Sustained Serine Proteases Activity by Prolonged Increase in pH Leads to Degradation of Lipid Processing Enzymes and Profound Alterations of Barrier Function and Stratum Corneum Integrity

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We showed recently that short-term increases in stratum corneum (SC) pH are accompanied by minor alterations in permeability barrier homeostasis and SC integrity/cohesion. Since prolonged SC neutralization more closely mirrors clinical situations (i.e., neonatal skin, occupational dermatitis conditions), we assessed here whether sustained elevations of SC pH by long-term application of 1,1,3,3-tetramethylguanidine superbase provoke profound alterations in SC function. Sustained SC neutralization altered not only barrier recovery kinetics but also basal permeability barrier function. These abnormalities were attributable to a decrease in β-glucocerebrosidase (β-GlcCer'ase) and acidic sphingomyelinase (aSMase) catalytic activity and enzyme degradation consequent to a pH-induced sustained serine protease (SP) activity. The role of SP in this process was shown by the normalization of enzyme activities/content by co-applied SP inhibitors (SPI). To address whether lipid-processing enzymes are potential substrates for the stratum corneum chymotryptic enzyme (SCCE), protein extracts from human SC were treated for 2 h at 37°C with recombinant active SCCE at pH 7.2. Recombinant SCCE induced a significant decrease in the immunoblotting of both β-GlcCer'ase or aSMase compared with control experiments performed in the absence of the active SCCE. Finally, with sustained SC neutralization, SC integrity/cohesion deteriorated, attributable to SP-mediated degradation of corneodesmosomes (CD) as well as CD constituent proteins, desmoglein 1. These abnormalities were again reversed by co-applied SPI. In conclusion, prolonged SC neutralization provokes profound abnormalities in SC function, due to pH-induced high SP activity that, in turn, degrades lipid processing enzymes and CD proteins.

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As the primary interface with a desiccating external milieu, the primary function of the stratum corneum (SC) is to limit excess transcutaneous water loss from the organism's aqueous interior. Yet, in addition, the SC also protects against mechanical/desquamatory insults, UV-irradiation, oxidant injury, and the ingress of xenobiotes (Elias, 1989). The acidic surface mantle of the skin, first described 76 y ago, has long been thought to mediate cutaneous antimicrobial activity (Korting *et al*, 1990; Korting and Braun-Falco, 1996). Recent studies point to other pH-dependent functions of the SC acid mantle (Fluhr *et al*, 2001; Behne *et al*, 2002). For example, permeability barrier recovery is delayed when: (a) perturbed skin sites are exposed to neutral pH buffers (Mauro *et al*, 1998) and (b) certain endogenous acidifying mechanisms are blocked or genetically knocked out (Fluhr *et al*, 2001; Behne *et al*, 2002). Moreover, short-term application of superbases, compounds that are ten times more basic than in 1 N sodium hydroxide, delay permeability barrier recovery following acute barrier disruption by tape stripping or acetone treatment, although basal function remains normal (Hachem *et al*, 2003). The mechanism responsible for the barrier abnormality in these prior examples involves the requirement of an acidic pH for the maximal catalytic activity of certain lipid-processing enzymes, i.e., β-glucocerebrosidase (β-GlcCer'ase) (Vaccaro *et al*, 1985; Hachem *et al*, 2003).

A third, potentially pH-mediated function is SC integrity, defined as the resistance of the SC to mechanical tape stripping, and a related parameter SC cohesion, defined as the amount of protein removed per stripping (Fluhr *et al,* 2001; Hachem *et al,* 2003). Both parameters are altered by: (a) short-term exposure of intact skin to neutral pH buffers (Fluhr *et al*, 2001); (b) elevation of SC pH by blockade of an

Abbreviations: aSMase, acidic sphingomyelinase; β-GlcCer'ase, beta-glucocerebrosidase; CD, corneodesmosomes; DSG1, desmoglein 1; OsO_4, osmium tetroxide; PBS, phosphate-buffered saline; RuO_4, ruthenium tetroxide; SC, stratum corneum; SD, standard deviation; SCCE, stratum corneum chymotryptic enzyme; SCTE, stratum corneum tryptic enzyme; SG, stratum granulosum; Skh1/hr, hairless mice; SP, serine protease; SPI, serine protease inhibitor; STI, soybean trypsin inhibitor; TEWL, transepidermal water loss; TMG, 1,1,3,3-tetra-methyl-guanidine

endogenous acidifying mechanism (Fluhr *et al*, 2001; Behne *et al*, 2002); or (c) short-term applications of superbases (Hachem *et al*, 2003). The compromise in SC integrity/cohesion was further shown to be attributable to accelerated corneodesmosome (CD) degradation (Fluhr *et al*, 2001; Hachem *et al*, 2003). The deleterious effects of an elevated pH were further linked to high serine protease (SP) activity, because co-applied serine protease inhibitors (SPI) normalized both SC integrity/cohesion and CD degradation, even in the face of an elevated pH (Hachem *et al*, 2003).

Because potentially confounding variables can occur with either buffers, inhibitor treatment, or knockout animals (i.e., hydration, non-specific toxicity, and unrelated, downstream effects, respectively), we recently developed a method whereby SC pH could be experimentally altered, independent of these variables (Hachem *et al*, 2003). In this hairless mouse (Skh1/hr) model, short-term (3 h) neutralization of SC was achieved with a single, topical application of a superbase. Whereas these studies demonstrated that even brief elevations in SC pH alter barrier recovery kinetics and SC integrity/cohesion, basal permeability barrier function remained unchanged (Hachem *et al*, 2003). Because a sustained increase in SC pH is characteristic of most if not all chronic inflammatory dermatoses (Chikakane and Takahashi, 1995; Ohman and Vahlquist, 1998), we assessed here whether more prolonged elevations in SC pH might provoke profound functional abnormalities that could more closely mimic these clinical situations. By repeatedly applying the "superbase", 1,1,3,3-tetramethyl-guanidine (TMG), we could sustain a neutral SC pH indefinitely without the development of toxicity. Yet, maintenance of a neutral pH was accompanied by profound abnormalities in both basal barrier functions and SC integrity/cohesion that were due to sustained SP activity, rather than just pH-induced changes in enzyme catalytic activity. Indeed, such prolonged pH elevations lead to progressive degradation of critical enzymes and structural proteins that mediate these key functions.

Results

Neither toxicity nor inflammation results from prolonged sc neutralization Before assessing the consequences of increased pH on epidermal function, we measured SC pH after twice-daily application of TMG superbase for 5–16 d. The surface pH of Skh1/hr skin, assessed daily before TMG application, showed a sustained and significant increase in SC pH in comparison with sites treated with neutralized superbase (nTMG) or vehicle (Fig 1*A*). The increase in pH extended to all levels of the SC, and it remained significantly higher, in TMG- than in nTMG-treated sites (Fig 1*B*). Yet, despite the sustained elevations in surface pH, there was no evidence of cutaneous abnormalities or inflammation in histologic sections, taken 5 or 16 d following TMG (or nTMG) treatment (Fig 2).

Sustained SC neutralization provokes abnormalities in SC integrity/cohesion, attributable to sustained SP activity After 5 d of repeated exposure of normal skin to TMG, both SC integrity and cohesion deteriorated significantly in comparison with the parameters in skin sites treated with the nTMG (Figs 1*C–E*). SC integrity and cohesion in nTMG-treated sites were comparable to vehicle-treated and untreated skin (not shown). Co-applications of SPI (soybean trypsin inhibitor (STI) or phenylmethanesulfonyl fluoride (PMSF)) blocked the deleterious effects of the superbase on both SC integrity (Fig 1*C* and *D*) and cohesion (Fig 1*E*). We next performed albumin + TMG-treated controls to exclude whether the effects of SPI co-application reflect non-specific mechanisms (e.g., adsorption, protein–protein competition for access to sites of SP activity, or partial neutralization of TMG). SC integrity was assessed on TMG $\pm$ 0.5% albumin-treated sites and compared with nTMG after 5 d of treatment ($n = 8$). No significant differences in SC integrity (transepidermal water loss (TEWL) measurements after three consecutive SC tape stripping) were observed on albumin + TMG-treated flanks (TEWL $= 53.39 \pm 4.72$ g per m^2 per h) *versus* TMG alone (TEWL $= 50.16 \pm 4.83$ g per m^2 per h). Yet, TMG $\pm$ albumin still showed significant alterations in SC integrity in comparison with nTMG + albumin (TEWL $= 32.03 \pm 3.75$ g per m^2 per h). Neither SPI alone, nor co-applications of SPI with the superbase altered surface pH, i.e., the superbase induced an increase in SC pH, even in the presence of an SPI.

