

Topical apigenin improves epidermal permeability barrier homeostasis in normal murine skin by divergent mechanisms

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Abstract: The beneficial effects of certain herbal medicines on cutaneous function have been appreciated for centuries. Among these agents, chrysanthemum extract, apigenin, has been used for skin care, particularly in China, for millennia. However, the underlying mechanisms by which apigenin benefits the skin are not known. In this study, we first determined whether topical apigenin positively influences permeability barrier homeostasis, and then the basis thereof. Hairless mice were treated topically with either 0.1% apigenin or vehicle alone twice daily for 9 days. At the end of the treatments, permeability barrier function was assessed with either an electrolytic water analyzer or a Tewameter. Our results show that topical apigenin significantly enhanced permeability barrier homeostasis after tape stripping, although basal permeability barrier function remained unchanged. Improved barrier function correlated with enhanced filaggrin expression and lamellar body production, which was paralleled by elevated mRNA levels for the epidermal ABCA12. The mRNA

levels for key lipid synthetic enzymes also were upregulated by apigenin. Finally, both cathelicidin-related peptide and mouse beta-defensin 3 immunostaining were increased by apigenin. We conclude that topical apigenin improves epidermal permeability barrier function by stimulating epidermal differentiation, lipid synthesis and secretion, as well as cutaneous antimicrobial peptide production. Apigenin could be useful for the prevention and treatment of skin disorders characterized by permeability barrier dysfunction, associated with reduced filaggrin levels and impaired antimicrobial defenses, such as atopic dermatitis.

Abbreviations: ABCA12, ATP-binding cassette transporter 12; CAMP, cathelicidin-related peptide; mBD3, mouse beta-defensin 3.

Key words: antioxidant – apigenin – barrier – differentiation – transepidermal water loss

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Introduction

Herbal medicines have been used widely, especially in Asia, for the prevention and treatment of a variety of disorders, including inflammatory dermatoses, largely due to their considerable efficacy and improved side effect profiles in comparison with Western medicines. Well-controlled clinical studies have demonstrated that herbal medicines are effective in treating cardiovascular disease (1,2), renal disorders (3,4), respiratory disease (5,6), as well as some cancers (7,8). Similarly, herbal medicines have shown beneficial effects in inflammatory dermatoses, such as psoriasis, acne, contact dermatitis and atopic dermatitis, not only in murine disease models, but also in humans (9–13). Our recent studies show that topical applications of several herbal extracts improve epidermal permeability barrier function, in part by stimulating keratinocyte differentiation and lipid production, in addition to upregulating epidermal antimicrobial peptide expression in murine models (14,15), perhaps accounting for the clinical impact of some of these preparations.

Chrysanthemum, also known as *Bracteantha bracteata*, is a common ingredient in some of these herbal medicines. It has proved effective in treating skin disorders in China for millennia. For instance, topical chrysanthemum extract alleviated diaper dermatitis in infants and newborns with erythema venenatum

(16,17). Improvement of certain cutaneous drug reactions also has been reported with chrysanthemum (18). Commercialized chrysanthemum tea, as well as chrysanthemum extracts contained in skin care products, is claimed to increase skin hydration and to provide a protective barrier (http://fr.provitalgroup.com/en/products_botanical/moisturizing/chrysanthemum-oil).

Apigenin is an active constituent that is present in large quantity in chrysanthemum extract. Certain benefits of apigenin on cutaneous function have already been documented. For example, apigenin exhibits preventive activity against UVB-induced skin tumors, apparently through inhibition of cyclooxygenase-2 (COX-2) expression (19–21). In a murine model, an apigenin-enriched diet attenuated the development of atopic dermatitis-like lesions (22), and one clinical study showed that an apigenin-containing cream inhibited cutaneous inflammation (23). Some of these benefits of apigenin, such as UVB protection and cancer prevention, could be due to the antioxidant properties of apigenin (24,25).

Recent studies suggest that antioxidant defense and epidermal permeability barrier could be interrelated functions. Oxidative stress inevitably occurs in skin disorders with epidermal permeability barrier abnormalities (26–28), while conversely, applications of antioxidants improve epidermal permeability barrier

function (15,29–31). However, whether the antioxidant, apigenin, improves epidermal permeability barrier function and the underlying mechanisms responsible for such changes remain undefined. In this study, we evaluated the effects of apigenin on epidermal permeability barrier function in normal murine skin. After first finding that apigenin improved barrier function, we assessed the responsible mechanisms, including the impact of apigenin on keratinocyte differentiation, lipid secretion and antimicrobial peptide expression.

Materials and methods

Materials

Six- to eight-week-old female hairless mice (h/h) were purchased from Charles River Laboratories (Wilmington, MA, USA) and fed mouse diet (Ralston-Purina Co., St Louis, MO, USA) and water *ad libitum*. Apigenin powder was purchased from Sigma Chemical Co (St Louis, MO, USA). Affinity-purified, rabbit anti-mouse antibodies to loricrin, involucrin and filaggrin were purchased from Covance (Emeryville CA, USA) for immunohistochemistry.

