Peroxisome Proliferator-Activated Receptor-α Enhances Lipid Metabolism in a Skin Equivalent Model

Michel Rivier, Isabelle Castiel*, Irina Safonova, Gérard Ailhaud,† and Serge Michel

GALDERMA R&D, Sophia-Antipolis cedex, Valbonne, France; *L'OREAL Life Sciences Research, Centre C. Zviak, Clichy, France; †Laboratoire de Biologie du Tissu Adipeux, Centre de Biochimie (C.N.R.S.), Faculté des Sciences, Parc Valrose, Nice, France

Peroxisome proliferator-activated receptors are involved in certain cell types such as adipocytes and hepatocytes, in the control of several pathways of lipid synthesis or catabolism by regulating the gene expression level of key lipid metabolizing enzymes. As the epidermis exhibits an extensive lipid metabolism necessary for the establishment of the barrier function, we have examined the role of peroxisome proliferator-activated receptor-α activation in this process. Living skin equivalents were treated with Wy 14,643, a selective peroxisome proliferator-activated receptor-α ligand, which enhanced greatly the synthesis of membrane coating granules, the organelles specialized in the processing of stratum corneum lipids. Also, the overall stratum corneum neutral lipid content assessed by Oil red O staining was increased. A detailed analysis of the lipid species present in the reconstructed epidermis showed that peroxisome proliferator-activated receptor-α activation increased the synthesis of ceramides and cholesterol derivatives, thought to be essential structural components of the permeability barrier. A synergistic effect was observed on lipid synthesis when peroxisome proliferator-activated receptor-α and retinoid X receptor were simultaneously activated by selective ligands. Furthermore, activation of peroxisome proliferator-activated receptor-α led to increased mRNA expression of several key enzymes of ceramide and cholesterol metabolism. An increase of serine-palmitoyl transferase and of β-glucocerebrosidase enzymatic activity was also demonstrated. Altogether, these results show that peroxisome proliferator-activated receptor-α is a key transcription factor involved in the control of the epidermal lipid barrier. ***Key words: keratinocytes/peroxisome proliferator-activated receptor-α/lipids. J Invest Dermatol 114:681–687, 2000***

The epidermis is an active site of lipid synthesis. Except for essential fatty acids that are obtained from the circulation, keratinocytes are able to synthesize all lipid species necessary for the proper functioning of the epidermis. During the migration of keratinocytes from the basal layer to the stratum corneum, a substantial shift in lipid composition takes place (reviewed by Schurer and Elias, 1991; Wertz and Downing, 1991). These changes include a progressive depletion in phospholipids and glycosphingolipids, concomitant with an enrichment in ceramides, cholesterol, free fatty acid, and small amounts of cholesterol sulfate and cholesterol esters (Lampe *et al*, 1983). These specialized lipids, which constitute the structural components of the epidermal permeability barrier, are secreted into the extracellular space by specific cell organelles: the lamellar bodies or membrane coating granules (MCG) (Landmann, 1986). These organelles also contain hydrolytic enzymes including acid phosphatases, lipases (phospholipase A, sphingomyelinase, steroid sulfatase) and a family of glycosylases.

Manuscript received November 13, 1999; revised January 7, 2000; accepted for publication January 11, 2000.

Reprint requests to: Dr. S. Michel, GALDERMA R&D, 635 route des Lucioles, B.p. 87, 06902 Sophia-Antipolis cedex, Valbonne, France. Email: serge.michel@galderma.com

Abbreviations: eFABP, epidermal fatty acid binding protein; MCG, membrane coating granule; NHK, normal human keratinocytes; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SPT, serine palmitoyl transferase.

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors that belong to the steroid/thyroid/retinoid receptor superfamily. Three different subtypes (α, δ, and γ) have been described in man. They heterodimerize with the retinoid X receptor (RXR) and bind specific *cis*-acting elements, called peroxisome proliferator-activated receptor response elements, located in the promoter of target genes. In target cells or tissues, PPAR are involved in the control of lipid homeostasis. On one hand, PPAR-γ is one of the main factors controlling (i) terminal differentiation of preadipocytes to adipocytes, and (ii) the lipogenesis process which includes the intracellular transport, activation, and esterification of fatty acids as triglycerides (Kliewer *et al*, 1994; Tontonoz *et al*, 1994; Lehmann *et al*, 1995; Braissant *et al*, 1996). In cells of the immune system, PPAR-γ is involved in the control of the inflammatory responses (Ricote *et al*, 1998). PPAR-α is implicated in peroxisomal metabolism and fatty acid oxidation in tissues such as liver, heart, muscle, kidney, and intestine (Issemann and Green, 1990; Kliewer *et al*, 1994).

A recent study from our laboratory has shown that all PPAR subtypes are expressed in human epidermal keratinocytes (Rivier *et al*, 1998) and that the expression of PPAR-α and -γ is increased in the course of keratinocyte differentiation. Until now, however, only PPAR-α has been shown to be functional (Rivier *et al*, 1998). Another study has demonstrated that clofibrate, a selective but weak PPAR-α activator, can accelerate when applied to rat fetal skin explants the formation of the epidermal permeability barrier (Hanley *et al*, 1997). Moreover, clofibrate can increase, in cultured

keratinocytes, the expression of keratinocyte differentiation markers (Hanley *et al*, 1998). This suggests that PPAR-α may play a part in epidermal differentiation as well as in the formation and maintenance of a functional lipid barrier.

In this study, we have investigated the effect of PPAR-α activation, with the selective ligand Wy 14,643, on lipid synthesis in a skin equivalent model. We have also determined the regulatory role of PPAR-α, on the expression of epidermal MCG and mRNA encoding lipid metabolizing enzymes.

MATERIALS AND METHODS

Retinoid 4-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydro-naphthalen-2-yl-sulfanyl)-benzoic acid (CD2624) was synthesized at GALDERMA R&D (Valbonne, France).

Reconstructed epidermis on a type I collagen dermal equivalent Adult interfollicular epidermal cells, isolated from human breast skin obtained after plastic surgery, were amplified and stored in liquid nitrogen. The cells were defrozen and seeded on a type I collagen dermal equivalent (Asselineau *et al*, 1985). The cultures were first kept submerged in the culture medium for 1 wk to obtain a confluent monolayer (day 0) and were then raised at the air–liquid interface to produce a stratified and keratinized epithelium. The culture medium consisted of minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal bovine serum, epidermal growth factor (10 ng per ml), hydrocortisone (0.4 μg per ml), and cholera toxin (10^{-9} M). Medium was changed three times a week. The compounds solubilized at 10^{-2} M in dimethyl sulfoxide were added to the culture medium to give the final concentration indicated in the legends for figures. The dimethyl sulfoxide concentration did not exceed 0.02% (vol/vol). The morphology of the reconstructed skin was evaluated by staining vertical paraffin section with hemalum–phloxin–saffron.

Oil Red O staining Briefly, frozen sections were washed for 5 min in water and stained for 15 min with 0.3% (wt/vol) oil red diluted in a mixture of isopropanol/water (60/40, vol/vol). The sections were quickly rinsed in water and counterstained for 5 min with hematoxylin.

