Deficiency of PPARβ/δ in the Epidermis Results in Defective Cutaneous Permeability Barrier Homeostasis and Increased Inflammation

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In cultured human keratinocytes or murine epidermis, peroxisome proliferator-activated receptor β/δ (PPARβ/δ) (NR1C2) activators (1) stimulate keratinocyte differentiation; (2) decrease keratinocyte proliferation; (3) accelerate permeability barrier repair; (4) increase epidermal lipid synthesis; and (5) reduce cutaneous inflammation. Since these results suggest that PPARβ/δ could play an important role in cutaneous homeostasis, we assessed here the skin phenotype of mice deficient in PPARβ/δ. Gross cutaneous abnormalities were not evident, and both stratum corneum (SC) skin hydration and surface pH were normal. However, the epidermis was thickened and proliferating cell nuclear antigen (PCNA) staining was increased, indicating increased cell proliferation. No change in apoptosis was observed but the expression of differentiation markers, such as filaggrin, involucrin, and loricrin, was slightly increased in PPAR$\beta/\delta^{-/-}$ mice. Although basal permeability barrier function was normal, PPARβ/δ knockout (KO) mice show a significant delay in barrier recovery rates following acute barrier disruption by either acetone treatment or tape-stripping. Delayed barrier recovery correlated with decreased production and secretion of lamellar bodies (LBs), and with reduced numbers of extracellular lamellar membranes in the SC. Finally, PPARβ/δ KO mice displayed increased inflammation in response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment. Together, these results further demonstrate that PPARβ/δ in the epidermis: (1) is required for permeability barrier homeostasis; (2) regulates keratinocyte proliferation; and (3) modulates cutaneous inflammation.

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INTRODUCTION

There are three peroxisome proliferator-activated receptor (PPAR) isoforms, α (NR1C1), β/δ (NR1C2), and γ (NR1C3). These receptors heterodimerize with RXR and are activated by fatty acids, prostaglandins, eicosanoids, and other lipid metabolites; and hence, may function as ''liposensors'' (Mangelsdorf and Evans, 1995; Kersten *et al.*, 2000; Chawla *et al.*, 2001). Braissant and Wahli, using *in situ* hybridization, reported that both PPARα and PPARβ/δ but not PPARγ are expressed in the epidermis during fetal development in rats, whereas in adult rats, these investigator did not observe the expression of PPARs in epidermis (Braissant *et al.*, 1996; Braissant and Wahli, 1998). However, both Rivier *et al.* (1998) and Westergaard *et al.* (2003) found that all three PPAR isoforms were expressed in human epidermis. Based on the studies of Westergaard *et al.* (2003) it appears that PPARβ/δ is the most abundantly expressed PPAR in human epidermis. Moreover, PPAR β/δ expression increases in hyperproliferative conditions such as psoriasis, following 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment, and with inflammatory stimuli such as cytokines, lipopolysaccharide and UV light (Michalik *et al.*, 2001; Tan *et al.*, 2001; Westergaard *et al.*, 2003; Schmuth *et al.*, 2004).

Studies by our group and others have shown that activation of PPAR α, β/δ, or γ has major effects on epidermal/keratinocyte function. First, PPARα, β/δ, or γ ligand treatment of cultured human keratinocytes and/or topical application of PPAR ligands to murine skin results in the increased expression of differentiation related proteins, such as involucrin, loricrin, profilaggrin, and transglutaminase 1 (Hanley *et al.*, 1998; Komuves *et al.*, 1998, 2000a; Westergaard *et al.*, 2001; Man *et al.*, 2004; Schmuth *et al.*, 2004). Second, activation of PPARα, β/δ, or γ inhibits keratinocyte proliferation *in vivo* and/or *in vitro* models (Ellis et al., 2000; Komuves *et al.*, 2000b; Demerjian *et al.*, 2006;

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Abbreviations: KO, knockout; LB, lamellar body; LOR, loricrin; PCNA, proliferating cell nuclear antigen; PPAR, peroxisome proliferator-activated receptor; SC, stratum corneum; SG, stratum granulosum; TPA, 12-O-tetradecanoylphorbol-13-acetate

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Kim *et al.*, 2006). Third, PPARα, *β/δ*, or *γ* ligands are anti-inflammatory, decreasing the inflammation evoked in response to TPA treatment, a model of irritant contact dermatitis (Sheu *et al.*, 2002; Man *et al.*, 2004; Schmuth *et al.*, 2004). Finally, topical treatment of murine skin with PPAR α, or *β/δ* and to a lesser extent *γ* ligands improves permeability barrier homeostasis, resulting in an acceleration of barrier recovery following acute disruption (Komuves *et al.*, 2000a; Man *et al.*, 2004; Schmuth *et al.*, 2004). Associated with the PPAR ligand-induced improvement of permeability barrier homeostasis is an increase in (a) epidermal cholesterol, fatty acid, and sphingolipid synthesis, (b) lamellar body (LB) number and secretion, and (c) *β*-glucocerebrosidase activity, all of which could contribute to the enhanced barrier homeostasis (Man *et al.*, 2006).

