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PPARβ/δ selectively induces differentiation and inhibits cell proliferation

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Abstract

Peroxisome proliferator-activated receptor (PPAR) β-null mice exhibit exacerbated epithelial cell proliferation and enhanced sensitivity to skin carcinogenesis, suggesting that ligand activation of PPARβ will inhibit keratinocyte proliferation. By using of a highly specific ligand (GW0742) and the PPARβ-null mouse model, activation of PPARβ was found to selectively induce keratinocyte terminal differentiation and inhibit keratinocyte proliferation. Additionally, GW0742 was found to be anti-inflammatory due to inhibition of myeloperoxidase activity, independent of PPARβ. These data suggest that ligand activation of PPARβ could be a novel approach to selectively induce differentiation and inhibit cell proliferation, thus representing a new molecular target for the treatment of skin disorders resulting from altered cell proliferation such as psoriasis and cancer.

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Abbreviations: ADRP, adipocyte differentation-related protein; MPO, myeloperoxidase; PPAR, peroxisome proliferator-activated receptor; SPR, small proline-rich protein; TG-I, transglutaminase-I

Introduction

During the past 5 years, a number of potential roles for peroxisome proliferator-activated receptor (PPAR) β (also referred to as PPARδ) in epithelial homeostasis have been described including the regulation of keratinocyte differentiation,[1–3] apoptosis[4] and cell proliferation,[3,5] inflammation[2] and wound healing.[6] However, the data supporting these roles for PPARβ in the skin remain highly controversial, as there is inconsistent evidence in the literature based on results from the skin, keratinocyte and other cell/tissue models. Recent observations suggest that activation of PPARβ may be of benefit for the treatment of dyslipidemias[7,8] and studies in PPARβ-null mice have suggested a possible role for this receptor in cancer,[9,10] thus illustrating the need to accurately determine the specific biological functions mediated by PPARβ ligands so that the safety of these potentially therapeutic drugs can be assessed. The use of highly specific ligands for PPARβ in conjunction with the PPARβ-null mouse model is the most definitive approach for these purposes, and was used for these studies.

Results

Application of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to mouse skin results in increased expression of PPARβ that is coincident with enhanced expression of mRNAs encoding proteins that are markers for keratinocyte differentiation.[11] However, increased expression of differentiation marker mRNAs in skin occurs *in vivo* in response to TPA in the absence of PPARβ expression.[5] Similar to results obtained *in vivo*, treatment of primary keratinocytes derived from either wild-type or PPARβ-null mice *in vitro* with TPA results in morphological changes indicative of differentiation (Figure 1a), coupled with increased expression of mRNAs encoding both early and late markers of differentiation (Figure 1b). In particular, a 2–6-fold induction of mRNAs encoding TG-I, involucrin, small proline-rich protein (SPR)1A, SPR2H was observed after 6 h of treatment, while induction of loricrin did not occur until after 48 h of treatment; a pattern of early and late markers of differentiation typically observed in primary keratinocytes.[12,13] Using the classic approach of increasing culture medium Ca^{2+} to induce keratinocyte differentiation, similar results were obtained as morphological changes indicative of differentiation (Figure 1c) and increased expression of mRNAs encoding both early and late markers of differentiation was observed in keratinocytes derived from either wild-type or PPARβ-null mice (Figure 1d).

Despite the increase in PPARβ expression coincident with induction of keratinocyte differentiation, the results in Figure 1 suggest that PPARβ is not required for differentiation induced by TPA or Ca^{2+} signaling. To further examine this hypothesis, primary keratinocytes were cultured in medium containing GW0742 at a concentration that specifically activates PPARβ (see below Figure 3a). Stratified cells that are indicative of differentiation (Figure 1c) were not found in keratinocytes cultured in GW0742 in low Ca^{2+} medium (Figure 2a), a result that is not unexpected since the terminal differentiation of primary keratinocytes requires Ca^{2+} for the formation of the