Experimental protocols and functional studies

All animal procedures were approved by the Animal Studies Subcommittee (IACUC) of the San Francisco Veterans Administration Medical Center and performed in accordance with their guidelines. Both flanks of mice were treated topically with 60 μ l of 0.1% apigenin or ethanol twice daily for 9 days. Basal epidermal permeability barrier function was assessed by measuring transepidermal water loss (TEWL) using TM300 connected to MPA5 (C&K, Cologne, Germany). For barrier recovery, TEWL was measured using an electrolytic water analyzer (Meeco, Warrington, PA) at 0, 2 and 4 h after tape stripping (10-fold increase in TEWL), and per cent barrier recovery was calculated as described earlier (32).

Cell culture

Second-passage keratinocytes isolated from newborn foreskins were cultured in serum-free keratinocyte growth medium containing 0.07 mM calcium (Clonetics, San Diego, CA, USA). Cells at 80–90% confluence were switched to a medium containing 1.2 mM calcium and treated with either 10 μ M apigenin or vehicle alone (0.05% ethanol). After 24 and 48 h of treatment, keratinocytes were collected for Western blot and Q-PCR analysis.

Western blot analysis of epidermal differentiation proteins

Epidermis from mice treated topically with 0.1% apigenin or vehicle alone for 9 days was obtained by EDTA separation (15). Epidermis was prepared in radioimmunoprecipitation assay buffer and was resolved by electrophoresis on 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA). Resultant bands were blotted onto polyvinylidene fluoride membranes and were subsequently probed with monoclonal anti-mouse β -actin antibody (Sigma), polyclonal antifilaggrin (against the 37 kD, monomer; Covance, Emeryville CA, USA), polyclonal anti-involucrin (against the 56 kD; Covance) or polyclonal antiloricrin (against the 57 kD; Covance), and the corresponding bands were detected by enhanced chemiluminescence (Thermo Fisher Sci., Rockford, IL, USA) and quantitated by scanning densitometry. For keratinocyte cultures, polyclonal antifilaggrin (against the 37 kD, monomer), monoclonal anti-involucrin and polyclonal antiloricrin were purchased from Abcam, Cambridge, MA. Results were presented as percentage of vehicle-treated control, setting vehicle treated as 100%. β -actin was used to normalize changes in expression levels. Results

were presented as percentage of vehicle-treated control, setting vehicle treated as 100%.

Q-PCR for mRNA expression of filaggrin and lipid synthetic enzymes

Total RNA was isolated from cultured human keratinocytes using TRI Reagent (Sigma). First-strand cDNA was synthesized from 1 μ g of total RNA with the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). The real-time PCR contained 20 ng of reversed transcribed total RNA, 450 nM forward and reverse primers, and 10 μ l of 2 $\times$ LightCycler 480 SYBR Green I Master in a final volume of 20 μ l in 96-well plates using M $\times$ 3000P™ real-time PCR system (Stratagene, La Jolla, CA, USA). Quantification was performed by the comparative C_T method with cyclophilin A used for normalization. The primers for filaggrin and lipid synthetic enzymes, including 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCoA), serine palmitoyltransferase 1 (SPT1), fatty acid synthase (FAS), the lipid transporter [ATP-binding cassette A12 (ABCA12)], as well as cyclophilin A are listed in Table S1. Relative expression of the mRNAs compared to control mRNA was calculated. Data are expressed as percentage of control (as 100%).

Immunohistochemistry

Immunohistochemical staining for changes in epidermal differentiation was performed as described previously (33). Briefly, 5- μ m paraffin sections were incubated with the primary antibodies at the dilutions of 1:2000 for filaggrin, 1:1000 for involucrin and 1:500 for loricrin overnight at 4°C. After washes $\times$ 3, sections were incubated with the secondary antibody for 30 min. Staining was detected with ABC-peroxidase kit from Vector Lab (Burlingame, CA, USA), and sections were then counterstained with haematoxylin. All antibodies for differentiation markers were from Covance. For assessment of changes in two antimicrobial peptides, mouse beta-defensin 3 (mBD3) and cathelicidin-related peptide (CAMP), expression was performed in 5- μ m frozen and paraffin sections, respectively [primary antibodies to mBD3 from Alpha Diagnostics; mouse cathelicidin (CAMP) antibody was a gift from Dr. Richard Gallo (UCSD)] (14). Sections were examined with a Zeiss fluorescence microscope (Jena, Germany), and digital images were captured with AxioVision software (Carl Zeiss Vision, Munich, Germany). All pictures were taken with the same exposure times.

Electron microscopy

Skin biopsies from both vehicle and apigenin-treated mice were taken for electron microscopy (15). Briefly, samples were minced to <0.5 mm³, fixed in modified Karnovsky's fixative overnight and postfixed in either 0.2% ruthenium tetroxide or 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide. After fixation, all samples were dehydrated in a graded ethanol series and embedded in an Epon-epoxy mixture. Ultrathin sections were examined, with or without further contrasting with lead citrate, in a Zeiss 10A electron microscope (Carl Zeiss, Thornwood, NJ, USA), operated at 60 kV. Number of lamellar body was counted in every 30 cm² area in the first layer of stratum granulosum on micrograph with magnification of 25 000 times. The data were expressed as number of lamellar body per 30 cm² area.