Immunofluorescence staining Unfixed tissue samples were rinsed in phosphate-buffered saline, embedded in Tissue-Tek OCT compound (Miles, Naperville, IL), frozen in liquid nitrogen, and stored at –80°C. Frozen sections (5 μm) were briefly rinsed in phosphate-buffered saline, and then incubated with the monoclonal antibody AE17, a kind gift of Dr. T.T. Sun (New York University, New York), directed against human membrane coating granules (O'Guin *et al*, 1989). After washing with phosphate-buffered saline, the sections were incubated with rabbit anti-mouse fluorescein isothiocyanate labeled polyclonal antibody (F0232, DAKO S.A, Trappes, France).

Sections were also incubated with a rabbit polyclonal antibody directed against human PPAR-α, a generous gift of Dr. J. Najib (LBRE U325 INSERM, Institut Pasteur, Lille, France). In this case, the secondary antibody was a swine-anti-rabbit fluorescein isothiocyanate labeled polyclonal antibody (F02025 DAKO S.A, Trappes, France).

High performance thin-layer chromatography (HPTLC) Epidermal lipids were extracted using the method of Bligh and Dyer (1959), dissolved in chloroform/methanol (2:1, vol/vol) and stored into liquid nitrogen until use. Lipids were separated by one-dimensional HPTLC on 10 × 20 cm glass plates coated with silica gel (Kieselgel 60, Merck) as described elsewhere (Ponec *et al*, 1988). For quantitation, lipid standards consisting of cholesterol, cholesteryl oleate, cholesterol-3 sulfate, oleic acid, tripalmitin, 1,2-1,3 diolein, 1,3 diolein, ceramide *III*, ceramide *IV*, cerebroside *II* (Sigma), were run in parallel. The quantitation was performed by scanning densitometry (Camag) after charring of the lipids (Ponec *et al*, 1988).

RNA isolation Total RNA from reconstructed epidermis was prepared as described by Chomczynski and Sacchi (1987) and stored at –80°C until use.

Reverse transcription–polymerase chain reaction (reverse transcription–PCR) and semiquantitative PCR The oligonucleotide primers for PCR were synthesized by Gibco BRL (Cergy Pontoise, France) and were as follows: GAPDH (GenBank accession number M33197) sense oligonucleotide (5′-AATCCCATCACCATCTTCCA-3′) and anti-sense oligonucleotide (5′-GTCATCATATTTGGCAGGTT-3′); Acyl CoA oxidase (GenBank accession number U07866) sense oligonucleotide (5′-GCCTATGCCTTCCAGTTTGT-3′) and anti-sense oligonucleotide (5′-TGGGCAGGTCGTTCAAATAG-3′); long chain acyl-CoA synthase (GenBank accession number D10040) sense oligonucleotide (5′-AAGGATACGGACAGACAGAG-3′) and anti-sense oligonucleotide (5′-TGTAACCAGCCGTCTTTGTC-3′); epidermal fatty acid binding protein (eFABP) (GenBank accession number M94856) sense oligonucleotide (5′-CAAGCCAGATTGTATCATCA-3′) and anti-sense oligonucleotide (5′-TTCATAGATCCGAGTACAGG-3′); β-glucocerebrosidase (GenBank accession number M16328) sense oligonucleotide (5′-CTTCTGCTGGGCTGTTGAGT-3′) and anti-sense oligonucleotide (5′-GAGGTTCGTGATGATGCTGT-3′); ceramide glucose synthase (GenBank accession number D50840) sense oligonucleotide (5′-GCTTTGCTGCCACCTTAGAG-3′) and anti-sense oligonucleotide (5′-AAACTGGCAACAAAGCATTC-3′); serine palmitoyl transferase (SPT2) (GenBank accession number U15555) sense oligonucleotide (5′-TGTCAGGAGCAACCATTAGA-3′) and anti-sense oligonucleotide (5′-CCCATCATAACATCCACATC-3′); sphingo-myelinase (GenBank accession number M59916) sense oligonucleotide (5′-CGGATA-CTGGGGCGAATACA-3′) and anti-sense oligonucleotide (5′-TGGGGAAAGAGCATAGAACC-3′); steroid sulfatase (GenBank accession number M16505) sense oligonucleotide (5′-CACCCTCCT-TACCCTTGCTG-3′) and anti-sense oligonucleotide (5′-GCTCCC-TGGTCCGATGTGAA-3′); 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (GenBank accession number M11058) sense oligonucleotide (5′-ACTTCACGAGTATTTCCCTG-3′) and anti-sense oligonucleotide (5′-ACCACCCACCGTTCCTATCT-3′); HMG CoA synthase (GenBank accession number X66435) sense oligonucleotide (5′-GTTCCCTTGCATCTGTTCT-3′) and anti-sense oligonucleotide (5′-CCGAGCGTAAGTTCTTCTGT-3′). The amplification products were predicted to be 558 bp for GAPDH, 367 bp for acyl-CoA oxidase, 244 bp for long chain acyl-CoA synthase, 280 bp for eFABP, 407 bp for β-glucocerebrosidase, 347 bp for ceramide glucose synthase, 310 bp for SPT2, 390 bp for sphingomyelinase, 451 bp for steroid sulfatase, 413 bp for HMG CoA reductase and 352 bp for HMG CoA synthase.

Reverse transcription–PCR and semiquantitative PCR were carried out using 5 μg of total RNA extracted from reconstructed epidermis as previously described (Rivier *et al*, 1998).

SPT and β-glucocerebrosidase assay Reconstructed epidermis was cut into small pieces, homogenized and sonicated at 4°C in assay buffer (100 mM HEPES, pH 8.3, 5 mM dithiothreitol, 2.5 mM ethylenediamine tetraacetic acid). The tissue homogenate was centrifuged at 10,000 × *g* for 10 min. To prepare a microsomal fraction, the resulting supernatant was centrifuged at 35,000 × *g* for 120 min. The microsomes were then resuspended in storage buffer [50 mM HEPES, pH 7.4, 5 mM dithiothreitol, 20% (vol/vol) glycerol]. The assay for SPT activity was performed using 100 μg of microsomal protein as described (Holleran *et al*, 1990), except that the specific activity of [^{3}H]L-serine was 30,000 dpm per nmol. SPT activity was expressed as pmoles of 3-ketosphinganine formed per min and per mg microsomal protein.

β-glucocerebrosidase activity was determined exactly as described by Holleran *et al* (1992). Results were expressed as nmoles of 4-methyl-umbelliferone synthesized per min and per mg protein.

Statistical analysis The results are presented as mean ± SEM of the replicates (n = 3) of three different independent experiments. They were analyzed using the two-sided Student's t test. Differences at $p < 0.05$ (*) were considered to be significant. Differences at $p < 0.01$ (**) or $p < 0.005$ (***) were also indicated.

