Peroxisome-Proliferator-Activated Receptor (PPAR)-γ Activation Stimulates Keratinocyte Differentiation

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Previous studies demonstrated that peroxisome-proliferator-activated receptor (PPAR)-α or PPAR-δ activation stimulates keratinocyte differentiation, is anti-inflammatory, and improves barrier homeostasis. Here we demonstrate that treatment of cultured human keratinocytes with ciglitazone, a PPAR-γ activator, increases involucrin and transglutaminase 1 mRNA levels. Moreover, topical treatment of hairless mice with ciglitazone or troglitazone increases loricrin, involucrin, and filaggrin expression without altering epidermal morphology. These results indicate that PPAR-γ activation stimulates keratinocyte differentiation. Additionally, PPAR-γ activators accelerated barrier recovery following acute disruption by either tape stripping or acetone treatment, indicating an improvement in permeability barrier homeostasis. Treatment with PPAR-γ activators also reduced the cutaneous inflammatory response that is induced by phorbol 12-myristate-13-acetate, a model of irritant contact dermatitis and oxazolone, a model of allergic contact dermatitis. To determine whether the effects of PPAR-γ activators are mediated by PPAR-γ, we next examined animals deficient in PPAR-γ. Mice with a deficiency of PPAR-γ specifically localized to the epidermis did not display any cutaneous abnormalites on inspection, but on light microscopy there was a modest increase in epidermal thickness associated with an increase in proliferating cell nuclear antigen (PCNA) staining. Key functions of the skin including permeability barrier homeostasis, stratum corneum surface pH, and water-holding capacity, and response to inflammatory stimuli were not altered in PPAR-γ-deficient epidermis. Although PPAR-γ activators stimulated loricrin and filaggrin expression in wild-type animals, however, in PPAR-γ-deficient mice no effect was observed indicating that the stimulation of differentiation by PPAR-γ activators is mediated by PPAR-γ. In contrast, PPAR-γ activators inhibited inflammation in both PPAR-γ-deficient and wild-type mouse skin, indicating that the inhibition of cutaneous inflammation by these PPAR-γ activators does not require PPAR-γ in keratinocytes. These observations suggest that thiazolidindiones and perhaps other PPAR-γ activators maybe useful in the treatment of cutaneous disorders.

Key words: inflammation/nuclear hormone receptor/permeability barrier function/stratum corneum
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Three peroxisome-proliferator activated receptors (PPAR) have been identified: α (NR1C1), δ (also known as β) (NR1C2), and γ (NR1C3). PPAR-α is predominately expressed brown adipose tissue and liver, and is also present in the kidney, heart, skeletal muscle, and the epidermis (Desvergene and Wahli, 1999; Kliewer *et al*, 2001). PPAR-α is an important regulator of lipid catabolism, mediating fatty acid oxidation, fatty acid uptake, and lipoprotein assembly and transport (Schoonjans *et al*, 1996; Desvergene and Wahli, 1999; Kliewer *et al*, 2001). PPAR-δ is ubiquitously expressed, including in the epidermis, and is predominately found in the intestine, kidney, and heart (Desvergene and Wahli, 1999; Kliewer *et al*, 2001). It is expressed at higher levels during embryogenesis, and studies suggest that PPAR-δ may participate in embryo implantation and decidualization (Basu-Modak *et al*, 1999; Kliewer *et al*, 1999; Lim *et al*, 1999; Michalik *et al*, 2001). Very recent studies have indicated a role for PPAR-δ in regulating fatty acid oxidation (Wang *et al*, 2003). PPAR-γ is most highly expressed in adipose tissue, and to a lesser extent in the colon, adrenal glands, spleen, and epidermis, where it mediates storage of fatty acids and regulates lipogenic pathways (Brun *et al*, 1997; Kliewer *et al*, 2001).

Although the PPAR classically are seen as regulators of lipid metabolism, activation of the PPAR produces additional and varied effects on the epidermis. PPAR-α stimulates differentiation and apoptosis and decreases proliferation both in cultured human keratinocytes and

Abbreviations: KGM, keratinocyte growth medium; PCNA, proliferating cell nuclear antigen; PPAR, peroxisome-proliferator-activated receptor; TEWL, transepidermal water loss; TPA, phorbol 12-myristate-13-acetate; TUNEL, Tdt-mediated dUTP nick end labeling

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in vivo when applied topically to mouse skin (Hanley *et al*, 1997, 1998; Komuves *et al*, 2000a). The pro-differentiating, anti-proliferative effects of PPAR-α activators also occur in animal models of epidermal hyperproliferation (Komuves *et al*, 2000b). Moreover, topical treatment with PPAR-α activators accelerates permeability barrier recovery following acute barrier disruption (Komuves *et al*, 2000a). Lastly, treatment with PPAR-α activators inhibits cutaneous inflammation in animal models of irritant contact dermatitis (phorbol 12-myristate-13-acetate (TPA) treatment) and allergic contact dermatitis (oxazalone treatment) (Sheu *et al*, 2002). With regard to PPAR-δ, we have recently shown that activation with GW 1514, a highly specific PPAR-δ activator, stimulates differentiation both *in vitro* in cultured human keratinocytes and *in vivo* when applied topically to normal and hyperproliferative mouse skin (Schmuth *et al*, 2004). Other investigators have also shown that PPAR-δ activators stimulate differentiation in cultured keratinocytes (Westergaard *et al*, 2001). Additionally, over-expression of PPAR-δ induced differentiation in cultured keratinocytes and protected against cell death *in vitro* (Tan *et al*, 2001; Di-Poi *et al*, 2002). In contrast to PPAR-α activators, however, PPAR-δ activators neither inhibit keratinocyte proliferation nor stimulate apoptosis (Schmuth *et al*, 2004). Similar to PPAR-α, PPAR-δ activators stimulate permeability barrier homeostasis and inhibit TPA-induced cutaneous inflammation (Schmuth *et al*, 2004). Moreover, PPAR-δ-deficient mice display an increased susceptibility to phorbol ester-induced epidermal hyperplasia, suggesting that PPAR-δ plays an important role *in vivo* in modulating proliferation (Peters *et al*, 2000). Lastly, Michalik *et al* (2001) observed a delay in wound healing in heterozygote PPAR-δ-deficient mice.

