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Topical Hesperidin Enhances Epidermal Function in an Aged Murine Model

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TO THE EDITOR

As skin ages, the epidermis is thinner with reduced epidermal proliferation, abnormal differentiation, impaired lipid synthesis, and elevated skin surface pH. These alterations have profound consequences for barrier function, skin cohesion, antimicrobial defense, inflammatory threshold, and cutaneous wound healing (Ghadially *et al.*, 1995; Mauro *et al.*, 1998; Choi *et al.*, 2007; Rodriguez-Martin *et al.*, 2011). These abnormalities have been linked, in part, to reduced epidermal IL-1α expression (Ye *et al.*, 2002), reduced epidermal expression of CD44 and its ligand, hyaluronic acid (Bourguignon *et al.*, 2013), and reduced epidermal lipid synthesis.

Among these many changes, much attention has been paid to the epidermal permeability barrier, because of its dominant role in regulating cutaneous homeostasis. Studies have demonstrated that epidermal permeability barrier regulates epidermal proliferation, differentiation, lipid production, and innate immunity. Therefore, strategies that enhance epidermal proliferation, differentiation, and/or lipid production, while also reducing stratum corneum (SC) pH, could prove to be useful for preventing and/or treating the functional abnormalities, including permeability barrier homeostasis, in aged skin. Our previous studies demonstrated that topical applications of a readily available herbal ingredient, hesperidin, improve epidermal permeability barrier function in young mice by stimulating epidermal proliferation, differentiation, and lamellar body formation/secretion (Hou *et al.*, 2012), all of which are likely independent of the antioxidant properties of hesperidin. Here, we show that topical applications of hesperidin improve multiple key epidermal functions in aged mouse skin. After 9 days of treatment, the gross appearance of mouse skin treated with vehicle and hesperidin appeared similar. Histological analysis showed that aged epidermis was thinner than young epidermis; whereas proliferating cell nuclear antigen (PCNA) staining indicated that aged epidermis displayed less robust proliferative activity as compared with young epidermis; hesperidin treatment did not stimulate epidermal proliferation in aged skin, as indicated by PCNA-positive cells per cm epidermal length (2.70 ± 0.10 vs. 2.45 ± 0.13 for vehicle-treated vs. hesperidin-treated skin, NS; 3.46 ± 0.17 for young skin; young vs. vehicle- or hesperidin-treated aged skin, $P<0.001$). These results indicate that topical hesperidin does not stimulate epidermal proliferation in aged mice.

After 9 days of topical hesperidin treatment, baseline SC hydration in hesperidin-treated mice also was no

Abbreviations: ABCA12, ATP-binding cassette transporter 12; FAS, fatty acid synthase; hBD2, human beta-defensin 2; HMGCoA, 3-hydroxy-3-methyl-glutaryl-CoA reductase; mBD3, mouse beta-defensin 3; NHE1, sodium/hydrogen exchanger 1; Nrf2, nuclear factor (erythroid-derived 2)–like 2; PCNA, proliferating cell nuclear antigen; Q-PCR, quantitative reverse transcriptase in real time; SC, stratum corneum; sPLA2, secretory phospholipase A2; SPT, serine palmitoyltransferase 1