Statistics

GraphPad Prism 4 software was used for all statistical analyses (GraphPad Software, Inc., La Jolla, CA, USA). An unpaired *t*-test with Welch's correction was used for comparisons between two groups. Data are expressed as mean $\pm$ SEM.

Results

Topical apigenin improves epidermal permeability barrier homeostasis in normal murine skin

We first assessed whether topical apigenin improves epidermal permeability barrier function in normal murine skin. After 9 days of topical apigenin treatment, there were no changes in gross appearance of mouse skin. Baseline skin surface pH and TEWL rates did not differ significantly in apigenin- versus vehicle-treated mice (Fig. 1a,c; $P = 0.3411$ for Fig. 1a and $P = 0.0963$ for Fig. 1c). But stratum corneum (SC) hydration was slightly but significantly lower in apigenin-treated as compared to vehicle-treated mice (Fig. 1b). In contrast to basal TEWL, topical apigenin accelerated barrier recovery, a change that was highly significant at 4 h after acute barrier disruption (Fig. 1d). These results demonstrate that topical apigenin improves epidermal permeability barrier homeostasis in normal murine skin.

Topical apigenin stimulates filaggrin expression in the murine model

Because differentiation-related structural proteins are a key determinant of normal barrier function, we next assessed whether topical apigenin influences the expression of epidermal differentiation-related proteins, potentially providing one potential mechanism whereby apigenin could improve barrier function. As showed in Fig. 2, epidermal filaggrin immunostaining became much more prominent following topical apigenin treatment (Fig. 2d versus Fig. 2a), but neither involucrin nor loricrin immunostaining differed in apigenin- versus vehicle-treated skin (Fig. 2b,c versus Fig. 2e,f). To further confirm the immunohistochemical results, Western blotting was employed to quantitate the changes in expression of the differentiation-related proteins. Apigenin again significantly and selectively stimulated epidermal filaggrin expression, without significantly altering either involucrin or loricrin expression (Fig. 2g and Fig. S1).

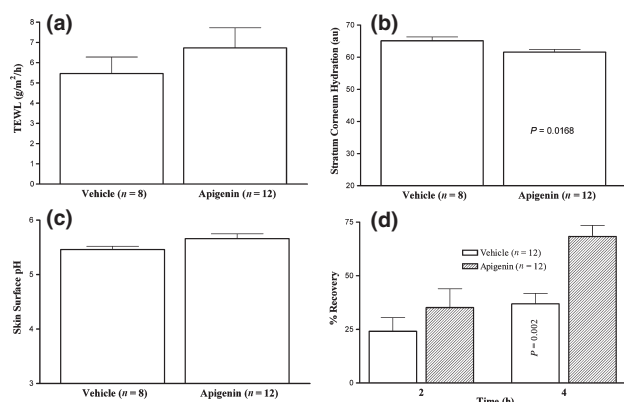


Figure 1. Topical apigenin improves epidermal permeability barrier homeostasis in normal murine skin: hairless mice were treated topically with 60 μ l of 0.1% apigenin in 100% ethanol or ethanol alone twice daily for 9 days. Basal epidermal permeability barrier function, skin surface pH and stratum corneum (SC) hydration were assessed with a MPA5 (CK electronic GmbH, Cologne, Germany) connected to TM 300, pH905 and Corneometer 825. Two readings were taken from each mouse for basal transepidermal water loss (TEWL), hydration, as well as pH. For barrier recovery, TEWL was measured with an electrolytic water analyzer (Meeco, Warrington, PA, USA) at 0, 2 and 4 h after tape stripping, which results in a 10-fold increase in TEWL, and per cent barrier recovery rates were calculated as described earlier (32). Figure (a) is basal TEWL; (b), SC hydration; (c), skin surface pH; (d), barrier recovery. Numbers and significances are indicated in the figures.

To further validate the *in vivo* data, the effect of exogenous apigenin (10 μ M) on keratinocyte differentiation was also assessed in cultured human keratinocytes. Filaggrin expression was slightly elevated at 24 h following exogenous apigenin treatments (Fig. 2h and Fig. S2) and significantly elevated at 48 h (Fig. 2h and Fig. S3). In contrast to the *in vivo* results, apigenin induced a significant reduction in loricrin expression at both 24 and 48 h (Fig. 2h), while involucrin expression was reduced at 24 h, but increased at 48 h (Fig. 2h). To determine whether the apigenin-induced elevation in filaggrin occurs at transcriptional levels, filaggrin mRNA expression was assessed in human keratinocyte cultures. The results showed that apigenin induced a significant increase in filaggrin mRNA expression at both 24 and 48 h (vehicle 100 + 9.22 vs apigenin 204 + 7.52, $P < 0.0001$, for 24 h; vehicle 100 + 10.20 vs apigenin 160 + 16, $P < 0.05$, for 48 h). Together, these results indicate that topical apigenin selectively upregulates epidermal filaggrin expression, thereby providing one mechanism by which apigenin could improve epidermal permeability barrier homeostasis.