Since two SP with known neutral pH optima, stratum corneum chymotryptic enzyme (SCCE) and stratum corneum tryptic enzyme (SCTE), regulate SC desquamation through CD degradation (Egelrud and Lundstrom, 1991; Backman *et al,* 1999; Johnson *et al*, 2003), we next assessed SP activity in TMG $\pm$ SPI (either STI or aprotinin)-*versus* nTMG-treated skin sites by *in situ* zymography. As shown in Fig 3, sustained SC neutralization enhanced SP activity (Fig 3*A*), an increase that could be inhibited by co-applications of either STI (Fig 3*C*) or aprotinin (not shown). To assess the target SC protease activated by sustained neutralization, *in vitro* zymography was performed on SC extract from 5 d superbase-treated skin in the presence of a panel of protease inhibitors, namely: (a) pepstatin A (acidic protease inhibitor) and phenanthroline monohydrate (metalloprotease inhibitor) or (b) pepstatin A, phenanthroline monohydrate, and STI. Compared with vehicle-treated skin, TMG application correlates with a significant increase in SP activity of SC extracts even in the presence of pepstatin A and phenanthroline monohydrate (Fig 3*D* and *E*). Yet, addition of STI overrides TMG-induced proteolysis (Fig 3*D* and *E*). Together, these results show that a prolonged SC neutralization results in deterioration of SC integrity and cohesion and, that these functional changes are due to an increase in SP activity.

Sustained SC neutralization accelerates SP-mediated degradation of CD We next assessed the structural and biochemical basis for the abnormalities in SC integrity and cohesion that result from sustained SC neutralization. Twice-daily applications of TMG for 5 d again raised SC pH, accompanied by a significant decrease in the density of CD in the lower SC, extending even to the stratum granulosum (SG) interface, assessed by quantitative electron microscopy. These effects were more pronounced than previously observed with short-term (3h) SC neutralization (Fig 4, see also Hachem *et al*, 2003). Not only the density of

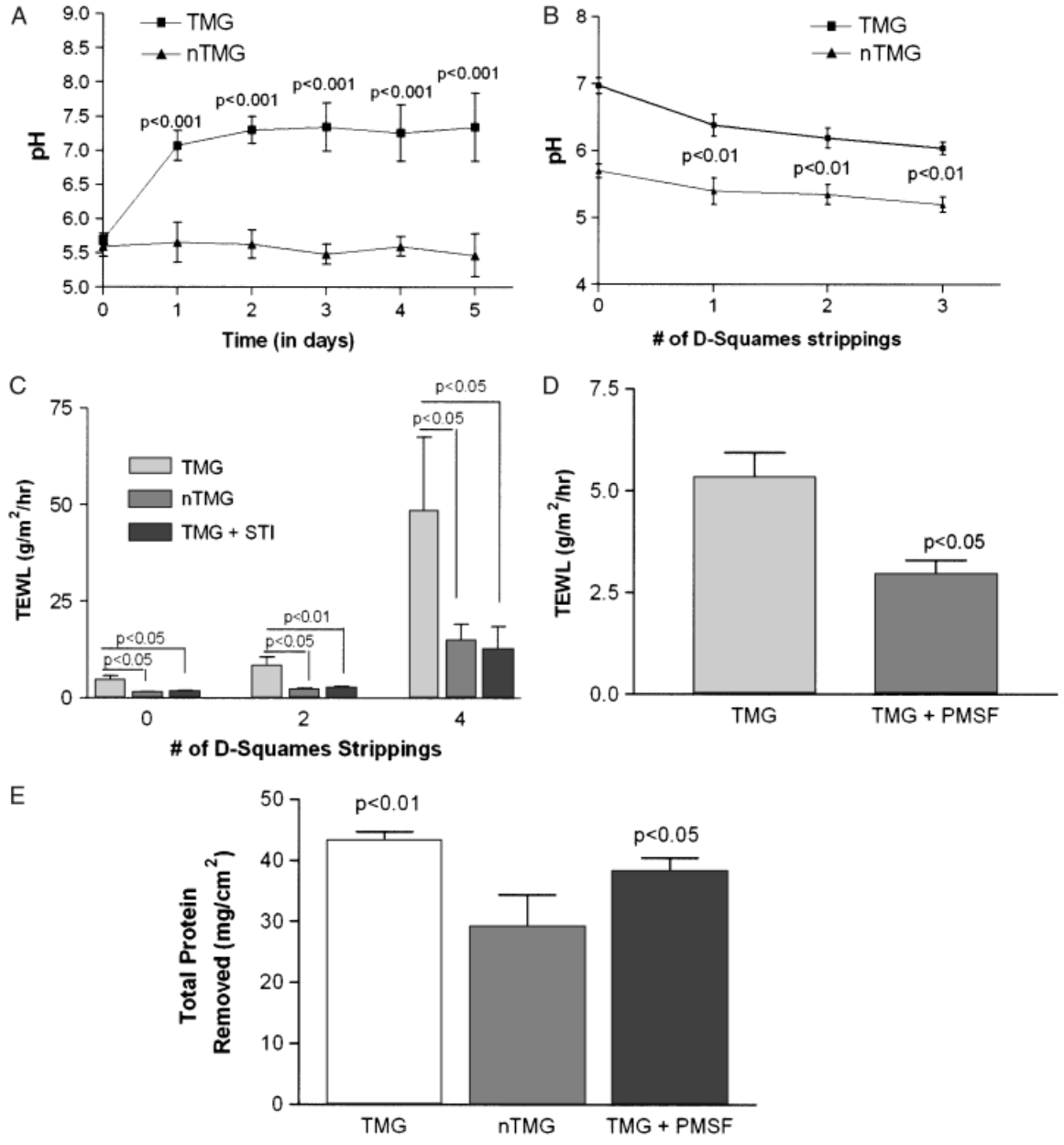

Figure 1

Repeated applications of superbase result in sustained stratum corneum (SC) neutralization and alterations in SC integrity/cohesion. (*A*) Topical application of tetra-methyl-guanidine (TMG: 6.5/100; vol/vol in propylene glycol:ethanol) induces sustainable neutralization of SC pH over 5 d in comparison with neutralized tetra-methyl-guanidine (nTMG). (*B*) At day 5 after TMG applications, sequential tape strippings show that the increase in pH is sustained, remaining significantly different at all levels of SC. (*C–E*) SC integrity and cohesion decline after 5 d of TMG (6.5:1000 vol/vol in propylene glycol ethanol 7:3) but not nTMG treatment. Co-applications of either soybean trypsin inhibitor (STI, 0.5%) or phenylmethanesulfonyl fluoride (10 mM) prevent these alterations. Results shown are the mean ± SD (n = 4–6).

