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Activators of PPARs and LXR Decrease the Adverse Effects of Exogenous Glucocorticoids on the Epidermis

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Abstract

While glucocorticoids (GC) exert beneficial effects (anti-inflammatory), they also have adverse effects on the epidermis including decreased epidermal differentiation, decreased keratinocyte proliferation, and decreased cutaneous permeability barrier homeostasis. Thus, the purpose of this study was to develop strategies to prevent these GC toxicities using simultaneous topical treatments in clobetasol-treated mice. While a triple-lipid mixture of stratum-corneum lipids (ceramide, free fatty acid, and cholesterol) was previously shown to reverse the GC-induced abnormality in cutaneous barrier function (Kao, et al. 2003), this lipid mixture did not prevent the GC-induced abnormalities in either keratinocyte proliferation or differentiation. Since activators of PPAR α , β/δ , γ , and LXR, regulate keratinocyte proliferation and differentiation and improve permeability barrier homeostasis, we next assessed the effects of these activators during concurrent GC treatment. Co-application of either ciglitazone (PPAR γ activator), clofibrate (PPAR α activator), or 22R (OH) cholesterol (LXR activator) with clobetasol prevented the decrease in involucrin, filaggrin and loricrin expression. In contrast, a PPAR β/δ activator (GW501516) normalized only the expression of involucrin and filaggrin, but not loricrin. Moreover, topical application of PPAR α , β/δ , or LXR activators partially prevented the decrease in keratinocyte proliferation in GC-treated murine skin, as measured by PCNA, while no effect was seen after co-treatment with PPAR γ activators. Finally, PPAR γ and PPAR β/δ activators but not PPAR α and LXR activators improved permeability barrier homeostasis in GC treated mice. Together, these studies demonstrate that PPAR and LXR activators can prevent several of the adverse effects of topical GC on the epidermis.

Keywords

keratinocyte proliferation; epidermal differentiation; permeability barrier; involucrin; loricrin; filaggrin; stratum corneum

Introduction

Glucocorticoids (GC) have been the treatment of choice for a large number of skin diseases, mainly due to their high anti-inflammatory potency (1,2). However, long-term topical GC therapy is well known to induce a wide range of adverse effects, including skin fragility, bruising, and atrophy (3,4). In the epidermis topical treatment with GC leads to decreased keratinocyte differentiation and decreased epidermal thickness due to an inhibition of

keratinocyte proliferation (5-8). Moreover, recent studies have demonstrated that even short term GC treatment impairs permeability barrier recovery after acute barrier disruption, thus impairing permeability barrier homeostasis (9). The delay in epidermal permeability barrier repair after GC treatment is secondary to a decrease in lamellar body (LB) generation attributed to the inhibition of epidermal lipid synthesis (9). Accordingly, topical physiologic lipid replacement reverses the GC-induced abnormalities in permeability barrier homeostasis (9).

The outermost layer of the epidermis, the stratum corneum (SC), is comprised of corneocytes derived from the terminal differentiation of keratinocytes, embedded in a lipid-enriched extra-cellular matrix, that together provide one of the major functions of the skin: the epidermal permeability barrier (10). In addition to changes in ionic gradients that play a major role epidermal homeostasis (11), PPAR α , β/δ γ , and LXR activators are also known to have numerous, diverse and specific effects on epidermal structure and function (12-16). First, both the addition of PPAR/LXR activators to cultured human keratinocytes and the topical application of PPAR/LXR activators to normal mouse skin stimulate expression of keratinocyte differentiation related proteins, such as involucrin, loricrin, profilaggrin, and transglutaminase 1, which together enhance cornified envelope formation (17-21). Second, PPAR/LXR activators increase cholesterol sulfotransferase activity (22), which would increase the synthesis of cholesterol sulfate, (14) and cholesterol sulfate, is itself a potent stimulator of epidermal differentiation and an important inhibitor of corneocyte desquamation (23). Third, topical treatment of murine skin with PPAR/LXR activators improves permeability barrier homeostasis, due to increased: a) epidermal cholesterol, fatty acid, and sphingolipid synthesis, b) lamellar body density and secretion, c) beta-glucocerebrosidase activity, and d) expression of ABCA12, a membrane transporter required for the delivery of glucosylceramides into lamellar bodies (24,25). These mechanisms all separately enhance permeability barrier homeostasis. Lastly, PPAR and LXR activators regulate keratinocyte proliferation (17,19,20,26). Given these positive attributes the aim of this study was to assess whether activators of PPARs and LXR could prevent the adverse effects of glucocorticoids in normal murine epidermis.