Despite the major effects of PPAR ligands on epidermal/keratinocyte structure and function, animals deficient in PPARs display few cutaneous changes. Specifically, in PPARα knockout (KO) mice there are no changes in skin physiology or general morphology, but a very modest decrease in the epidermal expression of differentiation markers, such as profilaggrin and loricrin (Lee *et al.*, 1995; Komuves *et al.*, 2000a; Schmuth *et al.*, 2002). Additionally, a thinner stratum granulosum (SG) layer was observed with a decrease in keratohyalin granules. Of note, electron microscopy revealed no abnormalities in the LB secretory system, which is consistent with the normal permeability barrier function in PPARα KO mice (Komuves *et al.*, 2000a). Similarly, mice with a localized KO of PPAR*γ* in the epidermis exhibit few changes in cutaneous structure and function (Man *et al.*, 2004). A modest increase in epidermal thickness occurs in PPAR*γ*-deficient mice, which is associated with increased proliferating cell nuclear antigen (PCNA) staining indicating keratinocyte hyperproliferation. Apoptosis, the expression of keratinocyte differentiation markers, and the LB secretory system, evaluated by electron microscopy, were unchanged in the PPAR gamma epidermal KO mice. Finally, stratum corneum (SC) pH, SC water content, basal permeability barrier function, barrier recovery following acute disruption, and the cutaneous inflammatory responses were all normal in these PPAR*γ*-deficient mice.

PPAR$\beta/\delta^{-/-}$ mice are difficult to obtain due to frequent gestational lethality from placental defects, but independent lines of PPAR-*β/δ*-targeted mice have nevertheless been generated (Peters *et al.*, 2000; Barak *et al.*, 2002). Previous studies to assess genetic loss of PPAR*β/δ* function in skin utilized PPAR-$\beta^{-/-}$ mice as described by Peters and co-workers. Notably, however, these PPAR-*β/δ*-targeted animals transcribe residual PPAR-*β/δ* RNA encoding a 60-amino-acid C-terminus deletion of the ligand-binding domain (Peters *et al.*, 2000). Whether this genetic disruption retains some residual PPAR *β/δ* activity, or could function in a dominant-negative manner, remains unsettled. Nevertheless, these animals are smaller, and display an increased susceptibility to phorbol ester-induced epidermal hyperplasia and reduced adipose stores (Peters *et al.*, 2000) and a delayed wound healing response (Michalik *et al.*, 2001). Epidermal differentiation is not altered in these animals.

Although a more complete knockout of PPAR *β/δ* also causes reduction of adipose stores and earlier embryonic lethality (Barak *et al.*, 2002), cutaneous function has not yet been carefully examined in these animals. The purpose of this study was to determine if there are any changes in skin physiology, ultrastructure, epidermal lipid synthesis, as well as cutaneous inflammatory response in animals with a complete deficiency of PPAR *β/δ* due to genetic disruption of the receptor's DNA-binding domain.

RESULTS

Epidermal structure and function in PPAR*δ* KO mice

The skin of PPAR*β/δ KO* mice appeared grossly normal. Additionally, there were no alterations in basal skin physiology such as surface pH, water holding capacity, or permeability barrier function in PPAR*β/δ* KO mice (Table 1). However, increased epidermal thickness was seen in the PPAR*β/δ* KO epidermis (Figure 1). Increased epidermal thickness, in turn, was associated with an increase in epidermal PCNA immunostaining (Figure 1; Figure S1). Additionally, in the PPAR*β/δ* KO mice, there also was increased PCNA-positive staining in the dermis, localized to hair follicles. The increase in keratinocyte proliferation in the epidermis of PPAR*β/δ* KO mice was further demonstrated by increased staining of proliferation makers k14 and P63 in the basal layer (Figure S2). In contrast, there was no change in the level of apoptosis, as detected by TUNEL staining in PPAR*β/δ* KO mice (Figure S3). Finally, expression of keratinocyte differentiation markers, particularly involucrin and loricrin, was moderately increased in PPAR*β/δ* KO mice in comparison to wild type mice (Figure S4). In addition, the mRNA levels of loricrin were increased 225±32% in PPAR*β/δ* KO compared to wild type mice. The mRNA levels of PPAR α and PPAR *γ* were not changed in PPAR *β/δ* KO epidermis. PPAR*β/δ* mRNA was present in wild type mice but as expected was not seen in PPAR*β/δ* KO mice.

Increased skin inflammatory response in PPAR*δ* KO mice

To determine the role of PPAR*β/δ* in regulating cutaneous inflammation, we next measured changes in ear thickness in KO *versus* wild type mice following TPA treatment.

