Peroxisome Proliferator-Activated Receptor (PPAR)-β/δ Stimulates Differentiation and Lipid Accumulation in Keratinocytes

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Peroxisome proliferator-activated receptor (PPAR) are nuclear hormone receptors that are activated by endogenous lipid metabolites. Previous studies have demonstrated that PPAR-α activation stimulates keratinocyte differentiation *in vitro* and *in vivo*, is anti-inflammatory, and improves barrier homeostasis. Recent studies have shown that PPAR-β/δ activation induces keratinocyte differentiation *in vitro*. This study demonstrated that topical treatment of mice with a selective PPAR-β/δ agonist (GW501516) *in vivo* had pro-differentiating effects, was anti-inflammatory, improved barrier homeostasis, and stimulated differentiation in a disease model of epidermal hyperproliferation. In contrast to PPAR-α activation, PPAR-β/δ *in vivo* did not display anti-proliferative or pro-apoptotic effects. The pro-differentiating effects persisted in mice lacking PPAR-α, but were decreased in mice deficient in retinoid X receptor-α, the major heterodimerization partner of PPAR. Furthermore, *in vitro* PPAR-β/δ activation, aside from stimulating differentiation-related genes, additionally induced adipose differentiation-related protein (ADRP) and fasting induced adipose factor (FIAF) mRNA in cultures keratinocytes, which was paralleled by increased oil red O staining indicative of lipid accumulation, the bulk of which were triglycerides (TG). Comparison of differentially expressed genes between PPAR-β/δ and PPAR-α activation revealed distinct profiles. Together, these studies indicate that PPAR-β/δ activation stimulates keratinocyte differentiation, is anti-inflammatory, improves barrier homeostasis, and stimulates TG accumulation in keratinocytes.

Key words: barrier function/fasting/lipid/nuclear hormone receptor/stratum corneum
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The peroxisome proliferator-activated receptors (PPAR) belong to a subset of the nuclear hormone receptor (NHR) superfamily (Class II receptors), which heterodimerize with the retinoid X receptor (RXR) (Mangelsdorf *et al*, 1995; Kersten *et al*, 2000; Chawla *et al*, 2001) Several members of this subset of NHR have been shown to modulate cutaneous homeostasis. For example, thyroid receptor (TR), retinoid acid receptor (RAR), and vitamin D receptor (VDR) are well-known regulators of epidermal growth and differentiation (Pillai *et al*, 1991; Blumenberg *et al*, 1992; Torma *et al*, 1993; Fisher *et al*, 1996), and activators of the latter two receptors are currently employed to treat a variety of skin diseases (Fritsch *et al*, 1992; Kragballe *et al*, 1992; Orfanos *et al*, 1997; Saurat *et al*, 1999). Further support for the importance of class II NHR in cutaneous homeostasis derives from a mouse model with spatially and temporally controlled deficiency of RXR-α, the major heterodimerization partner for class II NHR. These mice display abnormal skin development, epidermal hyperplasia, cutaneous inflammation, and alopecia (Li *et al*, 2000, 2001). Therefore, substantial evidence supports the importance of class II NHR as critical regulators of cutaneous homeostasis.

A number of NHR were first described before their ligands had been identified, which coined the term "orphan" receptors. After ligand identification, it became clear that many ligands of class II "orphan" NHR are lipid metabolites and therefore they are "liposensors". Medium- to long-chain fatty acids, eicosanoids, and isoprenoids derived from cholesterol synthesis, such as farnesol, are activators of the PPAR (Schoonjans *et al*, 1996; Forman *et al*, 1997; Hanley *et al*, 2000). Previous studies have demonstrated that the epidermis is an active site of cholesterol and fatty acid synthesis (Feingold, 1991; Hurt *et al*, 1995), raising the possibility that endogenous lipids produced in keratinocytes might activate PPAR and thereby regulate gene expression. In support of this hypothesis, results from our laboratory

Abbreviations: ADRP, adipose differentiation-related protein; CE, cornified envelope; FIAF, fasting induced adipose factor; HPTLC, high-performance thin layer chromatography; IL, interleukin; LPS, lipopolysaccharide; NHR, nuclear hormone receptor; PCNA, anti-proliferating cell nuclear antigen; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; TEWL, transepidermal water loss; TG, triglycerides; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

have demonstrated that activation of PPAR-α by fatty acids, isoprenoids, and drugs accelerates fetal skin development, induces differentiation in keratinocytes, inhibits inflammation, and improves permeability barrier function (Hanley *et al*, 2000; Komuves *et al*, 2000a, b). Importantly, these effects did not occur in PPAR-α−/− mice (Hanley *et al*, 2000), and fetal skin development was delayed in PPAR-α−/− mice (Schmuth *et al*, 2002), demonstrating that these effects are receptor mediated.

PPAR-β/δ is expressed ubiquitously (Braissant *et al*, 1996, 1998). A drug that activates PPAR-β/δ (GW501516) has recently been shown to markedly increase serum HDL levels whereas lowering serum triglyceride (TG) levels in obese rhesus monkeys (Oliver *et al*, 2001). The increase in serum HDL is thought to be due to the increased expression of ABCA1, a transporter that is key in the efflux of cholesterol out of cells to circulating HDL (Oliver *et al*, 2001). Furthermore, some but not all studies have suggested a role for PPAR-β/δ in regulating the differentiation of adipocytes (Bastie *et al*, 2000; Barak *et al*, 2002), and PPAR-β/δ is a potent inhibitor of ligand-induced transcriptional activity of PPAR-α (Barak *et al*, 2002). Pro-differentiating effects have been reported recently in cultured human keratinocytes treated with PPAR-β/δ agonists, such as tetradecylthioacetic acid and L165041 (Westergaard *et al*, 2001). Additionally, over-expression of PPAR-β/δ also induced differentiation in cultured keratinocytes (Tan *et al*, 2001) and protected against cell death *in vitro* (Tan *et al*, 2001; Di-Poi *et al*, 2002). But little is known about the role of PPAR-β/δ in cutaneous homeostasis, and the effects of PPAR-β/δ activators on the epidermis *in vivo*. PPAR-β/δ−/− mice are difficult to obtain due to gestational lethality (Peters *et al*, 2000; Michalik *et al*, 2001; Barak *et al*, 2002), and surviving animals have residual PPAR-β/δ RNA transcripts (Peters *et al*, 2000). Nevertheless, despite partial expression, these animals display an increased susceptibility to phorbol ester induced epidermal hyperplasia (Peters *et al*, 2000), and a delayed wound healing response, whereas epidermal differentiation and hair cycling are normal (Michalik *et al*, 2001).

The purpose of this study was to evaluate the role of exogenous PPAR-β/δ activation on cutaneous homeostasis and to delineate, if present, differences to PPAR-α activation. First, we determined the effects of topical PPAR-β/δ activation on proliferation, differentiation, epidermal cell death, and permeability barrier function in normal skin. Second, we tested the effects of PPAR-β/δ activation in disease models of cutaneous hyperproliferation and inflammation. Third, we identified other genes and functions that are regulated by PPAR-β/δ activation in cultured keratinocytes. The results indicated both similarities and differences in the effects of PPAR-β/δ activation to those exerted by PPAR-α activation on the epidermis.

Results

Effects of topical PPAR-β/δ activators on normal murine epidermis Previous studies by our laboratory demonstrated that topical PPAR-α activators inhibit keratinocyte proliferation and stimulate epidermal cell death and keratinocyte differentiation in murine epidermis (Hanley *et al*, 2000; Komuves *et al*, 2000). We therefore first assessed the effects of PPAR-β/δ activators on normal mouse skin *in vivo*. After 6 d of topical application of GW501516, epidermal thickness appeared slightly increased on light microscopy of hematoxylin and eosin stained paraffin sections (Fig 1*a*) but no significant increase in epidermal thickness was detectable when the distance between the basement lamina and the lower stratum corneum (SC) was quantitated (Fig 1*b*). While the pool of proliferating cells transiently increased after 3 d of treatment, it was unchanged after 6 d (Fig 1*c*), whereas epidermal cell death detected by TUNEL staining was unchanged (Fig 1*h*). These results contrast with the known anti-proliferative and pro-apoptotic effects of topical PPAR-α (Komuves *et al*, 2000).