CD but also the size of individual junctions in the lowermost SC layer was markedly reduced (Fig 4*A–C*). In contrast, repeated nTMG applications altered neither CD density (Fig 4*A–C*) nor the size of CD in comparison to either vehicle-treated or untreated SC (Fig 4*A–C*; untreated data not shown). The decrease in CD could be further ascribed to a superbase-induced increase in SP activity (see above zymography results), because co-applications of STI, along with TMG, prevented the decrease in CD density and size (Fig 4*A–D*). Finally, we assessed whether the superbase-induced decrease in CD density was attributable to loss of its constituent proteins, assessed as desmoglein 1 (DSG1) content in western blots of SC from TMG ± STI- and nTMG-treated SC. DSG1 immunoblotting indicated extensive proteolysis of DSG1 after 5 d of sustained neutralization of normal SC, a change that was again blocked by co-applications of STI (Fig 4*E–F*). In addition, DSG1 proteolysis again was more marked after 5 d of SC neutralization than occurred with 3 h of acute alkalization (Fig 4*F*, see also Hachem *et al*, 2003). Together, these results show that the decline in SC integrity and cohesion from prolonged SC neutralization can be ascribed to sustained SP activity, leading to progressive degradation of CD and its constituent proteins.

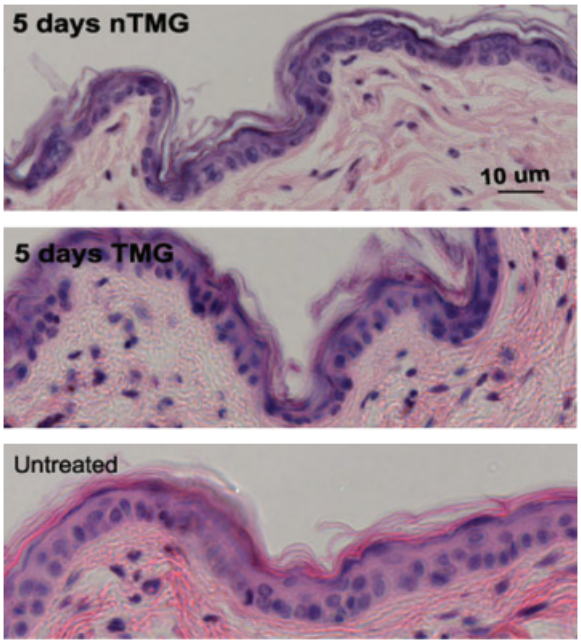

Figure 2
A sustained increase in pH does not alter skin histology. Hematoxylin- & eosin-stained paraffin sections 5 d after either 1,1,3,3-tetramethyl-guanidine (TMG) (6:1000 vol/vol in propylene glycol ethanol 7:3) (*A*), neutralized tetra-methyl-guanidine (nTMG) (*B*), or no treatment (*C*) of hairless mice show no changes in cellular or epithelial structures, or evidence of dermal inflammation, with repeated superbase applications. *Scale bar* = 10 μm.

Sustained elevations in SC pH not only delay barrier recovery but also increase basal TEWL Although our prior studies showed that a short-term increase in SC pH does not alter basal barrier function (Hachem *et al*, 2003), the sustained elevation in SC pH induced by repeated superbase applications produced a more profound barrier abnormality indicated by increased basal TEWL levels (4.75 ± 0.52 g per m^2 per h *vs* 1.62 ± 0.04 g per m^2 per h on TMG- *vs* nTMG-treated sites, $p < 0.01$) without affecting the level of SC hydration (55.19 ± 1.50 *vs* 56.38 ± 1.48 on TMG *vs* nTMG-treated sites, in relative units). The superbase-induced abnormality in basal TEWL was again prevented by co-applications of STI (1.7 ± 0.12 g per m^2 per h). To determine whether sustained SC neutralization also alters the kinetics of barrier recovery, we next compared barrier recovery after repeated tape stripping (TEWL > 6–8 g per m^2 per h) of flanks treated for extended periods with either TMG or nTMG ± STI. As shown in Figure 5, superbase (TMG)-treated sites exhibited a marked delay in barrier recovery in comparison with control (nTMG)-treated sides. Finally, SC hydration levels remained unchanged in all treatment groups, indicating that SC neutralization does not affect SC hydration. These results show that sustained neutralization of SC alters both basal barrier function, and the kinetics of barrier recovery.

Abnormal lipid processing accounts for the barrier abnormality To assess the structural basis for the sustained superbase-induced abnormalities in permeability barrier homeostasis, we next compared SC extracellular lamellar membrane structures in TMG- and control (nTMG)-treated skin, with or without a co-applied SPI. Under basal conditions, prolonged SC neutralization resulted in striking structural defects, i.e., delayed membrane maturation in the extracellular domains of the lower SC (Fig 6). In contrast, membrane structures were normal in sites treated repeatedly with nTMG, and in sites treated with SPI + TMG (Fig 6). Together, these results show that sustained SC neutralization results in abnormal lipid processing, linked to prolonged SP activity, which, in turn, explains the abnormalities in permeability barrier homeostasis.

Abnormal lipid processing from sustained neutralization is attributable to degradation of lipid-processing enzymes To further explore the mechanisms that account for both abnormal barrier homeostasis and impaired lipid processing, we next assessed the *in situ* activity of two key lipid-processing enzymes with known acidic pH optima (Vaccaro *et al*, 1985; He *et al*, 1999); β-GlcCer'ase and acidic sphingomyelinase (aSMase). Enzyme activity was assessed in TMG- and nTMG ± SPI (aprotinin or STI)-treated skin sites by *in situ* zymography. Both β-GlcCer'ase and aSMase activities decreased markedly in skin sites that were neutralized for 5 d (Figs 7*A–E* and 8). To ascertain whether the decreased activity reflects changes in enzyme catalytic activity alone, as occurred with acute pH increase (Hachem *et al*, 2003), we next assessed whether enzyme activities were normalized by re-acidification. In contrast to acute elevations in SC pH, where enzyme activity can be restored by re-acidification (Hachem *et al*, 2003), β-GlcCer'ase and aSMase activity did not recover when sites that were neutralized for several days were re-acidified (Figs 7*C* and 8*C*). Yet, despite a sustained neutral pH, SPI co-applications completely reversed the TMG-induced changes in β-GlcCer'ase and aSMase activity, but only when sections from TMG + STI-treated skin were also re-acidified (Figs 7*D* and 8*D*). Since these results suggest that SP-mediated enzyme degradation could be occurring, with prolonged neutralization, we next assessed enzyme content by both immunohistochemistry and western immunoblotting. Both immunostaining (Fig 7*F–H*) and immunoblotting (Fig 9*A*) for β-GlcCer'ase and immunoblotting (Fig 9*B*) for aSMase showed a decline in enzyme mass subsequent to prolonged neutralization of SC. Here again, co-application of SPI reversed the observed decrease of processing enzyme mass (Figs 7–9). Since SCCE is believed to be a central target activated by the prolonged elevation of SC pH, we analyzed whether β-GlcCer'ase and aSMase proteins are substrates for these proteases *in vitro*. Protein extracts from human SC were therefore treated for 2 h at 37°C with SCCE at pH 7.2, and the incubated extracts were analyzed by immunoblotting with either anti-β-GlcCer'ase or anti-aSMase antibodies (Fig 10). Recombinant SCCE induced a decrease in the immunoblotting of both β-GlcCer'ase or aSMase compared with control experiments performed in the absence of the active SCCE (Fig 10). These results further demonstrate that proteolytic degradation of both β-GlcCer'ase aSMase occurs with a sustained increase in SC pH, and show that SCCE produces a catalyzed proteolysis of key lipid processing secondarily responsible for the observed permeability barrier abnormalities.

