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TOPICAL ANTIHISTAMINES DISPLAY POTENT ANTI-INFLAMMATORY ACTIVITY LINKED IN PART TO ENHANCED PERMEABILITY BARRIER FUNCTION

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Abstract

Systemic antagonists of the histamine type 1 and 2 receptors (H1/2r) are widely used as anti-pruritics and central sedatives, but demonstrate only modest anti-inflammatory activity. Because many inflammatory dermatoses result from defects in cutaneous barrier function, and because keratinocytes express both Hr1 and Hr2, we hypothesized that H1/2r antagonists might be more effective, if they were used topically to treat inflammatory dermatoses. Topical H1/2r antagonists

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additively enhanced permeability barrier homeostasis in normal mouse skin by: i) stimulation of epidermal differentiation, leading to thickened cornified envelopes; and ii) enhanced epidermal lipid synthesis and secretion. Since barrier homeostasis was enhanced to a comparable extent in mast cell-deficient mice, with no further improvement following application of topical H1/2r antagonists, H1/2r antagonists likely oppose mast cell-derived histamine. In four immunologically-diverse, murine disease models, characterized by either inflammation alone (acute irritant contact dermatitis, acute allergic contact dermatitis), or by prominent barrier abnormalities (subacute allergic contact dermatitis, atopic dermatitis), topical H1/2r agonists aggravated, while H1/2r antagonists improved inflammation and/or barrier function. The apparent ability of topical H1r/2r antagonists to target epidermal H1/2r could translate into increased efficacy in the treatment of inflammatory dermatoses, likely due to decreased inflammation and enhanced barrier function. These results could shift current paradigms of antihistamine utilization from a predominantly-systemic to a topical approach.

Keywords

Antihistamines; epidermal differentiation; epidermal barrier function; epidermal lipid synthesis; H1 receptor; H2 receptor; treatment (therapy)

INTRODUCTION

Because of their common embryologic origin, epidermis not surprisingly expresses multiple neuroreceptors, neurotransmitters and neurohormones that mediate important functions in the central nervous system (Denda, 2002, Denda, *et al.*, 2007). One of these mediators is histamine (Travis, *et al.*, 2000), an aminergic neurotransmitter that is produced not only by neurons, but also by mast cells, eosinophils and basophils (Endo, *et al.*, 1992, Endo, *et al.*, 1995, Yamaguchi, *et al.*, 2000). In contrast to the limited number of cell types that synthesize histamine, one or more of four histamine receptors (H1r-4r), belonging to the superfamily of G-protein-coupled receptors, are ubiquitous and modulate a variety of pathophysiological responses, including pruritus and inflammation in the skin (Hill, *et al.*, 1997, Oda, *et al.*, 2000). Yet, though systemic antihistamines are widely deployed in clinical settings, they demonstrate only modest anti-inflammatory activity in diseases such as atopic dermatitis (Klein and Clark, 1999, Diepgen, 2002, Kawashima, *et al.*, 2003, Buddenkotte, *et al.*, 2010, Eschler and Klein, 2010). Moreover, their utilization can be limited by important side effects, including sedation and cardiotoxicity, particularly in the elderly [rev. in (Greaves, 2005)]. The reason for the limited anti-inflammatory activity of systemic antihistamines is not known, but it seems plausible that either their bioavailability to peripheral tissues could be limited at current dosage levels, or that they could be substantially metabolized prior to their arrival in the skin. Because the H1r and H2r are strongly expressed in epidermis [(Gschwandtner, *et al.*, 2011) and these studies], and because one prior study showed that topical H1/2r antagonists improve barrier function in normal skin (Ashida, *et al.*, 2001), we hypothesized here that the bioavailability and efficacy of antihistamines could be enhanced, were they deployed as topical, rather than as systemic agents to treat inflammatory dermatoses, with or without associated barrier abnormalities.

Epidermis mediates a set of protective functions, including maintenance of permeability barrier homeostasis. This critical function, which allows survival in a terrestrial environment, is mediated by the unique two-compartment organization of the stratum corneum (SC) into anucleate corneocytes embedded in an expanded, lipid-enriched extracellular matrix [rev. in (Elias and Menon, 1991)]. Inflammatory dermatoses are now increasingly recognized to result from inherited abnormalities that compromise epidermal barrier function (Sandilands, *et al.*, 2009, Irvine, *et al.*, 2011). A likely pathogenic sequence that leads to inflammation invokes both increased allergen penetration, through a genetically-impaired barrier, leading to Th2 inflammation (Irvine, *et al.*, 2011) and an epidermis-initiated ‘cytokine cascade’ that recruits downstream inflammation (Wood, *et al.*, 1992, Nickoloff and Naidu, 1994). Accordingly, immune abnormalities, once seen as the primary disease instigator, are now increasingly considered as downstream or disease-modifying participants (Elias, *et al.*, 2008a, Elias and Steinhoff, 2008). Then, inflammation once established, can further aggravate the barrier abnormality by multiple mechanisms, establishing an ‘outside-to-inside, back-to-outside’ pathogenic circle (Elias, *et al.*, 2008a, Elias and Steinhoff, 2008, Elias and Schmuth, 2009). Accordingly, disorders such as atopic dermatitis, psoriasis, and the inherited ichthyoses are increasingly being treated with various forms of topical ‘barrier repair therapy,’ strategies that can themselves be anti-inflammatory by multiple mechanisms [rev. in (Elias and Wakefield, 2011)]. We show here that topical antihistamines could comprise effective therapy not only through enhanced anti-inflammatory activity, but also in part through their ability to improve epidermal structure and function. Since mast cell deficient mice (MCDM) displayed a comparable enhancement in barrier function, and since topical H1/2r antagonists provided no further benefits, the H1/2r antagonists likely oppose mast cell-derived histamine. Finally, the H1r and H2r antagonists markedly improved inflammation in four different inflammatory dermatoses models, characterized by either inflammation alone and/or a prominent barrier abnormality.