Apigenin stimulates the mRNA expression of three key barrier-related lipid synthetic enzymes

Epidermal permeability barrier function requires synthesis of three key lipids, ceramides, cholesterol and fatty acids. We next quantitated changes in mRNA levels of the three rate-limiting enzymes for synthesis each of these key lipids, that is, HMGCoA, SPT1 as well as FAs, after addition of exogenous apigenin to cultured

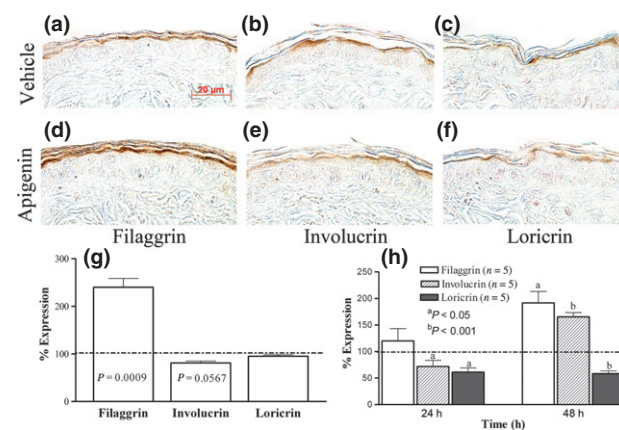


Figure 2. Topical selectively apigenin stimulates filaggrin expression in normal mouse epidermis and keratinocyte cultures: 5 μ m paraffin sections were incubated with respective antibodies. The sections were visualized with a Zeiss microscope. Figure (a) and (d) are filaggrin staining; (b) and (e) are involucrin staining; c and f are loricrin staining. Figure (a)–(c) are vehicle-treated samples and (d)–(f) are apigenin-treated samples. Magnifications are the same for all figures. Magnification bars represent 20 μ m (a–f). For Western blot analysis *in vivo*, differentiation-related protein from mouse epidermis was isolated and quantitated by scanning densitometry, as described in Materials and Methods. ($n = 5$ for each group). For Western blot analysis in human keratinocyte cultures, second-passaged human keratinocytes isolated from newborn foreskins were cultured in serum-free keratinocyte growth medium containing 0.07 mM calcium. Cells at 80–90% confluence were switched to medium containing 1.2 mM calcium and treated with either 10 μ M apigenin or vehicle alone (0.05% ethanol). After 24 and 48 h of treatment, keratinocytes were collected for Western blotting. The corresponding protein bands were detected by enhanced chemiluminescence and quantitated by scanning densitometry. Figure (g) and (h) displays the quantitative changes of expression in mouse epidermis and human keratinocyte cultures, respectively. Results were presented as percentage of vehicle-treated control, setting vehicle treated as 100% as indicated by dotted line. Significances are indicated in the figures.

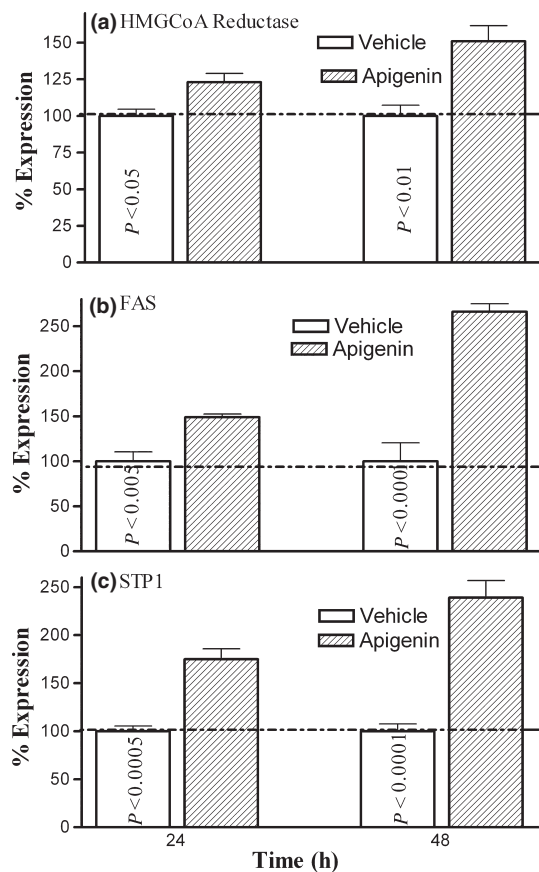


Figure 3. Apigenin upregulates mRNA expression of lipid synthetic enzymes *in vitro*: total RNA was isolated from cultured human keratinocytes (detailed in Materials and methods) and further purified by RNeasy RNA purification kit. cDNA was prepared using the reverse transcription kit. Levels of mRNA expression were measured by PCR using SYBR Green Master Mix. Relative expression of the mRNAs compared to GAPDH control mRNA was calculated. Data are normalized to vehicle control (setting vehicle control as 100% indicated by dotted line on figures). Figure (a) is the levels of HMGCoA mRNA expression, and Fig. 3b represents the levels of FAS mRNA expression. Figure (c) is SPT1 mRNA expression. Significances are indicated in the figures ($n = 5$ for each group).