Table 1. Basal skin physiology in PPAR *β/δ* KO and wild-type mice

Groups	Surface pH	Skin capacitance (arbitrary unit)	TEWL (mg/cm^2/hour)
Wild type (*n*=5)	5.02±0.07	56.20±1.70	0.18±0.03
PPAR *β/δ* KO (*n*=5)	5.17±0.05	57.30±1.29	0.16±0.03
Significance	NS	NS	NS

LOR, loricrin; KO, knockout; NS, not significant; PPAR, peroxisome proliferator-activated receptor; TEWL, transepidermal water loss.
The animals were 6–8 weeks old. Measurement was obtained from both flanks of wild-type (*n*=5) and PPAR*β/δ* KO (*n*=5) mice, one reading per flank. The unit for capacitance is arbitrary unit, and for TEWL is mg/cm^2/hour. The results are presented as mean±SEM.

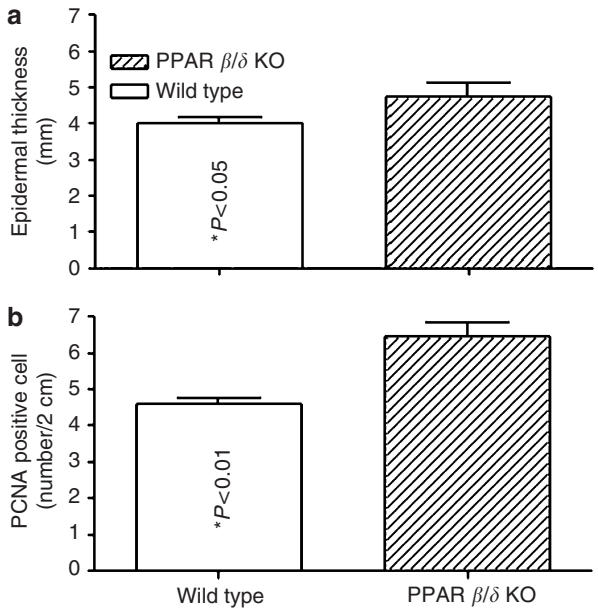

Figure 1. Increased epidermal thickness accompanied by increased PCNA staining is observed in PPAR*β/δ* KO mice. Epidermal thickness of the nucleated cell layer was measured in 100 × hematoxylin & eosin-treated sections. The measurement was taken at every 2 cm points along the epidermis. The data are presented as the mean of all measured points (**a**, $n=31$ for both groups). The number of PCNA-positive cells was counted on every 2 cm segment along the epidermis (**b**, $n=11$ for both groups). The data are presented as the mean of all segments counted ± SEM.

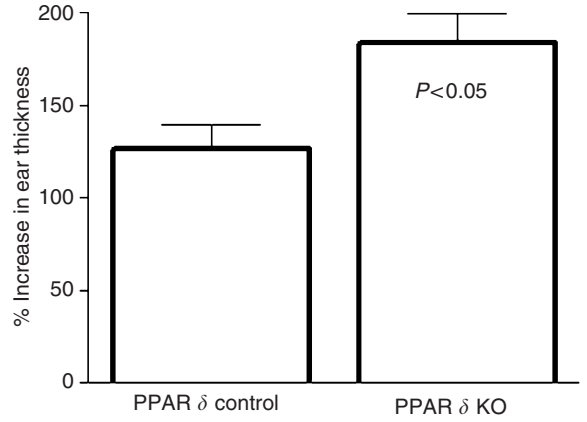

Figure 2. Increased inflammatory response to TPA treatment in PPAR*β/δ* KO mice. A 10 μl volume of 0.03%TPA in ethanol was applied to the inner and outer surfaces of the right ears, and the left ears were treated with ethanol alone. Ear thickness was measured before and 18 hours after TPA or ethanol applications. Ethanol alone did not alter ear thickness. Data are expressed as mean ± SEM ($n=11$ for each group).

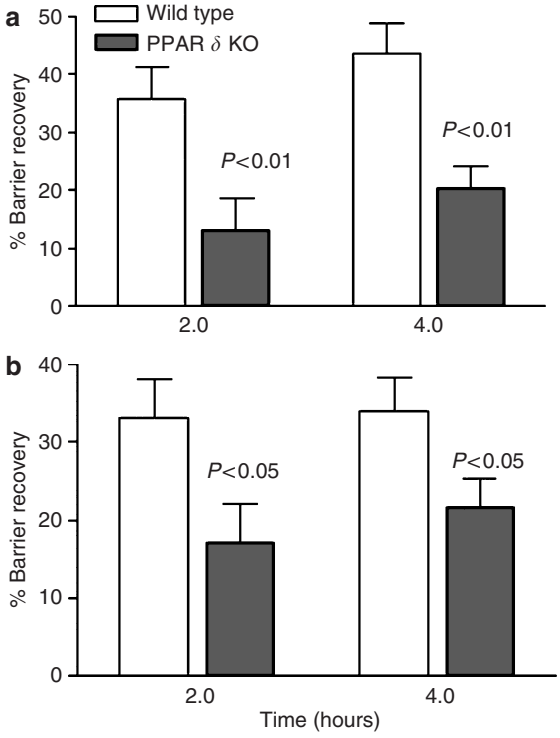

Figure 3. Permeability barrier recovery is delayed in PPAR*β/δ* KO mice. Permeability barrier was disrupted with either (**a**) tape-stripping (**b**) or acetone. Transepidermal water loss was measured 2 and 4 hours after barrier disruption. A significant delay in barrier recovery was observed at both 2 and 4 hours in both models in PPAR*β/δ* KO animals. Data are expressed as percent of barrier recovery and mean ± SEM ($n=9$–12 for wild type, $n=10$–11 for KO).