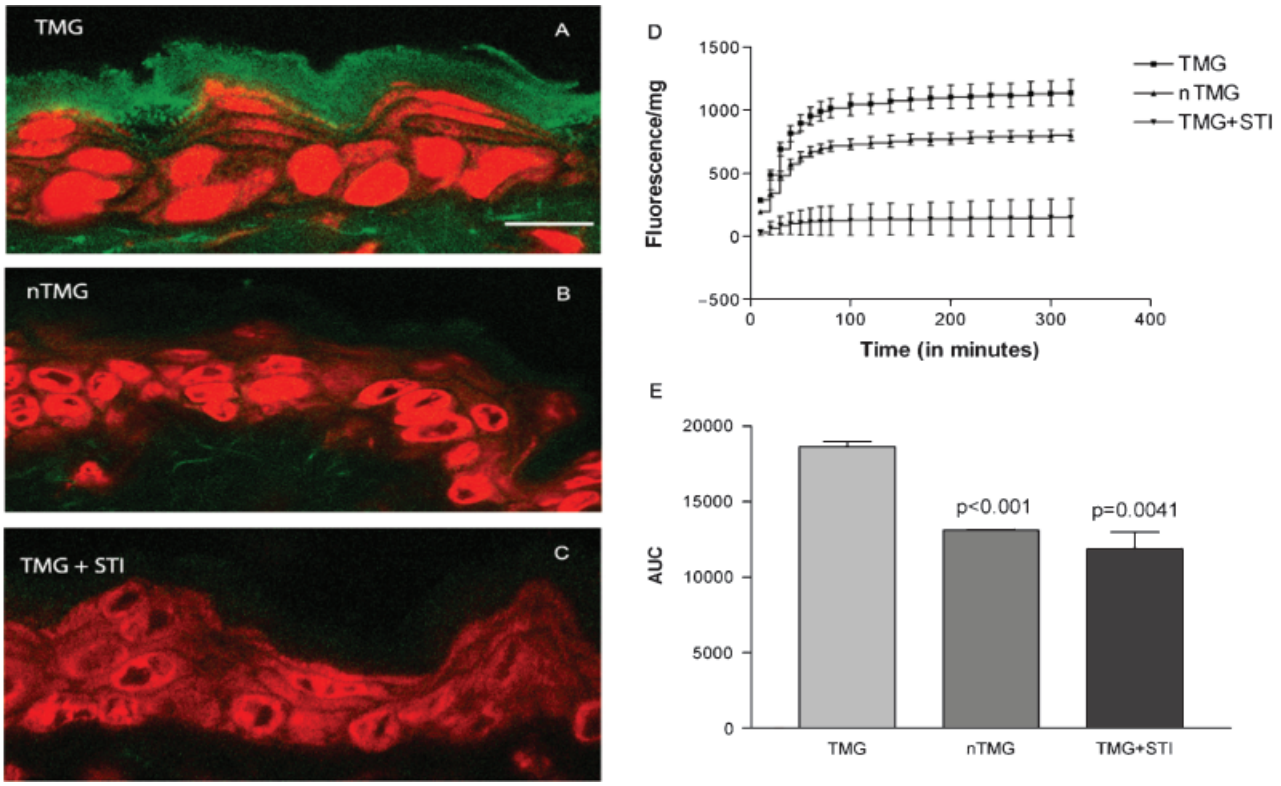

Figure 3
Sustainable stratum corneum (SC) neutralization induces increased serine protease (SP) activity, inhibited by soybean trypsin inhibitor (STI). (*A–C*) *In situ* zymography 5 d after repeated superbase treatments shows an increase in SP activity in TMG (6.5:1000 vol/vol in propylene glycol ethanol 7:3) (*A* and *D*) *versus* neutralized 1,1,3,3-tetra-methyl-guanidine (nTMG) (*B* and *E*)-treated sites. Co-application of either STI (0.5%) (*C*) with TMG inhibits pH-induced SP activity. *Scale bar* = 10 μm. (*D*, *E*) *In vitro* zymography performed on SC extracts 5 d after repeated superbase treatments shows a TMG-induced increase in protease activity in the presence of pepstatin A and phenanthroline monohydrate [TMG + (a)] compared with vehicle treatment [nTMG + (a)]. Unlike acidic and metallo-protease inhibitors, addition of STI [TMG + (b)], a serine protease inhibitor, to the reaction substrate downregulated TMG-induced proteolysis. Results shown in *E* are the mean of the areas under the curve (AUC) ± SD calculated from the data in *D*. The latter are measured as emitted fluorescence at an excitation/emission wavelength of 485/530 per mg sample protein (n = 4–6).

Discussion

Previous studies have demonstrated that acute transient increases in SC pH induce abnormalities in the recovery of permeability barrier function after acute barrier disruption without inducing abnormalities in basal TEWL (Hachem *et al*, 2003). This abnormality in permeability barrier homeostasis was due to impaired extracellular lipid processing in the SC, indicated by the presence of immature lamellar membranes in the SC. The pH-induced processing defect could be further attributed to decreased catalytic activity, but not enzyme mass, of a key lipid-processing enzyme with acidic pH optima, β-GlcCer'ase (Hachem *et al*, 2003). In addition, transient neutralization of SC also induced abnormalities in SC integrity and cohesion that were linked to increased SP-mediated degradation of CD.

Whereas the abnormalities induced by such transient increases in SC pH are significant, they may not be of great clinical relevance. Inflammatory dermatoses, such as psoriasis, atopic dermatitis, and seborrheic dermatitis, demonstrate an in SC pH that parallels disease activity (Beare *et al*, 1959; Jolly, 1960; Yosipovitch *et al*, 1993). Moreover, the increase in pH in these disorders is paralleled by increased SP activity (Murahata *et al*, 1988; Chikakane and Takahashi, 1995). Hence, we asked here whether more substantial abnormalities would emerge if a neutral SC pH could be sustained experimentally for longer time periods and whether a high SP activity is critical for these abnormalities. Indeed, our results demonstrate that sustained pH alterations produce more profound abnormalities in permeability barrier function and SC integrity/cohesion. These are found with transient elevations. The more profound nature of the functional abnormalities is shown by the elevations in basal TEWL induced by the sustained increase in SC pH. Likewise, the SC integrity abnormality is more profound, with a further degradation of CD, extending even to the SG–SC interface.

Since sustained increases in SC pH produced alterations that were even more profound than those produced by transient pH increase, intrinsic homeostatic mechanisms were not sufficiently upregulated to correct the pH-induced functional disturbances, which are assessed here, i.e., barrier function and SC integrity/cohesion. Yet, it is important to note that toxicity or inflammation did not emerge, suggesting that some or yet undetermined responses protect the skin from inflammation (Hachem *et al*, 2002). The lack of a sufficient homeostatic response to pH contrasts with the effects produced by alterations in humidity, which progressively modify epidermal barrier function to a level appropriate for the environmental insult (Denda *et al*, 1998).