human keratinocytes. As seen in Fig. 3, apigenin treatment significantly elevated the mRNA levels of HMGCoA, SPT1 and FAS. Together, these results suggest that apigenin stimulates epidermal lipid production, which could provide another mechanism by which apigenin improves epidermal permeability barrier homeostasis.

Topical apigenin increases production of lamellar bodies

Formation of the epidermal permeability barrier function requires not only the synthesis of lipids, but also production and secretion of lamellar bodies as a means to deliver lipids to the SC (34,35). Hence, we next evaluated whether topical apigenin accelerates lamellar body formation and secretion. Indeed, the density of lamellar bodies in intact epidermis significantly increased after topical apigenin treatment (Fig. 4a–c). As these ultrastructural studies strongly suggested that topical apigenin treatment increases lamellar body production, we next assessed changes in mRNA levels of the ATP-binding cassette transporter 12 (ABCA12), a transmembrane glycosylceramide transporter, required for lamellar body formation (36–39). As predicted, topical apigenin induced a

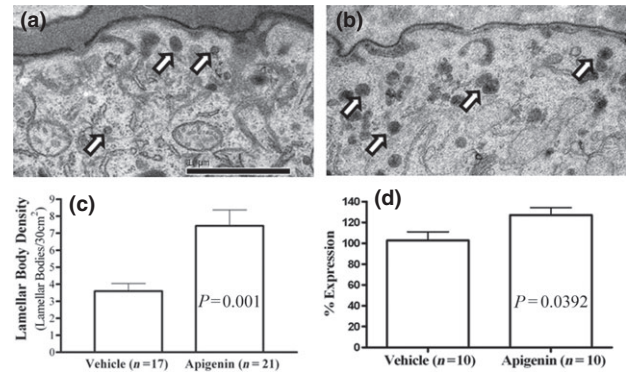


Figure 4. Topical apigenin stimulates lamellar body production in normal mouse epidermis, paralleled by increase ABCA12 mRNA expression in cultured human keratinocytes: skin biopsies from both vehicle- and apigenin-treated mice were taken for electron microscopy and processed as described in Materials and methods. Figure (a) and (b) display the density of lamellar bodies (see arrows) in vehicle-(a) or apigenin-(b)-treated epidermis. Figure (c) quantitatively displays changes in lamellar body density in vehicle- and apigenin-treated epidermis. Figure (d) exhibits changes in the levels of ABCA12 mRNA expression after addition of apigenin ($10 \mu\text{M}$) to cultured keratinocytes growing under differentiating conditions (1.2 mM calcium). Magnification bars, numbers (N) and significant differences are indicated in the figures.

marked increase in ABCA12 mRNA expression in human keratinocytes cultured in 1.2 mM calcium (Fig. 4d, $P = 0.0186$). Thus, apigenin appears to accelerate the delivery of newly synthesized lipids into nascent lamellar bodies.

Topical apigenin increases immunostaining for epidermal CAMP and mBD3

Previous studies demonstrated that two antimicrobial peptides, cathelicidin-related peptide (CAMP) and mBD3, are packaged within and secreted by lamellar bodies, and that CAMP is crucial not only for antimicrobial defense, but also for epidermal permeability barrier function (40–43). As our recent studies showed that topical applications of another herbal extract improved epidermal permeability barrier function in parallel with increased epidermal antimicrobial peptide expression (14), we next asked whether apigenin treatment also could increase epidermal CAMP and/or mBD3 expression. As shown in Fig. S4, immunostaining for both CAMP and mBD3 markedly increased following 9 days of topical apigenin treatment. These results suggest that topical apigenin enhances epidermal CAMP and mBD3 expression.

Discussion

An increasingly long line of evidence has demonstrated that certain herbal medicines benefit inflammatory dermatoses characterized and driven by abnormalities in barrier function. Yet, until recently, the influence of herbal medicines on epidermal permeability barrier function has not been well studied. In the present study, we demonstrated first that topical applications of apigenin, an extract from chrysanthemum, improve epidermal permeability barrier homeostasis. Although the exact mechanisms by which apigenin accelerates epidermal permeability barrier recovery are not clear, it is a known antioxidant (25), and topical or systemic administrations of other antioxidants, such as vitamin C, vitamin E as well as epigallocatechin gallate, improve epidermal permeability barrier function (25,29,44,45). Hence, it is possible that the apigenin-induced improvement in epidermal permeability barrier homeostasis could result from its antioxidant properties. Whether or not antioxidant mechanisms are involved, a most

noteworthy finding in the present study was a selective, apigenin-induced upregulation of filaggrin expression, shown both *in vivo* and *in vitro*. Moreover, we show here that the increased filaggrin is likely, at least in part, to be due to upregulation of its mRNA expression. The importance of filaggrin in epidermal permeability barrier function is shown by the observation that filaggrin mutations compromise epidermal permeability barrier function (26,46). Pertinently, increased epidermal filaggrin expression has been proposed to account in part for the enhanced epidermal permeability barrier function induced by topical peroxisome proliferator-activated receptor and liver X receptor activators (32,47–49).