As expected, ethanol (vehicle for TPA) treatment did not increase ear thickness. However, TPA treatment resulted in a greater increase in ear thickness in PPAR*β/δ* KO as compared to wild-type mice (Figure 2). This result, coupled with previous studies (Peters *et al.*, 2000; Schmuth *et al.*, 2004), provides further evidence that PPAR*β/δ* regulates cutaneous inflammation.

PPAR*δ* KO mice exhibit delayed permeability barrier recovery after acute injury

To determine the role of PPAR*β/δ* in permeability barrier homeostasis, we next measured barrier recovery following acute disruption induced by either acetone treatment or repeated tape-stripping. As shown in Figure 3, a significant delay in barrier recovery at both 2 and 4 hours post-disruption was seen in PPAR*β/δ* KO mice following either tape-stripping or acetone treatment. These results demonstrate the importance of PPAR*β/δ* in regulating epidermal permeability barrier homeostasis.

Epidermal lipid synthesis rate in PPAR*β/δ* KO epidermis

We next asked whether PPAR*β/δ* regulates epidermal lipid synthesis. Cutaneous permeability barrier recovery following acute disruption requires augmented production of lipids for the formation of additional LBs. Epidermal lipid synthesis provides these lipids, and inhibition of epidermal cholesterol, fatty acids or sphingolipid synthesis delays barrier recovery (Feingold *et al.*, 1990; Holleran *et al.*, 1991; Mao-Qiang *et al.*, 1993). The synthesis rates of cholesterol, fatty acids, and ceramides was comparable in PPAR*β/δ* KO and wild-type mouse epidermis under basal conditions (Figure 4). In addition, no decrease in epidermal lipid synthesis in PPAR*β/δ*

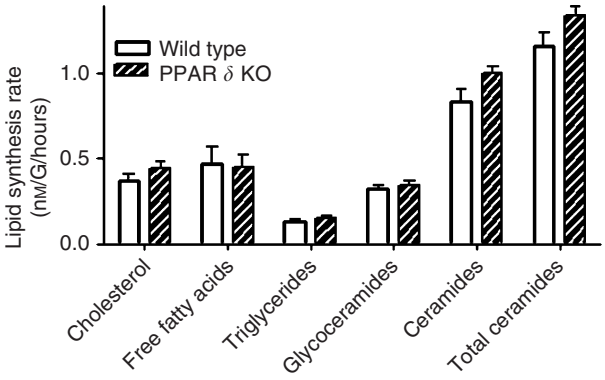

Figure 4. Epidermal PPAR β/δ KO does not alter epidermal lipid synthesis. Full-thickness skin was incubated with 40 μCi acetate for 2 hours at 37°C, and the incorporation of ^{14}C acetate into lipids was determined as described in Materials and Methods. The total ceramides includes both ceramides and glycoceramides. Data are expressed as nM/g wet epidermal weight/hour) and are mean ± SEM ($n = 5$ for each group).

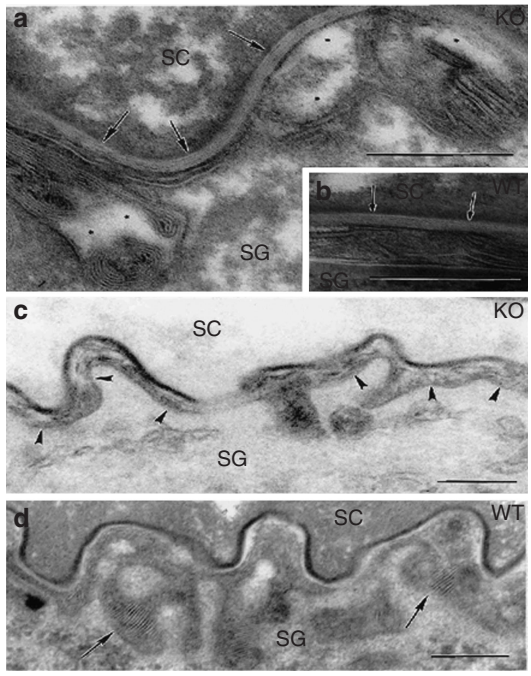

Figure 5. Secretion of LB contents is decreased, but post-secretory lipid processing is normal in PPARβ/δ KO epidermis. Skin biopsies from 6- to 8-week-old wild-type and PPARβ/δ KO mice were fixed in Karnovsky's fixative overnight, and post-fixed with 0.25% ruthenium tetroxide or 1% aqueous osmium tetroxide. Ultrathin sections were visualized with an electron microscope. Post-secretory processing of secreted LB contents in lamellar membranes (**a** and **b**, arrows) begins at the SG–SC interface in both PPARβ/δ KO and wild-type (wt) mice. Decreased quantities of secreted LB contents are apparent in (**c**) KO (arrowheads) *versus* (**d**) wt mice (arrows). (**a, b**) ruthenium tetroxide post-fixation; (**c, d**) osmium tetroxide post-fixation. Bar = 0.2 μm.