RESULTS

Topical Antihistamines Enhance Cutaneous Permeability Barrier Homeostasis By Opposing Mast Cell-Derived Histamines

As recently reported by Gschwandtner, *et al.*, (2011), we initially found that only the H1r and H2r are expressed in abundance in normal mouse epidermis (suppl. Fig. 1). We utilized the H1r and H2r antagonists, diphenhydramine and cimetidine here, because they most-potently improved barrier function among several agents tested in preliminary studies. Twice-daily topical applications of these H1/2r antagonists to intact skin produced only modest changes in basal barrier function, SC hydration, and surface pH, which all fell within the normal range (Figs. 1a-c). In contrast, when skin sites, previously-treated with H1/2r antagonists, were disrupted by sequential tape stripping, permeability barrier restoration accelerated (by $\approx 100\%$) in comparison to vehicle-treated sites (Fig. 1d). Moreover, co-applications of the H1r and H2r antagonists additively improved barrier function at two hours post-barrier disruption, but no additive or synergic effects were observed at four hours after barrier abrogation (Fig. 1d). Finally, when the H1r and H2r antagonists were applied unilaterally (vehicle alone applied to the opposing, similarly tape-stripped flank), barrier homeostasis improved only on the antagonist-treated side (not shown), indicating that the

H1/2r antagonists enhance epidermal function locally, rather than after prior systemic absorption.

The putative source for endogenous histamine in the skin are cutaneous mast cells, which are present in substantial numbers in normal skin (Janssens, *et al.*, 2005). To assess whether the H1/2r antagonists improve barrier function by opposing mast cell-derived histamine, we next compared barrier homeostasis in mast cell-deficient mice (MCDM, *kit^W/kit^{W-v}*) vs. age-matched, same-strain (WBB6F1), mast cell-replete mice. Although basal barrier function, hydration and surface pH did not differ in MCDM vs. wild-type mice, barrier recovery accelerated significantly ($\approx 160\%$) in MCDM at 2 hrs vs. control mouse skin (Fig. 1e; $p < 0.002$), an enhancement of barrier homeostasis that was comparable to that produced by the topical antihistamines. But, no further improvements in permeability barrier homeostasis occurred when MCDM were treated topically with either the H1r or H2r antagonist (Suppl. Fig. 2). Interestingly, barrier disruption provoked a modest, though significant increase in the density of mast cells (Suppl. Fig. 3). Together, these results show that: 1) topical H1r and H2r antagonists improve permeability barrier homeostasis in acutely-perturbed, but otherwise normal mouse skin [see also (Ashida, *et al.*, 2001)]; 2) improvement is due to local effects, ruling out efficacy due to prior systemic absorption; 3) mast cell-derived histamine is likely the primary source of ligand opposed by the H1/2r antagonists; 4) barrier disruption stimulates proliferation of mast cells in the dermis; and 5) H1/2r antagonists improve barrier function specifically by opposing mast cell-derived histamine.

Mechanisms Whereby Topical Antihistamines Enhance Barrier Function

Corneocytes and extracellular lipids together mediate epidermal permeability barrier homeostasis (Elias, 2006, Feingold, 2007). To explore the mechanistic basis for enhanced permeability barrier function, we first assessed whether these agents alter epidermal *proliferation* after barrier disruption. In H+E stained sections, both H1r and H2r antagonists modestly stimulated epidermal hyperplasia (suppl. Fig. 4a–c), but the increase in thickness achieved statistical significance only in H2r antagonist-treated skin (suppl. Fig. 4d–g). Likewise, epidermal proliferation, assessed as the density of PCNA-positive cells in the basal layer, increased more in H2r- than in H1r-antagonist treated skin (suppl. Fig. 4h).

We next assessed whether one or both of these agents enhance expression of epidermal *differentiation-related proteins*. Applications of the H1/2r antagonists to intact skin enhanced expression of involucrin, loricrin, and particularly filaggrin in immunohistochemical preparations (Figs. 2a–i). Accordingly, epidermal mRNA levels, assessed by real-time quantitative PCR [rt(Q)-PCR] (see suppl. Table 1 for list of all primers used in the studies), increased after H1/2r antagonist applications (Fig. 2j). Finally, we assessed whether increased differentiation-linked protein expression translated into altered *corneocyte structure*. Electron microscopy demonstrated an apparent increase in the thickness of cornified envelopes in both H1r and H2r antagonist-treated epidermis (Fig. 3a–c), validated further in quantitative studies, utilizing randomized, coded micrographs ($\approx 40\%$ increase; $p < 0.0001$ for both H1/2r antagonists) (Fig. 3d). Together, these studies show that the H1r/H2r antagonist-induced improvements in barrier function can be attributed in part to enhanced epidermal differentiation, leading to more-robust corneocytes.

Epidermal permeability barrier function requires both the synthesis and ultimately the secretion of hydrophobic lipids from epidermal keratinocytes into the SC extracellular matrix (Feingold, 2009). Therefore, we next asked whether topical H1r and H2r antagonists enhance lipid production and/or secretion in normal epidermis. To assess the global impact of the antihistamines on lipid *production*, we initially compared fluorescence intensity after applications of Nile red, a fluorophore that selectively depicts lipids, to frozen sections of topical H1/2r antagonist-treated normal skin. Both H1r and H2r antagonists markedly enhanced the overall lipid content of epidermis (Figs. 4a-c, arrows; H2r>H1r).