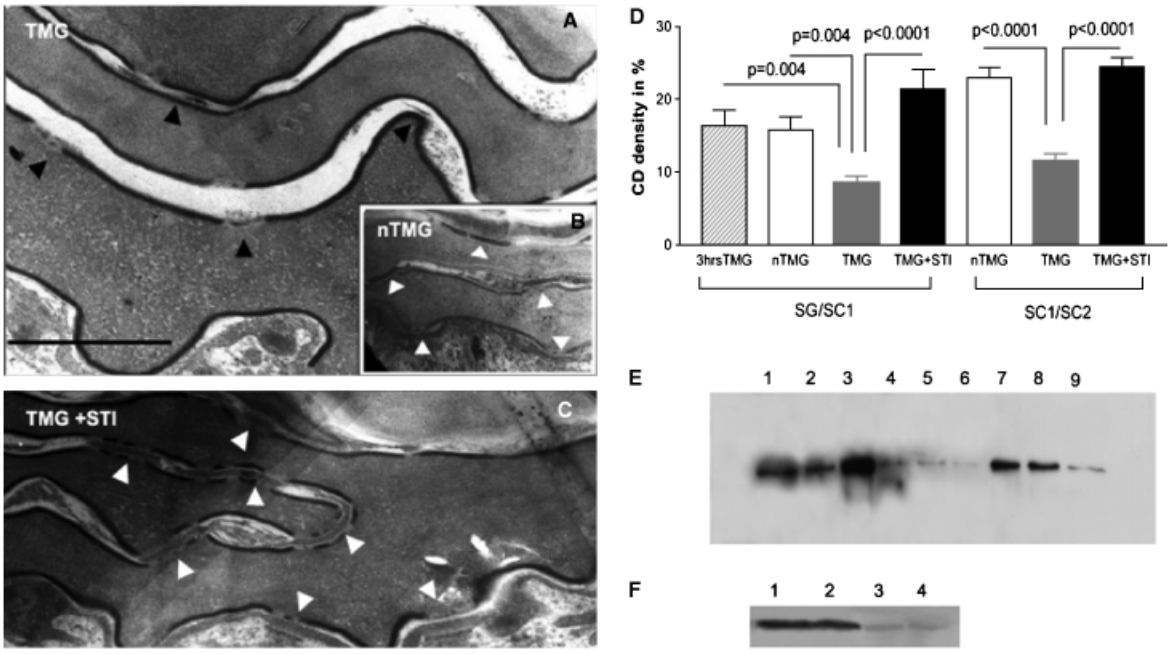

Figure 4
Corneodesmosome (CD) density declines after sustained stratum corneum (SC) neutralization paralleled by serine protease-dependent degradation of desmoglein I (DSG1). (*A–C*) 1,1,3,3-tetra-methyl-guanidine (TMG)-treated skin (6.5:1000 vol/vol in propylene glycol ethanol 7:3) shows a progressive decrease in CD density and length (*A*) in comparison with neutralized TMG (nTMG) treatment (*B*). Co-applications of soybean trypsin inhibitor (STI, 0.5%) with TMG prevent the superbase-induced loss of CD (*C*). *Scale bars* = 0.25 μm. (*D*) Quantitative electron microscopy analysis shows a significant decrease in CD density after 5 consecutive days of TMG applications in the lower SC, between the first (SC1) and the second (SC2), extending even to the stratum granulosum interface (SG/SC1). Compared with 3 h neutralization, the CD density is significantly more pronounced at the SG1/SC1 in the long-term neutralization model (5 d). CD density is normalized in superbase-treated skin by co-applications of STI (0.5%) with TMG (6.5:1000 vol/vol in propylene glycol ethanol 7:3). Results shown are the means ± SD (n = 30 micrographs in each experimental group). (*E*, *F*) Protein extracts from SC were loaded onto Tris-glycine PAGE gels, and after transfer onto a nitrocellulose membrane, incubated further with DSG1 antibody at 4°C overnight. Blot *E* shows a decrease in DSG1 immunostaining on TMG-treated (4–5) *versus* nTMG (1–3) or TMG + STI-treated sites (7–9). Blot *F* shows a relative decrease in DSG1 immunostaining with long-term (5 d; 3 and 4) TMG application sites compared with short-term application (3 h; 1 and 2)

Two SC SP with a convincing link to desquamation have been described to be present in the SC: SCCE and SCTE (Caubet *et al*, 2004). Yet, SCCE, which is found in both human and murine SC (Hansson *et al*, 1994; Backman *et al*, 1999), has been shown to be implicated in pro-inflammatory epidermal responses (Nylander-Lundqvist and Egelrud, 1997) and may contribute to disease pathogenesis and/or maintenance (Ekholm and Egelrud, 1999; Hansson *et al*, 2002; Ny and Egelrud, 2004). Hence, all of the pH-induced abnormalities in SC function can be further linked to enhanced SP activity, because inhibition of SP activity blocked the abnormalities in both SC integrity/cohesion and permeability barrier homeostasis. Although increased SP activity accounted for all of these functional changes, different downstream mechanisms were responsible for each functional abnormality. In the case of the abnormalities in SC integrity/cohesion, increased SP activity leads to accelerated degradation of DSG1, a structural protein required for CD formation, resulting in degradation of CD, which accounts for decreased SC integrity/cohesion, as with transient pH increase (Hachem *et al*, 2003), but more global in case of sustained pH elevations. As for permeability barrier homeostasis, increased SP activity leads to degradation of both β-GlcCer'ase and aSMase, enzymes that are essential for the extracellular lipid processing required for normal permeability barrier homeostasis (Holleran *et al*, 1994; Jensen *et al*, 1999).

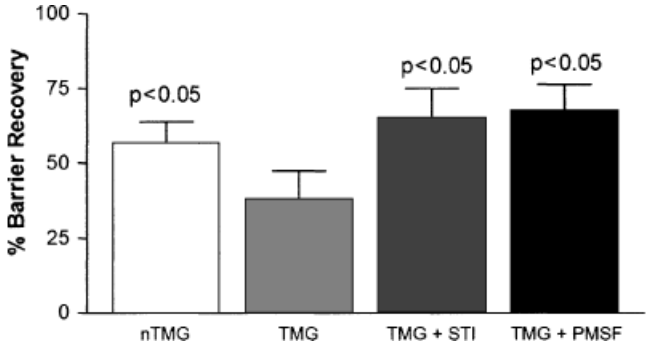

Figure 5
Barrier recovery normalizes when superbase-treated skin is co-treated with serine protease inhibitor (SPI). Transepidermal water loss was measured prior and following acute barrier disruption by repeated cellophane tape stripping at the end of 5 d of treatment, using an electrolytic water analyzer (MEECO). Barrier recovery kinetics (expressed in % of barrier recovery) assessed 3 h after acute disruption is delayed as a result of sustainable SC neutralization with twice-daily applications of 1,1,3,3-tetra-methyl-guanidine (TMG) *versus* neutralzed TMG (nTMG). The TMG-induced delay is reversed by the co-application of SPI (soybean trypsin inhibitor and phenylmethanesulfonyl fluoride). Results shown are mean ± SD (n = 4 in each group).

Although we have demonstrated that the degradation of key structural and enzymatic SC proteins is accelerated by the increase in SP activity that results from an increased SC pH, it is possible that other, as yet unidentified SC proteins

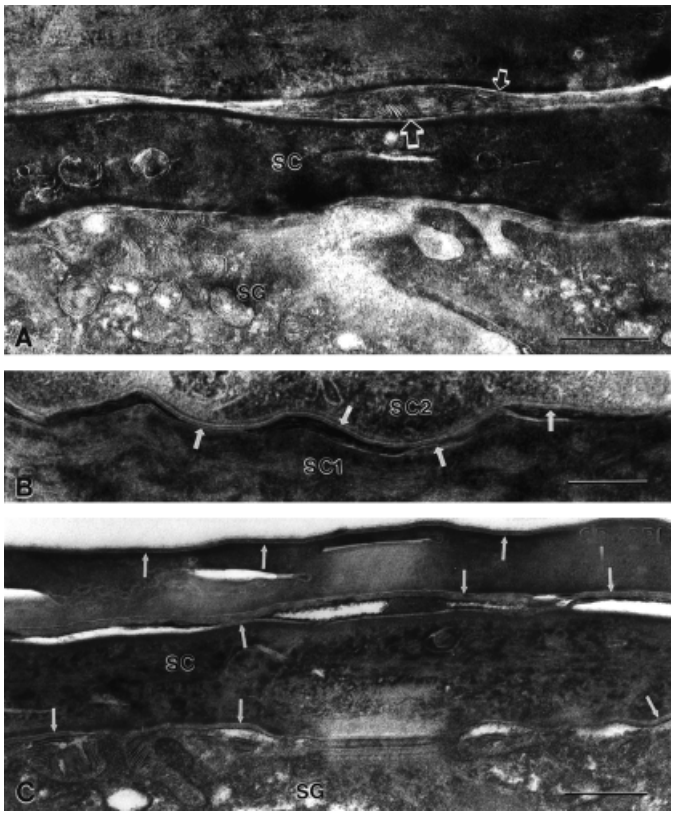

Figure 6
Superbase-induced lipid processing abnormality is reversed by a co-applied serine protease inhibitor (SPI). (*A*) Daily application of the superbase 1,1,3,3-tetra-methyl-guanidine (TMG), results in the persistence of unprocessed (immature) lamellar membranes (*open arrows*) above the stratum granulosum (SG)–stratum corneum (SC) interface. (*B*) Sites treated similarly with the neutralized superbase (nTMG) reveal mostly processed lamellar membranes (*solid arrows*) within the intercellular domains between the first (SC1) and second (SC2) cornified layers. (*C*) Co-applications of a soybean trypsin inhibitor (SPI) with the superbase reveal normal membrane processing (*solid arrows*) at all levels of the SC. *Scale bars* = 0.25 μm. (*A–C*), RuO_4 post-fixation.

could also be degraded, which could have adverse effects on crucial SC functions. Thus, stimulation of SP activity by prolonged increases in SC pH could provoke effects in addition to those described in this study. Finally, it should be recognized that some of these pH-induced effects could occur independent of increased SP activity. It is possible that an increased SC pH could alter the activity of one or more of these additional enzymes, independent of SP activation, thereby altering SC function, as we have shown for β-GlcCer'ase and aSMase, whose catalytic activities were changed with pH alterations alone.