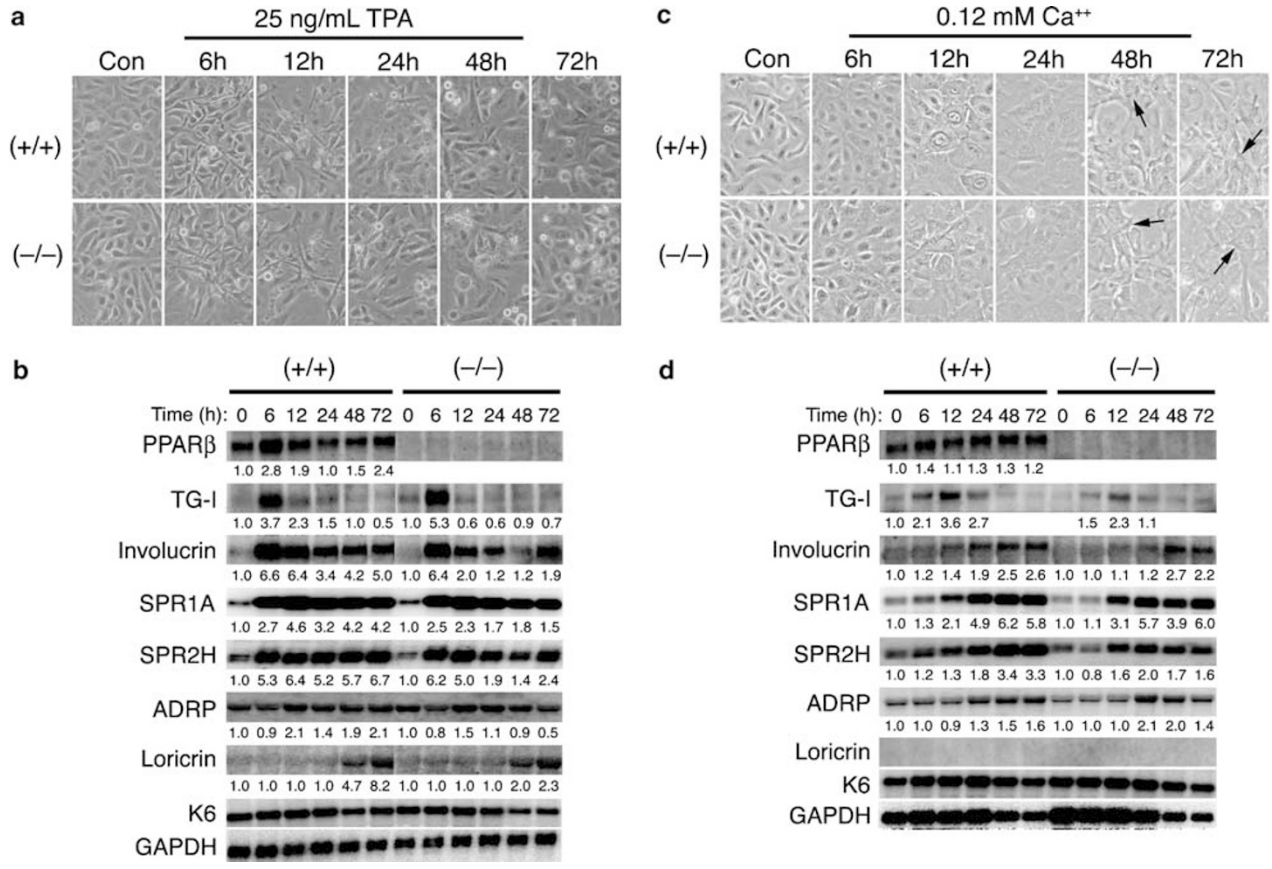

Figure 1 PPARβ does not influence TPA or Ca^{2+}-induced keratinocyte differentiation. Morphology of differentiating keratinocytes from wild-type (+/+) or PPARβ-null (−/−) mice is similar when cultured in either 25 ng TPA/ml of medium (**a**) or 0.12 mM Ca^{2+} (**c**). Note the dendritic-like cells indicative of TPA-induced differentiation typically observed in keratinocytes in both genotypes after 6–12 h (**a**). Note the overt changes in morphology and stratified cells (arrows), indicative of differentiation, in both genotypes in response to high Ca^{2+} medium (**c**). Early and late mRNA markers of differentiation are similar in keratinocytes from (+/+) and (−/−) mice when differentiation was induced with either 0.12 mM Ca^{2+} (**b**) or 25 ng TPA/ml of medium (**d**). The fold-induction of normalized hybridization signals is presented below each representative Northern blot. *Significantly different than controls, $P \leq 0.05$

cornified envelope.[14] However, ligand activation of PPARβ resulted in a PPARβ-dependent increase in SPR1A, SPR2H and adipocyte differentation-related protein (ADRP) mRNA expression (Figure 2b). Interestingly, the expression of ADRP was markedly lower in PPARβ-null cells (Figure 2b). Increased expression of mRNAs encoding TG-I and loricrin was not observed in response to GW0742, while increased expression of involucrin was observed after 24 h of treatment in both genotypes (Figure 2b). To determine whether ligand activation of PPARβ increases the expression of mRNAs encoding differentiation markers *in vivo*, a dose response experiment was performed to identify a concentration of GW0742 that specifically activates PPARβ in skin after topical application (Figure 2c). Consistent with results observed *in vitro*, topical administration of GW0742 with a stock concentration that specifically activates PPARβ (50 μM), resulted in increased expression of mRNAs encoding markers of keratinocyte differentiation including, TG-I, involucrin, SPR1A, SPR2H and ADRP in wild-type mouse skin 8 h post-treatment, and these increases were absent in PPARβ-null mouse skin (Figure 2d). To determine whether the increases in mRNAs encoding proteins required for terminal differentiation induced by ligand activation of PPARβ caused a functional increase in keratinocyte terminal differentiation, cornified envelopes were quantified. Indeed, administration of 50 μM GW0742 caused an increase in the average number of cornified envelopes in wild-type mouse skin as compared to controls (Figure 2e). In contrast, the average number of cornified envelopes was lower in PPARβ-null mouse skin, and treatment with GW0742 had no effect on terminal differentiation in the absence of PPARβ expression (Figure 2e). Collectively, these results suggest that the induction of differentiation mediated by TPA and high culture medium Ca^{2+} involves a number of other transcriptional regulators (e.g. AP-1) that modulate similar target genes (e.g. SPRs, ADRP) and prevents detection of the functional role of PPARβ-dependent transcriptional modulation of differentiation. Importantly, these results also demonstrate that ligand activation of PPARβ can selectively induce the expression of mRNAs encoding proteins that mediate lipid accumulation and terminal keratinocyte differentiation.