We next assessed whether topical H1/2r antagonist treatments enhance epidermal *lipid synthesis*. After four days of topical treatment, both the H1r and H2r antagonists significantly stimulated both epidermal non-saponifiable lipid and cholesterol synthesis, but only the H1r antagonist upregulated saponifiable lipid (i.e., fatty acid) synthesis (Fig. 4d). To assess the *basis* for *enhanced lipid synthesis*, we next compared changes in expression of several key lipid synthetic and lipid-modifying enzymes in epidermis two hrs after topical H1/2r antagonist applications. By rt(Q)-PCR, mRNA levels of HMGCoA reductase and serine palmitoyl transferase, the rate-limiting enzymes of cholesterol and sphingoid base (ceramide) synthesis, respectively, increased significantly after topical H1/2r antagonist applications (Figs. 4e&f). Moreover, mRNA levels for two key enzymes that modify fatty acid structure, the α -hydroxylating enzyme, fatty acid 2-hydroxylase (FA2H), and the elongation enzyme (elongation of very long-chain fatty acid-4, ELOVL4), which is required for acylceramide production, increased significantly after H1/2r antagonist applications (Figs. 4g&h). Together, these results demonstrate that both H1r and H2r antagonists stimulate epidermal lipid production by multiple pathways.

Newly-synthesized lipids are packaged into epidermal lamellar bodies, which then deposit their cargo at the stratum granulosum-SC interface. To determine whether the antihistamines stimulate lipid *secretion*, we next assessed changes in lamellar body formation after topical H1/2r antagonist applications to intact skin (Figs. 5a-f). While the density of lamellar bodies in the granular cell cytosol appeared to increase after treatment with the H2r antagonist, the H1r antagonist instead appeared to stimulate premature secretion of lamellar body contents between cells deep within the granular layer, a feature that was not evident following H2r antagonist applications (Figs. 5e vs. c). To determine the basis for enhanced lamellar body *production*, we next assessed mRNA levels of the epidermal-specific, transmembrane transporter, ABCA12, which delivers glucosylceramides into nascent lamellar bodies. Transporter mRNA levels increased significantly after H2r antagonist applications, a finding that correlated with increased lamellar body density in parallel samples (cf., Fig. 5e; increase after H1r antagonist applications did not achieve statistical significance, Fig. 5f). Finally, accelerated production, with or without premature secretion, correlated with enhanced deposition of lamellar body contents at the stratum granulosum-SC interface, a change more evident in H2r than in H1r antagonist-treated epidermis (Figs. 5b&d, open arrows). Together, these results suggest that the H1/2r antagonists also improve barrier function by stimulating lipid secretion.

Topical Antihistamines Improve Inflammation and Barrier Function in Diverse Murine Models

The studies above demonstrate that topical H1/2r antagonists substantially enhance epidermal structure and function in otherwise normal skin by multiple mechanisms. Many inflammatory skin diseases are characterized not only by inflammation, but also by permeability barrier abnormalities. A primary barrier abnormality can induce inflammation (Elias and Schmuth, 2009, Elias, 2010); or conversely, a primary immunologic abnormality, as in HIV (Gunathilake, *et al.*, 2010), can lead to abnormalities in barrier function that further stimulate inflammation (Elias, *et al.*, 1997, Elias, *et al.*, 1999, Elias and Feingold, 2001). Hence, we next asked whether topical H1/2r antagonists could have favorable effects in four different mouse models of cutaneous disease (suppl. Table 2).

Acute irritant contact dermatitis (AICD), induced by topical phorbol ester [12-O-tetradecanoylphorbol-13-acetate (TPA)] treatment, is characterized by inflammation, but barrier function remains within the range of normal, even after inflammation appears (2 hrs) (8.1 +/- 2.4 vs. 6.4 +/- 1.9 [SD]; n = 29-31; normal = 10) (suppl. Table 2). A single application of either the H1r or H2r antagonist, immediately after the phorbol ester application, significantly reduced inflammation in AICD, quantitated as reductions in ear thickness (Fig. 6a). In parallel, dermal inflammation and epidermal hyperplasia, assessed in H+E sections, declined markedly (suppl. Fig. 6). In contrast, pre-treatment with H1/2r antagonists prior to TPA application did not prevent inflammation (ear thickness, 0.32+0.01 for vehicle, 0.29+0.01 for H1r antagonist, and 0.34+0.01 for H2r antagonist).

Acute allergic contact dermatitis (AACD), produced by a single hapten (oxazolone, Ox) challenge, after prior sensitization, also induces inflammation, without provoking an immediate barrier abnormality (Sheu, *et al.*, 2002, Fowler, *et al.*, 2003) (3.9 +/- 1.7 vs. 3.4 +/- 1.5 [SD]; n = 26-31; p<0.5). Both H1r and H2r antagonists markedly reduced histological evidence of inflammation (suppl. Fig. 6), further quantified as a reduction in ear thickness in AACD (Fig. 6a). Yet, pretreatment again did not prevent development of inflammation (not shown). Together with the studies in AICD, these results demonstrate that topical H1/2r antagonists exhibit potent anti-inflammatory activity in dermatoses that lack a primary barrier abnormality.

Subacute allergic contact dermatitis (SACD), induced by repeated hapten challenges (3x), is characterized by both a substantial barrier abnormality (Fig. 6b), as well as inflammation. Treatment with both the H/2r antagonists significantly improved barrier function and decreased inflammation in the SACD model (Fig. 6b; but pretreatment with the antagonists again did not prevent the development of inflammation).