PPAR-γ is expressed in the epidermis and its expression increases with differentiation (Rivier *et al*, 1998). The role of PPAR-γ in the epidermis is not well understood. In normal human and psoriatic cultured keratinocytes, PPAR-γ activators are anti-proliferative (Ellis *et al*, 2000). Moreover, in a psoriatic mouse model, PPAR-γ activators reduce epidermal hyperplasia (Ellis *et al*, 2000). Additionally, in one clinical study, patients with chronic, stable plaque psoriasis demonstrated improvement after oral therapy with the PPAR-γ agonist, troglitazone (Ellis *et al*, 2000). Another study, however, did not demonstrate a beneficial effect of topical treatment with PPAR-γ activators in patients with psoriasis (Kuenzli and Saurat, 2003). The purpose of this study was to further characterize the role of PPAR-γ in the epidermis.

Results

Topical treatment with PPAR-γ activators stimulates differentiation Repeated topical application twice per day for 4 d with PPAR-γ agonists resulted in no consistent change in epidermal histology and epidermal thickness was similar in treated and control mice (data not shown). The PPAR-γ activators, however, slightly increased keratinocyte proliferation, as measured by the PCNA method (*supplemental* Fig S1a: ciglitazone: 16.32 ± 0.31 cells per inch, $p < 0.001$; troglitazone: 15.87 ± 0.22 cells per inch, $p < 0.01$; vehicle: 14.81 ± 0.21 cells per inch). This increase in proliferation was balanced by an increase in apoptosis, measured by the Tdt-mediated dUTP nick end labeling (TUNEL) assay (*supplemental* Fig S1b). We next determined whether topical treatment with PPAR-γ activators induce epidermal differentiation. Topical treatment with ciglitazone or troglitazone (10 mM), twice a day for 4 d, resulted in an increase in immunostaining of the differentiation markers, involucrin and loricrin in comparison to controls treated with vehicle alone (*supplemental* Fig S2a). Additionally, using *in situ* hybridization, an increase in filaggrin mRNA was observed with PPAR-γ treatment (*supplemental* Fig S2b). These results demonstrate that topical application of PPAR-γ activators to mouse skin stimulates epidermal proliferation, differentiation, and apoptosis with no net change in epidermal morphology.

Effect of PPAR-γ activators on cultured human keratinocytes To further demonstrate the role of PPAR-γ activation in stimulating keratinocyte differentiation we next determined the effects of ciglitazone *in vitro* on cultured human keratinocytes. As shown in Fig 1, ciglitazone treatment increased mRNA levels of both involucrin and transglutaminase 1 in keratinocytes grown in either low or high calcium. Of note is that the increase in both involucrin and transglutaminase 1 mRNA levels induced by ciglitazone treatment was greater than that observed with high calcium, the standard method for stimulating keratinocyte differentiation. These results confirm the *in vivo* studies in mice and demonstrate that a PPAR-γ activator stimulates human keratinocyte differentiation.

PPAR-γ activators improve permeability barrier homeostasis Because epidermal permeability barrier function is in part dependent on keratinocyte differentiation, we next examined the effect of PPAR-γ activators on barrier homeostasis. Following pre-treatment (twice a day for 4 d) with either ciglitazone or troglitazone, we observed that barrier recovery was increased at 6 h following disruption of the barrier by either tape stripping or acetone treatment (Fig 2). Thus, topical treatment with PPAR-γ activators improves permeability barrier homeostasis, the final product of epidermal differentiation.

PPAR-γ activators suppress cutaneous inflammation We next assessed the anti-inflammatory activity of PPAR-γ agonists. Employing the TPA model of irritant contact dermatitis, treatment with ciglitazone or troglitazone reduced the magnitude of the TPA-induced increase in both ear thickness and ear weight (ciglitazone: 46% and 44%; troglitazone: 69% and 68%, respectively, for ear thickness and weight (Fig 3). Remarkably, at these doses, ciglitazone and troglitazone displayed an anti-inflammatory effect comparable to that observed with the potent glucocorticoid, clobetasol. We next examined hematoxylin–eosin (H&E)-stained sections of the ears from the TPA-treated animals. As seen in *supplemental* Fig S3, vehicle treatment alone following TPA-induced inflammation resulted in a marked increase in ear thickness and an inflammatory infiltrate. Treatment with ciglitazone or troglitazone decreased ear thickness and the number of inflammatory cells compared to vehicle treatment alone. These results demonstrate that PPAR-γ activators are