It is well accepted that the induction of terminal differentiation is inversely correlated with cell proliferation,[14,15] as terminal differentiation of cells would in theory reduce the number of cells available for proliferation. Additionally, epithelial cell proliferation is exacerbated in the absence of PPARβ expression in response to TPA.[9] Together, these observations suggest that ligand activation of PPARβ could

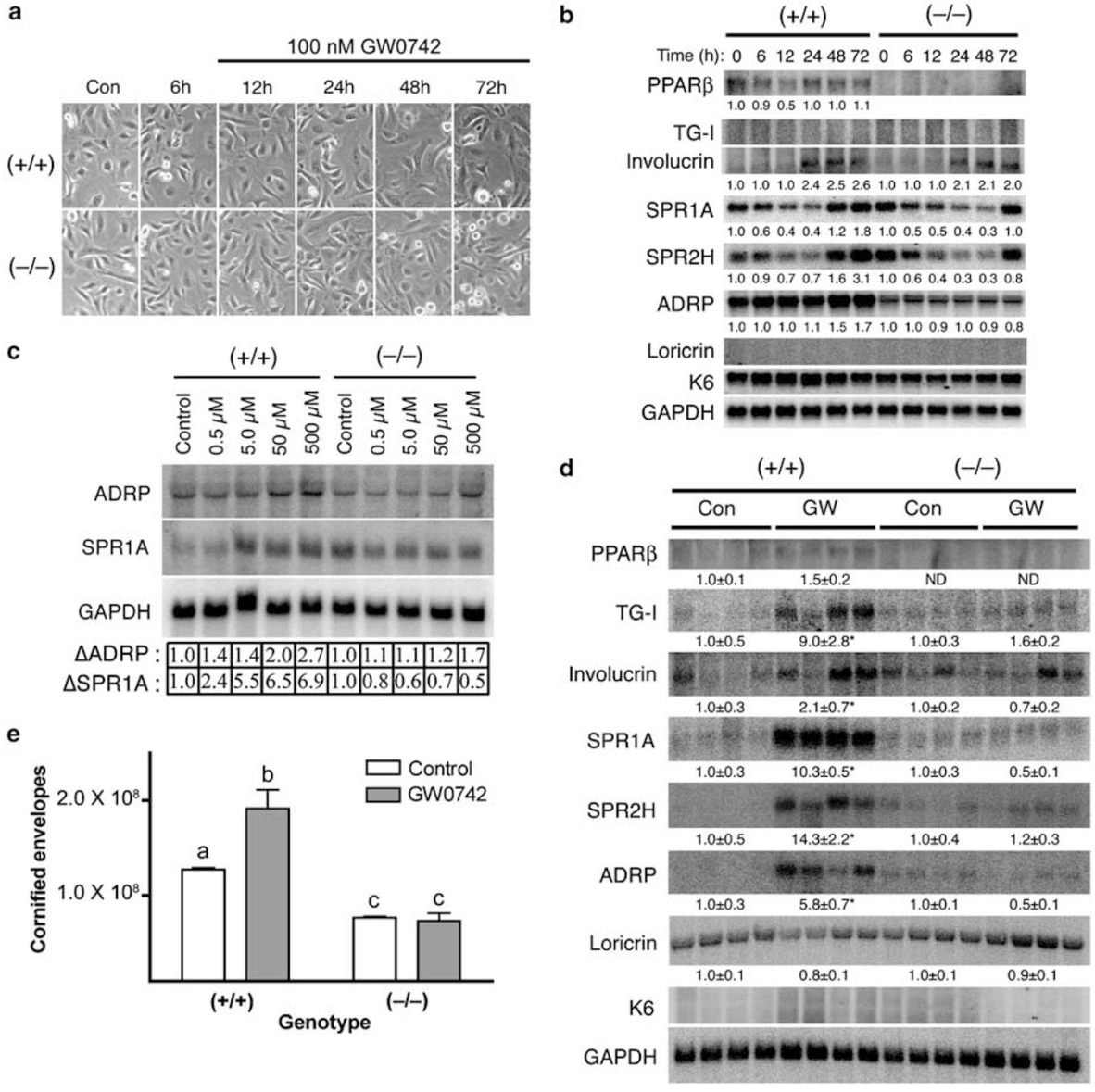

Figure 2 Ligand activation of PPARβ selectively regulates epithelial differentiation. (**a**) Morphology of differentiating keratinocytes from wild-type ($+/+$) or PPARβ-null ($-/-$) mice is similar when cultured in 100 nM GW0742. (**b**) mRNAs encoding SPR1A, SPR2H and ADRP mRNAs are selectively induced by 100 nM GW0742 *in vitro*. The fold-induction of normalized hybridization signals is presented below each representative Northern blot. (**c**) Induction of PPARβ target genes (ADRP and SPR1A) in skin after topical treatment with GW0742 occurs in ($+/+$) mice but not ($-/-$) mice using a stock concentration between 0.5 and 50 μM. (**d**) Ligand activaton of PPARβ *in vivo* causes induction of TG-I, involucrin, SPR1A, SPR2H and ADRP mRNAs in ($+/+$) but not ($-/-$) mouse skin. Mice were treated topically with 50 μM GW0742. Each lane represents mRNA isolated from an individual mouse. The fold-induction of normalized hybridization signals is presented below each representative Northern blot. *Significantly different than controls, $P \leq 0.05$. (**e**) PPARβ-dependent increase in keratinocyte terminal differentiation. The number of cornified envelopes was quantified as described in Materials and Methods