RESULTS

Effect of PPAR-α activation on MCG synthesis Immunofluorescence staining of reconstructed skin performed with a polyclonal antibody directed against human PPAR-α revealed that this receptor is expressed specifically in the nuclei of suprabasal keratinocytes (**Fig 1**).

The effect of PPAR-α activation on the regulation of epidermal lipid homeostasis was studied using a skin equivalent. In this model the characteristic features of keratinocyte differentiation are maintained which is not the case in classical submerged keratinocyte cultures (Asselineau *et al*, 1989). The PPAR-α selective ligand Wy 14,643 was added at different concentrations (from 25 to 75 μM) for 7 d after air exposure of the skin equivalent. Initially, the morphology of the reconstructed epidermis was assessed by histologic staining. No striking differences were

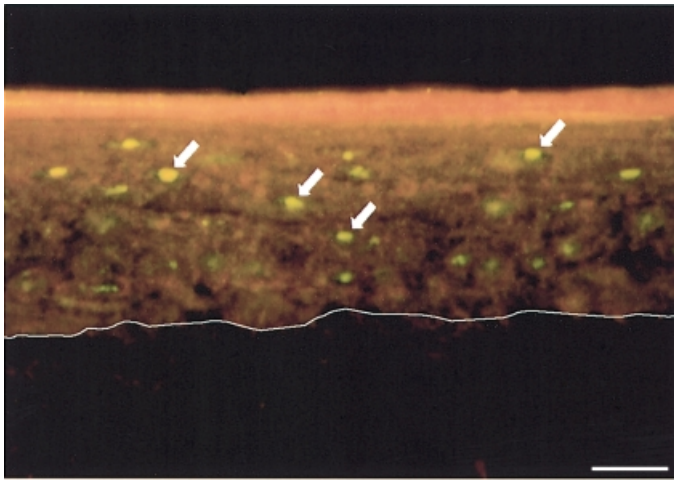

Figure 1. Localization of PPAR-α in reconstructed epidermis. Frozen sections of reconstructed skin were incubated with a polyclonal antibody directed against human PPAR-α. Labeled nuclei are indicated by arrows. *Scale bar*: 20 μm.

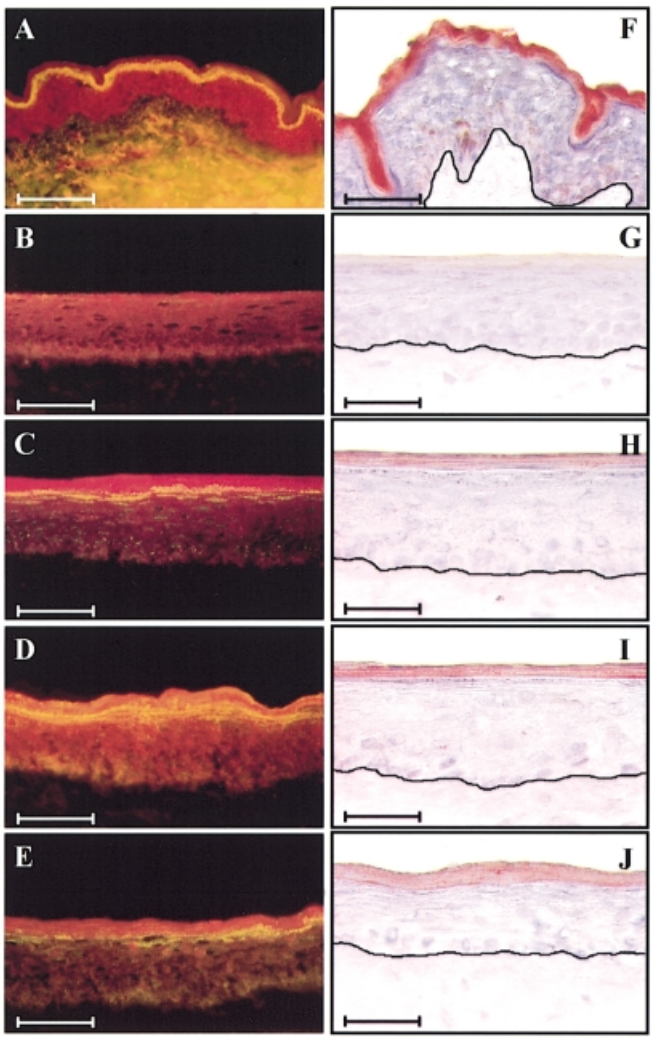

Figure 2. Effect of the selective PPAR-α activator Wy 14,643 on MCG and neutral lipid content of reconstructed epidermis. Frozen sections of human skin (*A*, *F*) or reconstructed epidermis treated with control dimethyl sulfoxide (*B*, *G*) or Wy 14,643 at different concentrations (*C*, *H*: 25 μM; *D*, *I*: 50 μM; *E*, *J*: 75 μM). Sections were reacted with the monoclonal anti-MCG antibody (*A–E*) or stained using Oil Red O (*F–J*). *Scale bars*: (*A–E*) 80 μm; (*F–J*) 40 μm.

observed between treated and control epidermis. A slight increase in the number of layers containing keratohyaline granules, however, was observed after treatment of the skin equivalent with 50 or 75 μM of Wy 14,643 (results not shown). This increase was accompanied by a thickening of the stratum corneum. Immunofluorescence staining with antibodies directed against keratin 1, transglutaminase type 1, and loricrin showed no marked differences between control and treated cells (results not shown). Immunofluorescence staining with a monoclonal antibody directed against MCG, however, revealed that treatment of skin equivalents with Wy 14,643 for 7 d led to a dose dependent increase in MCG content of the upper stratum spinosum and stratum granulosum (**Fig 2**). The localization of MCG was similar to that observed in normal skin (**Fig 2*A***).

Effect of PPAR-α activation on lipid quantity and composition To assess the effect of Wy 14,643 on the overall neutral lipid content of the reconstructed epidermis, frozen sections were stained with Oil Red O. As shown in **Fig 2**, the neutral lipid content increases in a concentration-dependent manner in response to Wy 14,643 treatment, which is consistent with the observed effect on MCG synthesis.