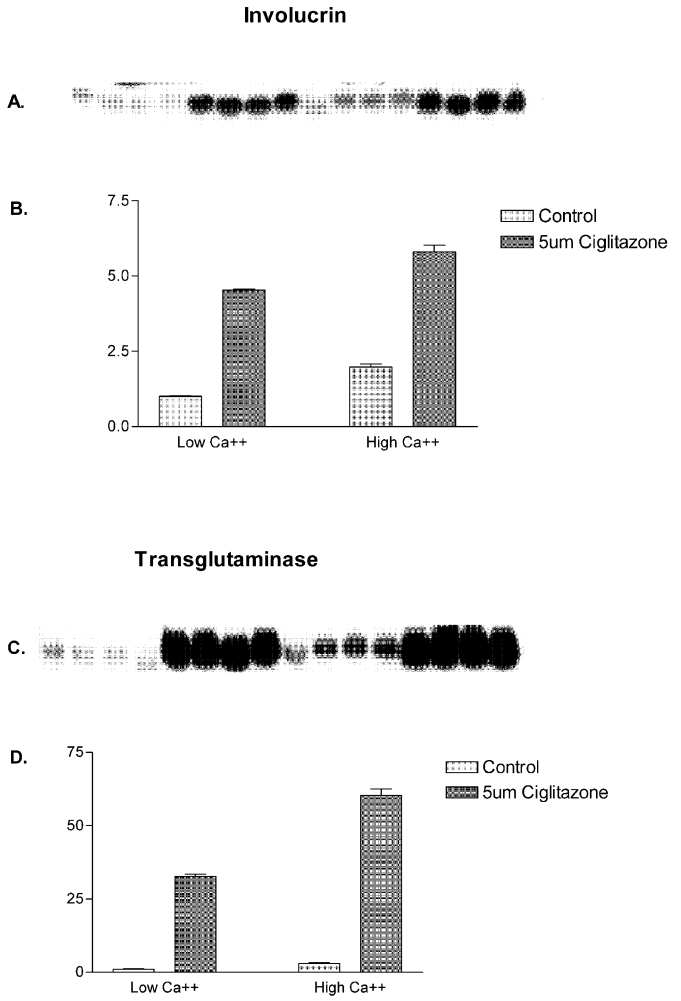

Figure 1
Effect of PPAR gamma activators on involucrin and transglutaminase 1 mRNA levels. Second-passage keratinocytes isolated from human newborn foreskins were cultured in serum-free keratinocyte growth medium Cells were treated with 5 μM ciglitazone at pre-confluence (60%–70%) in either low (0.3 mM) or high (1.2 mM) calcium conditions. Control keratinocytes were treated with vehicle (0.05% ethanol). Cells were harvested after 24 h. Involucrin and transglutaminase 1 mRNA levels were measured by northern blotting as described in the Materials and Methods section. (*A*). Northern blot probed with involucrin. (*B*). Quantitation of involucrin northern blot. (*C*). Northern blot probed with transglutaminase I. (*D*). Quantitation of northern blot probed with transglutaminase I. N = 4 for the groups.

effective in inhibiting cutaneous inflammation in a TPA-induced irritant contact dermatitis model.

We next examined the effects of the PPAR-γ agonists in the oxazolone model of allergic contact dermatitis. As seen in Fig 4, ear thickness and ear weight increased (1.31-fold) following topical oxazolone application to sensitized animals (vehicle controls), whereas topical treatment with either troglitazone or ciglitazone significantly reduced the oxazolone-induced inflammation, as shown by both decreased ear thickness (Fig 4) (31% and 27%, respectively, for troglitazone and ciglitazone) and decreased ear weight (Fig 4) (39% and 27%, respectively, for troglitazone and ciglitazone). Treatment with the potent glucocorticoid, clobetasol, produced a greater anti-inflammatory effect (78% reduction in ear thickness and weight, respectively) than did the PPAR-γ agonists at the concentrations used in this

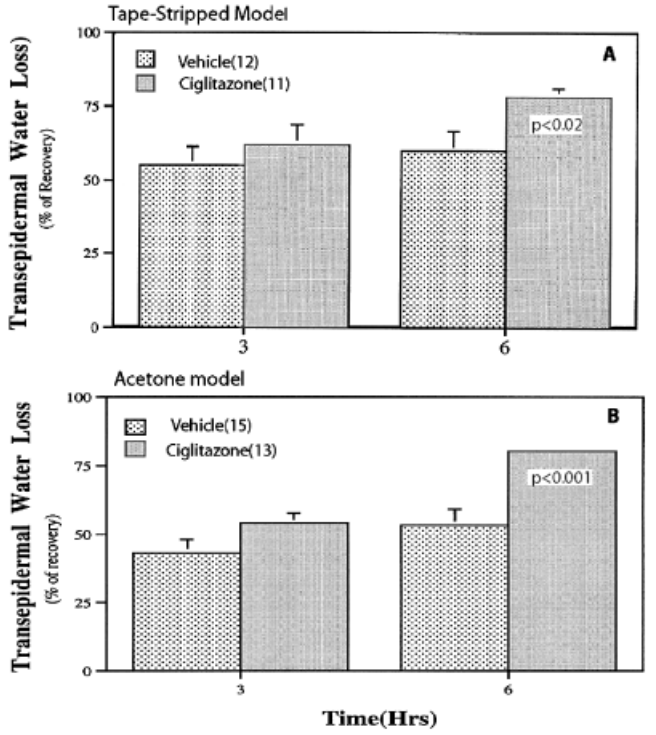

Figure 2
Effect of topical PPAR-γ activators on permeability barrier repair. Hairless mice were treated with vehicle or ciglitazone twice daily for 4 d. Subsequently, the animals were treated by either repeated application of cellophane tape stripping (*A*) or repeated acetone application (*B*) until the TEWL reached 6–8 mg per cm^2 per h. TEWL was measured at 3 and 6 h after barrier disruption. Recovery of barrier function is expressed as a percentage of time zero and data are presented as mean + SEM; N number and significance are shown in the figures.