inhibit keratinocyte proliferation in addition to inducing gene products required for terminal differentiation. To examine this hypothesis, cell proliferation was measured in primary keratinocytes from wild-type and PPARβ-null mice, in the presence or absence of GW0742 at concentrations that specifically activate PPARβ as revealed by a reporter gene transactivation assay (Figure 3a). Indeed, a dose-dependent inhibition of cell proliferation was observed in response to 10–100 nM GW0742 in wild-type cells, and this effect was not observed in keratinocytes from PPARβ-null mice (Figure 3b). The decrease in keratinocyte proliferation was consistent with the observed decrease in PCNA expression found in GW0742-treated wild-type cells and not in similarly treated PPARβ-null keratinocytes (Figure 3c). This observation is consistent with previous reports showing decreased cyclin A expression and inhibition of cell proliferation in keratinocytes treated with a PPARβ ligand.[1,3]

Others have shown an anti-inflammatory effect of a related PPARβ ligand, GW1514, in mouse epithelium.[2] Since inhibition of inflammation is known to reduce cell proliferative signaling, the role of PPARβ in modulating this anti-inflammatory effect was examined by treating wild-type and

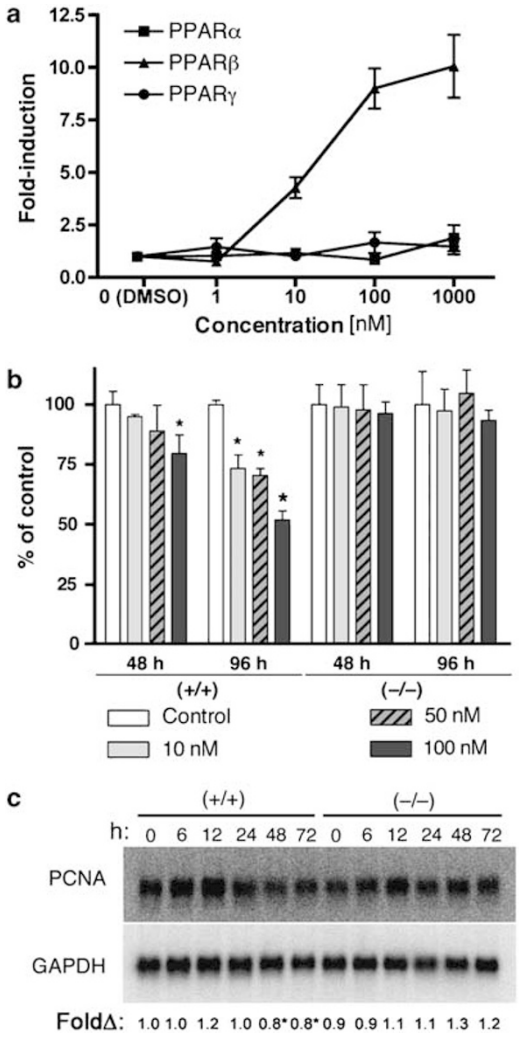

Figure 3 PPARβ-dependent inhibition of keratinocyte cell growth. (**a**) Specific activation of PPARβ by GW0742 at concentrations ranging from 10 to 100 nM based on trans-activation assay. (**b**) Inhibition of keratinocyte proliferation in wild-type (+/+) cells treated with 10–100 nM GW0742 that is not observed in similarly treated PPARβ-null (−/−) keratinocytes. *Significantly different than control, $P<0.05$. (**c**) Ligand activation of PPARβ leads to decreased mRNA encoding PCNA in wild-type keratinocytes, but not in those from PPARβ-null mice. Representative Northern blot from three independent experiments. Normalized hybridization signals are presented as fold change (Δ) as compared to control (+/+). Note significantly lower average PCNA mRNA levels in (+/+) cells treated with 100 nM GW0742 after 48 and 72 h of culture that is not found in (−/−) cells. *Significantly different than control, $P<0.05$