KO mice was observed even at 2 hours following tape-stripping (data not shown). These results suggest that the defective barrier homeostasis in PPARβ/δ KO mice is not due to the changes in epidermal lipid synthesis, because lack of PPARβ/δ does not significantly affect the rate of lipid synthesis in the epidermis.

Ultrastructural basis for defective barrier homeostasis in PPARβ/δ KO mice

Following barrier disruption, the events required for barrier restoration include not only epidermal lipid synthesis, but also accelerated secretion of preformed LBs, new LB formation, and post-secretory processing of secreted lipid, which ultimately leads to membrane bilayer maturation (Menon *et al.*, 1992; Holleran *et al.*, 1993). Since changes in lipid synthesis were not observed in PPARβ/δ KO mice, we next examined whether there were changes in LB formation, secretion, and/or lipid processing. Ultrastructurally there was no evidence of a lamellar membrane processing abnormality in PPARβ/δ KO mice (Figure 5a *vs* b). Additionally, *in situ* zymography demonstrated that the activity of β-glucocerebrosidase, a key enzyme required for lamellar membrane processing, was similar in PPARβ/δ KO compared to wild-type mice (Figure S5). In contrast, a significant decrease in LB density was observed in PPARβ/δ KO mice (Figure 6). In addition, the secreted material at the SG/SC (SG/SC) interface was reduced in PPARβ/δ KO *versus* wild-type mice (Figure 5c and d). Thus, changes in either LB formation or secretion could account for the delay in recovery of permeability barrier function in PPARβ/δ KO mice.

DISCUSSION

In contrast to our previous studies of PPARα- and PPARγ-deficient mice, in which we observed minimal changes in epidermal structure and function (Komuves *et al.*, 2000a; Schmuth *et al.*, 2002; Man *et al.*, 2004), in this study we observe that mice devoid of PPARβ/δ display a number of cutaneous abnormalities, including (1) increased epidermal thickness due to increased keratinocyte proliferation; (2) increased inflammatory response to TPA treatment, a model of irritant contact dermatitis; and (3) delayed permeability barrier repair following acute barrier disruption due to decreased LB formation and secretion. Studies have suggested that PPARβ/δ is the most abundant of the PPARs in the epidermis and with inflammation the levels of this receptor increase while the levels of the other PPARs decrease (Braissant and Wahli, 1998; Peters *et al.*, 2000; Kippenberger et al., 2001; Schmuth *et al.*, 2004). For example, in epidermis from TPA-treated mouse skin or patients with psoriasis the expression of PPARβ/δ is increased (Rivier *et al.*, 1998; Westergaard *et al.*, 2003; Kim *et al.*, 2006). Additionally, treatment of cultured keratinocytes with cytokines, lipopolysaccharide, or UV light increase PPARβ/δ and decrease the other PPARs (Michalik *et al.*, 2001; Tan *et al.*, 2001; Schmuth *et al.*, 2004). This increased abundance and upregulation of PPARβ/δ in response to inflammation and injury may contribute to alterations in epidermal structure and function in mice deficient in PPARβ/δ. In PPARα- and PPARγ-deficient mice it is possible that the presence of the other PPARs could in part compensate for the absence of the receptor, whereas in PPARβ/δ-deficient mice, such redundancy cannot totally compensate, resulting in cutaneous abnormalities.

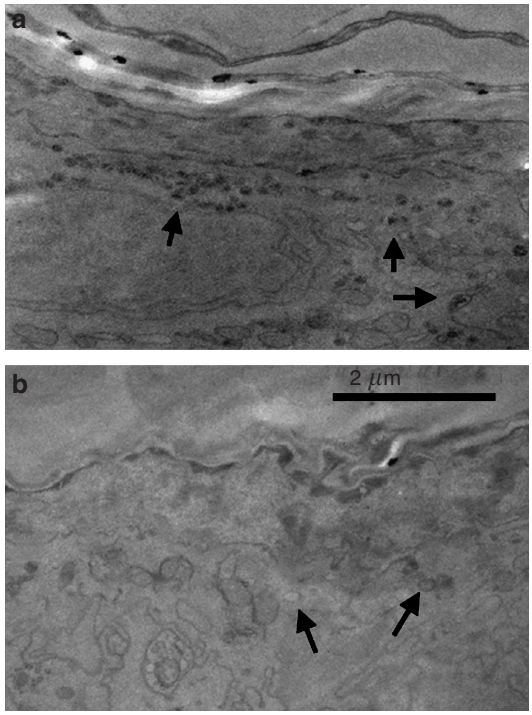

Figure 6. The number of LB is decreased in PPAR*β/δ* KO epidermis. Skin samples from both wild-type and PPAR*β/δ* KO mice were fixed with osmium tetroxide as described in Materials and Methods. (**a**) There is abundant LB in the SG in wild-type epidermis (arrows). (**b**) In contrast, there are only a few partially filled LB found in PPAR*β/δ* KO epidermis (arrows). Bar = 2 μm.