study. Moreover, H&E-stained sections demonstrated an increase in ear thickness and inflammatory cells in the oxazolone-treated animals treated with vehicle alone, whereas topical treatment with either ciglitazone or troglitazone

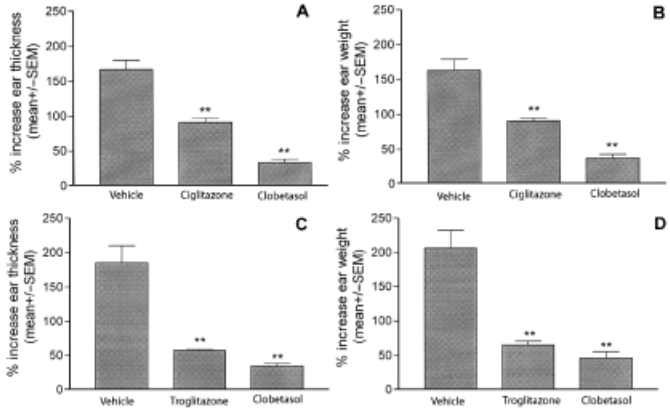

Figure 3
Effect of PPAR-γ activators on cutaneous inflammation as assessed by ear weight and ear thickness in TPA-induced irritant model. Both inner and outer surface of both ears were topically treated with 10 μL of 0.03% phorbol 12-myristate-13-acetate (TPA). Forty-five minutes and 4 h after TPA application, 20 μL of test compounds were topically applied to both inner and outer surface of the left ear, and the right ear was treated with acetone alone. The ear thickness was measured at 18 h following TPA application. The results are expressed as percentage increase in ear thickness (*A*, *C*) or in ear weight (*B*, *D*)

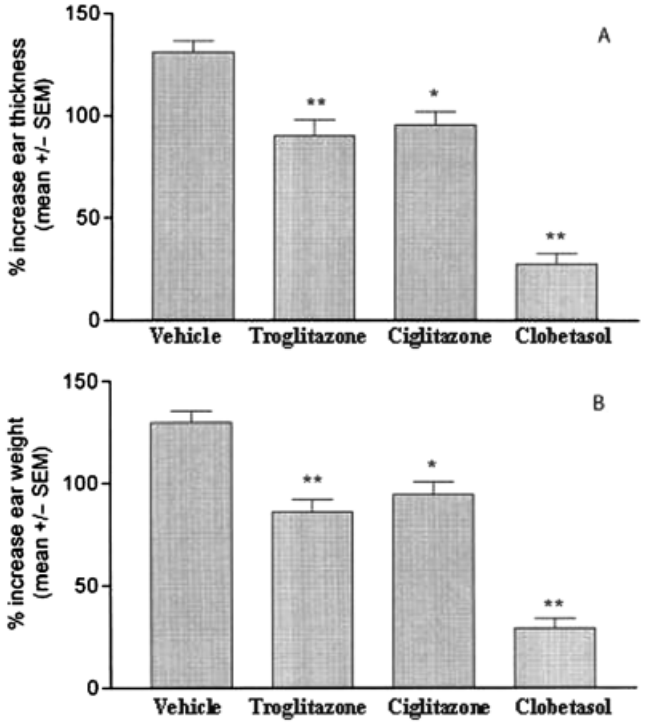

Figure 4
Effect of PPAR-γ activators on cutaneous inflammation as assessed by ear weight and ear thickness in oxazolone-induced allergic model. Both inner and outer surface of both ears were topically treated with 10 μL of 2% (wt/vol in acetone) oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) 1 wk following primary sensitization with 20 μL of 15% oxazolone. Forty-five minutes and 4 h after application of 2% oxazolone, 10 μL of test compounds was topically applied to both inner and outer surface of the left ear, and the right ear was treated with acetone alone. The ear thickness was measured at 18 h following oxazolone application. The results are expressed as percentage increase in ear thickness (*A*) or in ear weight (*B*)

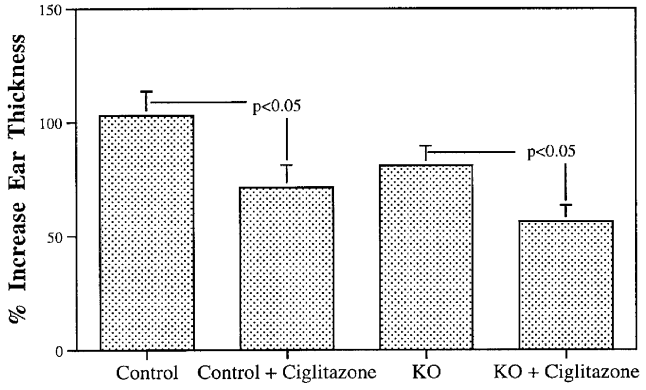

Figure 5
Effect of PPAR-γ activators on cutaneous inflammation in PPAR-γ-deficient mice. Both inner and outer surface of both ears were topically treated with 10 μL of 0.03% phorbol 12-myristate-13-acetate (TPA). Forty-five minutes and 4 h after TPA application, 20 μL of test compounds were topically applied to both inner and outer surface of the left ear, and the right ear was treated with acetone alone. The ear thickness was measured at 18 h following TPA application. The result was expressed as percentage increase (mean ± SEM, n = 20)

reduced both the ear thickness and the amount of inflammatory infiltrate compared to the vehicle-treated animals (*supplemental* Fig S4). Thus, activators of PPAR-γ reduce inflammation in a cutaneous model of allergic contact dermatitis.