Materials and Methods

Materials

Adult male and female hairless mice (Skh1), 8 to 10 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). All animals had free access to food and water ad libitum. Hydroxy acyl ceramides were purchased from Matreya (Pleasant Gap, PA). Palmitic acid, cholesterol, clofibrate, WY-14643 and 22(R) hydroxycholesterol were from Sigma-Aldrich (St. Louis, MO). GW501516 was a gift from Dr Timothy Willson (GlaxoSmithKline, NC, USA). Ciglitazone and TO901317 were from Cayman chemical (Ann Arbor, MI). Affinity purified rabbit primary antibodies specific for mouse filaggrin, loricrin and involucrin were purchased from BabCo (Richmond, CA). Secondary biotinylated goat anti-rabbit IgG, ABC-peroxidase kit and diaminobenzidine were purchased from Vector laboratories (Burlingame, CA). The anti-proliferating cell nuclear antigen (PCNA) was purchased from CalTag Laboratories (Burlingame, CA).

Animal model and tissue preparation

Mice were treated for three consecutive days, twice daily, with a topical application of 35 μ l of either vehicle (propylene glycol: ethanol, 7:3 v:v) or 0.05% clobetasol alone. A third group of mice were treated with both clobetasol and a PPAR/LXR ligand (PPAR α ligand, clofibrate (1 mM) or WY-14643 (10mM); PPAR β/δ ligand, GW501516 (4mM); PPAR γ ligand ciglitazone (10mM), or LXR ligand 22(R)hydroxycholesterol (10mM) or TO901317

(10mM)). PPAR and LXR activators were applied 10 min after the clobetasol solution. In separate experiments, a mixture of palmitic acid, cholesterol, and ceramides was applied in a 1 : 1 : 1 mole ratio in a propylene glycol:ethanol (7 : 3 vol/vol) vehicle, as described previously (27,28).

Barrier recovery measurements

Epidermal barrier disruption was achieved by repeated applications of cellophane tape on the flanks until transepidermal water loss reached 4 to 6 mg/cm²/hr, as determined with an electrolytic water analyzer (Meeco, Warrington, PA). Evaporative water loss was then measured immediately and then at 3, 6 and 24 hours after barrier disruption on three separate marked areas on the mouse flank where barrier disruption was achieved. Experiments were repeated at least twice to confirm results.

Immunohistochemistry

Paraffin-embedded sections were incubated with purified rabbit primary antibodies specific for involucrin, filaggrin and loricrin following blockade of endogenous peroxidase activity and incubation in blocking buffer. After application of secondary biotinylated antibody, staining for these proteins was detected using ABC-peroxidase and revealed by diaminobenzidine as the substrate. Experiments were repeated at least twice to confirm results.

Detection of proliferating cells

Proliferating keratinocytes were quantitated on paraffin-embedded tissue. After incubation in antigen retrieval solution, blockade of endogenous peroxidase activity and incubation in blocking buffer, sections were incubated with anti-PCNA antibody. Staining was revealed by colorimetric reaction using the ABC-peroxidase-diaminobenzidine system. PCNA positive cells were counted in the basal and supra-basal layers on 3 to 5 pictures taken from each section at a 20X magnification. Experiments were repeated at least twice to confirm results.

Measurement of epidermal thickness

Thickness of the epidermal nucleated cell layers was measured on 126X micrographs taken every 2 cm along the epidermis in biopsies from normal control, GC plus vehicle and GC plus ligand-treated skin. The normal epidermal thickness is set at 100% and others are calculated as percentage of normal control. The data presented represents the mean of all measured points +/- SEM. (n=31-45).

Microscopy and imaging

Skin samples were fixed in 4% formalin. Pictures of each section were taken on 5µm thick paraffin sections and examined under a Zeiss Axioplan 2 light microscope (Jena, Germany) at 20X magnification.

Statistics

All data are given as mean ± S.E.M. Statistical analyses were determined using the Student's t test with GraphPad Prism 4 software for Macintosh (version 4.0b, June 2004).