Next, we examined the effects of GW1514 on epidermal differentiation. Increased expression of filaggrin and loricrin protein was detected by immunohistochemistry in mouse skin topically treated with GW1514 for 6 d (Fig 1*d*). Treatment with GW1514 for shorter periods of time also increased mRNA levels of filaggrin, a keratinocyte differentiation marker, with increases observed after 3 d of treatment (Fig 1*e*). Thus, similar to PPAR-α activators, PPAR-β/δ activators stimulate keratinocyte differentiation.

Because many putative PPAR-specific activators promiscuously activate other PPAR, we next assessed whether the effects of topical GW1514 on differentiation were due to activation of PPAR-α. Similar to wild-type animals, the PPAR-β/δ activator, GW1514, increased expression of loricrin and filaggrin in the epidermis of PPAR-α−/− animals (Fig 1*f*). This observation demonstrates that GW1514-induced stimulation of keratinocyte differentiation *in vivo* is not mediated by activation of PPAR-α.

Since PPAR-β/δ null mice were not available for study, we next assessed the effects of PPAR-β/δ activation in mice with a keratinocyte-targeted disruption of RXR-α, the common heterodimerization partner of all PPAR, including PPAR-β/δ. In comparison to wild-type and PPAR-α−/− mice, the GW1514-mediated increase in filaggrin and loricrin expression was blunted in RXR-α deficient mice (Fig 1*g*). Additionally, the increase in differentiation markers induced by PPAR-α activators also declined in RXR-α deficient mice in comparison to control mice (not shown). Together, these results indicate that both PPAR-α and PPAR-β/δ activators utilize RXR-α to stimulate keratinocyte differentiation. Moreover, the ability of PPAR-β/δ activators to stimulate differentiation in PPAR-α deficient mice, but not in RXR-α deficient mice suggests that the stimulation of differentiation induced by GW1514 is mediated by PPAR-β/δ, and not by activation of PPAR-α.

PPAR-β/δ activation accelerates epidermal permeability barrier repair The SC, as the outermost layer of the epidermis, is the end product of keratinocyte differentiation and mediates epidermal permeability barrier function (Jackson *et al*, 1993). To test the potential functional relevance of the effects of GW1514 on epidermal differentiation we next determined the effect of topical treatment with PPAR-β/δ activators on the ability to restore barrier homeostasis after barrier perturbation. After 3 d of topical application of GW1514, we experimentally disrupted

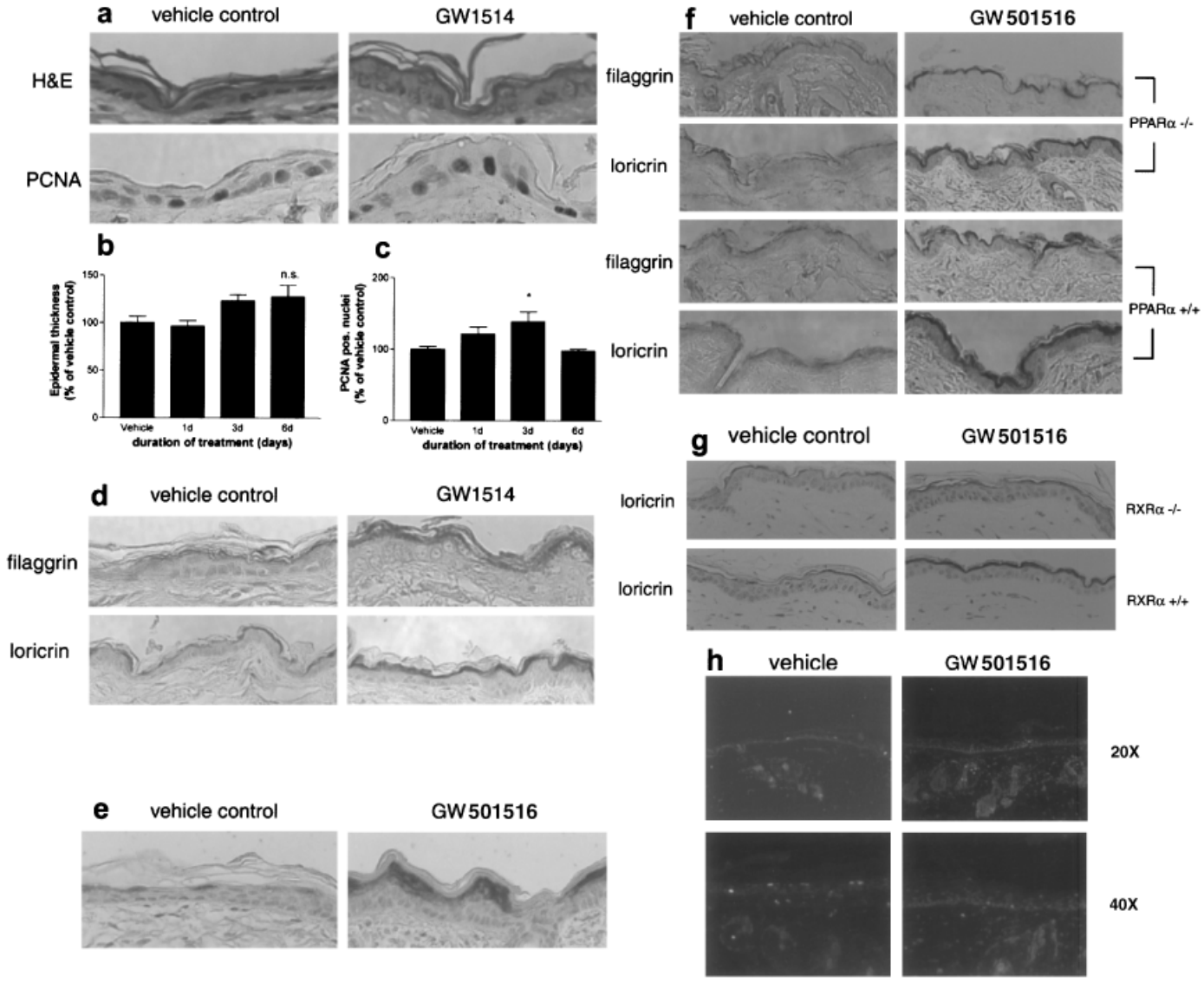


Figure 1
Effects of a peroxisome proliferator-activated receptor (PPAR)-β/δ agonist on epidermal morphology and differentiation. Skin of mice was topically treated twice daily for 1–6 d with vehicle (acetone) or with GW1514. (*a*) Hairless mouse skin (wild-type), hematoxylin and eosin staining (upper row) and anti-proliferating cell nuclear antigen (PCNA) staining (second row) at day 6. (*b*) Measurements of epidermal thickness. (*c*) Quantification of PCNA positive nuclei data are presented as mean ± SEM (n = 3–4), $^{*}p < 0.05$. (*d*) Filaggrin and loricrin protein expression assessed by immunohistochemistry after topical application of GW1514 at day 6. (*e*) Filaggrin expression assessed by *in situ* hybridization after topical application of GW1514 at day 3. (*f*) PPAR-α−/− mice and wild-type, filaggrin and loricrin expression was assessed by immunohistochemistry at day 3. (*g*) Retinoid X receptor (RXR)-α−/− mice and wild-type mice, loricrin expression was assessed by immunohistochemistry at day 3. (*h*) Hairless mouse skin (wild-type) at day 3 of topical treatment, TUNEL staining, scale bar 1 cm = 50 μm × 20, upper row; 1 cm = 25 μm × 40, lower row.

permeability barrier function either mechanically (tape stripping), by solvent extraction (repeated topical acetone), or by detergent treatment (repeated topical SDS), and measured the recovery of TEWL over time. Regardless of the method of barrier perturbation, barrier recovery was accelerated (Fig 2), indicating a beneficial effect on permeability barrier homeostasis.