PPARβ-null mice with TPA in the presence or absence of GW0742. Myeloperoxidase (MPO) activity (a marker of inflammation in skin that correlates with neutrophil activation and the induction of cell proliferation signaling)[16] was markedly induced by TPA in wild-type mouse skin, and administration of GW0742 (4 mM) resulted in a significant decrease in TPA-induced MPO activity (Figure 4a). Consistent with the known exacerbation of epithelial inflammation in PPARβ-null mice in response to TPA,[5] MPO activity was significantly greater as compared to similarly treated wild-type mice, and administration of GW0742 caused a significant reduction in MPO activity in this genotype, indicating that the anti-inflammatory effect of GW0742 does not require PPARβ (Figure 4a). To distinguish between alterations in protein levels *versus* direct inhibition of enzyme activity, an enzyme assay was performed using recombinant MPO and the results from these experiments revealed that GW0742 is an uncompetitive inhibitor of MPO activity (Figure 4b). Interestingly, ligands for PPARα and PPARγ also inhibited MPO activity (Figure 4b). Administration of 50 μM GW0742, a concentration that specifically activates PPARβ (Figure 2d), did not result in a statistically significant inhibition of MPO activity in either wild-type or PPARβ-null skin after TPA treatment (Figure 4c). It is important to note that inhibition of MPO activity by GW0742 occurs at concentrations that are significantly greater than those required for specific PPARβ activation (Figures 2d and 3a). This demonstrates that the anti-inflammatory effect of GW0742 occurs through a PPARβ independent mechanism, and at concentrations that could potentially activate PPARα or PPARγ.[17] This observation also illustrates that ligand activation of PPARβ by GW0742 at concentrations that do not activate PPARα or PPARγ (e.g. ≤1 μM effectively inhibit keratinocyte cell growth and induce differentiation independently of the influence of decreased MPO activity. Collectively, these data provide strong evidence that ligand activation of PPARβ could represent a novel molecular target to inhibit keratinocyte cell proliferation for various hyper-proliferative skin diseases.

Discussion

These studies provide *in vitro* and *in vivo* evidence that activation of PPARβ can induce expression of gene products required for terminal differentiation and lipid accumulation in keratinocytes. Others have shown that specific PPARβ ligands can induce mRNA markers of differentiation in keratinocytes,[1–3] but these studies did not determine the specificity of ligand activation of PPARβ as demonstrated by the present studies. The current work extends the earlier observations by demonstrating that increased expression of differentiation marker mRNAs induced by the PPARβ ligand GW0742 is dependent on a functional receptor. For example, the present studies demonstrate that induction of TG-I, involucrin, SPR1A, SPR2H and ADRP requires PPARβ, and is consistent with previous work showing induction of SPR1B, SPR2C, SPR3 and ADRP in primary keratinocytes in response to the PPARβ ligand GW1514.[2] Additional studies are still necessary to demonstrate that SPRs and ADRP represent bona fide PPARβ target genes in keratinocytes by functional analysis of peroxisome proliferator response elements (DR-1) in their respective promoter regions. However, the requirement of PPARβ for induction of ADRP in keratinocytes is consistent with work showing that ADRP is a PPARβ target gene in macrophages using GW1516 and PPARβ-null cells.[18] That the PPARβ-dependent induction of mRNAs encoding differentiation markers leads to functional increases in keratinocyte differentiation was demonstrated by the increase in cornified envelopes, the end product of keratinocyte terminal differentiation. This is consistent with earlier studies showing improved barrier homeostasis of the stratum corneum after treatment with the PPARβ ligand GW1514.[2] It is of interest that similar mRNA targets