PPAR-γ deficiency minimally alters cutaneous structure and function The gross cutaneous appearance of mice deficient in PPAR-γ in the epidermis was normal except for patchy hair loss in older animals (in young animals, <16 wk of age no abnormalities were observed). Next, we examined the skin by light microscopy. H&E-stained sections revealed a modest increase in epidermal thickness in the PPAR-γ-deficient mice (*supplemental* Fig S5a) (0.636 ± 0.0113 *vs.* 0.529 ± 0.0225 μm; $p < 0.05$; $n = 5$). In concordance with the increase in epidermal thickness, PCNA staining was increased in the PPAR-γ-deficient mice, indicating increased keratinocyte proliferation (*supplemental* Fig S5b). Apoptosis, however, as measured by the TUNEL assay, was unchanged in the PPAR-γ-deficient mice (data not shown). Similarly, the expression of differentiation markers was also not altered in PPAR-γ-deficient mice (*supplemental* Fig S6). We next examined several key functions of the epidermis. In PPAR-γ-deficient mice, stratum corneum pH, basal transepidermal water loss (TEWL), and stratum corneum water-holding capacity were all similar to wild-type mice (data not shown). Additionally, permeability barrier recovery following acute disruption by tape stripping was normal in PPAR-γ-deficient skin, as was the cutaneous inflammatory response to TPA treatment (control $103\% \pm 10.5\%$ and PPAR-γ knockout (KO) $81\% \pm 8.6\%$ increase in ear thickness). Lastly, electron microscopy revealed that the number and appearance of lamellar bodies and the lamellar secretory system were similar in PPAR-γ and wild-type mice (data not shown). Thus, except for slight epidermal hyperplasia and patchy hair loss with aging, the structure and function of PPAR-γ-deficient murine skin appears normal.

The role of PPAR-γ in mediating the effects of PPAR-γ activators We next utilized these PPAR-γ-deficient mice to determine the specificity of the effects of the PPAR-γ activators on the epidermis. As shown in *supplemental* Fig S6, loricrin and filaggrin staining were similar in PPAR-γ-deficient and wild-type mice. In the wild-type mice, topical ciglitazone treatment again increased the staining for both loricrin and filaggrin (*supplemental* Fig S6). In contrast, in PPAR-γ-deficient epidermis, topical treatment with ciglitazone did not increase either loricrin or filaggrin-staining, indicating that the stimulation of differentiation induced by PPAR-γ activators requires PPAR-γ. Finally, we determined the effect of PPAR-γ activators on inflammation in the PPAR-γ-deficient mice. As shown in Fig 5, ciglitazone inhibited inflammation in the PPAR-γ-deficient and wild-type skin to a similar degree (control 30% and PPAR-γ KO 31%), indicating that the inhibition of cutaneous inflammation by these PPAR-γ activators does not require PPAR-γ expression in keratinocytes.

Discussion

This study demonstrates that activators of PPAR-γ stimulate epidermal differentiation both *in vitro*, using cultured human keratinocytes, and *in vivo*, when applied topically to mouse skin. That this stimulation of differentiation is

mediated by PPAR-γ is shown by the absence of an effect in mice lacking PPAR-γ in the epidermis. The stimulation of epidermal/keratinocyte differentiation by PPAR-γ activators is very similar to previous observations by this laboratory using activators of PPAR-α, PPAR-δ, and LXR (Hanley *et al*, 1998, 2000; Komuves *et al*, 2000a, 2002; Schmuth *et al*, 2004). Lipid compounds activate PPAR (fatty acids) and LXR (oxidized cholesterol) and hence these nuclear hormone receptors are liposensors that are capable of monitoring intracellular lipid levels and then regulating the expression of a variety of different genes (Chawla *et al*, 2001b). During epidermal differentiation, abundant fatty acids and cholesterol are required for the formation of lamellar bodies in stratum granulosum cells (Proksch *et al*, 1993). Prior to terminal differentiation, these lamellar bodies are secreted into the extracellular spaces of the stratum corneum, where they form the lamellar membranes that mediate the barrier to the movement of water and solutes (Elias and Feingold, 2001). In parallel with the formation of lamellar bodies and lamellar membranes, keratinocytes differentiate into corneocytes which are encased by a cornified envelope consisting of loricrin, involucrin, and other structural proteins that are extensively cross-linked by transglutaminases (Fuchs, 1990; Eckert *et al*, 1997; Elias and Feingold, 2001). The cornified envelope provides the rigidity and mechanical strength to the stratum corneum. Although the formation of the extracellular lipid component of the stratum corneum and the corneocytes are usually considered as separate independent processes that occur simultaneously during keratinocyte differentiation, it is possible that these processes are coordinately regulated. The increase in lipids in stratum granulosum cells could activate PPAR and/or LXR, thereby stimulating the expression of filaggrin, loricrin, involucrin, transglutaminase 1, and perhaps other proteins required for corneocyte formation (Fig 6). Such cross-talk regulation would be advantageous to allow for the coordinate formation of both the corneocytes and extracellular lamellar membranes that comprise the stratum corneum.