PPAR-β/δ activation stimulates differentiation in a model of epidermal hyperproliferation In view of the beneficial effect of GW1514 on barrier repair, we next asked whether the cutaneous effects of GW1514 would translate into a potential therapeutic benefit using an animal model of skin disease. As seen in previous studies (Denda *et al*, 1996), repeated disruption of the permeability barrier of hairless mice by twice-daily topical acetone applications over 3 d induced epidermal hyperplasia and impaired differentiation. Topical treatment with GW1514 also stimulated an increase in the expression of the differentiation markers, loricrin and filaggrin (Fig 1*a*) in the hyperproliferative epidermis of those mice. But again and in contrast to PPAR-α activators, the PPAR-β/δ activator did not alter epidermal proliferation or epidermal thickness (Fig 3*a–c*). Thus, as in normal mouse skin, activation of PPAR-β/δ stimulates differentiation without affecting proliferation in this model.

Increased PPAR-β/δ expression in keratinocytes treated with cytokines and UV-B irradiation Previous studies reported decreased PPAR-α expression in UV-exposed epidermis and in skin from patients with psoriasis (Rivier

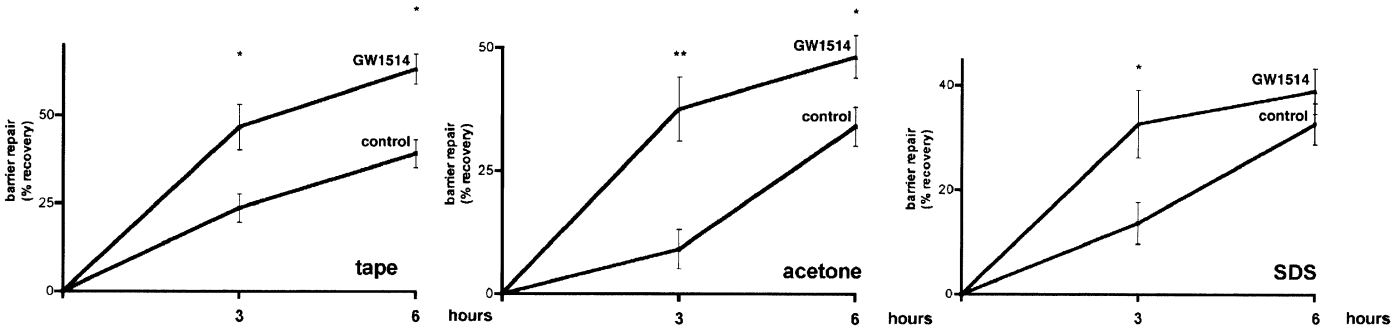


Figure 2
Effect of topical peroxisome proliferator-activated receptor-β/δ activation on permeability barrier repair. Hairless mice were treated with vehicle or GW1514 twice daily for 3 d. Subsequently, the animals were treated by either repeated application of cellophane tape stripping, repeated acetone application, or by repeated sodium dodecyl sulfate (SDS) treatment until the transepidermal water loss (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 = 12), $^*p < 0.05$, $^{**}p < 0.01$.

et al, 1998; Kippenberger *et al*, 2001). We therefore determined whether PPAR-β/δ expression changes after keratinocytes are exposed to LPS cytokines or UV-B irradiation. Northern analysis revealed an increase in PPAR-β/δ mRNA levels under all of these pro-inflammatory conditions (Fig 4*a*), whereas the expression of PPAR-α and γ was reduced (not shown). These observations suggest that PPAR-β/δ may be selectively altered during keratinocyte inflammation.

PPAR-β/δ activation exerts cutaneous anti-inflammatory activity Since prior studies demonstrated that PPAR-α activators are anti-inflammatory in an animal model of irritant contact dermatitis (Sheu *et al*, 2002), and PPAR-β/δ

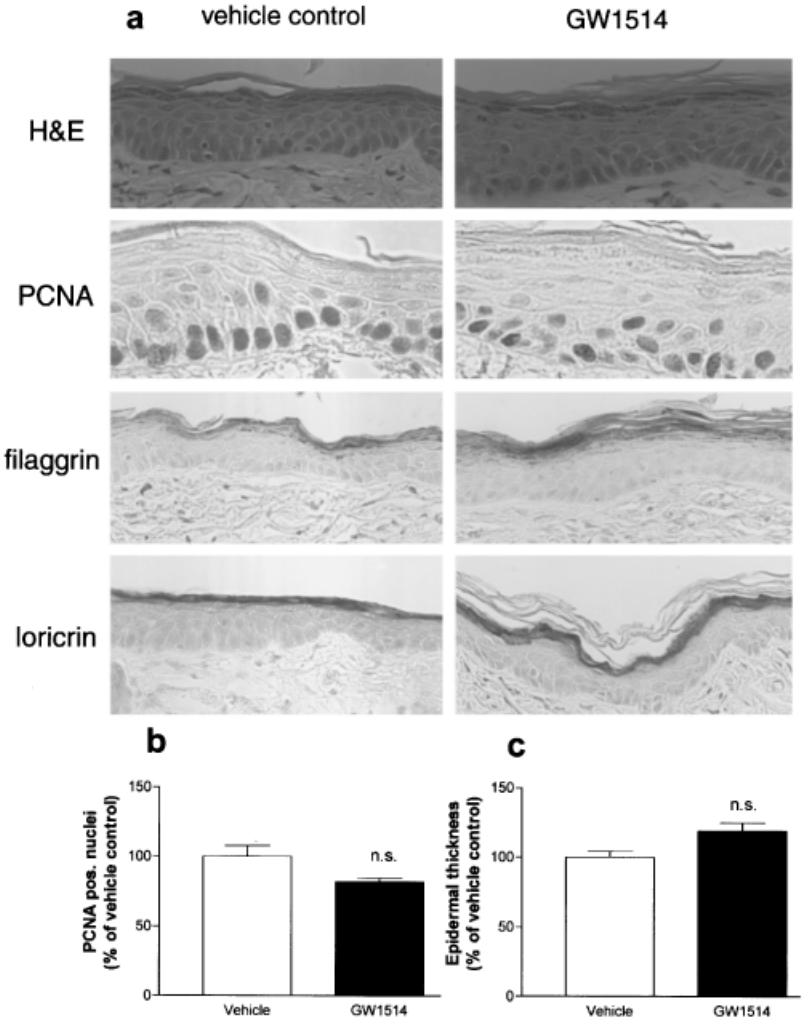


Figure 3
Effects of a peroxisome proliferator-activated receptor-β/δ activation in mouse model of epidermal hyperplasia and impaired differentiation. Hairless mice underwent repeated barrier disruption by topical acetone treatment for 3 d. Subsequent GW1514 was applied topically twice daily for 3 d. (*a*) Epidermal morphology including hematoxylin and eosin staining, proliferating cell nuclear antigen (PCNA) staining and immunohistochemistry for filaggrin and loricrin. (*b*) Quantification of PCNA positive nuclei. (*c*) Measurements of epidermal thickness. Data are presented as mean ± SEM (n = 3).