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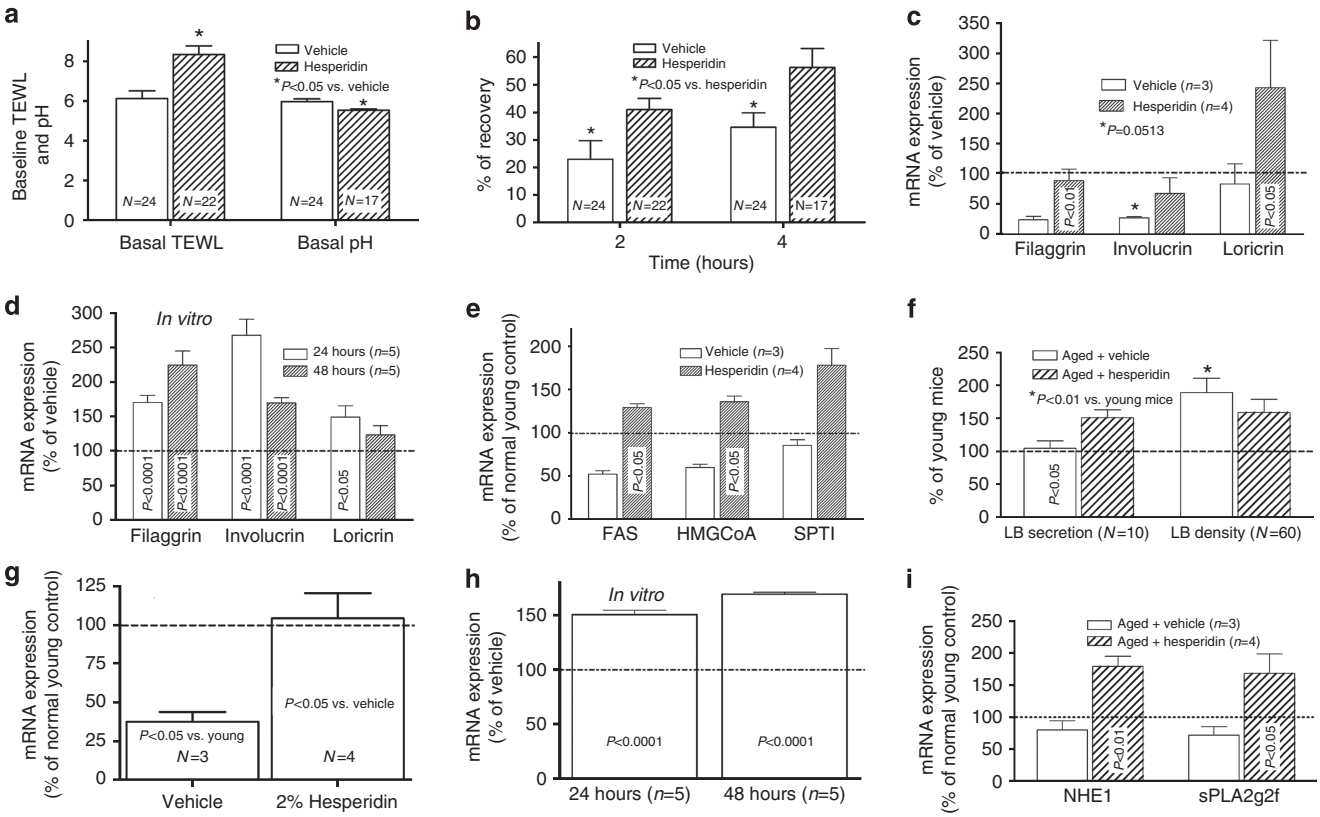

Figure 1. Topical hesperidin improves epidermal permeability barrier homeostasis in aged murine skin. The values present the data *in vivo* in mice unless otherwise specified. (**a**) Displays basal transepidermal water loss (TEWL) and skin surface pH in mice; (**b**) shows barrier recovery in mice; (**c**) shows the levels of epidermal mRNA in mice; (**d**) exhibits the levels of mRNA expression *in vitro* in human keratinocyte cultures, expressed as the % of vehicle-treated samples setting the levels of vehicle treated as 100% (dotted line); (**e**) shows epidermal mRNA levels in mice, expressed as the % of normal young mice setting the levels of young mice as 100% (dotted line); (**f**) shows the results of quantitative analysis of lamellar body density and secretion in mice; (**g** and **h**) present the expression levels of ATP-binding cassette transporter 12 (ABCA12) in mice and *in vitro* in human keratinocyte cultures, respectively; (**i**) shows the expression levels of epidermal sodium/hydrogen exchanger 1 (NHE1) and secretory phospholipase A2 (sPLA2) in mice. Significances and numbers of samples are indicated in the figures.

different from that in vehicle-treated mice (60.77 ± 1.32 for vehicle-treated vs. 58.80 ± 2.27 for hesperidin-treated). However, skin surface pH significantly declined in hesperidin-treated skin compared with vehicle-treated skin (Figure 1a). Although basal transepidermal water loss rates increased slightly in hesperidin-treated skin as compared with vehicle-treated skin (Figure 1a), these levels still fell well within the normal range of young skin. Consistent with previous findings in young mice (Hou *et al.*, 2012), topical hesperidin significantly accelerated barrier recovery at both 2 and 4 hours after acute barrier disruption of aged skin (Figure 1b). These results demonstrate that topical hesperidin improves epidermal permeability barrier homeostasis, while also lowering skin surface pH in aged murine skin.