The increase in epidermal thickness and keratinocyte proliferation is consistent with previous observations (Tan *et al.*, 2001; Kim *et al.*, 2006). Other investigators have shown that treatment of cultured keratinocytes with PPAR*β/δ* activators inhibits keratinocyte proliferation (Kim *et al.*, 2005, 2006), and thus it is not surprising that the absence of PPAR*β/δ* in the epidermis results in an increase in epidermal thickness. The increase in epidermal thickness in PPAR*β/δ*-deficient mice is due to increased keratinocyte proliferation, as shown by the increased PCNA staining, and the absence of alterations in apoptosis by TUNEL staining. However, others have observed no difference in epidermal proliferation between PPAR*β/δ*-deficient and wild-type mice in postnatal day 1–4 animals (Di-Poi *et al.*, 2005). This difference in results between adult and neonatal animals may reflect the different roles of PPAR*β/δ* during different developmental stages. Of note is that while treatment of either mouse skin or cultured keratinocytes with PPAR*β/δ* activators stimulates the expression of key genes required for keratinocyte differentiation, including loricrin, involucrin, filaggrin, and transglutaminase 1 (Westergaard *et al.*, 2001; Schmuth *et al.*, 2004), the expression of these differentiation related proteins was not greatly altered in mice deficient in PPAR*β/δ* in the epidermis. Moreover, it has been shown that in cultured keratinocytes derived from PPAR*β/δ*-deficient mice, the expression of differentiation markers following treatment with either high calcium or TPA increased similar to what is observed in wild-type keratinocytes (Kim *et al.*, 2006). In this study, we paradoxically noted a slight increase in the expression of involucrin and loricrin, which may have been secondary to the increase in epidermal thickness. However, Kim *et al.* (2006) noted a decrease in cornified envelope formation in PPAR*β/δ*-deficient mice. Taken together, these observations indicate that while PPAR*β/δ* plays a key role in regulating keratinocyte proliferation, the effects of PPAR*β/δ* on differentiation are not crucial, and the absence of PPAR*β/δ* in the epidermis can be compensated for by other regulatory pathways.

The increase in cutaneous inflammation in response to TPA is also consistent with previous studies (Peters *et al.*, 2000; Michalik *et al.*, 2001). First, as noted above, inflammation increases PPAR*β/δ* expression in keratinocytes, while decreasing the expression of other PPARs (Rivier *et al.*, 1998; Tan *et al.*, 2001; Schmuth *et al.*, 2004). Second, previous studies have shown that PPAR*β/δ* activators reduce cutaneous inflammation (Schmuth *et al.*, 2004; Kim *et al.*, 2006). Finally, studies on PPAR*β/δ*-deficient mice have shown increased epidermal thickness in response to TPA treatment (Peters *et al.*, 2000). In this study, we show that mice with complete deficiency of PPAR*β/δ* have an increase in ear thickness following TPA treatment compared with wild-type mice, indicative of an enhanced inflammatory response. Thus, PPAR*β/δ* plays a key role in regulating the response of the skin to inflammatory stimuli.

In our previous studies, we have shown that topical treatment with PPAR*β/δ* activators for three days before acute barrier disruption accelerated permeability barrier recovery (Schmuth *et al.*, 2004). This acceleration in barrier recovery was associated with an increased number of LBs in SG cells, which resulted in the increased secretion of lamellar material following barrier disruption (Man *et al.*, 2006). The mechanism accounting for the increase in LB number with PPAR*β/δ* treatment is unknown, but we did observe an increase in epidermal lipid synthesis, which is required to provide the structural lipid components required for LB formation. Additionally, the activity of *β*-glucocerebrosidase, a key enzyme required for the extracellular processing of glucosylceramides to ceramides, is increased in PPAR*β/δ* ligand-treated animals (Man *et al.*, 2006). This enzyme is incorporated into LBs for secretion into the extracellular spaces.