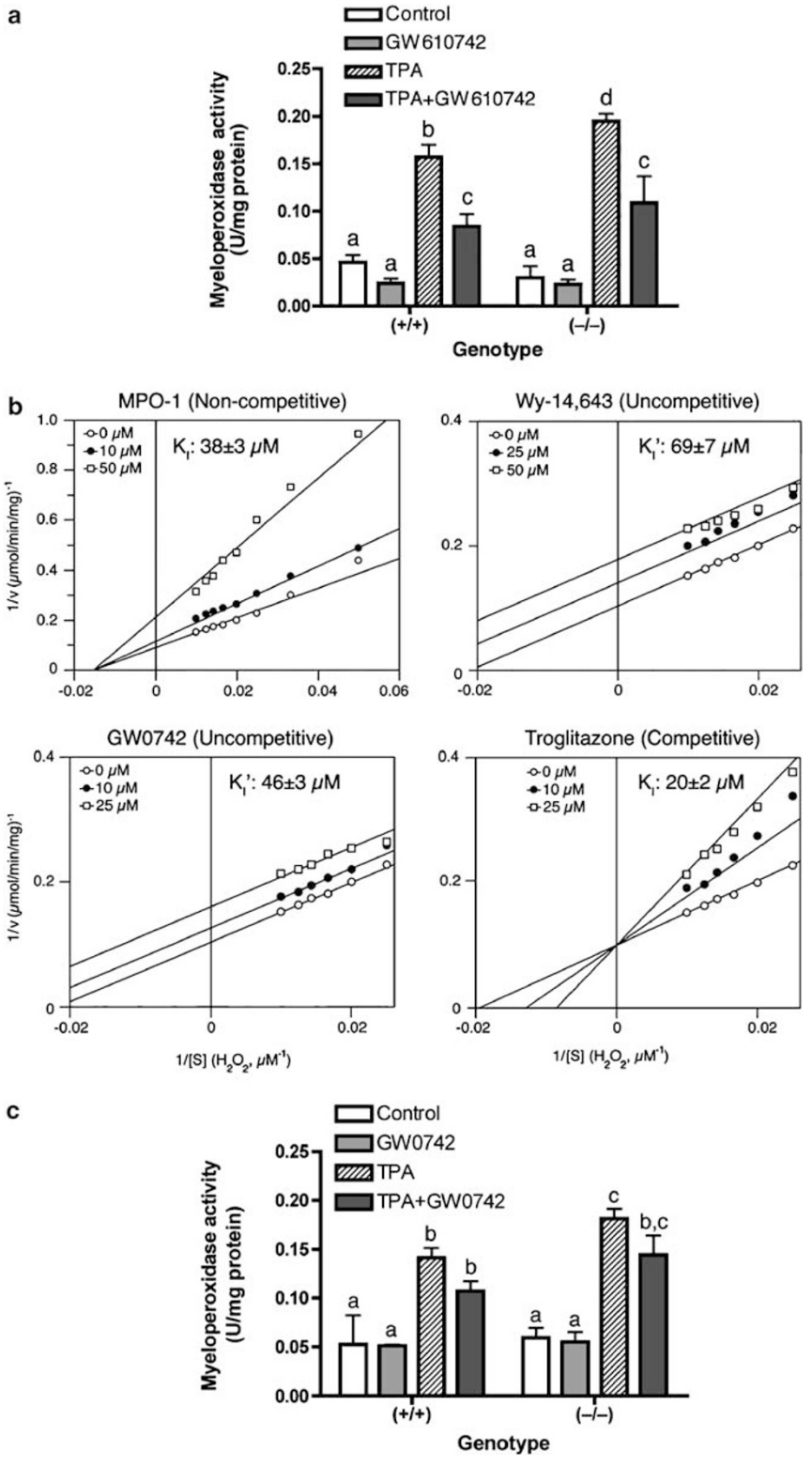

Figure 4 PPARβ-independent inhibition of myeloperoxidase (MPO) activity. (**a**) Inhibition of MPO activity in both wild-type ($+/+$) and PPARβ-null ($-/-$) mouse skin treated with TPA and 4 mM GW0742. *Values with different superscripts are significantly different at $P<0.05$. (**b**) Recombinant MPO was used to measure activity in the presence of either the positive control MPO inhibitor (MPO-I; Calbiochem), the PPARα ligand Wy,14,643, the PPARβ ligand GW0742, or the PPARγ ligand troglitazone. K_I values represent the mean from regression curves obtained from five to eight different concentrations of inhibitor, as described in Materials and Methods. For uncompetitive inhibitors, the K_I represents the K_I' as calculated for this type of inhibition. (**c**) MPO activity in both ($+/+$) and ($-/-$) mouse skin treated with TPA and 50 μM GW0742. *Values with different superscripts are significantly different at $P<0.05$

(e.g. involucrin, loricrin, TG-I, etc.) are induced by ligand activation of PPARα and PPARγ in skin,[19–21] and some of these changes in gene expression appear to require the presence of either PPARα or PPARγ.[20,21] The specificity for PPAR ligand-induced changes in epithelial differentiation deserves further investigation to determine if there is cross talk between the different PPARs, since earlier studies used stock concentrations of PPAR ligands (e.g. mM) that could effectively activate other PPAR isoforms.[19–21]

Results from the current studies demonstrate that ligand activation of PPARβ can selectively induce SPR1A and SPR2H, gene products that only appear to function as precursors of the cornified envelope composed of terminally differentiated keratinocytes, after covalent linkage mediated by TG-I. However, the present studies also demonstrate that induction of terminal keratinocyte differentiation by TPA or Ca^{2+} signaling (including SPR1A, SPR2H, TG-I, involucrin, etc.) is not dependent on PPARβ because similar changes in gene expression are observed in PPARβ-null keratinocytes. This suggests that while TPA and/or increased Ca^{2+} signaling likely results in increased intracellular levels of natural PPARβ ligands that function to attenuate cell proliferation and induce differentiation, other transcription factors 'override' these PPARβ-dependent functional changes. There are likely a number of transcription factors that can modulate gene expression required for terminal differentiation of keratinocytes including AP-1, and results from these studies suggest that PPARβ functions to potentiate this response. Since PPARβ-null mice exhibit no overt differences in their epithelium, this also suggests that PPARβ is not required for terminal differentiation *per se*. However, it is noteworthy that PPARβ-null neonates have significantly fewer cornified envelopes. Others have suggested that TPA-induced differentiation is mediated by PPARβ.[1] This conclusion was based on the observation that the induction of mRNAs encoding involucrin, TG-I and K6 by TPA was attenuated in cultured primary keratinocytes isolated from PPARβ-null mice. However, there are several issues that raise some uncertainty for these studies. For example, TG-I mRNA is increased maximally within 6 h post-TPA treatment, and within 10 h in response to increasing culture medium Ca^{2+} in primary keratinocytes,[12] findings that are consistent with results from the present studies. In contrast, increased TG-I mRNA was not observed until 24 h post-TPA treatment when passaged keratinocytes were used to determine the role of PPARβ in this process.[1] More importantly, it is well documented that cultured primary keratinocytes constitutively express K6, and that expression of this keratin is used as a marker of hyperproliferation rather than differentiation.[22,23] While results from the present studies are consistent with these findings, the passaged keratinocytes used to suggest that PPARβ mediates TPA-induced increases in differentiation markers lacked constitutive expression of K6.[1] Additionally, an increase in the expression of mRNA encoding K6 was also observed 6 h post-TPA treatment.[1] Thus, the expression pattern of standard mRNA markers in the passaged keratinocytes[1] is inconsistent with that typically observed in primary keratinocytes using the well-characterized methods used to study keratinocyte-specific mechanisms of regulation.[24] This suggests that the observed attenuation of TPA-induced changes in differentiation marker mRNAs[1] could reflect differences that are dependent on changes associated with cells that have undergone alterations after many passages. Lastly, the morphological appearance of primary keratinocytes cultured in either TPA or high Ca^{2+} medium in the present studies, is indistinguishable between genotypes, which is inconsistent with the hypothesis that PPARβ mediates TPA-induced differentiation as suggested by others.[1] Collectively, results from the present studies show that the expression pattern of mRNA markers of differentiation in control and PPARβ-null cells is consistent with historical observations, suggesting that PPARβ is not required to mediate terminal differentiation induced by TPA and Ca^{2+}. However, since exogenous treatment with a potent PPARβ ligand can selectively induce keratinocyte differentiation and inhibit proliferation in the absence of cell signaling that functions to stimulate cell growth such as that occurs in response to tumor promotion, ligand activation of PPARβ may represent a new molecular target that could be used for the treatment of hyperproliferative skin diseases (Figure 5). Whether the observed inhibition of keratinocyte cell proliferation is mediated by PPARβ-dependent modulation of unidentified target genes that attenuate cell growth, or is simply due to the induction of terminal differentiation, which is known to be associated with inhibition of cell proliferation, remains to be determined (Figure 5).