Sustained elevations in SC pH are an appropriate model for neonatal skin, which displays normal basal barrier function, but abnormalities in both barrier recovery and SC integrity/cohesion. In addition, the sustainable increase in SC pH may be a model for occupational diseases, such as irritant or allergic contact dermatitis, with prolonged contact with alkaline substances. Whereas contact dermatitis is aggregated when the allergen or irritant is applied at an elevated pH (Murahata *et al*, 1988), it is well recognized that alkaline detergent-induced cutaneous disorders are characterized by an increased SC pH (Baranda *et al*, 2002) and display disturbances in permeability barrier function (Hachem *et al*, 2002). It is possible that the sustained increase in SC pH in these cutaneous disorders further aggravates and sustains abnormalities in SC function that, in turn, could further worsen barrier abnormalities (a positive cycle of worsening disease leading to increased SC pH leading to worsening of disease), and/or make the occupational dermatoses more resistant to therapy.

In summary, this study demonstrates that sustained increases in SC pH, by stimulating SP activity, adversely affect a range of SC functions, including a decrease in SC integrity/cohesion, impaired permeability function, and the related mechanisms to these aberrations.

Materials and Methods

Materials Male Skh1/Hr, 6–8-wk old, were purchased form Charles River Laboratories (Wilmington, Massachsetts) and fed Purina mouse diet and water *ad libitum*. Propylene glycol, ethanol, and HCl were from Fisher Scientific (Fairlane, New Jersey), whereas TMG, STI, aprotinin PMSF, pepstatin A, and phenanthroline monohydrate were from Sigma Chemicals (St Louis, Missouri). Rabbit antibodies against mouse DSG1 and β-GlcCer'ase were gifts from Dr John Stanley (University of Pennsylvania) and Dr Ellen Sidransky (National Institutes of Mental Health, Bethesda), respectively. Anti-aSMase antibody (mouse, rat, and human specific) was purchased from Santa Cruz Biotechnology (Santa Cruz, California). Goat anti-rabbit Alexa-labeled secondary antibodies were purchased from Molecular Probes (Eugene, Oregon). Horseradish peroxidase-conjugated, anti-rabbit IgG was purchased from Vector Labs (Burlingame, California). The active form of the human SCCE was a generous gift from Arexis AB (Göteborg, Sweden). The EnzChek Protease Assay Kit containing BODIPY-FL-Casein (green fluorescence), resorufin α–D-glucopyranoside, and Amplex Red reagent was purchased from Molecular Probes, and used for *in situ* zymography (see below). In all, 22 mm D-Squame-100 tapes were purchased from CuDerm (Dallas, Texas). Bradford protein assay kits (Bio-Rad Protein Assay Dye), as well as lyophilized, bovine plasma γ-globulin were purchased from Bio-Rad (Hercules, California).

All procedures were performed while mice were anesthetized with chloral hydrate (Morton Grove Pharmaceuticals, Morton Grove, Illinois), under a protocol approved by the VA Animal Care Committee.

SC neutralization

Maintenance of SC neutralization Normal Skh1/hr were treated topically with twice-daily applications of TMG (6,5/1000, vol/vol; pH = 12.8) in propylene glycol:ethanol (7:3 vol/vol; pH = 7.0) at 12 h intervals for 5–16 d on a 5–6 cm^2 area on each flank. Because superbases and superacids are, by definition, at least 10 order of magnitude more basic or more acidic than 1 N sodium hydroxyde (NaOH) and 1 N sulfuric acid, respectively (Oyama and Kondo, 2003), they can be used to manipulate pH locally at very low concentrations (Hachem *et al*, 2003). In addition, their lipophilic naphthalene structure favors absorption throughout the SC, so that the pH elevation extends to deeper SC layers (Hachem *et al*, 2003). Control Skh1/hr were treated similarly with HCl-neutralized TMG (nTMG) in propylene glycol:ethanol (7:3 vol/vol), or with vehicle alone (same concentrations and volumes).

For the SPI experiments, animals received co-applications of 0.5% STI, or PMSF 0.5% aprotinin coincidentally with TMG or nTMG treatments. Co-applied SPI did not alter the pH of the applied solutions. In another set of experiments, albumin (5 mg per mL) was co-applied with TMG in order to exclude non-specific mechanisms that could inhibit protease activity such as adsorption or protein–protein competition for access to sites of SP activity.

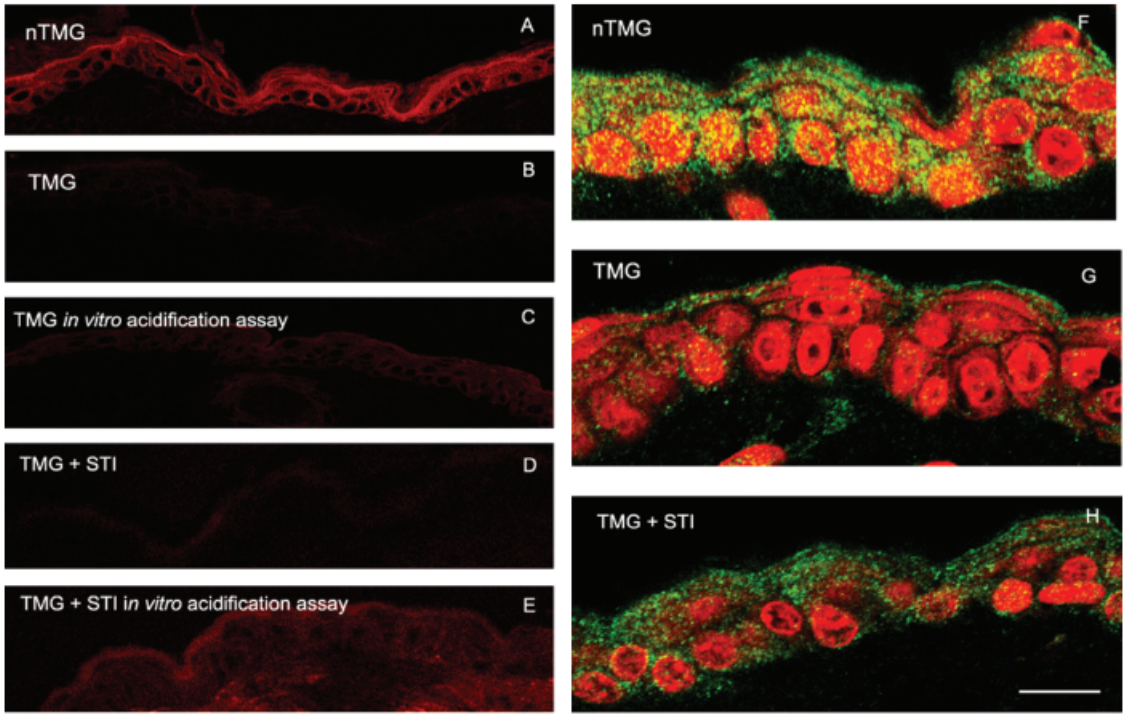

Figure 7
Defective barrier homeostasis from sustainable neutralization is attributable to both downregulation and degradation of a key lipid-processing enzyme β-glucocerebrosidase (β-GlcCer'ase). β-GlcCer'ase *in situ* zymography shows decrease in enzyme activity on sections from skin sites treated for 5 d with 1,1,3,3-tetra-methyl-guanidine ± serine protease inhibitor, namely soybean trypsin inhibitor (STI) *versus* neutralized TMG (nTMG) (*A*). β-GlcCer'ase enzyme activity is reversed by acidification of TMG + STI co-treated, but not with reacidification of sites treated with TMG alone (*C vs E*). These results suggest both a pH-dependent, downregulation of enzyme activity, and a protease-dependent degradation of β-GlcCer'ase enzyme with sustained superbase treatment. Similarly, immunostaining for β-GlcCer'ase decreased with SC neutralization by TMG (*G*), but not in parallel, nTMG-treated sites (*F*), nor in sites co-treated with STI (*H*).