Although increased filaggrin expression could account in part for the improved epidermal permeability barrier homeostasis induced by apigenin, we show here that apigenin also stimulated epidermal lipid synthesis and lamellar body production. The epidermal lamellar body is the only known organelle that delivers lipids and their respective postsecretory processing enzymes to the extracellular space of the SC. Previous studies showed that either abrogation of lamellar body formation or blockade of their secretion disrupts epidermal permeability barrier homeostasis (34). In the present study, we show that apigenin induced a marked increase in mRNA expression of not only key lipid synthetic enzymes, but also the lipid transporter protein, ABCA12, which is required for lamellar body formation. Hence, enhanced lipid production and lamellar body formation could further explain how apigenin improves epidermal permeability barrier homeostasis. Finally, the present study revealed that topical apigenin causes a marked elevation in two critical antimicrobial peptides, epidermal CAMP and mBD3 protein expression. In addition, CAMP is crucial for epidermal permeability barrier homeostasis. Prior studies have shown that CAMP-deficient mice display delayed barrier recovery and expression of both CAMP and mBD3 is regulated in parallel with changes in epidermal permeability barrier status (42,50). Thus, the accelerated barrier recovery could also be due in part to an upregulation of epidermal CAMP and mBD3 expression induced by apigenin. In the present study, a remarkable reduction in SC hydration was observed following apigenin treatment. While the exact mechanism for such a reduction is not clear, it could reflect inhibition of epidermal proteasomes, induced by apigenin (51,52). It is known that amino acid metabolites are the natural moisturizers in SC (53), and higher activity of proteasomal enzymes (which largely account for the degradation of proteins to amino acids) (Rev. in ref. 54) presents in the epidermis (55). Apigenin is a known proteasome inhibitor (51,52), which could progressively lower the amino acid content in SC, accounting for the low SC hydration in apigenin-treated skin. Additionally, it is also shown that filaggrin is the source of natural moisturizing factors (NMFs) and its degradation pathway to NMFs includes at least caspase-14 and bleomycin hydrolase (56,57). Caspase-14 degrades deiminated filaggrin into peptides, which is further catalysed into NMFs by bleomycin hydrolase (56). Filaggrin-deficient mice display dry skin with lower levels of NMFs (58). Therefore, it is possible that apigenin also inhibits the

pathway breaking down filaggrin into NMFs. However, further studies would be needed to elucidate the mechanisms by which apigenin reduces SC hydration.

The influence of antioxidants on cutaneous structure and function varies dramatically, according to the types of agents deployed. For example, vitamin E reduces lipid peroxidation and stimulates keratinocyte differentiation (59,60), while vitamin C stimulates both keratinocyte lipid synthesis and differentiation (61,62). Even within the same class of antioxidants, keratinocyte function may be regulated differently. Balasubramanian *et al.* showed that epigallocatechin gallate, a flavonoid, increases involucrin expression (63), whereas another flavonoid, curcumin, inhibits involucrin expression induced by epigallocatechin gallate (64). In contrast, hesperidin, also a flavonoid, increases epidermal filaggrin expression, without changing involucrin and loricrin expression (15), which is in agreement with data from the present study with apigenin *in vivo*, whereas the present *in vitro* study showed that apigenin reduced loricrin expression. These discrepant *in vivo* and *in vitro* results may reflect the difference in keratinocyte differentiation *in vivo* and *in vitro* (65). For example, treatment of keratinocytes with apigenin for 48 h may not suffice to stimulate loricrin, the later differentiation protein. Nevertheless, the present study demonstrates that apigenin upregulates filaggrin expression. Taken together, these results suggest further that antioxidants differentially impact cutaneous structure and function by a variety of mechanism. Accordingly, both the types of skin condition and the specific characteristics of each antioxidant should be taken into account when choosing the most appropriate agent for possible clinical deployment in dermatology. As apigenin selectively upregulates filaggrin expression and lipid production, it could be particularly useful for the treatment atopic dermatitis.

In summary, the present study demonstrates that topical apigenin improves epidermal permeability barrier homeostasis, stimulates lamellar body formation and upregulates filaggrin and lipid synthetic enzyme mRNA expression. Therefore, apigenin could be useful in treating skin disorders with permeability barrier dysfunction, especially those accompanied by reduced filaggrin expression, such as atopic dermatitis.