To analyze more thoroughly the changes in the lipid content, the different lipid species were separated by HPTLC. They were then quantitated by scanning densitometry after charring of the chromatography plates. **Figure 3** shows that after treatment of reconstructed skin with 75 μM of Wy 14,643, the amount of cholesterol sulfate (4.9-fold, $p < 0.01$), cholesteryl esters (3.7-fold, $p < 0.005$) and total ceramides (3.1 fold, $p < 0.05$) increased significantly. The triglyceride level seems also increased, but the difference is statistically not significant. The above changes were dependent of the Wy 14,643 concentration used. On the other hand, the amount of glucosylceramides, free fatty acids, cholesterol, and lanosterol remained unchanged. Also no modification of the total phospholipid content was observed (results not shown). A subsequent analysis of the different ceramides was performed (**Fig 4*A***) the amount of which increased from 1.9-fold to 9.2-fold except for ceramides 7. Of note is the increase of the atypical ω-OH acyl-ceramide (ceramide 1) (4.8-fold, $p < 0.05$). On the other hand, a significant decrease of ceramide 6 was observed. As

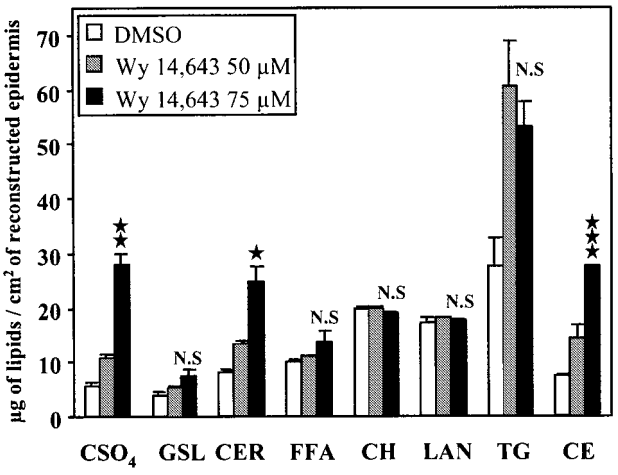

Figure 3. Effect of the selective PPAR-α activator Wy 14,643 on lipid synthesis by reconstructed epidermis. Reconstructed epidermis was treated for 7 d during air exposure with Wy 14,643 at 50 and 75 μM and HPTLC analysis of the total lipid content was performed. After charring of the chromatography plates, lipid species were quantitated by scanning densitometry. Abbreviations are: cholesterol sulfate (CSO_4), glycosphingolipids (GSL), ceramides (CER), free fatty acids (FFA), cholesterol (CH), lanosterol (LAN), triglycerides (TG), cholesteryl esters (CE). The results are presented as mean ± SEM of the replicates ($n = 3$) of two different experiments. Differences at $^*p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.005$ are indicated. N.S, nonsignificant.

shown in **Fig 4(*B*)**, and except for ceramides 1, 3, and 6, the percentage of each ceramide fraction in the total ceramide content of reconstructed epidermis did not change after treatment.

An RXR agonist (CD2624) increases the effect of PPAR-α activation on lipid composition In view of the fact that PPAR heterodimerize with RXRs to control gene expression, reconstructed skin was treated with an RXR agonist (CD2624) (see Spanjaard *et al*, 1997 for its retinoid receptor selectivity) in combination with Wy 14,643. The treatment was also performed with Wy 14,643 at the suboptimal concentration of 50 μM or with 1 μM CD2624 alone. **Figure 5** shows that, except for cholesterol esters, Wy 14 463 (see also **Fig 4**), or CD2624 had little effect on lipid synthesis. Treatment of reconstructed skin with a mixture of Wy 14,643 and CD2624, however, increased synergistically the level of ceramide (2.3-fold, $p < 0.05$). As shown in **Fig 5**, the major ceramides species,C1, C2, and C3, were increased from 1.8- to 4.6-fold. In addition, the two compounds displayed additive effects on the level of cholesterol sulfate (2.0-fold, $p < 0.05$).

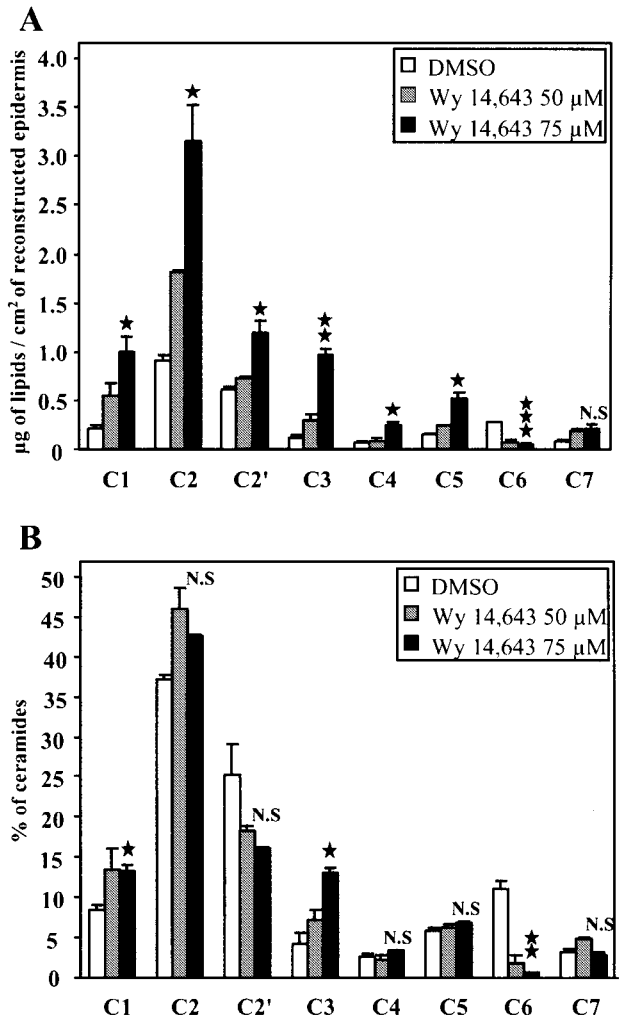

Figure 4. Activation of PPAR-α with the selective agonist Wy 14,643 increases the ceramide content of reconstructed epidermis without affecting the general ceramide profile. Reconstructed epidermis was treated for 7 d during air exposure with Wy 14,643 at 50 and 75 μM and analysis of the ceramide content was performed using HPTLC. After charring of the chromatography plates, lipid species were quantitated by scanning densitometry. Ceramides (C1–C7) were named as previously described (Ponec *et al*, 1997) except for ceramide 2 that was separated into two fractions (C2 and C2′). The results are presented as mean ± SEM of the replicates (n = 3) of two different experiments. Differences at $*p < 0.05$, $**p < 0.01$, or $***p < 0.005$ are indicated. N.S, nonsignificant. (*A*) The ceramides are expressed as μg of lipids per cm^2 of reconstructed epidermis. (*B*) The ceramides are expressed as percentage of the total ceramide content.