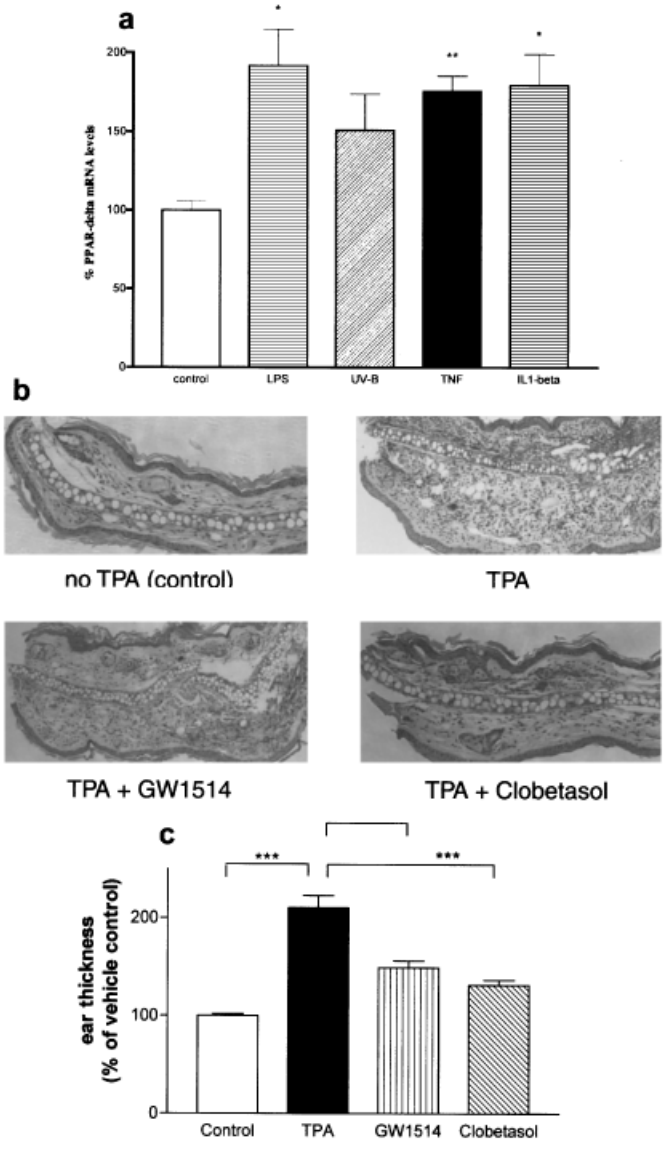


Figure 4
Peroxisome proliferator-activated receptor (PPAR)-β/δ expression and cutaneous inflammation. (*a*) Human keratinocytes were incubated with lipopolysaccharide (LPS), interleukin (IL)1-β, tumor necrosis factor (TNF)-α or exposed to UV-B irradiation and poly-A RNA was isolated for northern blot analysis. Data are presented as mean ± SEM ($n = 3$–4), $^{*}p < 0.05$, $^{**}p < 0.01$. (*b*) Hematoxylin and eosin-stained sections of normal mouse ear, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced irritant dermatitis, clobetasol treated and GW1514 treated. TPA treatment results in a marked increase in ear thickness and a prominent mononuclear cell infiltrate in the dermis. GW1514 treatment reduces ear thickness and the number of inflammatory cells. (*c*) Quantification of ear thickness measurements. Data are presented as mean ± SEM; $n = 4$; $^{***}p < 0.001$.

is increased after cytokine treatment, after UV-B exposure and in psoriasis (Rivier *et al*, 1998), we next assessed the potential anti-inflammatory effects of GW1514 in a model of inflammatory skin disease. We experimentally induced irritant contact dermatitis by topical applications of the phorbol ester, TPA, to the ears of CD1-mice. Both ear thickness (Fig 4*b*, *c*) and ear weight (not shown) markedly increased after a single TPA application (approximately 2-fold) accompanied by a mixed cellular infiltrate containing neutrophils on light microscopy (Fig 6*c*). Treatment with GW1514 significantly reduced the magnitude of these TPA-induced changes, indicating an anti-inflammatory effect of GW1514 (Fig 4*b*, *c*). Moreover, the extent of the reduction in inflammation produced by the PPAR-β/δ agonists approached the anti-inflammatory effects of the topical glucocorticoid, clobetasol, in this model (Fig 4*c*). Together, these results demonstrate that topical PPAR-β/δ activation exerts anti-inflammatory effects in a model of irritant contact dermatitis, comparable to those of a potent topical glucocorticoid.

Effects of PPAR-β/δ activation on keratinocytes *in vitro* The above *in vivo* studies in mice demonstrate important PPAR-β/δ effects on cutaneous homeostasis. To further explore which genes may be regulated in keratinocytes by PPAR-β/δ, we used spotted cDNA microarrays to assess the effects of PPAR-β/δ activation on keratinocyte gene expression in a comprehensive fashion. When primary cultured keratinocytes were treated with a PPAR-β/δ agonist, using arrays comprising a total of 42,432 cDNA sequences, representing 29,778 independent genes based on Unigene Build 155, we observed a 4-fold change (either up or downregulation) in the expression of 258 cDNAs at 6 h and a 4-fold change in the expression of 120 cDNAs at 48 h. These results indicate that PPAR-β/δ activation has highly selective effects on keratinocyte gene expression.

Using hierarchical cluster analysis (average linkage metric) (Eisen *et al*, 1998), we identified a number of keratinocyte differentiation-related genes, including involucrin, small proline-rich region protein (SPRR) 1B, SPRR 2C, SPRR 3, desmoplakin, envoplakin, cystatin A, and annexin A1, which increased with PPAR-β/δ activation (Table I*a*). These genes have in common that they encode for proteins that are cross-linked into the cornified envelope (CE) as keratinocytes transform into anucleate corneocytes during differentiation. Many of these genes also map to the same chromosomal region, the epidermal differentiation complex (EDC) on chromosome 1q21 (Volz *et al*, 1993). A representative cluster from these experiments, demonstrating changes in several differentiation-related genes is shown in Fig 5*a*. Notably, the changes in differentiation-related genes occur at later time points; i.e., 24–48 h (Fig 5*a*).

We used independent assays to confirm the results obtained on microarray hybridization. By both northern and western analyses, GW1514 treatment similarly increased mRNA and protein levels of involucrin, a marker of keratinocyte differentiation (Fig 5*c*, *d*), confirming previous results (Westergaard *et al*, 2001). Strikingly, the increase in involucrin by GW1514 was more effective than the effects of switching from low (0.03 mM) to high (1.2 mM) calcium concentrations in the medium (Fig 5*c*), a standard method to induce keratinocyte differentiation (44). Finally, GW1514 treatment resulted in an increase of PPAR-β/δ mRNA, indicating a positive feedback loop (not shown). These results indicate that activation of PPAR-β/δ increases a number of differentiation-related genes in cultured keratinocytes.

Effects of PPAR activation on keratinocyte lipid metabolism Whereas the microarray experiments demonstrated that PPAR-β/δ regulates a number of keratinocyte differentiation-related genes, in many instances the genes

Table I. PPAR-β/δ activation modulates differentiation-related genes in primary cultured keratinocytes

Description	GeneBank accession no.	PPAR-β/δ (fold change)
(a) Markers of keratinocyte differentiation		
Annexin A1	H63077	2.6
Cystatin A	W72207	2.7
Desmoplakin	H90899	2.1
Envoplakin	AA029418	2.0
Involucrin	AI417983	2.8
Keratin 1	AA706022	0.8
Keratin 10	AA42454	1.4
Periplakin	AI682360	0.6
SPRR 1B	AA447835	8.0
SPRR 2C	AA399647	6.7
SPRR 3	AI278521	2.2
Transglutaminase-1	AI652954	1.3
(b) Adipocyte genes		
ADRP	AA700054	68
FIAF	W30988	12.2
(c) Markers of sebocyte differentiation		
Keratin 7	AA489869	1.2
Keratin 19	AA464250	1.4
EMA	AA975768	1.1

Total RNA from keratinocytes was hybridized to a set of 42.432 human cDNAs in a microarray format. Log transformed and normalized ratios between the combined signals from GW1514 and vehicle-treated cells after 48 h of culture are shown. PPAR, peroxisome proliferator-activated receptor; ADRP, adipose differentiation-related protein; FIAF, fasting induced adipose factor; EMA, epithelial membrane antigen; ApoE, apoprotein E; SPRR, small proline-rich protein.