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Author contributions

MH performed keratinocyte culture, Western blotting *in vitro* and prepared the graphs and figures for publication; MH performed the immunohistochemical staining; PLK performed qPCR; RS assessed barrier function and prepared samples for morphology and Western blotting *in vitro*; KP performed Western blotting *in vivo*; DC carried out the ultrastructural studies; TKL and JLS assessed basal stratum corneum function and statistical analyses; TMM and PME designed the experiment, interpreted data, critically reviewed the manuscript and approved the final version; MQM designed the experiment, interpreted data and drafted the manuscript.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

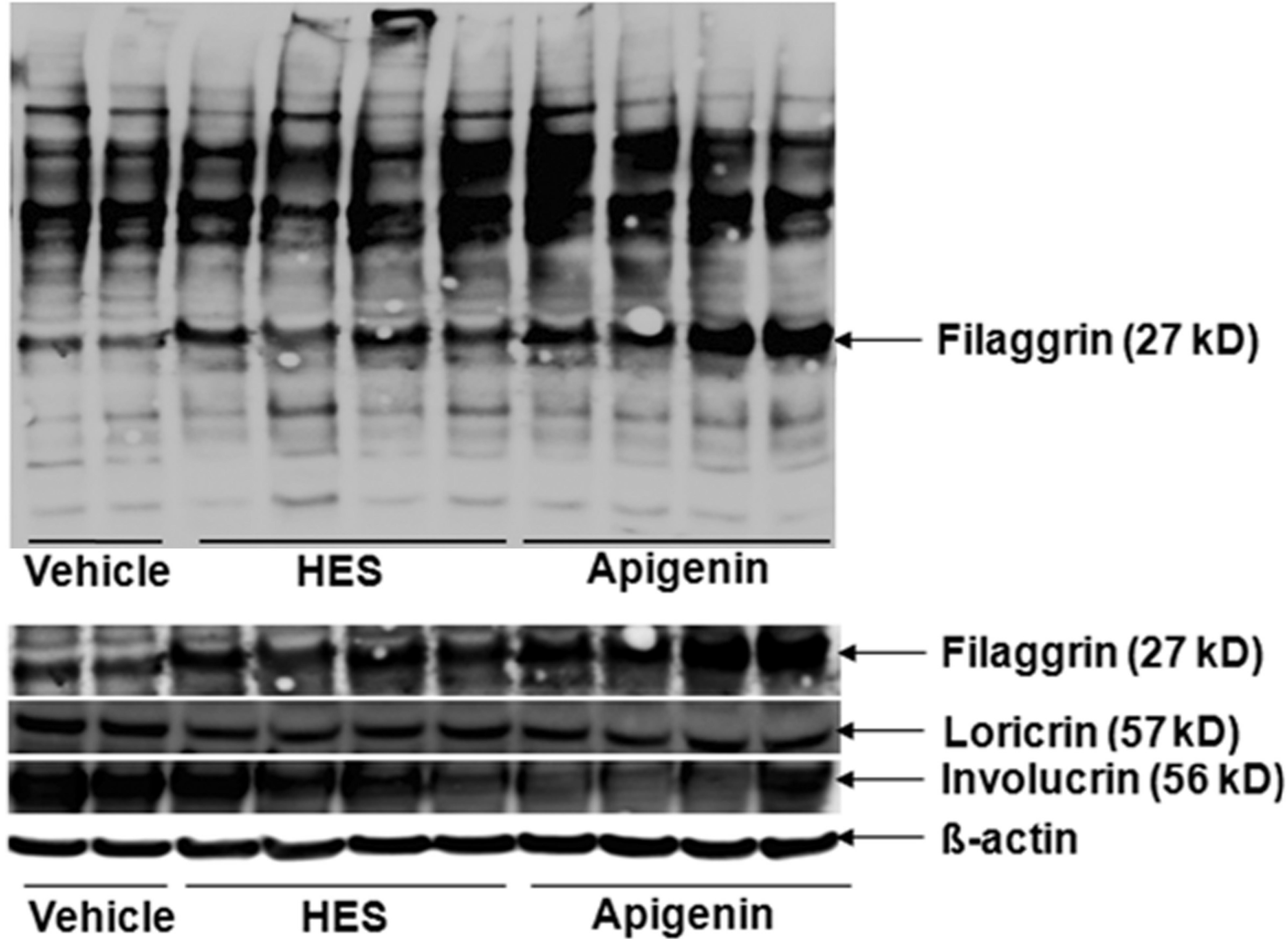
Figure S1. Effects of apigenin on filaggrin expression *in vivo*.

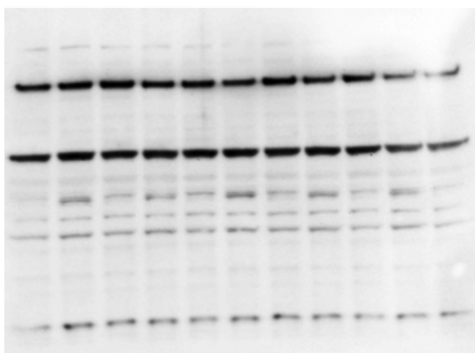
Figure S2. Effects of apigenin on filaggrin expression *in vitro* at 24 h after apigenin treatment.