PPAR-α activation increases the level of mRNAs encoding lipid metabolizing enzymes Next we examined the effect of PPAR-α activation on the expression by reconstructed epidermis of mRNA encoding enzymes involved in lipid homeostasis. Enzymes encoded by genes containing a peroxisome proliferator-activated receptor response element in their promoter, i.e., acyl-CoA oxidase and long chain acyl-CoA synthase (Lemberger *et al*, 1996) were used as positive controls. We also analyzed the mRNA level of enzymes involved in the different pathways of ceramide synthesis (SPT2, β-glucocerebrosidase, ceramide glucose synthase and sphingomyelinase) and enzymes involved in cholesterol metabolism (HMG CoA reductase, HMG CoA synthase, and steroid sulfatase). As it is known that the gene encoding adipocyte fatty acid binding protein contains a peroxisome proliferator-activated receptor response element in its promoter (Lemberger *et al*, 1996), we also determined the expression of eFABP mRNA. As shown in **Fig 6** the different mRNAs could be detected by reverse transcription–PCR in reconstructed epidermis. The quantitation of the mRNAs was performed by semiquantitative reverse transcription–PCR. To study the effect of PPAR-α activation on mRNA expression, cultures of reconstructed epidermis were treated for 24 h with Wy 14,643 either at day 4 or 8 after air exposure. The first treatment condition allowed the study of the effect of Wy 14,643 during reconstruction of the epidermis, whereas in the second treatment protocol the PPAR-α selective agonist was applied on the already reconstructed epidermis. As shown in **Fig 7(*A*)**, the mRNA level of the control markers acyl-CoA oxidase and long chain acyl-CoA synthase was increased 1.9-fold ($p < 0.01$) and 2.5-fold ($p < 0.01$) in treated reconstructing epidermis at day 4, but no effect was observed at day 8. As shown in **Fig 7(*B*)**, after treatment at day 4 with Wy 14,643, the mRNA level of the ceramide synthesizing enzymes β-glucocerebrosidase, ceramide glucose synthase, and SPT2, increased 2.5-fold ($p < 0.01$), 2.3-fold ($p < 0.05$), and 2.1-fold ($p < 0.01$), respectively. The mRNA expression of sphingomyelinase remained unchanged. No effect of Wy 14,643 was observed at day 8 on the enzyme mRNA levels. Also, the mRNA expression of eFABP was unchanged.

As shown in **Fig 7(*A*)**, mRNA expression of HMG CoA synthase was markedly increased by Wy 14,643 treatment at day 4 (3.5-fold; $p < 0.005$) and at day 8 (5.3-fold; $p < 0.005$), whereas that of HMG CoA reductase was slightly but significantly increased at day 4. The mRNA level of steroid sulfatase was not affected.

Activation of PPAR-α increases the activity of SPT and β-glucocerebrosidase As activation of PPAR-α led to an increase in ceramide synthesis, the activity of β-glucocerebrosidase and SPT, the two major enzymes responsible for ceramide metabolism, was assayed after treatment of reconstructed skin for 7 d with Wy 14,643 at 75 μM. As shown in **Fig 8** the specific activity of the two enzymes was significantly increased (1.7-fold for β-glucocerebrosidase and 2-fold for SPT, $p < 0.005$) following PPAR-α activation.

DISCUSSION

PPAR receptor subtypes, are involved, in target tissues, with the regulation of lipid homeostasis. PPAR-α and PPAR-δ bind and are activated by fatty acids whereas 15d-$\Delta^{12,14}$-prostaglandin J_2, an arachidonic acid metabolite, is a naturally occurring PPAR-γ ligand (Forman *et al*, 1995; Kliewer *et al*, 1995). We have previously demonstrated the expression of PPAR subtypes in keratinocytes (Rivier *et al*, 1998) and showed that the expression of PPAR-α and PPAR-γ is linked to the terminal differentiation program of keratinocytes. Consistent with this finding is the demonstration that PPAR-α is found in the nuclei of suprabasal cells of reconstructed human epidermis. In this study, we have also determined the effect

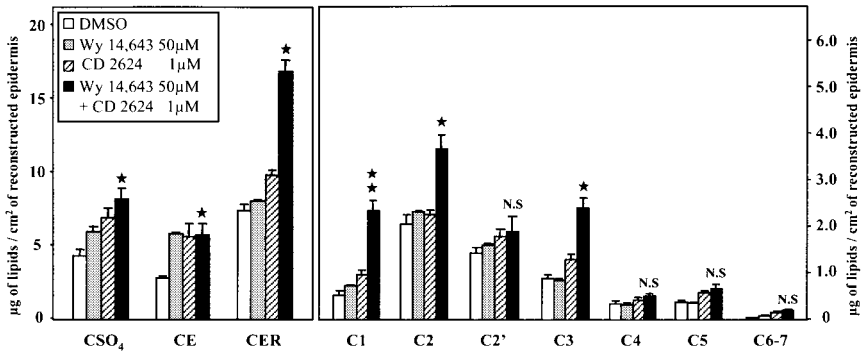

Figure 5. Effect of a combination of CD2624, an RXR agonist, and the selective PPAR-α activator Wy 14,643 on lipid synthesis by reconstructed epidermis. Reconstructed epidermis was treated for 7 d during air exposure with Wy 14,643 at 50 μM, or CD2624 at 1 μM or a combination of the two compounds and HPTLC analysis of the total lipid content was performed. After charring of the chromatography plates, lipid species were quantitated by scanning densitometry. Abbreviations are: cholesterol sulfate (CSO_4), ceramides (CER), cholesteryl esters (CE). Ceramides (C1–C7) were named as previously described (Ponec *et al*, 1997) except for ceramide 2 that was separated in two fractions (C2 and C2′). The results are presented as mean ± SEM of the replicates (n = 3) of two different experiments. Differences at $*p < 0.05$ or $**p < 0.01$ are indicated. N.S, nonsignificant.

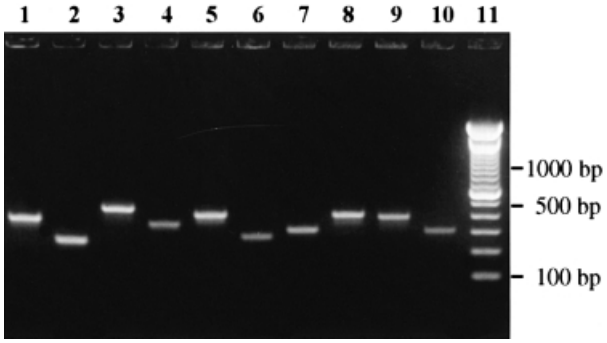

Figure 6. Expression of lipid metabolizing enzyme mRNA in reconstructed epidermis. Total RNA was prepared from untreated reconstructed epidermis and reverse transcription–PCR products were separated by gel electrophoresis. PCR products were as follow: acyl-CoA oxidase (*lane 1*, 367 bp), long chain acyl-CoA synthase (*lane 2*, 244 bp), steroid sulfatase (*lane 3*, 451 bp), HMG CoA synthase (*lane 4*, 352 bp), HMG CoA reductase (*lane 5*, 413 bp), eFABP (*lane 6*, 280 bp), ceramide glucose synthase (*lane 7*, 347 bp), β-glucocerebrosidase (*lane 8*, 407 bp), sphingomyelinase (*lane 9*, 390 bp), and SPT2 (*lane 10*, 310 bp). *Lane 11* represents 100 bp DNA ladder.