We next examined the basis for improved barrier function and acidification in aged epidermis. Our previous studies demonstrated that topical hesperidin stimulates epidermal differentiation, accounting in part for improved epidermal permeability barrier homeostasis in young mice. Hence, we next assessed whether topical hesperidin also stimulates epidermal differentiation in aged epidermis. As shown in Figure 1c, topical hesperidin significantly increased the mRNA levels of filaggrin and loricrin in aged mouse epidermis, consistent with the results of immunostaining (Supplementary Figure S1 online). Consistently, hesperidin also increased the mRNA levels of filaggrin, involucrin, and loricrin in adult keratinocyte cultures (Figure 1d). These results indicate that hesperidin stimulates epidermal differentiation, providing one potential mechanism whereby hesperidin improves barrier function in aged skin.

Epidermal lipid synthesis is required for the formation and maintenance of the epidermal permeability barrier. Synthesis of three key barrier-related lipids, cholesterol ceramides, and fatty acids requires their respective rate-limiting enzymes 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCoA), serine palmitoyltransferase 1 (SPT1), and fatty acid synthase (FAS). Basal mRNA levels for all three key lipid synthetic enzymes were lower in aged as compared with young epidermis (demonstrated by the dotted line in Figure 1e), consistent with the concept that the lower lipid synthesis rates in aged epidermis could reflect the reduced expression of their synthetic enzymes. Topical hesperidin treatment significantly increased the mRNA levels of HMGCoA, SPT1, and FAS in aged mouse epidermis,

as assessed by quantitative reverse transcriptase in real time (Q-PCR).

Epidermal permeability barrier function depends on newly synthesized epidermal lipids delivered to the SC through the secretion of lamellar bodies from the stratum granulosum. Therefore, we next assessed whether topical hesperidin stimulates lamellar body formation and/or secretion. As ATP-binding cassette transporter 12 (ABCA12), a trans-membrane glycosylceramide transporter, is required for normal lamellar body assembly (Thomas *et al.*, 2009), we next evaluated the changes in epidermal mRNA levels of ABCA12 in hesperidin-treated aged epidermis. Although untreated aged epidermis displayed lower levels of ABCA12 mRNA in comparison with young mice, topical hesperidin induced a marked increase in ABCA12 mRNA expression in aged mouse epidermis (Figure 1g) and adult keratinocyte cultures (Figure 1h). Although the density of lamellar bodies did not increase in aged epidermis after hesperidin treatment (Figure 1f), quantitative analyses revealed that the extent of lamellar body secretion was enhanced by topical hesperidin treatment (Figure 1f). In comparison with young epidermis, the increased number of lamellar bodies in aged epidermis is likely because of the retardation of secretion. Together, these results suggest that hesperidin induced an increase in ABCA12 mRNA expression that results in an apparent acceleration in the delivery of newly synthesized lipids to the SC.

Both epidermal sodium/hydrogen exchanger 1 (NHE1) and secretory phospholipase A2 (sPLA2; in particular SPLAg2f) are key factors that selectively influence the pH of the SC (Ilic *et al.*, 2014). Previous studies from our group have shown that aged skin exhibits higher pH (Choi *et al.*, 2007), at least partly owing to reduced NHE1 expression (Choi *et al.*, 2007). To determine whether the hesperidin-induced acidification of the pH of the SC results from the upregulation of NHE1 and/or the parallel acidifying mechanism, sPLA2g2f, we next assessed the changes in epidermal mRNA levels of these two genes in aged epidermis after herperidin treatments by Q-PCR. Topical hesperidin provoked a marked elevation in mRNA levels for both NHE1 and sPLA2g2f in aged epidermis (Figure 1i). These results suggest that hesperidin-induced acidification of aged epidermis results from stimulation of NHE1 and sPLA2g2f, accounting for the lower skin surface pH, and likely improved epidermal permeability barrier homeostasis in hesperidin-treated aged mouse skin.

Our prior studies demonstrated that epidermal permeability barrier and antimicrobial function are co-regulated and independent (Aberg *et al.*, 2008). Aged humans are predisposed to develop both cutaneous and extracutaneous infections, and expression of the epidermal cathelicidin antimicrobial peptide CAMP/LL37 is reduced in aged skin (Rodriguez-Martin *et al.*, 2011). To determine whether hesperidin enhances epidermal antimicrobial defense, we next assessed changes in the mRNA levels of mouse beta-defensin 3 (mBD3), a homolog of human beta-defensin 2 (hBD2), following hesperidin treatment. Hesperidin treatment significantly increased epidermal mBD3 mRNA levels (Supplementary Figure S2a online). To further validate these *in vivo* results, the effects of hesperidin on antimicrobial mRNA expression were evaluated in cultured keratinocytes from aged human skin. Although no changes in constitutive hBD2 mRNA expression were observed (Supplementary Figure S2c online), the addition of hesperidin to aged human keratinocyte cultures markedly upregulated not only hBD3 mRNA but also CAMP/LL37 expression (Supplementary Figure S2b and d online). These results demonstrate that hesperidin stimulates antimicrobial peptide mRNA expression in aged keratinocytes.