Atopic dermatitis [AD]-like dermatosis—With further hapten challenges (10x), atopic dermatitis-like inflammation develops, in which a prominent barrier abnormality is currently thought to ‘drive’ downstream inflammation, characterized by a prominent th2-dominant immunophenotype (suppl. Table 2) (Elias, *et al.*, 2008a, Elias and Steinhoff, 2008, Irvine, *et al.*, 2011). When we applied specific H1/2r receptor *agonists*, both exacerbated inflammation in the AD model (suppl. Figs. 6&7). In contrast, H1r and H2r *antagonists* reduced inflammation (suppl. Figs. 6&7), but only the H2r antagonist significantly improved

barrier function in this model (Fig. 6b). Since the H1/2r antagonists improved inflammation and barrier function only at sites of local application in both the SACD and AD models, systemic activity did not account for disease improvement. Yet again, neither antagonist exhibited preventive benefits in these models. These results show that H1/2r antagonists improve inflammation in the AD model, often with parallel improvements in barrier function.

DISCUSSION

Although histamine is a potent inflammatory mediator, whose levels increase markedly in inflammatory dermatoses [rev. in (Pavlinkova, *et al.*, 2003, Greaves, 2005)], systemic antihistamines have proven ineffective as anti-inflammatory therapy for these disorders (Belsito, *et al.*, 1990, Klein and Clark, 1999, Diepgen, 2002, Kawashima, *et al.*, 2003, Buddenkotte, *et al.*, 2010). Thus, these agents are largely deployed for their relatively-modest anti-pruritic or central sedating properties (Buddenkotte, *et al.*, 2010, Eschler and Klein, 2010). Yet, their use for these purposes can be limited by important side effects, particularly in the elderly (Greaves, 2005). Systemic antihistamines could be minimally effective, either because of poor peripheral bioavailability, and/or metabolism to inactive compounds prior to their arrival in the skin (Levi-Schaffer and Eliashar, 2009). Although topical antihistamines are widely used as anti-pruritics (Eschler and Klein, 2010, Baumer, *et al.*, 2011), whether the topical approach could provide further anti-inflammatory benefits has not yet been examined. After showing that H1r and H2r are highly-expressed in epidermis, we hypothesized that topical deployment of H1/H2r antagonists could provide a boost in anti-inflammatory activity, because of their greater bioavailability, and perhaps by improving barrier function. Pertinently, several inherited inflammatory dermatoses, including atopic dermatitis, inflammatory ichthyoses, and even psoriasis, are now seen as barrier-initiated (Sun, *et al.*, 2006, Schmuth, *et al.*, 2007, Tschachler, 2007, Elias, *et al.*, 2008b, Chen, *et al.*, 2009, Sandilands, *et al.*, 2009, Elias, *et al.*, 2010, Strange, *et al.*, 2010). Hence, after initially determining whether and how these agents improve barrier function in normal epidermis, we then assessed their efficacy in several unrelated mouse models of inflammatory dermatoses.

We described here a marked improved barrier function following topical H1r and H2r antagonist applications to normal skin, confirming prior studies (Ashida, *et al.*, 2001). We further demonstrated that H1r and H2r additively improved barrier function, at least at early time points. These results suggest that H1r and H2r could regulate epidermal function via different downstream mechanisms. Moreover, these agents appear to target the appropriate receptors, since H1/2r antagonist applications to mast cell deficient mice (MCDM) provided no further benefits for the barrier. Since the MCDM also demonstrated enhanced barrier function, and since topical H1/2r antagonists exert no further benefits, these results strongly suggest that mast cells must be the primary source of the ligand that is opposed by the H1/2r antagonists. Yet, these studies did not completely rule out other cell types as potential sources of histamine.

How these agents enhance epidermal structure and function is not yet known. We identified several mechanisms that account for enhanced permeability barrier in H1/2r antagonist-

treated normal skin. First, acute barrier disruption increased the density of mast cells in the dermis, raising the question whether recruitment of mast cells to the skin contributes to the development of inflammation in dermatoses characterized by barrier abnormalities. But perhaps more importantly, the topical H1/2r antagonists directly impact epidermal structure and function. Topical applications of H1r and H2r antagonists enhanced epidermal differentiation, the latter at both the mRNA and protein levels, which could reflect the ability of these agents to mobilize intracellular calcium (Koizumi and Ohkawara, 1999). Enhanced differentiation translated further into a significant increase in the thickness of cornified envelopes, which should yield more robust corneocytes, also a Ca^{++} -dependent process (Kim and Bae, 1998, Nemes and Steinert, 1999). The converse certainly proves this point -- effete cornified envelopes occur in several inherited disorders of cornification, including loss-of-function mutations in transglutaminase 1-deficient (lamellar) ichthyosis [rev. in (Schmuth, *et al.*, 2007)], that display subnormal barrier function. Thus, the more robust corneocytes in topical H1/H2r-antagonist-treated skin likely contribute to enhanced barrier function.

We also show that topical antihistamines enhance barrier function by stimulating the synthesis and secretion of epidermal lipids. Multiple steps in the initial synthesis, later modification, and subsequent secretion of epidermal lipids were stimulated by topical applications of the H1r and/or H2r antagonists. Pertinently, hepatic lipid synthesis is similarly enhanced in both H1r and H2r knockout mice (Wang, *et al.*, 2010). Yet, there were subtle differences in the effects of H1r and H2r antagonists on these metabolic pathways in normal epidermis. While both the H1r and H2r antagonists stimulate epidermal lipid *synthesis*, the H2r antagonists more-potently stimulate lamellar body *production*, which parallel enhancement of ABCA12 expression after H2r (but not H1r) antagonist applications. While the H1r antagonist displayed a lesser impact on organelle *production* (and ABCA12 expression), it instead appeared to accelerate lamellar body *secretion*. Thus, in addition to profound effects on epidermal differentiation, H1/2r antagonists strongly stimulate epidermal lipid synthesis, metabolism and secretion.