of PPAR-α activation on lipid metabolism using an *in vitro* reconstructed skin model (Asselineau *et al*, 1985). Wy 14,643 was used to activate PPAR-α as this compound is very specific and does not lead to the activation of the two other subtypes. Artificial skin, compared with normal human skin, is characterized by an abnormal lipid composition (Ponec *et al*, 1988; Williams *et al*, 1988; Ponec *et al*, 1995) and certainly lacks one or several factors involved in lipid synthesis regulation. It was thus interesting to test whether or not, activation of PPAR-α was leading to a lipid pattern most closely resembling that of normal skin. As a first approach, samples of reconstructed epidermis were treated with several concentrations of Wy 14,643 ranging from 25 to 75 μM. As end-point, the presence of MCG was studied by immunofluorescence staining using a specific monoclonal antibody (O'Guin *et al*, 1989). We also determined, by Oil Red O staining, the location and approximate amount of neutral lipids in treated reconstructed epidermis. The results obtained show that PPAR-α activation not only increases dramatically the presence of MCG in reconstructed epidermis, but also augments the overall amount of neutral stratum corneum lipids. This first observation raised the interesting possibility that PPAR-α is involved in the regulation of the synthesis of lipids essential for the formation of the epidermal permeability barrier. Among these lipids, ceramides, free fatty acids, cholesterol, and cholesterol derivatives are considered to play a major part, as they are increased after barrier disruption (Grubauer *et al*, 1987; Holleran *et al*, 1991). To assess the effect of PPAR-α activation on these specific lipids, a quantitative analysis was performed. Wy 14,643 treatment increased the overall ceramide, cholesterol sulfate, and cholesterol ester content of the reconstructed epidermis, whereas it did not seem to affect the synthesis of glucosylceramide, free fatty acids, cholesterol, lanosterol, and triglycerides. A detailed analysis of the ceramide fraction of the treated epidermis showed that, with the exception of ceramide 6 and 7, all species are increased and that the general ceramide profile is not affected. Compared with normal skin, the lipids of reconstructed epidermis are characterized by low levels of ceramide, cholesterol sulfate, and cholesteryl ester as well as a high triglyceride and lanosterol content (Ponec *et al*, 1988; Ponec, 1991). The increased presence of the two latter lipid species is certainly a consequence of an abnormal lipid metabolism (Ponec *et al*, 1995). PPAR-α activation tends to normalize the ceramide, cholesterol sulfate, and cholesteryl esters level of reconstructed epidermis. For a still unknown reason, however, ceramide 6 and 7, which represent a significant ceramide fraction in normal epidermis (Long *et al*, 1985) are not increased. It was shown recently that vitamin C treatment of skin equivalent raises the level of these two ceramide fractions (Ponec *et al*, 1997).

PPAR-α (and the two other PPAR subtypes) heterodimerize with RXR (Lemberger *et al*, 1996) for gene transactivation. The heterodimer can be activated by either a PPAR-α agonist or an RXR ligand and a combination of the two activators leads to a synergistic response (Kliewer *et al*, 1992). To demonstrate further that the effect of Wy 14,643 on lipid synthesis is a PPAR-α mediated process, reconstructed skin was treated with a combination of the PPAR-α agonist and CD2624, a selective RXR agonist. The two compounds when used alone at a suboptimal concentration had a slight effect on lipid synthesis. In contrast, the RXR ligand enhanced synergistically the level of ceramides induced by Wy 14,643 treatment. The increase in cholesterol sulfate level seems to result from additive effects. The response observed with the two compounds was very similar to the one obtained with high concentration of the PPAR-α agonist which further demonstrate the implication of PPAR-α on keratinocyte lipid synthesis.

The fact that PPAR-α activation leads to increased levels of ceramide, cholesterol esters, and cholesterol sulfate in reconstructed epidermis prompted us to study the mRNA level of key enzymes involved in the synthesis of these lipid species. With respect to ceramide synthesis, the mRNAs encoding SPT2 (the rate-limiting sphingolipid synthesizing enzyme), β-glucocerebrosidase (responsible for the conversion of glucosylceramide into ceramide), ceramide glucose synthase (involved in the formation of glucosylceramide from ceramide), and sphingomyelinase (catalyzing the conversion of sphingomyelin into ceramide) were quantitated (see **Fig 9**). We also attempted to quantitate the mRNA for cytochrome 4A11, the enzyme responsible for the ω-hydroxylation of fatty acids, but we were unable to amplify its cDNA using specific oligonucleotide primers selective for the kidney enzyme (Imaoka

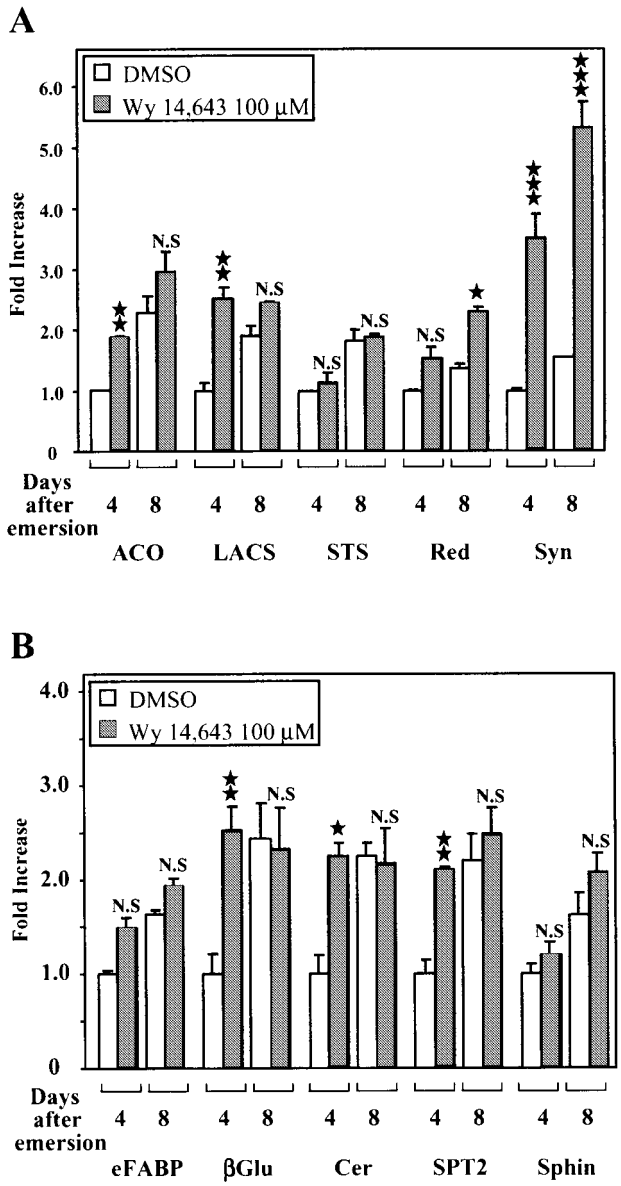

Figure 7. Effect of the selective PPAR-α activator Wy 14,643 on the expression of mRNA encoding lipid metabolizing enzyme. Reconstructed epidermis was treated for 24 h with Wy 14,643 either at day 4 or day 8 after air exposure. The expression level of the different lipid metabolizing enzyme mRNA and of eFABP was determined by semiquantitative reverse transcription–PCR. The abbreviations are as follow: acyl-CoA oxidase (ACO), long chain acyl-CoA synthase (LACS), steroid sulfatase (STS), HMG CoA reductase (Red), HMG CoA synthase (Syn), eFABP, β-glucocerebrosidase (βGlu), ceramide glucose synthase (Cer), serine palmitoyl transferase (SPT2), and sphingomyelinase (Sphin). The results are presented as mean ± SEM of the replicates (n = 3) of three different experiments and differences at $*p < 0.05$, $**p < 0.01$, or $***p < 0.005$ are indicated. N.S, nonsignificant.