Results and Discussion

SC triple-lipid mixture does not override the negative effects of GC on keratinocyte differentiation or proliferation

Previous studies have shown that a mixture of physiologic lipids, that includes palmitic acid, cholesterol, and ceramides, applied in a 1 : 1 : 1 mole ratio, prevents the adverse effects of GC on permeability barrier homeostasis (9). In contrast, co-application of the same mixture of physiologic lipids did not prevent the GC-induced decrease in expression of the keratinocyte differentiation markers, filaggrin, loricrin and involucrin (Fig.1A). Similarly, when keratinocyte proliferation was measured using PCNA immunohistochemical staining, the physiologic lipid mixture did not prevent the GC-induced decrease (Fig.1B). Thus, while a physiological lipid mixture is capable of improving permeability barrier function in GC treated animals, it does not prevent the full spectrum of epidermal abnormalities induced by GC treatment; i.e. keratinocyte differentiation or proliferation still decrease.

Improved permeability barrier homeostasis when PPAR β/δ or PPAR γ , but not PPAR α or LXR activators, are co-applied with GC

Short-term topical treatment with potent GC compromises permeability barrier homeostasis. We therefore next determined if co-application of PPAR and LXR activators also prevents the development of this abnormality. Interestingly, clofibrate and 22Rhydroxycholesterol, PPAR α and LXR activators, respectively, did not improve permeability barrier recovery following acute disruption (data not shown). However, the PPAR β/δ and PPAR γ activators, GW501516 and ciglitazone, prevented the negative effects of GC on permeability barrier homeostasis (Fig.2). Why PPAR β/δ and PPAR γ activators improve permeability barrier homeostasis, while PPAR α and LXR activators were ineffective is unknown.

PPAR α , PPAR γ and LXR activators normalize differentiation in GC- treated murine skin

Since nuclear hormone receptor activators are known to stimulate keratinocyte differentiation in normal skin, we next asked whether they could prevent the adverse effects of GC on the expression of differentiation markers in the epidermis. Topical co-applications of two classes of PPAR activators, PPAR α (clofibrate, Fig.3A) and PPAR γ (ciglitazone, Fig. 3B) or of the LXR activator (22R hydroxycholesterol, Fig.4) with clobetasol blocked the GC-induced decrease in the expression of the keratinocyte differentiation markers, involucrin, filaggrin and loricrin. In contrast, an activator of PPAR β/δ (GW501516) only prevented the decrease in filaggrin and involucrin expression, but did not prevent the decrease in the expression of loricrin when co-applied with clobetasol (Fig.5). These results demonstrate that PPAR and LXR activators can prevent, at least in part, the GC-induced decrease in keratinocyte differentiation.

PPAR α , PPAR β/δ , and LXR activators increase proliferation in GC- treated murine epidermis

Epidermal thinning is commonly observed in glucocorticoid treated skin as a result of decreased keratinocyte proliferation. Thus, we next assessed the ability of PPAR and LXR activators to prevent the decrease in keratinocyte proliferation induced by topical clobetasol treatment. Topical co-applications of the PPAR α activator, clofibrate, the PPAR β/δ activator, GW501516, or the LXR activator, 22R hydroxycholesterol, partially prevented the decrease in keratinocyte proliferation induced by GC-treatment (Fig.6 - A,B,C,E). In contrast, the PPAR γ activator, ciglitazone, did not affect the GC-induced decrease in keratinocyte proliferation (Fig.6D). Thus, activation of PPAR α , PPAR β/δ , and LXR can reduce the inhibition of keratinocyte proliferation induced by GC treatment. Finally, we measured the thickness of the epidermal nucleated cell layers after PPAR or LXR

application onto GC treated epidermis. The results paralleled those observed with the PCNA staining with an increase in epidermal thickness after PPAR α , β/δ and LXR treatment. However, while PPAR γ did not affect keratinocyte proliferation, it did induce a significant increase in epidermal thickness (Fig.6F).

In conclusion, a physiological triple lipid mixture can correct the GC induced defects in epidermal permeability barrier homeostasis but it does not reverse the GC-induced abnormalities in differentiation and proliferation (Table 1). In contrast, we show here that topical treatment with PPARs or LXR activators prevents the GC induced abnormalities in differentiation and proliferation, while only PPAR β/δ and PPAR γ activators improve the decrease in permeability barrier function induced by GC treatment. Further experiments are needed to assess the molecular mechanisms by which PPAR and LXR activators protect from the adverse effects of glucocorticoids and the mechanisms underlying the different effects observed with the SC triple lipid mixture, PPAR activators, and LXR ligands on barrier homeostasis, epidermal differentiation and keratinocyte proliferation, in both normal and glucocorticoid treated epidermis.