The final key observation from this study is that deficiency of PPAR*β/δ* in the epidermis results in a delay in permeability barrier recovery following acute disruption by either repeated tape-stripping or acetone treatment. This delay in repair was associated with a decreased number of LBs in SG cells, which resulted in the decreased secretion of LBs following barrier disruption, an essential step in the recovery of permeability barrier function. The decrease in lamellar membranes in the extracellular spaces in PPAR*β/δ*-deficient mice is likely due to the decrease in LBs, and could account for the delay in permeability barrier repair. However, epidermal cholesterol, fatty acid, and ceramide synthesis in the PPAR*β/δ*-deficient mice was similar to wild-type mice, indicating that a deficiency in bulk lipids was not the etiology for the decrease in LB formation in the SG cells. It is of course possible that

specific lipid subclasses that are required for LB formation are deficient in PPARβ/δ KO mice. Alternatively, structural proteins required for LB formation or transporters required for the entry of lipid into LBs could be limiting in PPARβ/δ-deficient mice. Recent studies have shown that ABCA12 is required for LB formation (Lefevre *et al.*, 2003; Hovnanian, 2005; Akiyama, 2006). However, in preliminary studies, mRNA levels of ABCA12 were not decreased in PPARβ/δ-deficient mice. Thus, at this time, the alterations that account for the decrease in LBs in PPARβ/δ-deficient mice are unknown. Nevertheless, a decrease in LBs would adversely affect permeability barrier homeostasis. The delay in repairing permeability barrier function following acute injury is consistent with studies that have shown delays in wound healing in PPARβ/δ-deficient mice (Michalik *et al.*, 2001).

Since the animals that we studied are deficient in PPARβ/δ in all tissues, we can not be certain whether the changes in epidermal structure and function are due to the absence of PPARβ/δ in the epidermis or other tissues. It is worth noting that studies by Matsuura *et al.* (1999) have shown, using *in situ* hybridization, that PPARβ/δ is expressed in the suprabasal layers of human epidermis, and it is therefore possible that the effects of PPARβ/δ on LB formation and permeability barrier function are direct effects.

In conclusion, this study demonstrates that PPARβ/δ plays important roles in the epidermis and is essential in regulating keratinocyte proliferation, the cutaneous response to inflammation, and permeability barrier homeostasis.

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.

Materials

Generation of PPARβ/δ-deficient animal. The mice used in this study were generated using a similar strategy as described previously (Barak *et al.*, 2002), differing only in that the wild-type exon 4 was replaced with a β-galactosidase gene, rather than a deletion, upstream of the PPARδ DNA-binding domain. Homozygous lacZ knock-in mice (PPARδ$^{ki/ki}$) are backcrossed four generations into the SV129 inbred strain. These mice lack almost the entire PPARδ gene product, including the DNA-binding domain. Male wild-type and PPARδ KO mice, 6–8 weeks of age, were used in this study. Animals were maintained on mouse diet (Ralston-Purina Co., St Louis, MO) and water *ad lib.*

Chemicals. Acetone was purchased from Fisher Scientific (Fairlane, NJ). TPA was purchased from Sigma (St Louis, MO). ^{14}C-labeled acetate (56 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (St Louis, MO).

Experimental protocols and functional studies

All measurements were made on the flanks of the mice 24 hours after shaving. Basal cutaneous permeability barrier function was determined by measuring transepidermal water loss with an electronic water analyzer (MEECO, Warrington, PA). The raw data for transepidermal water loss were in ppm/0.5 cm^2/hour and were converted to mg/cm^2/hour. The kinetics of barrier recovery were determined after acute barrier disruption either by sequential applications of cellophane tape (Scotch tape, 3 m) or repeated topical applications of acetone (transepidermal water loss ⩾ 2–8 mg/cm^2/hour) at 2 and 4 hours post-disruption, as described previously (Man *et al.*, 1997a, b). SC hydration was measured with the capacitance-based Corneometer CM 825 (Courage&Khazaka, Cologne, Germany), with values reported in arbitrary units (Man *et al.*, 1997a). Surface pH was measured with a flat, glass surface electrode from Mettler-Toledo (Giessen, Germany), attached to a pH meter (Skin pH Meter PH 900, Courage & Khazaka, Cologne, Germany).

Epidermal lipid synthesis

Skin samples were incubated for 2 hours at 37°C in 2 ml of 10 mM EDTA in PBS–calcium and magnesium free, containing 40 μCi ^{14}C-labeled acetate, as described previously (Mao-Qiang *et al.*, 1993). After stopping the reaction by placing on ice, the epidermis was separated from the dermis. The incorporation rates of [^{14}C-labeled acetate into lipids in epidermis were determined following Bligh/Dyer extraction, and thin-layer chromatography, as described previously (Holleran *et al.*, 1991; Mao-Qiang *et al.*, 1993). The sphingolipid components were separated by high-performance thin-layer chromatography developed in the following solvent mixtures: (1) chloroform/methanol/water (40:10:1, v/v) for 2 and 5 cm; (2) chloroform/methanol/acetic acid (47:2:05, v/v) for 8.5 cm; and (3) *N*-hexane/diethylether/acetic acid (30:15:0.5, v/v) for 9 cm. The plates for neutral lipids were developed in the following solvent systems: (1) Benzene/hexane(1:1, v/v) for 8 cm and (2) hexane/ether/acetic acid(70/30/1) for 5 cm. The corresponding lipid bands were collected and counted with a scintillation counter. The incorporation rates of ^{14}C-labeled acetate into lipids were expressed as nM/g wet epidermal weight/hour.