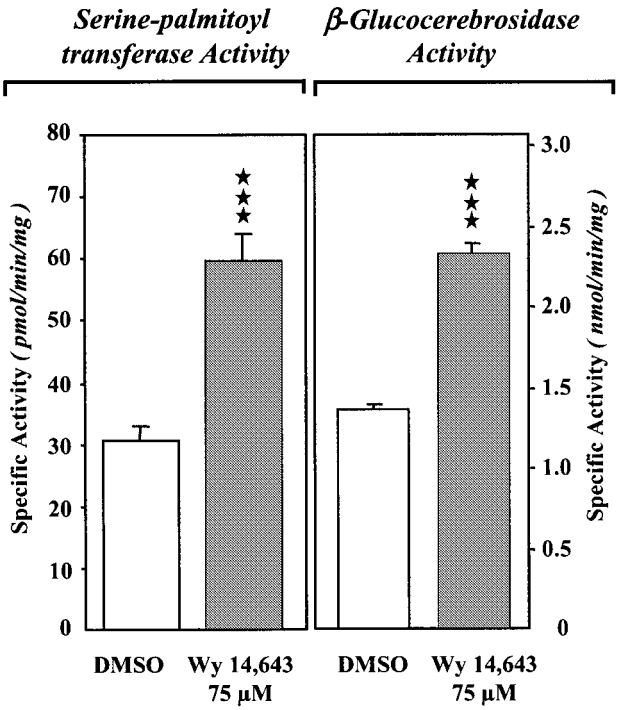

Figure 8. SPT and β-glucocerebrosidase activity is increased in reconstructed skin after treatment with the selective PPAR-α activator Wy 14,643. Reconstructed epidermis was treated for 7 d during air exposure with control dimethyl sulfoxide, Wy 14,643 at 50 µM or 75 µM. Fractions were prepared and assayed for SPT or β-glucocerebrosidase activity as described in *Materials and Methods*. The results are presented as mean ± SEM of the replicates (n = 3) of three different experiments and differences at $***p < 0.005$ are indicated.

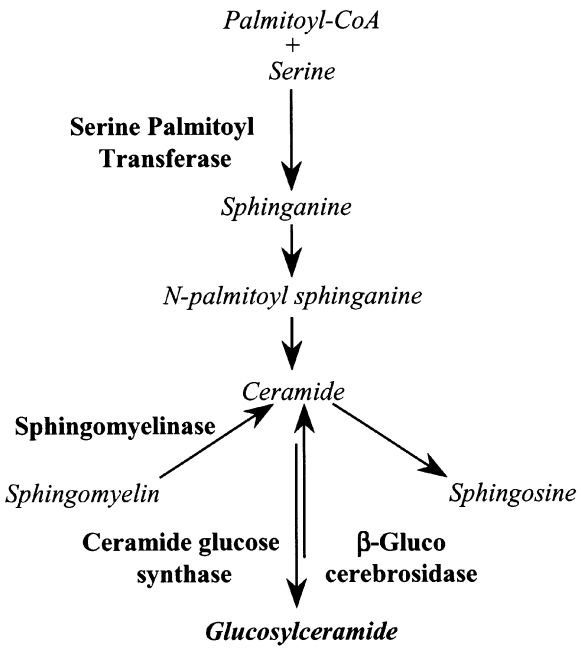

Figure 9. Enzymes involved in the ceramide pathways.

et al, 1993). This is certainly due to the expression of an epidermis specific enzyme certainly different in its cDNA sequence from the kidney enzyme. To account for cholesterol or cholesterol derivative synthesis, the mRNA level of HMG CoA reductase, HMG CoA synthase and steroid sulfatase, the enzyme responsible for cholesterol sulfate formation, were analyzed. For this purpose, reconstructed skin equivalent were treated with the PPAR-α agonist for only 24 h either during or after epidermal reconstruction. During the formation of the epidermis (after 4 d of air exposure), Wy 14,643 treatment increases the mRNA level encoding sphingolipid synthesizing enzymes, with the exception of sphingomyelinase. The observed increase of the mRNAs encoding SPT and β-glucocerebrosidase was confirmed at the level of the enzymatic activity. In the case of enzymes involved in the synthesis of cholesterol and cholesterol derivatives, only HMG CoA synthase mRNA was upregulated during and after reconstruction of the epidermis. The observed increase in enzyme mRNA levels were mostly consistent with the result described

above on lipid synthesis. The important raise in HMG CoA synthase mRNA, however, does not lead to a parallel increase in cholesterol synthesis. It is likely that *de novo* synthesized cholesterol is immediately converted into cholesterol derivatives. Our result on enhanced expression of β-glucocerebrosidase is consistent with the observation of Hanley *et al* (1997) who have shown that the activity of this enzyme increases after activation of PPAR-α in rat fetal skin. In the same study, however, an increase in the steroid sulfatase activity was also observed. This is not the case at the mRNA level in the work described here. The fact that PPAR-α activation leads to the rapid increased expression of the mRNA encoding enzymes involved in lipid metabolism could be explained by two hypothesis: (i) the receptor modulates the transcription of these enzymes by interacting directly with a peroxisome proliferator-activated receptor response element present in their gene promoter, or (ii) PPAR-α induces the expression of a transcription factor regulating lipid synthesis. This transcription factor is certainly different from sterol regulatory binding protein-2 as activation of this factor, as opposed to PPAR-α activation, led to an increase in HMG CoA reductase and fatty acid synthetic enzymes, but did not change expression of SPT2 (Harris *et al*, 1998).

In conclusion, our results show that PPAR-α, whose expression is increased during keratinocyte differentiation (Rivier *et al*, 1998), is involved in the control of epidermal lipid metabolism. This observation explains at the molecular level previous results showing that PPAR-α activation increases the permeability barrier function of fetal rat skin explants (Hanley *et al*, 1997). Several skin diseases, such as psoriasis and atopic dermatitis, are characterized by defects in skin barrier function caused by an abnormal stratum corneum lipid composition (Schäfer and Kragballe, 1991; Motta *et al*, 1994). In this respect, we have also shown that the expression of PPAR-α is downregulated in involved psoriatic epidermis (Rivier *et al*, 1998). Future studies should demonstrate if PPAR-α selective agonists may have a beneficial action in the treatment of these diseases.

Thanks are due to Dr. D. Quiec and to other members of the ''Biologie du soin'' Laboratory (C. Ch. Zviak, L'OREAL, Paris) for their help in lipid analysis.