Results from these studies also demonstrate that GW0742 is an uncompetitive inhibitor of MPO activity, and that this anti-inflammatory activity is not receptor dependent. Since the K_I is significantly greater than that required to specifically activate PPARβ, this suggests that the inhibition of inflammation through this mechanism should not interfere with future studies aimed at delineating the molecular events that mediate differentiation and in particular, inhibition of cell proliferation, as inflammation can significantly influence the latter process by increasing the production of cytokines and other signaling molecules. This is also supported by the observation that inhibition of MPO activity occurred after topical application of 4 mM GW0742 but not with 50 μM GW0742. Others have suggested that ligand activation of PPARβ results in an anti-inflammatory response,[2] but results from our studies demonstrate that this effect is not dependent on PPARβ and likely occurs in part through direct competition with MPO substrates. While the significance of this observation is uncertain, inhibition of MPO by the three PPAR ligands tested here suggests that this effect likely has impacted a number of previous studies examining the anti-inflammatory properties of PPAR ligands, especially those studies using ligands at concentrations that will effectively inhibit MPO. All three of the tested PPAR ligands are lipophillic acids and this shared property may be responsible for their effect on MPO activity at high concentrations. Whether this, or other receptor-independent activities of GW0742, modulates cellular processes deserves further evaluation.

The anti-inflammatory property and the ability of GW0742 to induce keratinocyte differentiation and inhibit cell proliferation, are remarkably similar to effects induced by PPARγ ligands (reviewed in Michalik *et al*.[25]). Thus, it will also be of great interest to determine whether ligand activation of PPARβ will inhibit carcinogenesis, similar to the hypothesis

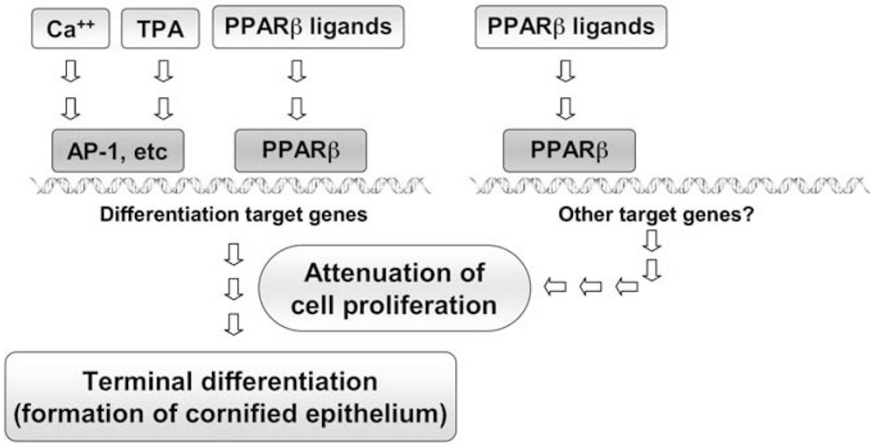

Figure 5 Hypothetical model of PPARβ-dependent regulation of differentiation and inhibition of cell proliferation. In the absence of other transcriptional regulators such as AP-1 that is known to mediate differentiation induced by Ca^{2+} and/or promote tumor formation and enhanced cell proliferation (e.g. TPA), PPARβ ligands can selectively induce differentiation through the regulation of proteins required for this process, and inhibit cell proliferation of keratinocytes. While the induction of keratinocyte terminal differentiation likely contributes to the inhibition of cell growth, it is also possible that other PPARβ-dependent target genes modulate this effect. Combined, these effects could possibly be applied to inhibit skin carcinogenesis or other hyper-proliferative skin disorders, and requires further examination

examined with clinical trials testing the efficacy of ligand activation of PPARγ as a chemopreventive/chemotherapeutic approach.[26,27]