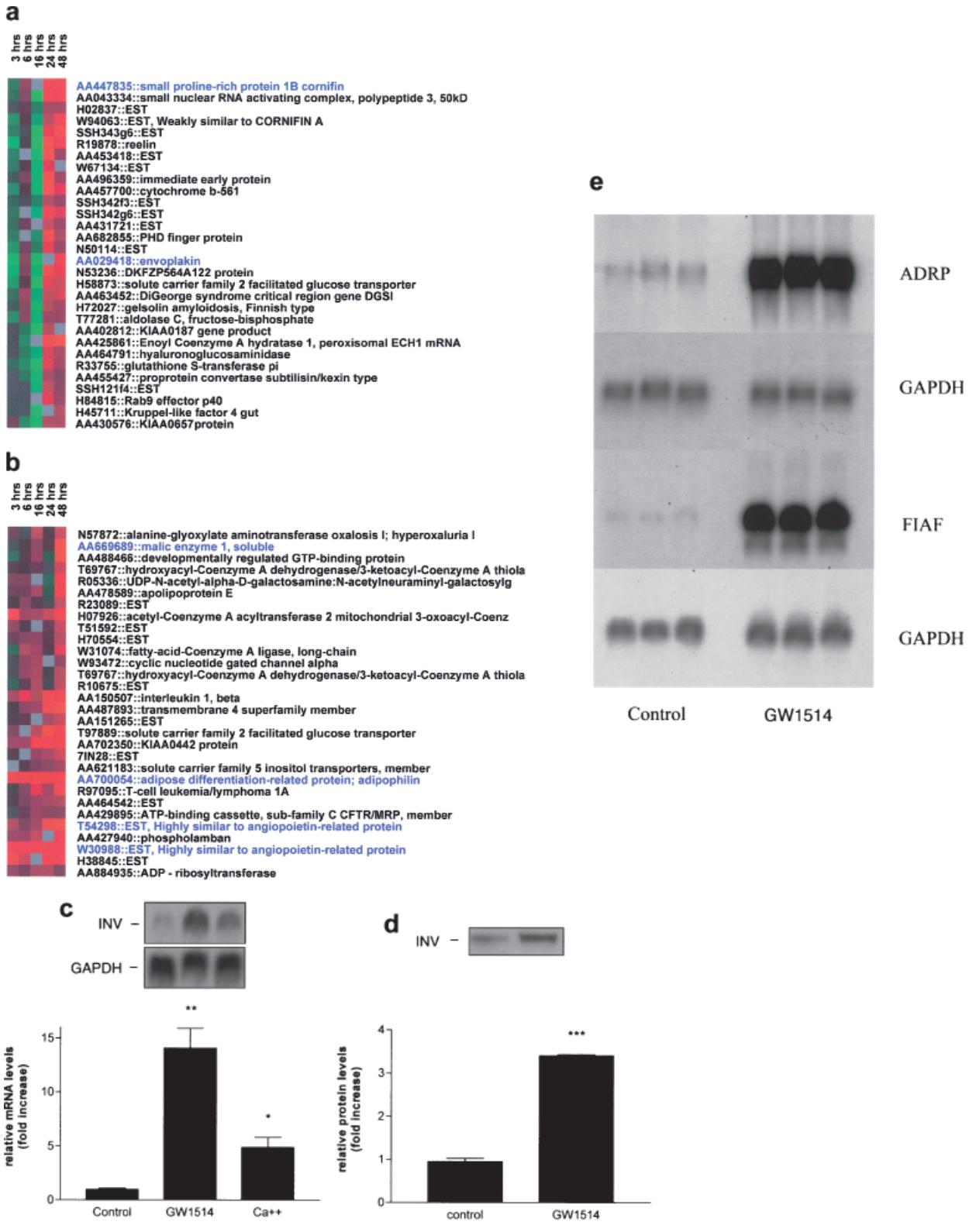
effected were EST with unknown functions. Yet, another category of genes, which we noted to consistently increase with PPAR-β/δ treatment, included adipose differentiation-related protein (ADRP) and fasting induced adipose factor (FIAF) mRNA, which were consistently increased at both early and late time points (6–48 h) after PPAR-β/δ activation (Table 1*b*, Fig 5*b*). The apparent stimulation of ADRP and FIAF again was confirmed by northern blotting (Fig 5*e*). A similar increase in ADRP and FIAF occurred when keratinocytes were treated with a PPAR-α activator (not shown).

ADRP is associated with intracellular lipid droplets (Brasaemle *et al*, 1997; Frolov *et al*, 2000; Corsini *et al*, 2003; Schmuth, 2003) and reportedly facilitates fatty acid binding and uptake in COS-7 cells (Gao *et al*, 2000), as well as the transfer of triacylglycerol from lipofibroblasts to surfactant producing epithelial cells in the lung (Schultz *et al*, 2002). FIAF was previously reported to be increased in liver and white adipose tissue during fasting but the function of this protein is unknown. FIAF levels decreased in the plasma with high fat feeding, an effect directly opposite that observed with leptin (Kersten *et al*, 2000). Because of the potential role of ADRP and FIAF in lipid metabolism, we next assessed lipid storage in PPAR-β/δ-activated keratinocytes, and compared these effects with PPAR-α activation. Lipids did not accumulate during the first 48 h of treatment with either PPAR-β/δ or PPAR-α agonist as indicated by oil red O staining (not shown). Keratinocytes treated for more prolonged periods (6 d) in the presence of either GW1514 or WY-14,643, however, showed increased oil red O staining in comparison to vehicle-treated controls grown in the presence of either palmitic acid or fetal calf serum (Fig 6*a*). HPTLC of keratinocytes treated for 6 d with GW1514 or Wy-14,643 showed an increase in TG (Fig 6*b*) without changes in other lipid species such as fatty acids, phospholipids or ceramides (not shown). Finally, to determine whether lipid accumulation also occurs in the epidermis *in vivo*, we treated hairless mice topically with either PPAR-α or PPAR-β/δ activators. Nile red staining of frozen skin sections from these animals revealed increased lipid staining in PPAR-β/δ and PPAR-α activator-treated animals *versus* vehicle-treated controls (Fig 6*c*). These results indicate increased lipid accumulation in keratinocytes exposed to either PPAR-α or PPAR-β/δ activators.

These results prompted us to ask whether keratinocytes acquire an adipocyte phenotype when exposed to PPAR activators. Classical markers of adipocyte differentiation such as aP2, adipsin, PPAR-γ, resistin, and leptin, however, were not found to be present on either microarray or northern blot analysis in keratinocytes treated with either PPAR-α or PPAR-β/δ activators (not shown). Yet, the epidermal cell that is most efficient in lipid storage is the sebocyte. Therefore, we next asked whether the increased accumulation of TG and the increased expression of ADRP and FIAF indicate differentiation of cultured keratinocyte towards a sebocyte lineage (Elias *et al*, 1987; Ferraris *et al*, 1997; Merrill *et al*, 2001) under the influence of PPAR activation. Searching our microarray database for markers of sebocyte differentiation (Zouboulis *et al*, 1999), we found keratin 7, keratin 19, and epithelial membrane antigen (EMA) to be essentially unchanged in our experimental system (Table I*c*), which in the case of keratin 7 was additionally confirmed on northern blot analysis (not shown). Thus, the increased storage of TG and the expression of ADRP and FIAF do not indicate transdifferentiation of keratinocytes into either sebocytes or adipocytes.

Figure 5
***In vitro* effects of peroxisome proliferator-activated receptor (PPAR)-β/δ activation on keratinocyte gene expression.** (*a*) Cluster containing differentiation-related genes. (*b*) Cluster containing adipose differentiation-related genes. Total RNA was reverse transcribed, fluorescent labeled and hybridized to a set of human cDNAs in a microarray format. Color-coded expression table of the genes selected by cluster analysis from various time points (representative of duplicate arrays) after PPAR-β/δ activation, visualized by TreeView (Eisen *et al*, 1998). Abscissa, time points; ordinate, genes. Increase, red; decrease, green. (*c*, *d*) Total cellular RNA or protein were isolated from cultured keratinocytes after treatment with GW1514 and involucrin levels were measured by (*c*) northern and (*d*) western blot analysis. Northern data are presented as ratio to GAPDH levels. Ca^{2+}, high calcium medium (1.2 mM). Data are presented as mean ± SEM ($n = 3$), $^{**}p < 0.05$, $^{***}p < 0.005$. (*e*) Total RNA was isolated from cultured keratinocytes 24 h after treatment with GW1514 and adipose differentiation-related protein (ADRP) or fasting induced adipose factor (FIAF) mRNA levels were measured by northern blot analysis and compared to GAPDH levels.