REFERENCES

Asselineau D, Bernard BA, Bailly C, Darmon M: Epidermal morphogenesis and induction of 67 kD keratin polypeptide by culture at the air liquid interface. *Exp Cell Res* 159:536–539, 1985

Asselineau D, Bernard BA, Bailly C, Darmon M: Retinoic acid improve epidermal morphogenesis. *Dev Biol* 133:322–335, 1989

Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911–917, 1959

Braissant O, Foufelle F, Scotto C, Dauca M, Wahli W: Differential expression of peroxisome proliferator-activated receptor (PPAR): tissue distribution of PPAR-α, -β, and -γ in the adult rat. *Endocrinology* 137:354–366, 1996

Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987

Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM: 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. *Cell* 83:803–812, 1995

Grubauer G, Feingold KR, Elias PM: Relationship of epidermal lipogenesis to cutaneous barrier function. *J Lipid Res* 28:746–752, 1987

Hanley K, Jiang Y, Crumrine D, *et al*: Activators of the nuclear hormone receptors PPARα and FXR accelerate the development of the fetal epidermal permeability barrier. *J Clin Invest* 100:705–712, 1997

Hanley K, Jiang Y, He SS, *et al*: Keratinocyte differentiation is stimulated by activators of the nuclear receptor PPARα. *J Invest Dermatol* 110:368–375, 1998

Harris IR, Farrell AM, Holleran WM, Jackson S, Grunfeld C, Elias PM, Feingold KR: Parallel regulation of sterol regulatory element binding protein-2 and the enzymes of cholesterol and fatty acid synthesis but not ceramide synthesis in cultured human keratinocytes and murine epidermis. *J Lipid Res* 39:412–422, 1998

Holleran WM, Williams ML, Gao WN, Elias PM: Serine-palmitoyl transferase activity in cultured human keratinocytes. *J Lipid Res* 31:1655–1661, 1990

Holleran WH, Feingold KR, Man MQ, Gao WN, Lee JM, Elias PM: Regulation of epidermal sphingolipid synthesis by permeability barrier function. *J Lipid Res* 32:1151–1158, 1991

Holleran WM, Takagi Y, Imokawa G, Jackson S, Lee JM, Elis PM: β-glucocerebrosidase activity in murine epidermis: characterization and localization in relation to differentiation. *J Lipid Res* 33:1201–1209, 1992

Imaoka S, Ogawa H, Kimura S, Gonzalez FJ: Complete cDNA sequence and cDNA-directed expression of CIP4A11, a fatty acid omega-hydroxylase expressed in human kidney. *DNA Cell Biol* 12:893–899, 1993

Issemann I, Green S: Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* 347:647–650, 1990

Kliewer SA, Umesono K, Noonan DJ, Heyman RA, Evans RM: Convergence of 9-cis and peroxisome signalling pathways through heterodimer formation of their receptors. *Nature* 358:771–774, 1992

Kliewer SA, Forman BM, Blumberg B, *et al*: Differential expression and activation of a family of murine peroxisome proliferator-activated receptor. *Proc Natl Acad Sci USA* 91:7355–7359, 1994

Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM: A prostaglandin J_2 metabolite binds peroxisome proliferator activated receptor γ and promotes adipocyte differentiation. *Cell* 83:813–819, 1995

Lampe MA, Williams ML, Elias PM: Human epidermal lipids: characterization and modulations during differentiation. *J Lipid Res* 24:131–140, 1983

Lanzmann L: Epidermal permeability barrier: transformation of lamellar-granule disks into intercellular sheets by a membrane-fusion process, a freeze-fracture study. *J Invest Dermatol* 87:202–209, 1986

Lehmann JM, Moore LB, Smith-Oliver TA, Wilkinson WO, Wilson TM, Kliewer SA: An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator activated receptors γ (PPARγ). *J Biol Chem* 270:12953–12956, 1995

Lemberger T, Desvergne B, Wahli W: Peroxisome Proliferator-Activated Receptors: A nuclear receptor signaling pathway in lipid physiology. In: Spudish JA, Gerhart J, Mcknight SL, Schekman R (eds). *Annual Review in Cell and Development Biology*. Palo Alto CA: Annual Reviews Inc., 1996, 12, pp 335–363

Long SA, Wertz PW, Strauss JS, Downing DT: Human stratum corneum polar lipids and desquamation. *Arch Dermatol Res* 277:284–287, 1985

Motta S, Monti M, Sesana S, Mellesi L, Ghidoni R, Caputo R: Abnormality of water barrier function in psoriasis. Role of ceramide fractions. *Arch Dermatol* 130:452–456, 1994

O'Guin W, Manabe M, Sun T: T: association of a 25K protein with membrane coating granules of human epidermis. *J Cell Biol* 109:2313–2321, 1989

Ponec M. Lipid metabolism in cultured keratinocyte. In: Elias P (ed.) *Skin Lipids, Advances in Lipid Research*. San Diego: Academic Press, 1991, 24, pp 83–117

Ponec M, Weerheim A, Kempenaar J, Mommaas AM, Nugteren DH: Lipid composition of cultured human keratinocytes in relation to their differentiation. *J Lipid Res* 29:949–961, 1988

Ponec M, Kempenaar J, de Weerheim A, Lannoy L, Kalkman I, Jansen H: Triglyceride metabolism in human keratinocytes cultured at the air–liquid interface. *Arch Dermatol Res* 287:723–730, 1995

Ponec M, Weerheim A, Kempenaar J, Mulder A, Gooris GS, Bouwstra J: The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C. *J Invest Dermatol* 109:348–355, 1997

Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK: The peroxisome proliferator-activated receptor-γ is a negative regulator of macrophage activation. *Nature* 391:79–82, 1998

Rivier M, Safonova I, Lebrun P, Griffiths CEM, Ailhaud G, Michel S: Differential expression of peroxisome proliferator-activated receptor subtypes during the course of keratinocyte differentiation. *J Invest Dermatol* 111:1116–1121, 1998

Schäfer L, Kragballe K: Abnormalities in epidermal lipid metabolism in patients with atopic dermatitis. *J Invest Dermatol* 96:10–15, 1991

Schurer NY, Elias PM. The biochemistry and function of stratum corneum lipids. In: Elias P (ed.). *Skin Lipids, Advances in Lipid Research*. San Diego: Academic Press, 1991, 24, pp 27–56

Spanjaard RA, Ikeda M, Lee PJ, Charpentier B, Chin WW, Eberlein TJ: Specific activation of retinoic acid receptors (RARs) and retinoid X receptors reveals a unique role for RARgamma in induction of differentiation and apoptosis of S91 melanoma cells. *J Biol Chem* 272:18990–18999, 1997

Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM: mPPARγ2, tissue-specific regulator of an adipocyte enhancer. *Genes Dev* 8:1224–1234, 1994

Wertz PW, Downing DT. Epidermal lipids. In: Golsmith LA (ed.). *Physiology, Biochemistry and Molecular Biology of the Skin*. New York: Oxford University Press, 1991:pp 205–236

Williams ML, Brown BE, Monger DJ, Grayson S, Elias PM: Lipid content and metabolism of human keratinocyte cultures grown at the air–medium interface. *J Cell Physiol* 136:103–110, 1988