Based upon the putative link between abnormalities in barrier function and downstream inflammation, we reasoned that topical antihistamines could reduce inflammation in inflammatory dermatosis, at least in part by improving barrier function, as we showed previously with activators of the liposensor subclass of nuclear hormone receptors (i.e., PPAR α , γ , β/δ and LXR). These agents not only improve barrier function in normal skin (Man, *et al.*, 2006, Schmuth, *et al.*, 2008), but also reduce inflammation in diverse inflammatory dermatosis models that may or may not be characterized by a barrier abnormality (Komuves, *et al.*, 2000, Sheu, *et al.*, 2002, Fowler, *et al.*, 2003). Indeed, our results strongly suggest that the benefits of the topical H1/2r antagonists extend beyond their impact on barrier function, because they also reduced inflammation in two models, where barrier function remained normal (i.e., AICD and AACD). Thus, the topical H1/2r antagonists exhibit potent anti-inflammatory activity that could operate independently of, or in parallel to improved barrier function. In contrast, the SACD and AD models display progressively more-severe barrier abnormalities [(Man, *et al.*, 2008, Hatano, *et al.*, 2009, Hatano, *et al.*, 2010) and these results]. Since both the H1r and H2r antagonists improve

barrier function in both of these models, it is tempting to argue that this result reflects the impact of the antagonists on barrier function. The H1r antagonist, though highly anti-inflammatory, did not significantly improve barrier function in the AD model. Hence, it is not possible to discriminate which of these two mechanisms (anti-inflammatory vs. barrier enhancement) predominates in reducing inflammation. Yet, even the anti-inflammatory benefits could reflect direct effects on the epidermis, because both H1r and H2r antagonists oppose production of multiple keratinocyte-derived cytokines (Shimizu, *et al.*, 2004, Matsubara, *et al.*, 2005, Kobayashi, *et al.*, 2009), independent of their well-known ability to stabilize histamine production by mast cells (Levi-Schaffer and Eliashar, 2009). Moreover, anti-inflammatory benefits can accrue with improved barrier function via a reduction in the barrier-initiated 'cytokine cascade' (Elias, *et al.*, 2008a, Elias and Steinhoff, 2008). Pertinently, topical H1/2r agonists instead aggravated inflammation, perhaps by direct pro-inflammatory effects, or by further compromising barrier function, as they do after topical applications to normal skin [(Ashida, *et al.*, 2001) and these studies].

Not only filaggrin-deficient atopic dermatitis (Sandilands, *et al.*, 2009), but also all of the inherited ichthyoses studied to date (Schmuth, *et al.*, 2007, Elias, *et al.*, 2008b, Elias, *et al.*, 2010), and most recently even psoriasis (Sun, *et al.*, 2006, Tschachler, 2007, Chen, *et al.*, 2009, Strange, *et al.*, 2010), appear to be provoked by primary genetic alterations that compromise epidermal structure and function. Since these dermatoses are often driven or accompanied by prominent barrier abnormalities, not surprisingly, recent studies show that a variety of 'barrier repair' strategies comprise effective (and inherently safer) therapy for these disorders (Elias and Wakefield, 2011). The topical H1/2r antagonists, if they prove equally effective when deployed topically for their human disease counterparts, could be added to this list. Nonetheless, it now seems reasonable to propose that H1r and H2r antagonists could be deployed topically to treat a broad range of inflammatory dermatoses.

MATERIALS AND METHODS

(Please read supplemental information for further details of Materials and Methods)

Materials

Female albino hairless (Skh1) mice, aged six-eight weeks, were from Charles River Laboratories (Wilmington, MA). Mast cell deficient mice (MCDM, Kit^W/Kit^{W-v} double heterozygous mice) and age- and gender-matched wild-type littermates (WBB6F1) were from Jackson Labs (Bar Harbor, ME). Ethanol and propylene glycol were from Fisher Scientific (Fairlane, NJ); diphenhydramine chlorhydrate and cimetidine were from Sigma (St Louis, MO), and affinity-purified, rabbit anti-mouse filaggrin, involucrin, and loricrin antibodies were from BabCo (Richmond, CA). Secondary biotinylated, goat anti-rabbit IgG and ABC-peroxidase kit were from Vector laboratories (Burlingame, CA). Anti-proliferating cell nuclear antigen antibody (PCNA, Ki-67) was from CalTag Laboratories (Burlingame, CA).

Experimental protocols and functional studies

Animal procedures were approved and performed in accordance with guidelines of the Animal Studies Subcommittee (IACUC), San Francisco VA Medical Center. Mice were maintained in temperature- and humidity-controlled rooms, and given standard laboratory food and tap water *ad libitum*. Barrier disruption on hairless mice was achieved by repeated tape-stripping until 10 fold increase in transepidermal water loss. Mice were treated topically on one or both flanks with 5% diphenhydramine or 5% cimetidine or vehicle alone (propylene glycol:ethanol:water = 1:2:2, volume) twice-daily for four days. Changes in transepidermal water loss (TEWL), measured with an electrolytic water analyzer (Meeco, Warrington, PA), were measured 0, 2 and 4 hrs after sequential tape stripping, resulting in a 10-fold increase in TEWL, and percent barrier recovery rates was calculated (Man, *et al.*, 1993, Man, *et al.*, 2006, Man, *et al.*, 2008). SC hydration was measured as changes in electrical capacitance, and surface pH with a flat surface electrode (Ibid.). For studies in MCDM, additional groups of WBB6F1 mice, treated with vehicle, served as controls.

Quantitation of Mast Cell Densities

Skin biopsies were taken from normal, 30 min, 3 hr and 6 hrs after barrier disruption. Mast cell infiltrates in the dermis were identified with 1% toluidine blue staining of 5 um paraffin sections. Pictures were taken at 20X with a Leica DM400B digital microscope, equipped with LAS v4.0 software. The density of mast cells was determined on every 25 cm² area at regions between basement membrane and 5 cm below basement membrane in printed micrographs.