Although PPAR-α, PPAR-δ, PPAR-γ, and LXR activators all stimulate keratinocyte differentiation there are differences in the effects of these receptors. Specifically, activation of PPAR-α and LXR inhibits keratinocyte proliferation leading to a decrease in epidermal thickness (Komuves *et al*, 2000a, 2002). In contrast, topical treatment with activators of PPAR-δ and PPAR-γ do not result in epidermal thinning (Schmuth *et al*, 2004 present study). In fact, in the present study activation of PPAR-γ caused a slight increase in PCNA staining indicating increased proliferation. Along similar lines PPAR-α and LXR activators increased apoptosis, whereas PPAR-δ activators had no effect. In the present study, PPAR-γ activators caused a slight increase in apoptosis. Importantly, activation of all the liposensors, PPAR-α, PPAR-δ, PPAR-γ, and LXR, improves permeability barrier homeostasis (Komuves *et al* 2000a, 2002; Schmuth *et al*, 2004). After disruption of the barrier, the ability of the epidermis to restore barrier function to normal is accelerated in animals treated with PPAR or LXR activators. The precise mechanism by which barrier homeostasis is improved is unknown but studies by Rivier *et al* (2000) have shown that PPAR-α activators stimulate the synthesis of the lipids that are required for the formation of lamellar bodies. It is, therefore, possible that activation of PPAR and LXR not only stimulate formation of the cornified envelope and filaggrin but also play a role in regulating the synthesis of the lipids required for the formation of lamellar bodies.

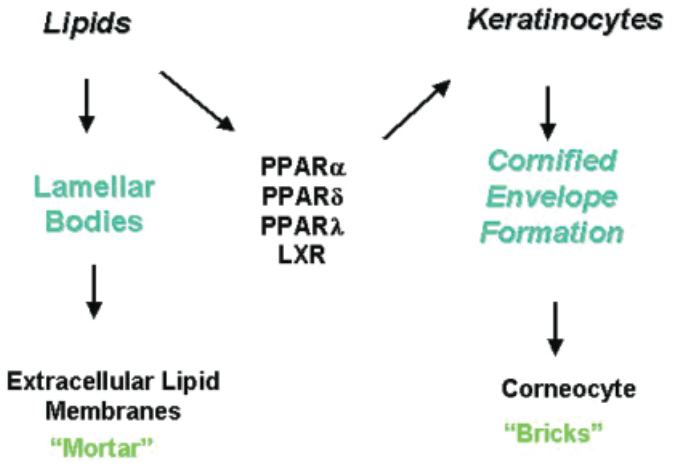

Figure 6
Role of lipids in coordinating SC formation.

Previous studies have shown that PPAR-α, PPAR-δ, and LXR activators inhibit cutaneous inflammation (Sheu *et al*, 2002; Fowler *et al*, 2003; Schmuth *et al*, 2004). In the present study, we demonstrate that PPAR-γ activators also inhibit the inflammation induced in animal models of both irritant and allergic contact dermatitis. The inhibition of inflammation, however, was not only observed in normal mice but was also observed in mice deficient in PPAR-γ in the epidermis demonstrating that this anti-inflammatory effect does not require PPAR-γ expression in the epidermis. In contrast, previous studies have shown that the inhibition of inflammation by PPAR-α and LXR activators did not occur in PPAR-α- or LXR-α/β-deficient mice, respectively, indicating that the anti-inflammatory effects are mediated by PPAR-α and LXR. Studies by other investigators have similarly shown that thiazolidindione PPAR-γ activators, similar to those used in the present study, have anti-inflammatory effects on macrophages independent of PPAR-γ (Chawla *et al*, 2001a). Thus, although the stimulation of differentiation by PPAR-γ activators is mediated by PPAR-γ, the inhibition of inflammation does not require PPAR-γ expression in the epidermis. Nevertheless, these thiazolidindione drugs, which are widely used in the treatment of Type 2 diabetes, maybe therapeutically useful either topically or orally in the treatment of inflammatory skin disorders. It has been reported that psoriasis is improved in diabetic patients being treated with troglitazone (Ellis *et al*, 2000).

Animals that are deficient in PPAR-γ die *in utero* (Barek *et al*, 1999). Therefore, in the present study, we employed animals that had a deficiency of PPAR-γ localized to the epidermis. Despite the absence of PPAR-γ in their epidermis, the gross cutaneous appearance of these animals was normal except for patchy hair loss in older animals. Similarly, the morphological appearance using both light and electron microscopy was normal except for a slight increase in epidermal thickness and an increase in PCNA staining. It may seem paradoxical that deficiency of PPAR-γ and activation of PPAR-γ both lead to increased PCNA staining in

the epidermis but such paradoxical responses are often seen with nuclear hormone receptors. For example, previous studies by our laboratory have shown that LXR deficiency and LXR stimulation both reduce epidermal thickness and PCNA staining (Komuves *et al*, 2002). Similarly, LXR activation increases cholesterol 7-α hydroxylase activity in the liver, whereas LXR deficiency also induces an increase in the activity of this enzyme in liver (Peet *et al*, 1998). It has been hypothesized that the basis for these seemingly paradoxical observations is that although activation of a nuclear hormone receptor will stimulate gene expression, the nuclear hormone receptor alone will inhibit gene expression. Thus, deficiency in the nuclear hormone receptor could result in an increase in gene expression. Finally, we did not observe any functional abnormalities in the skin of animals deficient in PPAR-γ in the epidermis. Specifically, stratum corneum pH, stratum corneum water-holding capacity, basal TEWL, and the ability to recover barrier function after acute disruption were not altered in PPAR-γ-deficient mice. These observations of a paucity of abnormalities in animals deficient in PPAR-γ in the epidermis is very similar to our previous observations in PPAR-α- and LXR-α/β-deficient animals in which we also observed minimal cutaneous alterations (Komuves *et al*, 2000a, 2002). It is likely that the effects of these receptors on the epidermis are redundant such that the absence of any single receptor has only minimal effects.