Materials and Methods

Differentiation analysis

Primary keratinocytes obtained from 2-day-old neonates from wild-type (+/+) or PPARβ-null (−/−) mice were cultured as previously described. [24] To induce differentiation, keratinocytes were cultured until 80–85% confluent at which time the medium was replaced with one containing either 0.12 mM Ca^{2+} or 0.05 mM Ca^{2+} and 25 ng TPA/ml of medium. To determine the specific effect of PPARβ ligand activation, keratinocytes were cultured in medium containing 0.05 mM Ca^{2+} and 100 nM GW0742. Representative photomicrographs of keratinocyte morphology were obtained and RNA was isolated from cultured cells after 6, 12, 24, 48, and 72 h of treatment. In all, 5 μg of total RNA was electrophoresed on a 1.0% agarose gel containing 0.22 M formaldehyde, transferred to a nylon membrane, and ultraviolet crosslinked to fix the RNA. Membranes were hybridized in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA) with random primed probes following the manufacturer's protocol and washed with salt/detergent solutions using standard procedures. The following previously described cDNA probes were used: PPARβ, transglutaminase-I (TG-I), involucrin, small proline-rich protein 1A (SPR1A), SPR2H, adipocyte differentiation-related protein (ADRP), loricrin, keratin 6 (K6) or GAPDH.[5,28] Hybridization signals were quantified (OptiQuant, version 4.0, Packard Instrument Co.) and are expressed as a fold-induction relative to wild-type control. The fold-induction of normalized hybridization signals is presented below each representative Northern blot. Statistical comparisons between groups were made using ANOVA (Prism 4.0b, GraphPad Software) and posthoc testing. To examine the effect of ligand activation of PPARβ *in vivo*, (+/+) and (−/−) mice were shaved of dorsal hair and treated with GW0742 (200 μl of either 0, 0.5, 5.0, 50, or 500 μM stock solution dissolved in acetone, per mouse). At 8 h post-treatment, skin was obtained and RNA isolated as described above. Northern blot analysis of keratinocyte differentiation marker mRNAs was as described above. To quantify keratinocyte terminal differentiation, 2-day-old neonates from wild-type or PPARβ-null mice were treated topically with 50 μM GW0742 and 24 h later, the number of cornified envelopes was determined using a standard method described by others.[29]

Transfection

To determine the concentration range capable of specifically activating PPARβ, mouse 3T3-L1 fibroblasts were transfected with a reporter construct containing both the ligand binding domain of mouse PPARα, PPARβ, or PPARγ fused with the DNA-binding domain of Gal4 under the control of the SV40 promoter, and the UAS-firefly luciferase reporter under the control of the Gal4 DNA response element.[30] Post-transfection, cells were cultured overnight and then treated with GW0742 at concentrations ranging from 1 nM to 1 μM for 24 h. After treatment with GW0742, luciferase activity was measured from triplicate cell lysates and normalized to the protein concentration of each sample. The fold induction of normalized luciferase activities was calculated relative to DMSO (vehicle)-treated cells, and represents the mean of at least three independent samples per treatment group.

Keratinocyte proliferation

Primary keratinocytes were cultured as described previously[31] in low calcium medium (0.05 mM). with and without GW0742 at a concentration from 10 to 100 nM, and cell growth was measured using a Z1 coulter® particle counter (Beckman Coulter Inc.,). Cumulative cell growth was determined by quantifying the number of attached and unattached cells and is presented as the mean percentage of control cells cultured in the absence of GW0742. Northern blot analysis was performed using total RNA isolated from keratinocytes cultured with GW0742 after 0 and 72 of treatment. Membranes were probed with random primed radio-labeled probes for PCNA or GAPDH and hybridization signals were normalized to GAPDH.

MPO assays

Wild-type or PPARβ-null mice were treated with either acetone, 5 μg of TPA dissolved in 200 μl of acetone, 200 μl of either 4 mM or 50 μM

GW0742 dissolved in acetone, or TPA and GW0742. For the latter treatment, mice were treated with 5 μg TPA as described, followed 1, 3, and 5 h later with 200 μl of either 4 mM or 50 μM GW0742. Cytosolic protein samples were obtained from the skin of mice and used for analysis of MPO activity as previously described.[16] Enzyme kinetics of MPO were performed using purified recombinant MPO (Sigma Chemical Co., St. Louis, MO, USA) in the absence and presence of specific PPAR ligands (Wy-14,643 for PPARα, GW0742 for PPARβ and troglitazone for PPARγ), and a specific MPO inhibitor (Calbiochem) at concentrations ranging from 1 to 50 μM. MPO activity was measured as described and the K_I or K_I' were determined by a global fit of the data from an entire experiment (GraFit, Version 5.0).

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