Comparison of PPAR-α *versus* PPAR-β/δ activators in keratinocytes Finally, in view of the overlapping effects of PPAR-α and PPAR-β/δ activators on the epidermis, we asked which genes respond similarly or differently to PPAR-α or PPAR-β/δ activators. To this end, using hierarchical cluster analysis (average linkage metric) (Eisen *et al*, 1998), comparing the 6 and 48 h time points, we observed 34 cDNAs selectively being decreased by PPAR-α (50% decrease) and two cDNAs by PPAR-β activation (50% decrease). In contrast, only five cDNAs were selectively increased by PPAR-α (2-fold increase) and two cDNAs by PPAR-β/δ activation (2-fold increase) (Table II). Thus,



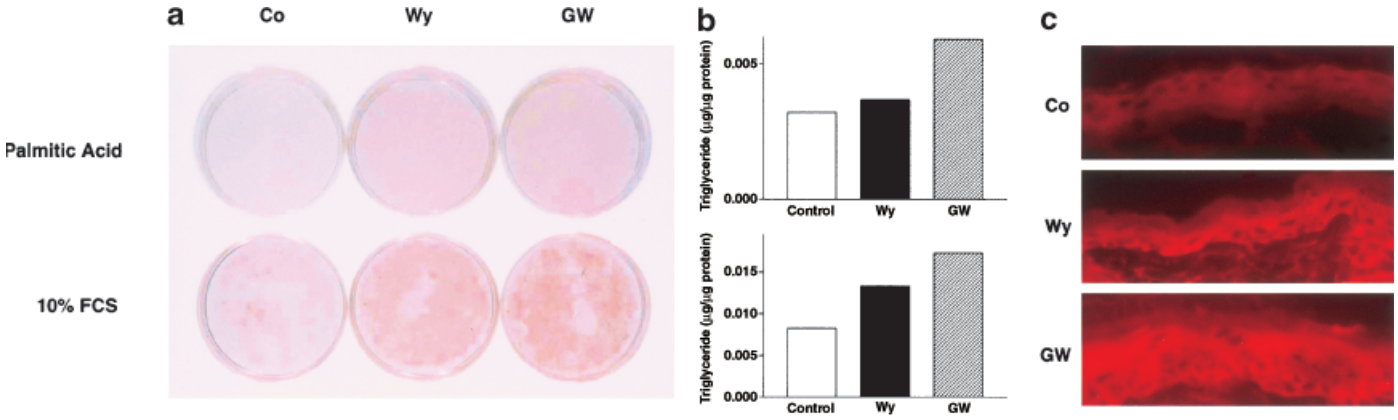


Figure 6
Effects of peroxisome proliferator-activated receptor activation on epidermal lipids. (*a*) Cultured, 40%–60% confluent keratinocyte were treated with 8 μM GW1514 or 20 μM Wy-14,643 for 6 d. Cells were fixed using 3.7% paraformaldehyde and incubated with oil red O (Sigma, 0.5 g in 100 mL isopropanol stock solution diluted 6 : 4 with water) for 1 h. (*b*) High-performance thin layer chromatography was used for the analysis of lipid extracts from cultured cells. One hundred micromolar palmitic acid (upper graph); 10% fetal calf serum (FCS) (lower graph). (*c*) For *in vivo* assessment, Nile red was applied to 5 μm frozen sections of skin that had been topically treated twice daily with 4 mM GW1514 for 3 d. Sections were examined using a fluorescence microscope equipped for epifluorescence.

whereas the patterns of genes induced and repressed by PPAR-α and PPAR-β/δ activation are largely overlapping, both PPAR-α and PPAR-β/δ also have selective effects on certain subsets of genes and these differential effects more commonly involve repression of gene expression. Together, these results indicate that there are distinct patterns of genes that are repressed or induced by PPAR-α and PPAR-β/δ activation in keratinocytes.

Discussion

Previous studies by other investigators have shown that activation of PPAR-β/δ stimulates the differentiation of keratinocytes in culture (Westergaard *et al*, 2001). In this study, we extend these observations and demonstrate that the topical treatment of mice with a PPAR-β/δ activator stimulates epidermal differentiation *in vivo*. The expression of loricrin and filaggrin, two well-recognized markers of keratinocyte differentiation, were increased by the topical application of GW1514, a selective PPAR-β/δ activator (Fig 1*d*–*f*). Moreover, we demonstrate that this increase in loricrin and filaggrin expression is receptor mediated. In RXR-α deficient mice the stimulation of differentiation induced by GW1514 treatment was blunted (Fig 1*f*). RXR-α is the most abundant isoform of RXR in the epidermis and RXR is an obligate partner required for the regulation of gene expression by PPAR. Hence, the decrease in the ability of GW1514 to induce differentiation in RXR-α deficient mice indicates that the effects of GW1514 are receptor mediated. Previous studies by our laboratory have shown that clofibrate and other PPAR-α activators stimulate epidermal differentiation in wild-type but not in PPAR-α deficient mice. In this study, we demonstrate that GW1514 stimulates differentiation in PPAR-α deficient mice indicating that the effect of GW1514 on differentiation is not mediated by PPAR-α. Thus, these experiments taken together strongly suggest that the stimulation of epidermal differentiation by GW1514 is mediated by PPAR-β/δ. Determining the effect of GW1514 in PPAR-β/δ deficient mice would be the ideal experiment to definitively prove that the effect of GW1514 is mediated by PPAR-β/δ, but unfortunately such animals are not available for study (homozygote PPAR-β/δ knockout mice have a high mortality rate and therefore animals with a localized deficiency of PPAR-β/δ need to be created).

The formation of a competent permeability barrier, which resides in the extracellular compartment of the SC, is the end product of keratinocyte differentiation (Eisinger *et al*, 1993; Jackson *et al*, 1993). Pre-treatment with GW1514 accelerated the restoration of permeability barrier function following barrier disruption by tape stripping, solvent treatment or detergent treatment (Fig 2). This improvement in permeability barrier homeostasis confirms the biological importance of the pro-differentiating effects of PPAR-β/δ activation. Additionally, GW1514 induced differentiation in an animal model of epidermal hyperplasia and abnormal differentiation (Fig 3). This ability of GW1514 to stimulate differentiation in abnormal epidermis suggests that PPAR-β/δ agonists may be useful in the treatment of a variety of skin disorders characterized by disturbed differentiation.

In addition to stimulating epidermal differentiation, this study also demonstrates that PPAR-β/δ activation is anti-inflammatory. In a mouse model of irritant contact dermatitis (TPA-induced inflammation of ears), treatment with GW1514 markedly decreased inflammation (Fig 4*c*, *d*). In line with these results, Peters *et al* (2000) have previously reported that PPAR-β/δ deficient mice fed with the NSAID sulindac have an increased inflammatory response to phorbol ester (TPA) treatment when compared to wild-type mice, which suggests that PPAR-β/δ plays a key role in downregulating inflammation. Together, these results suggest that PPAR-β/δ activators may be useful therapeutic agents to reduce inflammation in the skin and perhaps in other tissues. Pertinently, whereas agents that induce inflammation, such as cytokines and UV light, reduce the expression of PPAR-α and PPAR-γ in cultured human keratinocytes, the expression of PPAR-β/δ is increased (Fig 4*a*) (Tan *et al*, 2001). Moreover, in psoriatic skin, the expression of PPAR-β/δ is increased whereas the expression of PPAR-α

Table II. Differential gene regulation PPAR-α *versus* PPAR-β

Description	GeneBank accession no.
(a) Selectively decreased by PPAR-α	
3-α-hydroxysteroid dehydrogenase	AA916325
FXYD domain containing ion transport regulator 3	AA126115
EST	W68630
EST	R51067
EST	AA757672
Ephrin-A1	AA857015
EST	T56713
EST	N21217
EST	AA598615
EST	AA479954
ATP synthase, H+ transporting, vacuolar	R73402
Chromosome 20 open reading frame 155	N29545
Non-metastatic cells 5	AA453579
ATP synthase, H+ transporting, mitochondrial	AA431433
Ribosomal protein S19	H41165
EST	H97921
EST	AA157112
EST	AA487934
EST	AA399166
EST	H58959
EST	AA399372
ATPase, Na+/K+ transporting	AA598814
EST	H04815
EST	AA011400
EST	AA418593
cDNA DKFZp434P0235	AA169444
Hypothetical protein LOC55565	AA446446
EST	AA872402
HBV associated factor	AA447671
Janus kinase 1	AA284634
Selenoprotein W, 1	AA283629
EST	N91527
EST	AI053614
EST	AA983191
(b) Selectively decreased by PPAR-β	
Topoisomerase (DNA) II α 170 kDa	AA504348
EST	AA172076
(c) Selectively increased by PPAR-α	
EST	N48689
EST	H38977
EST	AA406059
EST	AA172076
EST	N32071
(d) Selectively Increased by PPAR-β	
SPRR 1B	AA447835
EST	W68630

EST, expressed sequence tags.

and PPAR-γ is decreased (Rivier *et al*, 1998). The increased expression of PPAR-β/δ during cutaneous inflammation makes it an attractive therapeutic target for the treatment of diseases that are characterized by cutaneous inflammation.