Figure S3. Effects of apigenin on filaggrin expression *in vitro* at 48 h after apigenin treatment.

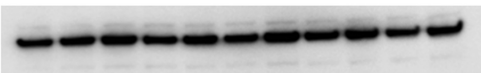
Figure S4. Topical apigenin increases immunostaining for epidermal antimicrobial peptides.

Table S1. Primer sequences.

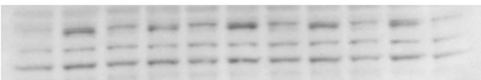




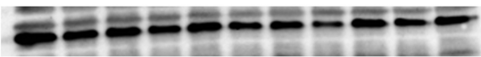
↑ Filaggrin 37KD



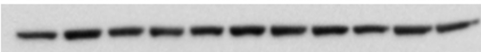
↑ Involucrin 120-170KD



↑ Filaggrin 37KD

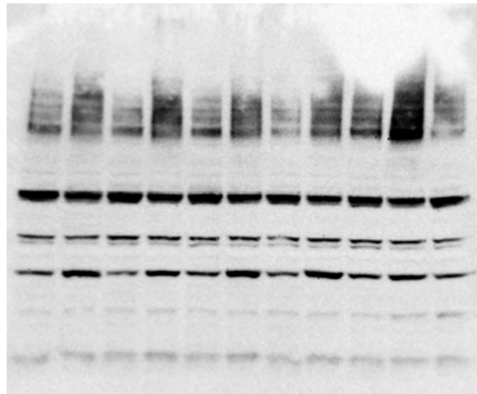


↑ Loricrin 26 KD

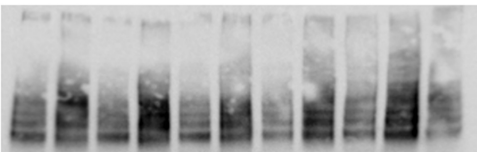


↑ β -actin

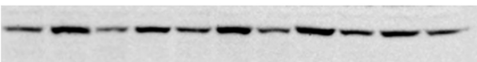
Vehicle
Apigenin
Vehicle
Apigenin
Vehicle
Apigenin
Vehicle
Apigenin
Vehicle
Apigenin



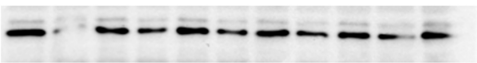
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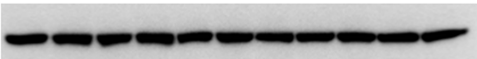
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↑ Filaggrin 37KD



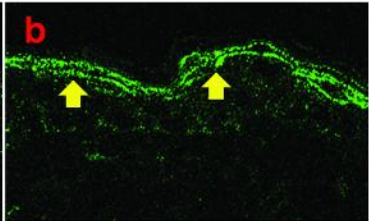
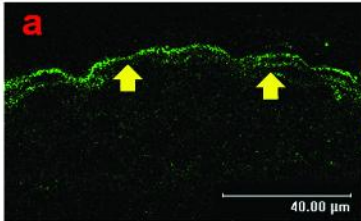
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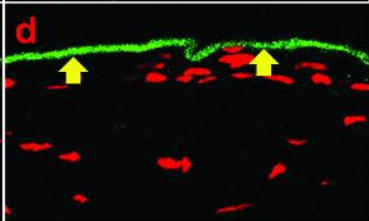
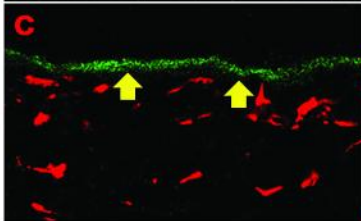
↑ β -actin

Vehicle Apigenin Vehicle Apigenin Vehicle Apigenin Vehicle Apigenin Vehicle Apigenin Vehicle

CAMP



mBD3



Vehicle

Apigenin

Supplemental Table 1. Primer Sequences

Sequences		
Human HMGC _o A	Forward	5' TTCAAAGGGTACAGAGAAAGCAC 3'
	Reverse	5' TATGCTCCCAGCCATGG 3'
Human FAS	Forward	5' GCATCAAAGAAGCCCATCTC 3'
	Reverse	5' GCTGCTCCACGAACTCAAAC 3'
Human SPT1	Forward	5' GCTGCCACCATGAGCGACCA 3'
	Reverse	5' GCTGCTACTGCCTGTGCTGCT 3'
Human ABCA12	Forward	5' ACAGGAATGGCCTTCATCAC 3'
	Reverse	5' AACATGGTGGCCTGAGAAAC 3'
Human Filaggrin	Forward	5'CCCCACCTTTGCGGAGA3'
	Reverse	5'CCCCACCTTTGCGGAGA3'
Human CycloA	Forward	5' TCTCCTTTGAGCTGTTTGCAG 3'
	Reverse	5' CACCACATGCTTGCCATC 3'