydration effects, TEWL was recorded only after levels had reached their lowest point (i.e. 10–15 min). The opposite, nonimmersed flank served as either an untreated, or an equivalently treated air-exposed control.

After barrier disruption with acetone, one flank was submerged in isotonic sucrose or Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free sodium phosphate-buffered saline (PBS), or with the indicated buffers, for the time intervals shown, at either pH 5.5 or 7.4. Additional animals were exposed to isotonic Ca<sup>2+</sup>- and K<sup>+</sup>-containing PBS (see legend to Fig. 4), isotonic *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), isotonic piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), and isotonic sodium citrate buffers over a range of pH

values (i.e. 5.5, 6.5, 7.4, 8.5) after equivalent degrees of barrier disruption. While anesthetized, the animals were suspended on a mesh netting to avoid contact with the Petri dish [16]. The solutions were maintained at 35°C for the entire procedure. After 2 h, and either 4 or 5 h, the animals were removed, excess fluid was gently blotted off the skin surface, and TEWL was remeasured. The percentage recovery of TEWL was calculated by comparing TEWL measurements prior to immersion (immediately after barrier perturbation with acetone) with measurements after 2, 4 or 5, and 24 h. The pH had not changed in any of the solutions at the end of the immersion experiments. TEWL recovery rates after barrier disruption and immersion in isotonic sucrose, with air exposure alone, and with normal, unperturbed skin immersed in neutral vs acidic pH buffers for 2.5 h served as additional controls. Free Ca<sup>2+</sup> concentrations in the solutions were measured using a Ca<sup>2+</sup> electrode.

### Transmission electron microscopy

Full-thickness skin samples were obtained from euthanized animals, minced to <0.5 mm<sup>3</sup>, and fixed in modified Karnovsky's fixative overnight. All samples were then divided and postfixed in the dark in either 0.5% ruthenium tetroxide or 2% aqueous osmium tetroxide, both containing 1.5% potassium ferrocyanide [12]. Thin sections were examined, with or without further contrast with lead citrate, in a Zeiss 10A electron microscope operated at 60 kV.

### In situ zymography

To assess the effect of pH on  $\beta$ -GlcCer'ase activity *in situ*, we developed an *in situ* enzyme assay method. Briefly, fresh human skin sections were obtained from surgical margins, immediately placed into keratinocyte growth medium, and snap-frozen in 10.24% polyvinyl alcohol/4.26% polyethylene glycol medium (OCT, Miles Laboratories, Elkhart, Ind.) within 30 min of excision, sectioned (20  $\mu$ m), and mounted onto poly-L-lysine-coated slides. Similar patterns were seen in mouse and human skin, but localization was better in human samples because of the increased number of cell layers in the epidermis and corneum [15]. Unfixed sections were equilibrated in the appropriate buffer, i.e. citrate/phosphate buffer (pH 5.5) or HEPES/Tris buffer (pH 7.4), for 30 min at 22–25°C. The pH of the buffer solutions was measured at the beginning and end of each incubation. Sections were incubated with substrate solution, containing 0.5 mM 4-MUG in the buffer solution for 30 min, covered with glass coverslips, sealed, and incubated for 16 h at 4°C (less diffusion of signal occurs at 4°C), and viewed on an inverted Zeiss (Thornwood, N.Y.) laser scanning confocal microscope (objective 40 $\times$ , aperture 1.2, brightness set to maximal and contrast adjusted to 329 arbitrary units, pinhole 22). Scans were subjected to signal analysis, representing relative rates of 4-MU release, using Zeiss imaging software. Controls included both inhibitor-treated samples, i.e. 100  $\mu$ M CBE [9, 10] at both pH values, and substrate-excluded samples.

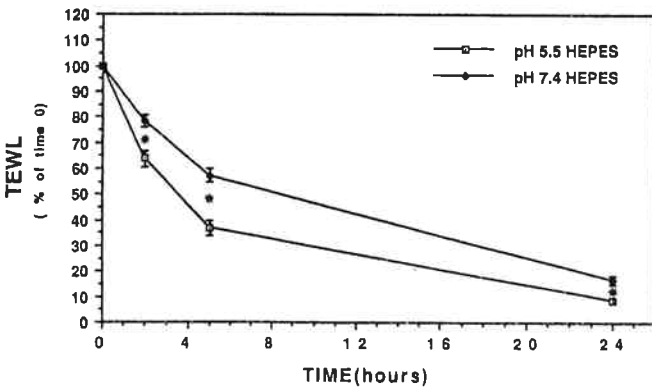
### Statistical analysis

All TEWL results are presented as the mean  $\pm$  SEM. A paired two-tailed Student's *t*-test was used to compare rates of recovery in the various experimental groups, based upon 100% abnormality in each animal.