To gain further insights into the effect of PPAR-β/δ activation on keratinocytes we carried out microarray experiments. As expected, we found that the expression of genes that are well known to increase during keratinocyte differentiation, were increased by treatment with GW1514. Specifically, we found that involucrin, SPRR 1B, SPRR 2C, SPRR 3, annexin A1, cystatin A, desmoplakin, and envoplakin were increased by PPAR-β/δ activation (Table I*a*). It should be noted that the increase in the expression of these differentiation-related proteins was a late effect (48 h) of PPAR-β/δ activation (Fig 5*a*). This suggests that the increased expression of these proteins is not a direct effect of PPAR-β/δ on the promoter of these genes, but rather was due to PPAR-β/δ activation stimulating other transcription factors. In our studies of PPAR-α activators, we observed that the increase in the expression of keratinocyte differentiation markers induced by PPAR-α activation also was a late effect (not shown). Furthermore, the PPAR-α induced increase in the expression of involucrin required an intact AP-1 response element in the involucrin promoter (−2011 to –2017) suggesting that the effect of PPAR-α was not a direct effect, but rather was mediated by a secondary increase in AP-1 activity (Hanley *et al*, 2000).

To our surprise the expression of two genes that are well known to be expressed in adipocytes, FIAF and ADRP, were markedly increased in keratinocytes treated with GW1514 (Table I*b*; Fig 5*b*, *e*). FIAF is expressed in adipocytes and its expression is increased during adipocyte differentiation, during fasting, and in mouse models of obesity and diabetes (Kersten *et al*, 2000). The biological function of FIAF is unknown but it is presumed to play a role in lipid metabolism. ADRP is a membrane-associated fatty acid binding protein that facilitates the transport of long chain fatty acids (Frolov *et al*, 2000; Gao *et al*, 2000). Its expression is induced during adipocyte differentiation but it is also expressed in other tissues that are involved in TG and fatty acid metabolism (Yuspa *et al*, 1988; Brasaemle *et al*, 1997). Cells that either store or transport lipid express ADRP and it has been proposed that ADRP associates with lipid vacuoles (Yuspa *et al*, 1988; Brasaemle *et al*, 1997). Recent studies have suggested that ADRP plays an important role in the up take of surfactant lipids by alveolar type II epithelial cells (Schultz *et al*, 2002). Studies have shown that there are NHR response elements in the promoter of ADRP and that PPAR activation directly stimulates ADRP expression (Eisinger *et al*, 1993). That ADRP is a direct downstream target of PPAR-β/δ is very compatible with the early increase in ADRP expression that we see with GW1514 treatment (Fig 5*b*).

Because of the link between the expression of FIAF, ADRP, and lipids we examined the effect of PPAR-β/δ activation on lipid accumulation. In both cultured human keratinocytes and mouse epidermis we found that treatment with GW1514 resulted in the increased accumulation of lipids that were predominantly TG (Fig 6) similar to previous results from macrophages (Vosper *et al*, 2001) and

keratinocytes exposed to irritants (Corsini *et al*, 2003). Since studies by other investigators have shown that activation of PPAR-β/δ stimulates adipocyte differentiation (Bastie *et al*, 2000), we assessed if treatment of keratinocytes with PPAR-β/δ activator was inducing an adipocyte phenotype in keratinocytes. To this end, we determined if the expression of other genes that are expressed in adipocytes were increased in GW1514-treated keratinocytes. Adipsin, resistin, leptin, and aP2, specific adipocyte markers, were not expressed in either GW1514-treated or control keratinocytes. Keratinocytes also have the potential to become sebocytes, which store and secrete lipids. To determine if PPAR-β/δ activation was stimulating the conversion of keratinocytes to sebocytes (Elias *et al*, 1987; Ferraris *et al*, 1997; Merrill *et al*, 2001), we determined if the expression of sebocyte markers were increased in keratinocytes treated with GW1514. Keratin 7, keratin 19, and EMA are sebocyte markers and the expression of these genes were not increased in keratinocytes by PPAR-β/δ activator treatment (Table I*c*). Thus, PPAR-β/δ activation stimulates lipid accumulation in keratinocytes, but it is not clear at this time why this occurs or its functional significance.

Previous studies by our laboratory have shown that PPAR-α activators stimulate keratinocyte differentiation, improve permeability barrier homeostasis, and are anti-inflammatory (Hanley *et al*, 2000; Komuves *et al*, 2000a, b). Moreover, in this study we observed that the PPAR-α activator, WY14,643, also stimulates the expression of ADRP and FIAF (not shown) and the accumulation of lipids (Fig 6). Additionally, as previously reported by others, PPAR-α activation increases lipid metabolism in a skin equivalent model (Rivier *et al*, 2000). But there are differences between the effects of PPAR-α and PPAR-β/δ activation. Although PPAR-α activation inhibits epidermal proliferation, PPAR-β/δ activation does not affect *in vivo* proliferation. Accordingly, when one examines the microarray data, although there are many similarities in the pattern of genes induced and repressed by PPAR-α and PPAR-β/δ activation there are also differences (Table II). Thus, although there will be a great deal of overlap and redundancy in the actions of PPAR-α and PPAR-β/δ on the epidermis there will also be differences.

In the epidermis, a multi-step process of keratinocyte differentiation occurs that ultimately forms the outermost layer of the skin, the SC, which consists of protein-enriched corneocytes embedded in a continuous, lipid-enriched, extracellular matrix (Jackson *et al*, 1993; Steinert, 2000). Thus, the SC consists of corneocytes (the bricks) and extracellular lipid membranes (the mortar) that serve as a barrier between the organism and the environment and a competent barrier is required for survival (Jackson *et al*, 1993). The lipid enriched extracellular lamellar membranes are derived from the secretion of lamellar bodies from stratum granulosum cells and provide the permeability barrier to water transit. The corneocytes form a CE by the enzyme transglutaminase extensively cross-linking loricrin, involucrin, and other proteins to form a rigid structure that provides strength and resistance to the SC. The protein versus lipid arms of keratinocyte differentiation and SC formation are traditionally viewed as concurrent, but independent processes. But the ability of lipids, such as long chain fatty acids, to activate PPAR-α and PPAR-β/δ and the recent data (including this paper) demonstrating that these transcription factors can stimulate keratinocyte proteins required for CE formation, suggest that there may be cross-talk between the two arms of keratinocyte differentiation (Hanley *et al*, 2000; Schmuth *et al*, 2002). One can speculate that as lipid precursors accumulate in stratum granulosum cells, these lipids or their metabolites could activate NHR, which in turn stimulate other aspects of keratinocyte differentiation, such as increasing the expression of the structural proteins required to form the CE. This would allow for the coordinated formation of the SC.

In summary the results of this and previous studies demonstrate that PPAR-α and PPAR-β/δ activation stimulates keratinocyte differentiation, improves permeability barrier homeostasis, increases lipid accumulation in keratinocytes, and is anti-inflammatory, whereas we also show differing effects on other parameters such as epidermal proliferation *in vivo*. It is likely that these profiles will be beneficial for the treatment of a variety of cutaneous disorders and therefore PPAR-α and/or PPAR-β/δ activators may be useful therapeutic agents for the treatment of skin diseases.

Materials and Methods

Cell culture Second-passage keratinocytes isolated from newborn foreskins were cultured in serum-free keratinocyte growth medium (Clonetics, San Diego, California). Cells were treated at 70%–100% confluency with either the PPAR-α activator Wy-14,643 (20 μM, Sigma, St Louis, Massachussets), the PPAR-β/δ activator GW1514 (8 μM, GlaxoSmithKline, Triangle Park, North Carolina), tumor necrosis factor (TNF)-α (100 ng per mL, R&D Systems, Minneapolis, Minnesota), interleukin (IL)1-β (50 ng per mL, R&D Systems), lipopolysaccharide (LPS, 100 μg per mL, Difco, Detroit, Michigan) or exposed to UV-B irradiation (30 mJ per cm^2, FS-20/T12 tubes). Control keratinocytes were treated with vehicle (0.05% ethanol) or sham irradiated.