In summary, in the present study we demonstrate that thiazolidindiones stimulate keratinocyte differentiation by activating PPAR-γ, and decrease cutaneous inflammation, a response that is not dependent on PPAR-γ in keratinocytes. These observations raise the possibility that thiazolidindiones and perhaps other PPAR-γ activators will be useful in the treatment of a variety of cutaneous disorders.

Materials and Methods

All animal procedures were approved by the Animal Studies Subcommittee of the San Francisco Veterans Administration Medical Center and were performed in accordance with their guidelines.

The Committee on Human Research at the University of California, San Francisco, California, approved studies with human keratinocytes.

Animals—production of PPAR gamma KO mice Mice harboring LoxP-flanked PPAR-γ alleles (PPAR-$\gamma^{L2/L2}$) that will be described in detail elsewhere, were bred with K14-Cre transgenic mice, in which the Cre recombinase is expressed under the control of the human keratin 14 (K14) promoter (Li *et al*, 2000). The resulting K14-Cre $^{(tg/0)}$/PPAR-$\gamma^{L2/L2}$ mice in which PPAR-γ is selectively disrupted in epidermal keratinocytes, and age- and sex-matched PPAR-$\gamma^{L2/L2}$ control littermates were analyzed.

***In vivo* studies** In order to study the effects of the PPAR-γ activators on differentiation, normal hairless mice (males, age 6–8 wk from Charles River, Wilmington, Massachusetts) or PPAR-γ keratinocyte selective KO mice and wild-type controls were treated on one side of the shaved flank (2.5 cm^2) with 40 μL of 10 mM ciglitazone (Cayman Chemical, Ann Arbor, Michigan), 10 mM troglitazone (BioMol, Plymouth Meeting, Pennsylvania), or with vehicle (propylene glycol: ethanol 7:3 ratio) twice a day for 4 d. Biopsies were obtained and tissue samples were fixed overnight at 4°C in freshly prepared 4% formaldehyde in phosphate-buffered saline (PBS), and embedded in paraffin for immunohistochemical analysis and *in situ* hybridization.

Epidermal proliferation Epidermal thickness was determined on H&E-stained sections using a 40x objective and a computer-generated micrometer (Carl Zeiss Vision, Munich, Germany). Epidermal thickness was defined as the distance between the basement lamina and the stratum granulosum–stratum corneum interface. For the detection of proliferating keratinocytes, paraffin sections (5 μm) were processed and stained using a biotinylated anti-proliferating cell nuclear antigen (CalTag Laboratories, Burlingame, California) as described previously (Komuves *et al*, 2000a, 2002). The proliferating pool of keratinocytes was determined by quantitating PCNA positive nuclei per inch of basement membrane.

Epidermal apoptosis The TUNEL assay was performed using the *in situ* cell death detection kit (Roche Molecular Diagnostics, Indianapolis, Indiana) according to the manufacturer's instructions using FITC-labeled dUTP.

Immunohistochemistry for epidermal differentiation markers In order to assess epidermal differentiation, paraffin-embedded sections (flank) (5 μm) were incubated overnight with affinity-purified rabbit antibodies, specific for involucrin (1:1000), filaggrin (1:2000), and loricrin (1:1000) (Babco, Berkeley, California) as described previously (Komuves *et al*, 2000a, 2002).

***In situ* hybridization for profilaggrin** Digoxigenin-labeled RNA probes for profilaggrin (coding region, 300 bases) (Yuspa *et al*, 1989) were made from linearized cDNA sequences (a gift from S. Yuspa, NIH) using reagents supplied by Boehringer-Mannheim (Indianapolis, Indiana). *In situ* hybridization was performed as described earlier (Komuves *et al*, 1998).

Microscopy and Imaging The paraffin sections (H&E, differentiation markers, PCNA, TUNEL) were examined with a Zeiss (Axioplan 2) microscope (Jena, Germany) using brightfield optics. Digital images were captured with AxioVision software 2.05 (Carl Zeiss Vision, Munich, Germany).

Electron microscopy Skin biopsies were taken and samples were minced to $<$0.5 mm3, fixed in Karnovsky's fixative overnight, and post-fixed with either 0.5% ruthenium tetroxide or 2% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide, and analyzed by electron microscopy as described previously (Hou *et al*, 1991).

Assessment of epidermal function Basal cutaneous permeability barrier function was determined by measuring TEWL with an electronic water analyzer (MEECO, Warrington, Pennsylvania). The kinetics of barrier recovery were then determined after acute disruption by sequential applications of cellophane tape (Shurtape, Shurtape Technologies, Hickory, North Carolina) or acetone treatment (TEWL $\geqslant$ 6–8 mg per cm^2 per h) at 3 and 6 h post-disruption, as described previously (Komuves *et al*, 2000a, b). Surface pH was measured with a flat glass electrode (Mettler-Toledo, Giessen, Germany), using a pH meter (skin pH Meter PH 900, Courage and Khazaka, Cologne, Germany). Stratum corneum hydration was measured with the capacitance-based Corneometer CM 825 (Courage and Khazaka), with values reported in arbitrary units.