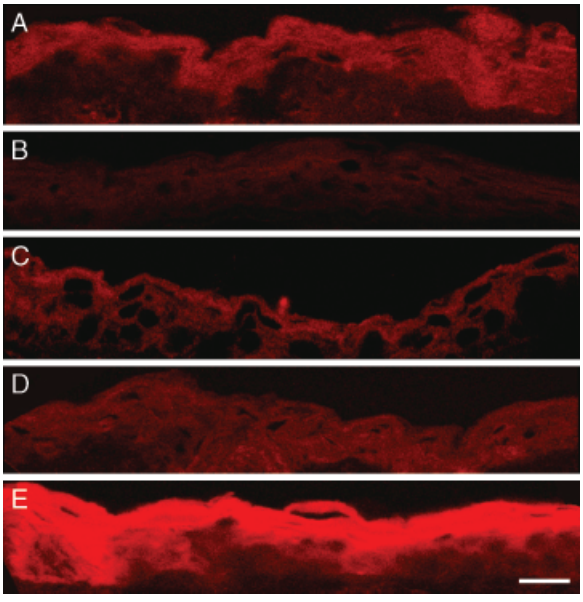

Figure 8
Defective barrier homeostasis from sustainable neutralization is attributable to both downregulation and degradation of a key lipid-processing enzyme acidic sphingomyelinase (aSMase)**.** aSMase *in situ* zymography shows decrease in enzyme activity on sections from skin sites treated for 5 d with 1,1,3,3-tetra-methyl-guanidine (TMG) ± serine protease inhibitor (*B*), namely Aprotinin (*C*) *versus* neutralized TMG (nTMG) (*A*). *C* and *D* show a small enzyme activity band in the stratum corneum compared with *A*. aSMase enzyme activity is reversed by acidification of TMG + aprotinin co-treated (*E*), but not with re-acidification of sites treated with TMG alone (*D*). These results suggest a pH-dependent downregulation and degradation of aSMase with sustained superbase treatment.

Acute neutralization model Normal Skh1/hr were treated topically with a single application of either TMG (1:100; vol/vol) in propylene glycol: ethanol (7:3 vol/vol) on 5–6 cm^2 areas on both flanks as described previously (Hachem *et al*, 2003). Controls were treated with HCl-neutralized TMG (nTMG) in propylene glycol:ethanol (7:3 vol/vol).

Assessment of epidermal function

SC pH Surface pH was measured with a flat, glass surface-electrode from Mettler-Toledo (Giessen, Germany), attached to a pH meter (Skin pH Meter pH Model 900; Courage & Khazaka, Cologne, Germany). Evaluation of skin surface pH was performed once daily immediately prior to treatment with either TMG, nTMG, or vehicle.

Permeability barrier function To assess epidermal permeability function, TEWL was measured daily immediately prior to the next application of TMG ± STI or nTMG. TEWL was measured both under basal conditions, as well as following acute barrier disruption by repeated cellophane tape stripping at the end of five days of treatment, using an electrolytic water analyzer (MEECO; Warrington, Pennsylvania). Barrier recovery kinetics were then assessed 3 h after acute disruption.

SC hydration SC hydration was determined by electrical capacitance, measured in arbitrary units with a Corneometer CM Model 810 (Courage & Khazaka), before and 5 d after applications of TMG ± STI, nTMG, or vehicle.

SC integrity Before stripping experiments, the skin surface was cleaned with a single ethanol wipe. D-Squame tapes then were placed sequentially onto the test areas for about 3 s each, removed with forceps, and stored at 4°C in glass scintillation vials. Sequen-

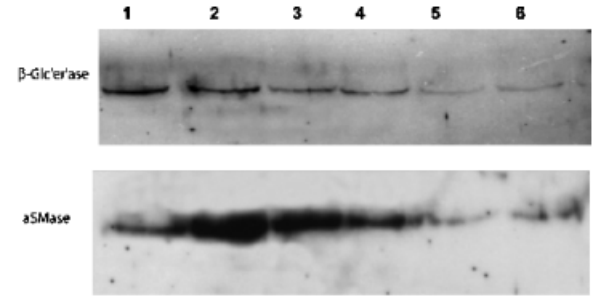

Figure 9
Sustainable stratum corneum (SC) neutralization induces a serine protease-dependent degradation of β-glucocerebrosidase (β-GlcCer'ase) and acidic sphingomyelinase (aSMase). Protein extracts (n = 2 each) from whole epidermis for and SC for aSMase and loaded onto Tris-glycine PAGE gels, and after transfer onto a nitrocellulose membrane, incubated further with either aSMase or β-GlcCer'ase antibody at 4°C overnight. Blots show a decrease in both β-GlcCer'ase and aSMase immunostaining on 1,1,3,3-tetra-methylguanidine (TMG)-treated (5–6) *versus* neutralized TMG (nTMG) (3 and 4) or TMG + aprotinin (1 and 2)-treated sites.

tial tape strippings were performed on the flanks of Skh1/hr 12 h after the last application of TMG ± STI or nTMG. TEWL was evaluated before and after each tape strip, and the rate of change in TEWL equated to SC integrity.

SC cohesion The amount of protein removed per D-Squame strip was measured as previously described (Dreher *et al*, 1998). This microassay system has been shown to be linear in the range of 1–10 μg per mL, using human SC removed from a heel callus as the protein source (calculated slope $R_f = 0.0297 \pm 0.0006$; Spearman coefficient: 0.999; $p < 0.0001$). The protein content per stripping was determined with the Bio-Rad Protein Assay Kit. Lyophilized, bovine gamma globulin was used as the standard in all assays, because it correlated best with human SC. Each tape was incubated with 1 mL of 1 M NaOH for 1 h at 37°C in an incubator shaker at 80 rpm, and neutralized thereafter with 1 mL of 1 M HCl in the scintillation vials. Subsequently, 0.2 mL of this solution was incubated in 0.6 mL distilled water plus 0.2 mL of the

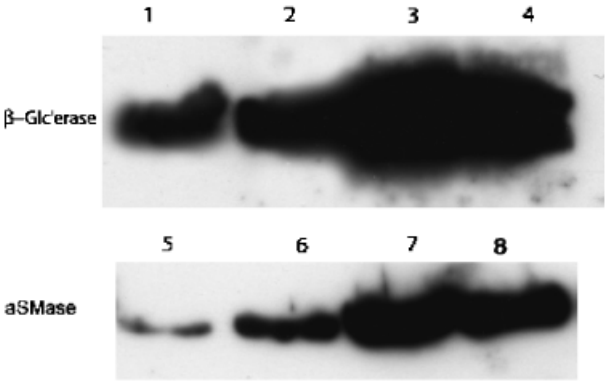

Figure 10
Stratum corneum (SC) chymotryptic enzyme induces proteolysis of β-glucocerebrosidase (β-GlcCer'ase) and acidic sphingomyelinase (aSMase) at pH 7.2. To evaluate the importance of stratum corneum chymotryptic enzyme (SCCE) in the degradation of β-GlcCer'ase and aSMase, SC protein extracts from six healthy individuals were pooled into two samples and incubated at 37°C in the presence (1–2 and 5–6) or absence (3–4 and 7–8) of recombinant active SCCE for 2 h at pH = 7.2. The incubated extracts were analyzed by immunoblotting with either anti-β-GlcCer'ase or anti-aSMase antibodies. A significant decrease in the immunostaining for both enzymes (1–2 and 5–6) is observed 2 h following incubation with SCCE.