Immunohistochemistry

The methods for assessment of both the differentiation markers and proliferation were carried out as published previously (Komuves *et al.*, 1998, 2000b). Briefly, after deparaffinization and blocking with 4% BSA, 5 μm paraffin sections were incubated with primary rabbit anti-mouse antibodies (Covance/BabCo., Berkely, CA) at dilutions of 1:2,000 for filaggrin, 1:1,000 for involucrin, and 1:500 for loricrin, for overnight at 4°C. After washing with 10 mM citrate buffer, sections were incubated with goat anti-rabbit antibody (1:400) for 30 minutes at room temperature, followed by ABC-peroxidase (Vector, Burlingame, CA) reaction. For PCNA staining, sections were incubated with biotinylated monoclonal anti-PCNA antibody (CalTag Laboratories, Burlingame, CA) for 2 hours at room temperature. For anti-K14 and anti-P63 stainings, methods described by Mikaelian and Bilal were followed (Bilal *et al.*, 2003; Mikaelian *et al.*, 2006). The sections were visualized with a Zeiss (Axioplan 2) microscope (Jena, Germany). Digital images were captured with AxioVision software 2.05 (Carl Zeiss Vision, Munich, Germany).

Keratinocyte apoptosis

TUNEL assay kit (Roche Molecular Diagnostics, Indianapolis, IN) was used to assess keratinocyte apoptosis, according to the manufacturer's instruction. Briefly, after deparaffinization, tissue sections were incubated with proteinase K (20 μg/ml) in 10 mM Tris-HCl for 30 minutes at 37°C. Then the sections were incubated with

Table 2. The mouse primer sequence for real-time PCR

Gene	Primer sequence
LOR	(F) 5′-GTGGAAAGACCTCTGGTGGA-3′
	(R) 5′-TGGAACCACCTCCATAGGAA-3′
PPARα	(F) 5′-CCTGAACATCGAGTGTCGAATAT-3′
	(R) 5′-GTTCTTCTTCTGAATCTTGCAGCT-3′
PPARδ	(F) 5′-CCACGAGTTCTTGCGAAGTC-3′
	(R) 5′-AACTTGGGCTCAATGATGTCA-3′
PPARγ	(F) 5′-CCACCAACTTCGGAATCA-3′
	(R) 5′-TTTGTGGATCCGGCAGTTA-3′
36B4	(F) 5′-GCGACCTGGAAGTCCAACTAC-3′
	(R) 5′-ATCTGCTGCATCTGCTTGG-3′

LOR, loricrin; PPAR, peroxisome proliferator-activated receptor.

TUNEL mix for 60 minutes at 37°C in dark. Sections were examined with a microscope as described above.

Real-time PCR

Real-time PCR was carried out as described previously (Jiang *et al.*, 2006). Briefly, following RNA isolation, cDNA was synthesized to measure the relative mRNA levels of target genes. The primer sequences for PCR are listed in Table 2. A mixture of individual PCR reaction contains cDNA, forward or reverse primers and 2 × SYBR Green Q-PCR Master Mix (BIO-RAD, Hercules, CA). The PCR reaction was performed at 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of amplification of melting at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds, respectively. The PCR reaction was performed in duplicate, with 4∼5 samples in each group ($n = 4 \sim 5$). Gel electrophoresis and melting curve analyses were performed to confirm accurate PCR product sizes and absence of nonspecific bands. The expression levels of each gene were normalized against 36B4 (an invariant transcript) using the comparative C_T method, and expressed as percentage of control, with the control as 100%.

Inflammatory response

A 10 μl volume of 0.03% TPA in ethanol was applied to the inner and outer surfaces of the right ears, and the left ears were treated with ethanol alone. Ear thickness was measured with a digital micrometer (Mitutuyo, Japan) before and 18 hours after TPA or ethanol applications (Sheu *et al.*, 2002). Data are expressed as percentage increase in ear thickness.

Electron microscopy

Skin biopsies of wild-type and PPARδ KO mice were fixed in Karnovsky's fixative overnight, and post-fixed with either 0.25%. ruthenium tetroxide or 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide, as described previously (Hou *et al.*, 1991). Ultrathin sections were examined using an electron microscope (Zeiss 10A, Carl Zeiss, Thornwood, NY) operated at 60 kV. LB density and secretion were assessed visually in randomly photographed, coded micrographs by Peter M. Elias, without knowledge of the experimental group.

Statistical analyses

All statistical analyses were performed using the two-tailed Student's *t*-test. Data were expressed as mean ± SEM.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Epidermal proliferation is increased in PPARβ/δ KO mice.

Figure S2. Both K14 and P63 staining are increased in PPARβ/δ KO mice.

Figure S3. No change in the level of apoptosis is detected in PPARβ/δ KO mice.

Figure S4. Expression of differentiation markers is slightly increased in PPARβ/δ KO mice.

Figure S5. Epidermal β-glucocerebrosidase activity in PPARβ/δ KO mice is similar to that in wild type.

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