Statistical Analyses

Data are expressed as the means \pm SEM. Unpaired two-tailed student t test with Welch's correction was used to determine significant differences when two groups were compared, and a one-way ANOVA with a post-Tukey Test or Dunnett post-correction was used to determine significant differences when three or more groups were compared.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AACD	acute allergic contact dermatitis
AD	atopic dermatitis
AICD	acute irritant contact dermatitis

CE	cornified envelope
Hr	histamine receptor
MCDM	mast cell deficient mice
Ox	oxazolone
rt(Q)-PCR	real time quantitative PCR
SACD	subacute allergic contact dermatitis
SC	stratum corneum
TEWL	transepidermal water loss
TPA	12-O-tetradecanoylphorbol-13-acetate

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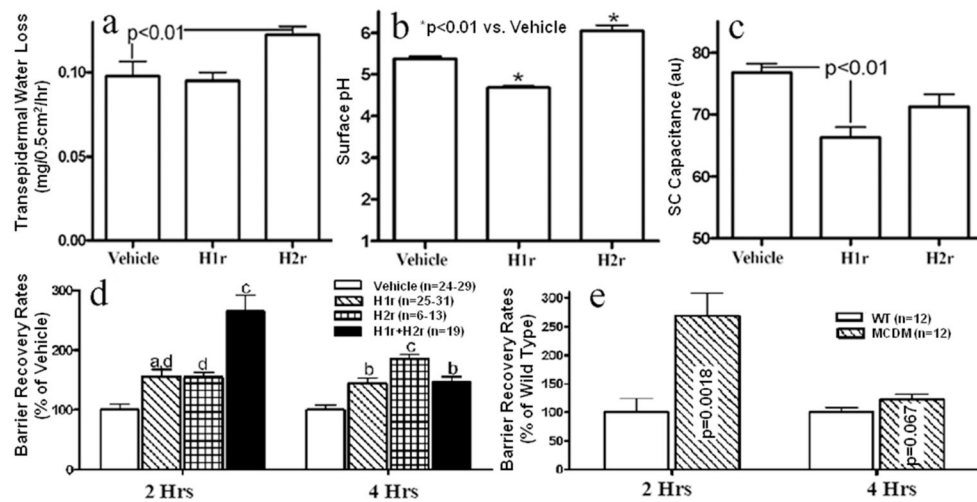


Figure 1. Topical H1/2r Antagonists Enhance Permeability Barrier Homeostasis in Normal Skin
 The flanks of hairless mice (n=4–5 each) were treated with topical applications of either diphenhydramine chlorhydrate (H1r), cimetidine (H2r), or ethanol (vehicle) twice-daily for four days (see Methods for further details). At the end of treatments, changes in basal barrier function, assessed as transepidermal water loss (TEWL, **a**), surface pH (**b**), and electrical conductance (SC hydration, **c**) were measured. (**d**) Barrier disruption was induced by sequential cellophane tape strippings until TEWL levels 10-fold increase over baseline, and barrier recovery rates were assessed two and four hrs later (a=p<0.05 vs. vehicle, b=p<0.01 vs. vehicle, c=p<0.001 vs. vehicle, and d=p<0.001 vs. H1r+H2r)66. **e**: Percent barrier recovery was compared in mast cell-deficient *Kit^W/Kit^{W-v}* double heterozygote mice (MCDM) vs. wild-type (WBB6F1) mice two and four hrs after tape stripping, as above.

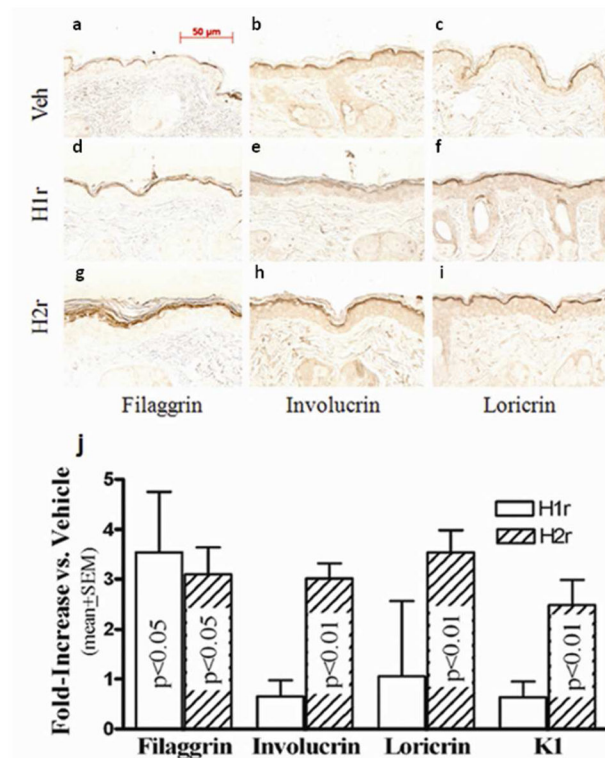


Figure 2. Topical H1/H2r Antagonists Stimulate Epidermal Differentiation

Hairless mice were treated as above, and paraffin-embedded sections (6 μm) then were immunostained to detect changes in filaggrin, involucrin, and loricrin content and localization (a-i). j: In parallel, mRNA was isolated from freshly-obtained epidermal sheets after treatments as in Fig. 1, above (n=4), and changes in mRNA levels for filaggrin, loricrin, and involucrin were assessed by rt(Q)-PCR (see Methods and suppl. Table 2 for further details). Scale bar = 50 μm.