These studies suggest that it will be possible to develop strategies to ameliorate the epidermal abnormalities that occur with topical GC treatment. If similar favorable results can be demonstrated in humans one maybe able to take advantage of the anti-inflammatory beneficial effects of topical GC treatment without the toxic side effects on the epidermis. Importantly, topical PPAR and LXR activators demonstrate potent anti-inflammatory activity in a variety of inflammatory models (18,29,30), so that the addition of such compounds, many of which are naturally occurring lipids, to topical GC preparations would likely enhance the anti-inflammatory effects of GC. Finally, preliminary studies in animal models and humans have suggested that PPAR activators may have beneficial effects on skin diseases, such as psoriasis and atopic dermatitis, suggesting that the addition of such compounds to GC could result in added clinical benefit (26,31-36).

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Abbreviations

GC	glucocorticoids
PPAR	peroxisome proliferators-activated receptor
LXR	liver X receptor
N	number of animals in each experimental group

Figure 1. SC triple-lipid mixture does not override the negative effects of GC on keratinocyte differentiation or proliferation

A mixture of physiologic lipids, that includes palmitic acid, cholesterol, and ceramides, applied in a 1 : 1 : 1 mole ratio, did not prevent the GC-induced decrease in expression of the keratinocyte differentiation markers, filaggrin, loricrin and involucrin (A). This lipid mixture also did not prevent the decrease in keratinocyte proliferation observed after GC treatment measured by PCNA staining (B- PCNA cell count: N= 3; PCNA staining: arrows indicate PCNA positive cells).

Figure 2. Improved permeability barrier homeostasis when PPAR β/δ or PPAR γ are co-applied with GC

At 3 and 6 hours after barrier disruption permeability barrier recovery was significantly improved when β/δ ligand (GW501516, 4mM) was co-applied with GC (A; N=8). Similar results were obtained at 3, 6 and 24 hours after PPAR γ ligand, ciglitazone co-application (B) (N=5).

Figure 3. PPAR α and PPAR γ activators normalizes differentiation in GC- treated murine skin
 As shown in immunohistochemical staining for the keratinocyte differentiation markers, co-application of clofibrate (PPAR α ligand)(1mM) with clobetasol normalizes the expression of filaggrin (FIL), involucrin (INV) and loricrin (LOR)(A). Similarly, co-application of ciglitazone (PPAR γ activator) (10mM) with clobetasol normalizes these differentiation markers (B). N= 3.

Figure 4. LXR activator normalizes differentiation in GC- treated murine skin

Immunohistochemical staining for the keratinocyte differentiation markers shows that co-application of the LXR activator 22R hydroxycholesterol (10mM) with clobetasol normalizes the expression of filaggrin (FIL), involucrin (INV) and loricrin (LOR). N= 3.

Figure 5. PPAR β/δ activator GW501516 inhibited the decrease in filaggrin and involucrin, but not loricrin expression, on GC- treated murine epidermis

Application of the PPAR β/δ activator GW501516 (4mM) on GC-treated murine epidermis normalized the levels of two keratinocyte differentiation markers, filaggrin (FIL) and involucrin (INV). However, this PPAR β/δ ligand did not prevent the decrease in loricrin (LOR) after GC treatment. N= 3.

Figure 6. PPAR α , PPAR β/δ , and LXR activators increase proliferation in GC-treated murine epidermis

Topical application of either PPAR α activator (clofibrate, 1mM)(A)(N=3), β/δ ligand (GW501516, 4mM)(B)(N=3) or LXR activator (22R hydroxycholesterol, 10mM)(C, E)(N=3) increased keratinocyte proliferation in GC-treated murine epidermis, as measured by PCNA. In contrast, no effect on proliferation was seen after ciglitazone (PPAR γ ligand) co-treatment (D)(N=3). All ligands significantly reversed the decrease in the thickness of the epidermal nucleated layers observed after GC treatment (F). The data presented represents the mean of all measured points $\pm$ SEM. (N=31-45).

Table 1
Effects of NHR ligands after glucocorticoid treatment on murine epidermal homeostasis

	GC	GC+Lipids	GC+PPAR α	GC+PPAR δ	GC+PPAR γ	GC+LXR
Barrier recovery	Decreased ^a	Improved^a	No change	Improved	Improved	No change
Differentiation: - INV	Decreased	No effect	Increased	Increased	Increased	Increased
- FIL	Decreased	No effect	Increased	Increased	Increased	Increased
- LOR	Decreased	No effect	Increased	No change	Increased	Increased
Mitogenesis (PCNA)	Decreased	No effect	Increased	Increased	No effect	Increased

^a) data from Kao et al., 2003.