Bio-Rad protein dye for 5 min in borosilicate tubes. After incubations, the reagents were transferred to polysterene cuvettes, and absorption was measured with a Genesys 5 spectrophotometer (Spectronic, Rochester, New York) at 595 nm. An empty D-Squame tape, as well as distilled water incubated with the Bio-Rad dye, served as negative controls. The amount of calculated protein was then normalized to the skin surface area (μg per cm^2). The amount of removed protein per D-Squame strip agreed with previous reports of SC from untreated skin of Skh1/hr (i.e., range 2.5–4 μg per strip) (Dreher *et al*, 1998; Fluhr *et al*, 2001).

Zymography assays

SP

(a) *In situ*: Skh1/hr flanks were excised from treated animals, and the subcutaneous fat was removed. Frozen sections (8 μm) were rinsed with a washing solution (1% Tween 20 in deionized water) and incubated at 37°C for 2 h with 250 μL of BODIPY-Fl-casein (1 μg per μL) in deionized water (2 μL per mL). The acidification-reversal experiments utilized the same fluorophores in 10 mM morpholino-ethanesulfonic (MES) acid, sodium buffer (pH 5.5) as the *in situ* incubation medium, again at 37°C for 2 h. All sections were then rinsed with the 1% Tween 20 washing solution, coverslipped, and visualized under a confocal microscope (Leica TCS SP, Heidelberg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

(b) *In vitro*: SC was isolated from treated Skh1/hr flanks using sequential D-Squame tape strippings until the glistening layer (= 1 remaining SC layer by EM) was reached. Hematoxylin and eosin staining was performed on paraffin sections from portions of all tape-stripped areas to insure equivalent depth of SC removal in all experiments. D-squame tapes were then incubated overnight at 4°C in 1% Triton X-100 in deionized water, and then sonicated for 5 min on ice to extract proteins from the tapes. 100 μL of extracted SC, in three duplicates from each treatment (n = 4), was incubated at 37°C with 100 μL BODIPY-Fl-casein (1 μg per μL) for 300 min in a microplate reader (Perkin-Elmer 1420 VICTOR multilabel plate reader, BE Perkin-Elmer, Brussels, Belgium) in the presence of either (i) pepstatin (acidic protease inhibitor, 2 mg per mL) and phenanthroline monohydrate (metalloprotease inhibitor, 1 mM) or (ii) pepstatin (2 mg per mL), phenanthroline monohydrate (1 mM) and STI (0.1% vol/vol). Fluorescence was monitored every 10 min at an excitation/emission wavelength of 485/530, and protease activity was determined as measured fluorescence per mg protein present in each sample.

β-GlcCer'ase Skh1/hr flanks were excised and sectioned as above. Frozen sections were washed with the 1% Tween 20 washing solution and incubated at 37°C for 2 h with 250 μL of 1 mM resorufin α-D-glucopyranoside in deionized water (Hachem et al, 2003). The acidification-reversal experiments were performed in 10 mM MES buffer (pH 5.5), as above. All sections were then rinsed with the washing solution, coverslipped, and visualized under the confocal microscope at an excitation wavelength of 588 nm and an emission wavelength of 644 nm.

aSMase Skh1/hr flanks were excised and sectioned as above. Frozen sections were washed with the 1% Tween 20 washing solution and incubated at 37°C for 2 h with 250 μL of 100 mM Amplex Red reagent (containing 2 U per mL HRP, 0.2 U per mL choline oxidase, 8 U per mL of alkaline phosphatase) in deionized water. All sections were then rinsed with the washing solution, coverslipped, and visualized under the confocal microscope at an excitation wavelength of 588 nm and an emission wavelength of 644 nm.

Immunofluorescence Skh1/hr skin was excised from treated animals, and the subcutaneous fat was removed. Paraffin tissue sections from TMG ± SPI or nTMG treated mice 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 24 h at room temperature with 1:100 dilution of a primary antibody against rabbit β-GlcCer'ase, diluted in blocking

buffer. The tissue sections were then washed with PBS, and incubated for 1 h at room temperature with the goat anti-rabbit antibody, diluted in blocking buffer. Tissue sections were then washed with PBS, and mounted before 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 For DSG1 and aSMase, SC was isolated from treated Skh1/hr flanks using sequential D-Squame tape strippings as described above (cfr Zymography assay; SP, *in vitro*), except that the overnight incubation at 4°C was performed in the presence of 1% Triton X-100 and a protease inhibitor cocktail (Complete Mini, EDTA-Free, Hoffmans La Roche, 1 tablet per 10 mL Hoffmans LaRoche, Brussels Belgium) in deionized water. For β-GlcCer'ase, 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% SDS containing a protease inhibitor cocktail). The protein content of tape stripping or epidermis extracts was then determined, as above. Equal amounts of protein from each experimental group were loaded onto 4%–12% Tris-glycine polyacrylamide gels (PAGEr Gold Precast Gels, BioWhittaker Molecular Applications, Rockland, Maine). After electrophoresis, proteins were transferred from the slab gel onto nitrocellulose membranes and immunoblotted to detect DSG1, β-GlcCer'ase, or aSMase, using the Western Lightening chemiluminescence kit (Perkin-Elmer Life Sciences, Boston, Massachusetts).

SCCE proteolysis experiments Proteolysis experiments were performed on SC extracts as described previously (Caubet *et al*. 2004) with the following modifications. Briefly, human SC was isolated from the heel of six healthy individuals (mean age $\pm$ SD 25 ± 5.3 y old), and proteins were extracted in PBS buffer (pH = 7.2, 1% Triton-X 100) at 4°C overnight. SC extracts from each three individuals were pooled into two separate samples and the protein content was then determined, as above. 50 μg of each sample was incubated in the presence or absence of 100 ng recombinant active SCCE for 2 h at 37°C. Reactions were stopped by boiling for 2 min in sample buffer. Samples were separated by SDS-PAGE and analyzed by immunoblotting for β-GlcCer'ase and aSMase as described above.

Light and electron microscopy (EM) Skin biopsy samples were taken 5 d after the initiation of TMG $\pm$ STI or nTMG treatments (n = 3 from each group) and processed for light and EM. Samples were minced to < 0.5 mm^3, fixed in modified Karnovsky's fixative overnight, and post-fixed in either 0.2% ruthenium tetroxide (RuO_4), or 1% aqueous osmium tetroxide (OsO_4), containing 1.5% potassium ferrocyanide, as described previously (Hou *et al*, 1998). After fixation, all samples were dehydrated in graded ethanol solutions, and embedded in an Epon–epoxy mixture. Ultrathin sections were examined, with or without further contrasting with lead citrate, in an electron microscope (Zeiss 10A; Carl Zeiss, Thornwood, New York) operated at 60 kV.

Statistical analyses and CD EM quantification Statistical analyses were performed using Prism 2 (GraphPad Software, San Diego, California). Two groups were compared with a Student's *t* test. In order to quantify CD density by EM, ten pictures of the outer epidermis were taken at random from each sample at 31.5K magnification. The ratio between the total lengths of all CD to the total length of cornified envelopes at the SG/SC junction, as well as surrounding the first SC cell layer, was determined using a planimeter. Non-parametric Mann–Whitney statistical analyses were performed to compare percent ratios between different groups of treatments (Morris 2000).

The medical ethical committees of the University of California, San Francisco, California, USA and Vrije Universiteit Brussel, Brussels, Belgium approved all described studies.

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