In the present study, we demonstrated that topical hesperidin improves a wide spectrum of functional abnormalities in aged epidermis, including abnormalities in epidermal permeability barrier function, epidermal differentiation, lipid production, and SC acidification. Although the molecular mechanisms of hesperidin-induced functional changes in aged skin is not clear, the antioxidant property of hesperidin could be involved. Aged skin displays lower antioxidant capacity and excessive accumulation of oxidative products, and hesperidin shows high antioxidant capacity (Pękal *et al.*, 2011). Hesperidin inhibits the production of reactive oxygen species in rat kidney and human hepatocytes, reduces plasma malondialdehyde levels, and increases superoxide desmutase activity in diabetic rats. Oral hesperidin administration increases blood glutathione peroxidase activity in type 1 diabetic patients, whereas antioxidants stimulate keratinocyte differentiation. Our recent study demonstrated that topical hesperidin applications increased epidermal mRNA levels of antioxidant enzymes such as glutathione reductase and superoxide dismutase in murine skin (Man *et al.*, 2014). Moreover, antioxidants such as vitamin E and C increase lipid production in keratinocyte cultures. Pertinent to antioxidant, nuclear factor (erythroid-derived 2)–like 2 (Nrf2), a transcription factor, regulates epidermal differentiation and antioxidant defense (Schäfer *et al.*, 2012). Nrf2 function is impaired in aged heart (Gounder *et al.*, 2012), and expression levels were lower in aged epidermis (unpublished observation by Man MQ and Elias PM). Hesperidin upregulates Nrf2 in the heart (Elavarasan *et al.*, 2012) and in the aged epidermis (unpublished observation by Man MQ and Elias PM). Hence, hesperidin-induced improvement of epidermal permeability barrier function in aged skin may be mediated via Nrf2. Nevertheless, this study indicates that hesperidin could be a valuable approach for antiaging of skin.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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A Spontaneous *KRT16* Mutation in a Dog Breed: A Model for Human Focal Non-Epidermolytic Palmoplantar Keratoderma (FNEPPK)

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TO THE EDITOR

The keratin 16 gene (*KRT16*) encodes an intermediate filament protein mainly expressed in palmoplantar epidermis. In humans, mutations in *KRT16* are responsible for pachyonychia congenita and focal non-epidermolytic palmoplantar keratoderma (FNEPPK; Smith *et al.,* 2000; McLean and Moore, 2011). One of the main symptoms is a painful thickening of the palms and soles. To understand molecular mechanisms involved in this keratoderma, Krt16 mutant mouse models have been developed, but only one reproduces fully the palmoplantar phenotype (Lessard and Coulombe, 2012). In this study, we present a spontaneous canine model of FNEPPK inherited as an autosomal recessive disorder in the Dogue de Bordeaux breed. Because of its population structure, which features genetic isolates, the purebred dog model has recently proven its utility in understanding the molecular mechanisms of hereditary cornification disorders, notably in humans and dog Autosomal Recessive Congenital Ichthyosis (Grall *et al.,* 2012).

We investigated a family of 130 dogs including 28 affected animals; no sex bias was observed among the 13 males and 15 females analyzed. The onset usually occurred between 10 weeks and 1 year of age. First described by Paradis (1992), affected dogs exhibit a painful thickening of the footpads with severe keratinous proliferations and fissures only at the ground contact locations similar to those observed in FNEPPK patients (Figure 1). Cracks predispose the dogs to secondary infections, leading to lameness, causing the dog to be reluctant to walk. Nails did not seem to be affected, as reported in some human FNEPPK patients and in Krt16-null mice models (Shamsher *et al.,* 1995; Smith *et al.,* 2000; Liao *et al.,* 2007; Lessard and Coulombe, 2012). Similarly, no other cutaneous sign such as oral leukoplakia, cysts, or follicular keratosis was reported. This is concordant with our results of quantitative reverse transcription PCR of messsengerRNA from unaffected dog biopsies, showing strong and specific expression of *KRT16* in the footpad, nose, and keratinocytes but not in body skin, oral mucosa, or other organs (data not shown).

Histopathological examinations of footpad biopsies revealed thick hyperkeratotic digital epidermis that was roughened by marked conical papillae with a prominent ''church

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