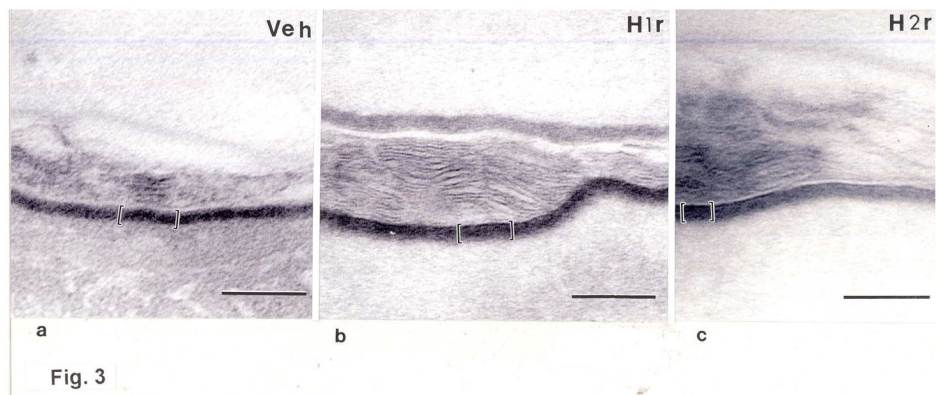


Fig. 3

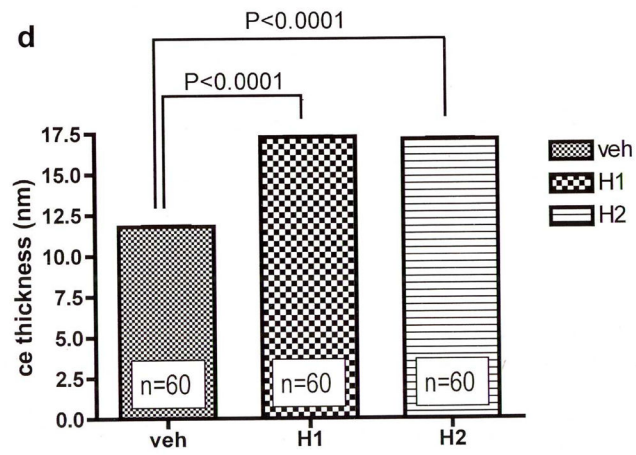


Figure 3. Topical H1r/H2r Antagonists Enhance Corneocyte Envelope Thickness

Electron micrographs of biopsies of H1/2r antagonist-treated skin were processed for electron microscopy (a-c), as in Methods. **d**: 10 micrographs each of perpendicular sections taken at random from 6 different biopsy samples. CE dimensions were measured directly on the electron microscope, as in Methods. **a-c**, Osmium tetroxide post-fixation; Scale bar = 100 μ m.

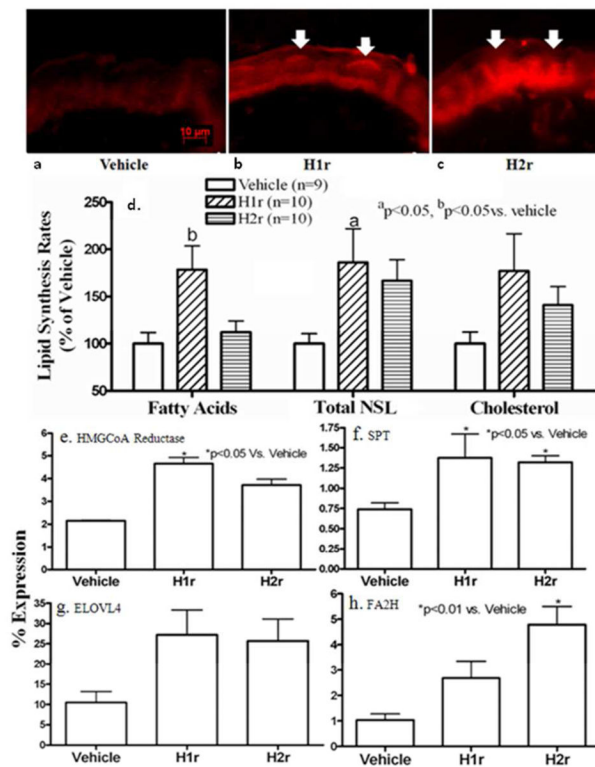


Figure 4. Global Stimulation of Epidermal Lipid Synthesis by Topical H1/2r Antagonists Hairless mice were treated as above (Fig. 1), and biopsies were snap-frozen in liquid nitrogen. Frozen sections (5 μ m) were incubated with either the fluorophore, Nile red, or vehicle, and viewed in a fluorescence microscope, as in Methods (a-c; arrows depict sites of enhanced staining for lipids in epidermis). **d**: Freshly-obtained, full-thickness skin biopsies were incubated with 14 C-acetate, followed by epidermal isolation, lipid extraction, saponification, fractionation by thin layer chromatography, and quantitation of changes in non-saponifiable (NSL) and saponifiable (total fatty acid) lipids, as in Methods. **e-j**: mRNA was isolated from epidermal sheets as above (n=4 mice each), and changes in mRNA levels for lipid synthetic enzymes (HMGCoA reductases, serine palmitoyl transferase [SPT], fatty acid modifying enzyme, fatty acid 2-hydroxylase [FA2H], and the acylceramide-generating elongation of very-long chain fatty acid-4 [ELOVL4]) were assessed by rt(Q)-PCR, as in Methods and suppl. Table 2. Scale bar = 10 μ m.