Animal models Animals employed in these studies were 6–8-wk-old male hairless mice (Simonsen Laboratories, Gilroy, California), CD-1 mice (Charles River, Wilmington, Massachusetts), PPAR-α−/− mice (Lee *et al*, 1995), and RXR-α−/− mice (Li *et al*, 2000, 2001). Age matched mice from the same genetic background were used as controls. The animal procedures were approved by the local Animal Studies Subcommittee and were performed in accordance with their guidelines. For topical treatment GW1514 was dissolved in an acetone or dimethyl sulfoxide (DMSO) vehicle at a concentration of 4 mM and topically applied to the flank of mice twice daily for 1–6 d. Barrier disruption was induced in hairless mice by application of acetone or sodium dodecyl sulfate (SDS)-soaked cotton balls until the transepidermal water loss (TEWL) reached 6–8 mg per cm^2 per h. Alternatively, the barrier was disrupted by repeated tape stripping with cellophane tape. TEWL recovery was determined at 3 and 6 h following disruption. Chronic barrier disruption was induced in hairless mice by twice daily application of acetone-soaked cotton balls until the TEWL reached 6–8 mg per cm^2 per h for 3 consecutive days. Subsequently, repeated acetone treatment was discontinued and the area was treated with 4 mM GW1514 as above. Irritant contact dermatitis was induced in CD1 mice by a single topical application of 10 μL of 0.03% (wt/vol in acetone) 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on the inner and outer surfaces of the left ears of male mice. The right ears were treated with vehicle alone (acetone). GW1514 was dissolved in acetone at a concentration of 4 mM and topically applied to both the left and right ears at 45 min and 4 h following the inflammatory insult with TPA in one group of animals. In a

separate set of animals acetone was applied to both left and right ears as a vehicle control. A total of 20 μl of GW1514 or vehicle was applied to each surface of each ear. At 18 h, when TPA-induced inflammation is maximal, ear thickness was measured with a digital caliper (Mitutoyo Corp., Tokyo, Japan), followed by 6 mm full skin biopsies for weight determinations. Ear swelling, measured by thickness and weight, was calculated according to the following equation: Ear swelling (%) = 100(a−b)/b; a = thickness or weight of treated left ear; b = thickness or weight of control right ear. Mouse skin sections (6 μm) were fixed in 4% formaldehyde and stained either with hematoxylin and eosin or used for immunohistochemistry (see below). Photographs were taken with a Zeiss microscope (Jena, Germany).

Northern blot analysis Poly-A RNA was purified using oligo(dT) cellulose (Amersham–Pharmacia, Piscataway, New Jersey) and hybridized with ^{32}P-labeled cDNA probes as described previously (Beigneux *et al*, 2000).

Microarray analysis Aliquots of 30 μg keratinocyte total RNA were reverse transcribed in the presence of amino-allyl- dUTP and coupled to fluorescent Cy-dyes (Amersham) as described previously (Hughes *et al*, 2001). We compared control RNA (vehicle-treated keratinocytes) to RNA from cells treated for 3, 6, 16, 24, and 48 h after addition of nuclear hormone receptor activators to the medium. A set of 42,432 human cDNA clones comprising 29778 genes as well as expressed sequence tags (EST) from Research Genetics (Huntsville, Alabama) were spotted onto polylysine-coated glass slides by a costum made arrayer robot kindly made available by Joe deRisi (University of California, San Francisco). Hybridization in a buffer containing 3 × SSC, 0.25% SDS, 0.75 mg per mL polyadenylic acid, and 0.5 mg per mL Cot-1 DNA, was carried out in a sealed hybchamber (GeneMachines, San Carlos, California) overnight at 63–65°C. Fluorescence intensity was detected using the GenePix 4000B Array Scanner (Axon Instruments, Union City, California). Resulting data files were evaluated by a series of quality-control criteria including replicate cDNA spots on the same slide, technical replicates (same RNA independently labeled and hybridized to different slides), and exclusion of array images with obvious blemishes (control procedures relating to the image itself). The results reported are comprised from experiments involving primary cultured keratinocytes from multiple donors (biological replicates), samples, and arrays. Computational analysis of the results was carried out as desribed below (Data analysis).

***In situ* hybridization** Digoxigenin-labeled RNA probes to detect filaggrin mRNA were made from linearized cDNA sequences using reagents supplied by Boehringer-Mannheim (Indianapolis, Indiana). The sections were hybridized at 45°C and binding of DIG-labeled probes to the endogenous mRNA was detected by anti-DIG-alkaline phosphatase (Boehringer-Mannheim), using 5-bromo-4-chloro-3-indolyl phosphate/nitrotetrazolium blue substrate. Hybridization with DIG-labeled sense control probes resulted in no staining.

Immunohistochemistry Antibodies to filaggrin and loricrin were purchased from BabCo (Richmond, California). For identification of proliferating keratinocytes, paraffin sections were stained with a biotinylated anti-proliferating cell nuclear antigen (PCNA) antibody (CalTag Laboratories, Burlingame, California). TUNEL assay was carried out using the *in situ* cell death detection kit from Roche Molecular Biochemicals (Indianapolis, Indiana) using FITC-labeled dUTP.

Western blot analysis Involucrin protein was detected by incubation overnight at 4°C with a monoclonal mouse anti-human involucrin antibody (Sigma) as described previously (Hanley *et al*, 2000).

Lipid detection Nile red and frozen tissue embedding compound (OCT) were from Miles Scientific Division (Naperville, Illinois). Nile red was applied to 5 μm frozen sections, which were examined using a Leitz fluorescence microscope equipped for epifluorescence (E. Leitz, Inc., Rockleigh, New Jersey). For visualization of lipids in cultured keratinocytes, confluent keratinocytes were cultured in the presence of 100 μM palmitic acid (in bovine serum albumin 3 : 1) or in the presence of 10% fetal calf serum for 6 d and then exposed to 8 μM GW1514 for 6 d. Subsequently, cells were fixed using 3.7% paraformaldehyde and incubated with oil red O (Sigma, 0.5 g in 100 mL isopropanol stock solution diluted 6 : 4 with water) for 1 h. Alternatively, lipids were extracted from cultured keratinocytes with the Bligh/Dyer method. They were spotted on high-performance thin layer chromatography (HPTLC) Plates (Merck, Gibbstown, NJ) along with known standards (Sigma Chemical Co.) using a Linomat II Auto spotter (CAMAG Scientific Inc., Wilmington, North Carolina). Neutral lipids (sterol ester, triglyceride, free fatty acid, and free sterol) were sub-fractionated in benzene : *n*-hexane (1 : 1, v/v) to 8 cm from the application site followed by *n*-hexane : diethylether : acetic acid (70 : 30 : 1, v/v) to 5 cm from the application site. Ceramides and glucosylceramides were sub-fractionated in chloroform : methanol : H_2O (40 : 10 : 1, v/v) to 2 cm from the application site, repeat to 5 cm, followed by chloroform : methanol : acetic acid (47 : 2 : 0.5, v/v) to 8.5 cm, and followed by *n*-hexane : diethylether : acetic acid (30 : 15 : 0.5, v/v) to 8.5 cm. Phosphatidylcholine and sphingomyelin were sub-fractionated in chloroform : methanol : 28% NH_4OH (32 : 12 : 2.5, v/v) to 8.5 cm from the application site. All separations were done in a vertical developing chamber at room temperature. The plate was then dipped in charring solution, charred, and quantitated with a CAMAG Scanning Densitometer (CAMAG Scientific Inc.).

Data analysis For northern and western blot results statistical significances were determined using the Student's *t* test or the Kruskal–Wallis test, as appropriate. For microarray experiments background subtraction and computation of ratios was carried out using GenePix 4.0 Software (Axon Instruments). Using NOMAD (http://www.microarrays.org/software.html) and Cluster (Eisen *et al*, 1998) the ratio of medians was normalized, and ratios underwent a logarithm transformation for all the observed gene-expression levels. Hierarchical clustering (average linkage metric) was used to organize the data (Eisen *et al*, 1998).

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