Anti-inflammatory models and studies Adult CD-1 male and female mice, 6–10 wk of age, purchased from Charles River Labs (Wilmington, Massachusetts), or PPAR-γ KO mice and wild-type controls were used in these studies. TPA (Sigma, St Louis, Missouri)-induced irritant contact dermatitis was instituted by the topical application of 10 μL 0.03% (wt/vol in acetone) TPA to both the inner and outer surface (20 μL total) of both ears. Forty-five minutes and 4 h after TPA application, 20 μL of test compounds, ciglitazone (10 mM) (Cayman Chemical, Ann Arbor, Michigan) or troglitazone (10 mM) (BioMol, Plymouth Meeting, Pennsylvania) were applied to both surfaces of the left ear and the right ear was treated with

acetone vehicle alone (40 μL total per ear). Identical treatments were performed with 20 μL of 0.05% clobetasol (1.1 mM), a topical anti-inflammatory glucocorticoid, which served as a positive control. All chemical compounds were dissolved in absolute acetone (reagent grade) vehicle.

Allergic contact dermatitis was induced by sensitization (for 2 d) on the shaved backs of CD-1 female mice with 20 μL of 15% (wt/vol in acetone) oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one), followed by challenge on day 7 with a single topical application of 10 μL oxazolone (2%) to the inner and outer surfaces of both ears. This challenge was followed by treatment with ciglitazone (10 mM), troglitazone (10 mM), clobetasol (0.05%), or acetone at 45 min and 4 h.

Eighteen hours after the inflammatory insult induced by either TPA or oxazolone, inflammation was assessed as the percent increase in ear thickness and/or ear weight in the treated left ear *versus* the vehicle-treated right ear. Ear thickness was measured with a digital caliper (Mitutoyo, Tokyo, Japan), followed by a 6 mm-punch biopsy to ascertain changes in ear weights.

After samples were obtained for assessment of ear thickness/weight, biopsies were obtained from adjacent sites and fixed in 4% freshly prepared formaldehyde in PBS and stained with H&E.

Cell culture, cell proliferation, and mRNA measurements Second-passage keratinocytes isolated from human newborn foreskins were cultured in serum-free keratinocyte growth medium (KGM; Clonetics, San Diego, California). Cells were treated with 5 μM ciglitazone at pre-confluence (60%–70%) in either low (0.3 mM) or high (1.2 mM) calcium conditions. Control keratinocytes were treated with vehicle (0.05% ethanol). Cells were harvested after 24 h. Total RNA was isolated using TriReagent (Sigma), electrophoresed, transferred to a nylon membrane (NytranN, Schleicher & Schuell, Dassel, Germany) and hybridized overnight at 65°C with ^{32}P-labeled cDNA probes for involucrin (gift from Dr Howard Green, Harvard University) or transglutaminase 1 (a gift from Dr Robert Rice). To control for equal loading blots were probed with GAPDH. Bands of the reported size were quantified by densitometry using QuantityOne Software (BioRad, Hercules, California).

Statistical analyses All statistical analyses were performed using Prism 3 software (Graph Pad Software, San Diego, California). Results were compared between multiple groups, using ANOVA, and expressed as mean ± SEM. When results between pairs were analyzed, the Student's *t*-test was used.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23235/JID23235sm.htm

Figure S1

Effects of PPAR-γ activators on epidermal proliferation and apoptosis. Skin of mice was topically treated twice daily for 4 d with vehicle (propylene glycol: ethanol, 7:3, vol/vol) or with ciglitazone or troglitazone. Epidermal proliferation was measured by PCNA-staining (*A*) and apoptosis was assessed by TUNEL staining (*B*).

Figure S2

Effect of topical PPAR-γ activators on epidermal differentiation. Skin of mice was topically treated twice daily for 4 d with vehicle (propylene glycol: ethanol, 7:3, vol/vol) or with the PPAR-γ activators, ciglitazone or troglitazone. Involucrin and loricrin expression were assessed by immunohistochemistry (*A*). Filaggrin expression was assessed by *in situ* hybridization (*B*).

Figure S3

Effect of PPAR-γ activators on cutaneous morphology in the TPA model of irritant contact dermatitis. Both inner and outer surface of both ears were topically treated with 10 μL of 0.03% phorbol 12-myristate-13-acetate (TPA). Forty-five minutes and 4 h after TPA application, 20 μL of test compounds were topically applied to both inner and outer surface of the left ear and the right ear was treated with acetone alone. The ear thickness was measured at 18 h following TPA application. Epidermal morphology was assessed by H&E staining.

Figure S4

Effect of PPAR-γ activators on cutaneous morphology in the oxazolone model of allergic contact dermatitis. Both inner and outer surface of both ears were topically treated with 10 μL of 2% (wt/vol in acetone) oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) 1 wk following primary sensitization with 20 μL of 15% oxazolone. Forty-five minutes and 4 h after application of 2% oxazolone, 10 μL of test compounds was topically applied to both inner and outer surface of the left ear, and the right ear was treated with acetone alone. The ear thickness was measured at 18 h following oxazolone application. Epidermal morphology was assessed by H&E staining.

Figure S5

Morphological changes in PPARγ-deficient mice. Mouse skin from both wilditype and PPAR-γ KO were obtained for paraffin sections. Both H&E staining (*A*) and PCNA staining (*B*) were used to analyze the morphological changes in PPAR-γ KO and wild-type mice.

Figure S6

Effect of topical PPAR-γ activators on epidermal differentiation in PPAR-γ-deficient mice. Both wild-type and PPAR-γ KO animals were topically treated with 40 μL of 10 mM ciglitazone in acetone for 4 d. A separate group of wild-type and PPAR-γ KO animals were treated with vehicle alone for 4 d and served as controls. Skin biopsy was obtained for paraffin sections. Both filaggrin (*A*) and loricrin (*B*) staining were used to analyze epidermal differentiation.

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