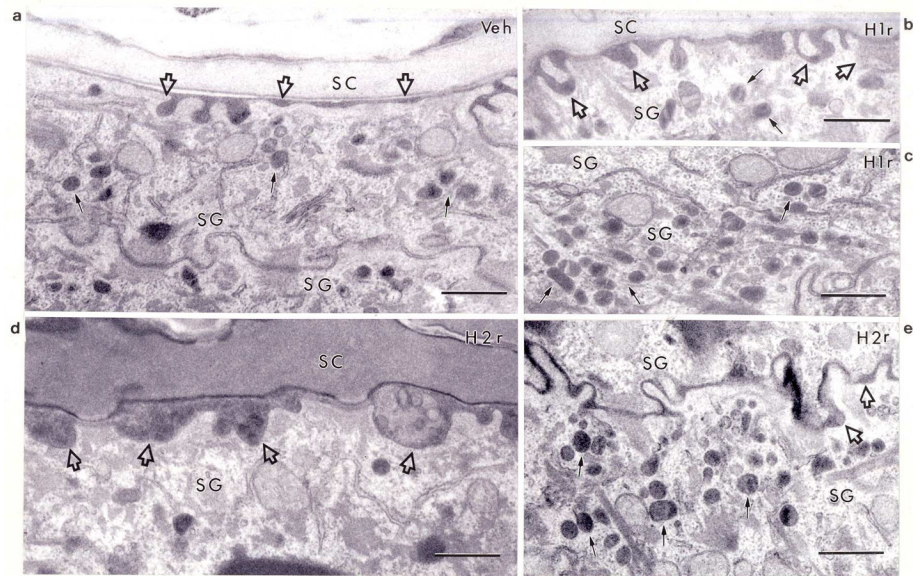


Fig. 5

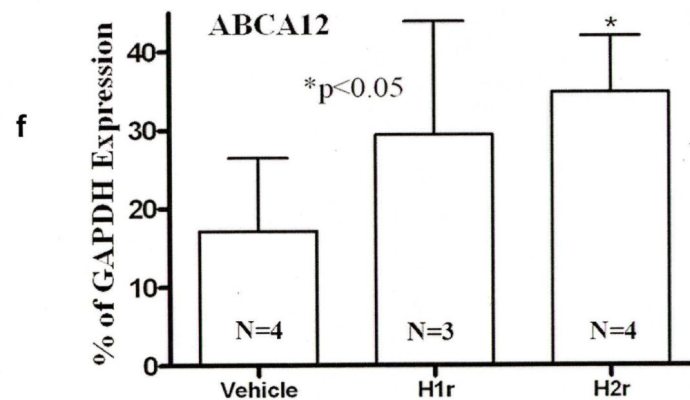


Fig. 5

Figure 5. Topical H1/2r Antagonists Stimulate Lamellar Production and/or Secretion

Biopsies of H1/2r antagonist- and vehicle-treated skin samples (as in Fig. 1) were processed for electron microscopy, as in Methods. Solid arrows point to individual or aggregated lamellar bodies, and open arrows depict changes in secreted contents at stratum granulosum (SG)-SC interface. Representative samples of outer epidermis of vehicle-treated (Veh)- (a), H1r antagonist (diphenhydramine chlorhydrate)-treated- (b+c), and H2r (cimetidine)-treated- (d) skin. Note increased organelle density in the SG of H2r-treated epidermis, and premature secretion of lamellar bodies in H1r antagonist-treated skin, (b-e), as well as enhanced secretion of lamellar body contents at the stratum granulosum (SG)-SC interface (H2r>H1r). f: Enhanced formation of lamellar bodies correlated with increased mRNA levels of ABCA12 in H2r antagonist-treated skin (n=4); the increase in ABCA12 mRNA following H1r antagonist treatments did not achieve statistical significance. a-e: Osmium tetroxide post-fixation. Scale bar = 0.5 μ m.

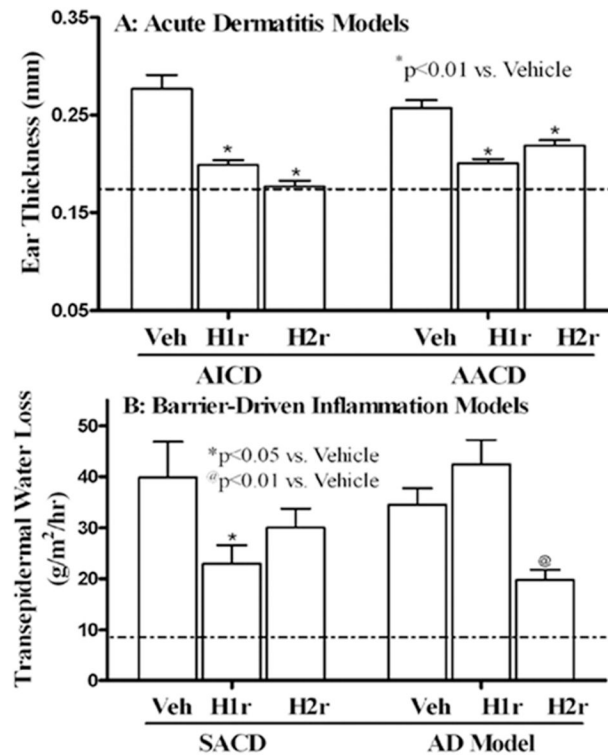


Figure 6. Topical H1/2r Antagonists Improve Inflammatory Dermatitis, Independent of Benefits for Barrier Function

a: Changes in ear thickness in acute irritant and acute allergic contact dermatitis (AICD and AACD, respectively) 16 hrs after prior topical application of the H1/2r antagonists. **b:** Changes in transepidermal water loss 16 hrs after a single topical application of the H1r or H2r antagonist or vehicle to opposing flanks of previously-sensitized and subsequently challenged mice (3× = subacute allergic contact dermatitis [SACD]); (atopic dermatitis [AD] =10 haptens challenges). Dotted line indicates upper level of water loss in normal mice.