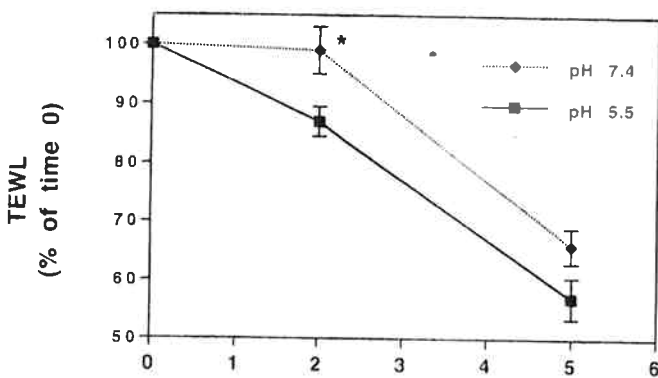
**Results**

**Neutral pH buffers retard the initiation of barrier recovery**

To test whether extracellular pH influenced barrier recovery, we compared the kinetics of recovery in acetone-treated mouse skin exposed to physiologic (pH 7.4) or acidic (pH 5.5) buffer solutions. Barrier repair proceeded significantly more quickly ( $P < 0.01$  at 2 and 4 h,  $P < 0.05$  at 24 h) in skin sites exposed to pH 5.5 HEPES buffer than in sites exposed to pH 7.4 (Fig. 1). Skin sites immersed in acidic buffer demonstrated a rate of barrier recovery comparable to either skin exposed to air or im-



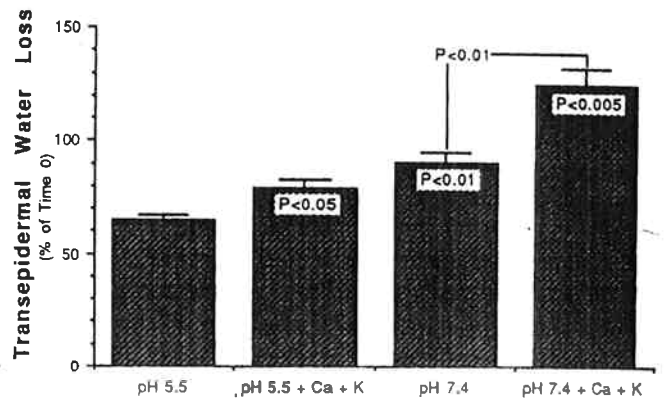
**Fig. 1** Barrier repair is inhibited by neutral pH buffers. Transepidermal water loss (TEWL) rates were measured in animals exposed to bathing solutions of acidic or neutral pH. The epidermal barrier was perturbed to  $\geq 5$  mg/cm<sup>2</sup> per h with gentle wiping of acetone, and the animals' flanks were immersed in 10 mM HEPES, buffered to either pH 7.4 or 5.5. \* $P > 0.01$  ( $< 0.05$  at 24 h). Values are means  $\pm$  SEM (three or four animals per group)



**Fig. 2** Secondary exposure to acidic buffer reverses the initial neutral pH-induced delay of barrier recovery. TEWL rates were measured in animals after the epidermal permeability barrier had been perturbed to  $\geq 5$  mg/cm<sup>2</sup> per h with acetone wipes. The flank skin of one set of animals was immersed in solutions buffered to pH 7.4 with 10 mM HEPES for 2 h, then switched to either fresh pH 7.4 buffer (not shown; cf. Fig. 1), or pH 5.5 (also buffered with 10 mM HEPES) (◆) for the indicated times. The control animals (■) were exposed to pH 5.5 throughout the experiment. \* $P < 0.05$ . Values are as means  $\pm$  SEM (three animals per group)

mersed in isotonic sucrose alone (data not shown; see references 16, 17). Skin sites immersed in PIPES at pH 7.4 or pH 5.5 or in phosphate demonstrated identical curves at 2 and 4 h, suggesting that this phenomenon was independent of the type of pH buffer used. Specifically, for HEPES buffer, the delay was 25%, 40%, and 8%, for PIPES buffer, 20%, 33% and  $< 5\%$ , and for phosphate, 32%, 38% and 10% at two, five and 24 h, respectively. Since the kinetics of recovery in each of the buffers were similar after 2 h, regardless of pH (i.e. the slopes for neutral pH and acidic pH were parallel), the delay at neutral pH appears to occur primarily during the initial phase of barrier recovery, i.e. in the first 2 h. Furthermore, when skin sites first exposed to phosphate buffer at pH 7.4 were returned subsequently to pH 5.5, barrier recovery normalized so that between 2 h and 5 h the recovery rates (i.e. slope of the recovery curve) were no longer significantly different from the rate of recovery of skin exposed to the pH 5.5 buffers alone (Fig. 2; compare Fig. 1).

The rapid normalization of recovery rates after 2 h, despite ongoing exposure to neutral pH, might have been a consequence of the restoration of a partial barrier, allowing the SC to regenerate an acidic pH. To test this hypothesis, we next measured barrier recovery in animals with a lesser initial barrier abnormality (i.e.  $\approx 1$  mg/cm<sup>2</sup> per h vs  $\leq 5$  mg/cm<sup>2</sup> per h, as in Fig. 1). The epidermal permeability barrier recovery was no different in skin exposed to pH 5.5 or pH 7.4 buffers when the barrier was only moderately disrupted (data not shown). Together, these experiments demonstrate that exposure of markedly permeabilized epidermis to neutral pH buffers results in a significant delay in the initiation of barrier recovery. This delay appears to be: (a) related to the extent of the initial insult; (b) not attributable to toxicity because it is rapidly and



**Fig. 3** pH influences barrier recovery independent of ions. The effects of maximally effective concentrations of Ca<sup>2+</sup> (1.0 mM) and K<sup>+</sup> (40 mM) added to acidic (pH 5.5) or neutral (pH 7.4) citrate buffer 100 mM are shown. The epidermal permeability barrier first was disrupted as described in the legend to Fig. 1, and the animals' flanks were then immersed in 100 mM citrate buffer with or without the ions for 0–4 h. TEWL rates were normalized to 100% at 0 h, and the results are presented as percentage recovery 4 h after barrier perturbation (means  $\pm$  SEM, three or four animals per group). The  $P$ -values shown are in relation to the value citrate buffer (pH 5.5) or as indicated