INVESTIGATIVE REPORT

Expression of Peroxisome Proliferator Activated Receptors (PPARs) in Human Hair Follicles and PPARα Involvement in Hair Growth

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Peroxisome proliferator-activated receptors (PPARs), which belong to the nuclear hormone receptor superfamily, have recently been described as potent key regulators of epidermal development. As 1,25-dihydroxyvitamin D$_3$, retinoic acid and triiodothyronine are known to exert effects on skin and hair follicle growth through similar receptors, we decided to investigate both the expression pattern of the PPARα, -δ and -γ subtypes and their role in human hair follicles. Using reverse transcriptase-polymerase chain reaction and immunohistochemistry, we established that PPARα, -δ and -γ were expressed in both dermal and epithelial human hair follicle cells. Additionally, we evaluated the dose effect of clofibrate, a PPARα ligand, on the survival of human hair follicles in culture. A beneficial effect was observed within a narrow range of concentrations. *Key words: human hair growth; clofibrate; nuclear receptor.*

We (1, 2) and others (3–7) have shown that retinoids, vitamin D$_3$ and triiodothyronine and their respective nuclear receptors, namely retinoic acid receptors, vitamin D receptor and triiodothyronine receptors, are implicated in the control of human hair growth both *in vivo* and *in vitro*. All these nuclear receptors belong to the same family, which is known as the steroid/thyroid/retinoid nuclear receptor superfamily. Peroxisome proliferator-activated receptors (PPARs) are also members of this nuclear hormone receptor superfamily (8). PPARs control a variety of target genes involved in the key steps of lipid metabolism (9) and were initially shown to be activated by agents which stimulate peroxisome proliferation (10, 11).

To date, at least 3 different members of the PPAR family have been found in human tissues: PPARα, PPARδ and PPARψ (12–15). PPARα is expressed preferentially in the liver and tissues with high fatty acid catabolism. It is involved in the fatty acid degradation and xenobiotic detoxification pathways. PPARα can also stimulate target gene expression in response to diverse signals, including hypolipidemic drugs of the fibrate class (10, 16, 17). Recently, leukotriene B4 (LTB4) was identified as one of the natural ligands of PPARα, therefore suggesting an additional role of the latter isoform in the inflammatory response (18). PPARδ (also called β or NUC1) is ubiquitously expressed in human tissues, although its biological function and ligand(s) are still unknown.

PPARψ is considered to be one of the key actors of the adipocyte differentiation process and its natural ligand 15-deoxy-Δ^{12,14} prostaglandin J2 is a potent adipogenesis inducer (19, 20). Thiazolidinedione-derived antidiabetics also bind to and activate PPARψ, leading to adipocyte differentiation and *in vivo* reduction in circulating lipid levels (21).

The presence of PPARs in human keratinocytes has recently been revealed (22), and several recent studies have suggested that PPARs may also be key factors in the regulation of epidermal development and keratinocyte differentiation (22–25). One report described the presence of PPARα and -δ in rat sebaceous glands (26). Recently, involvement of PPARs has been reported in rat preputial sebocyte differentiation (27). To further investigate PPARα, -δ and -ψ expression in human hair follicle, both immuno-histochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) were performed on different compartments of the human pilosebaceous unit. Furthermore, to gain insight into the role of PPAR ligands in hair follicle homeostasis, we studied their effects on human hair follicle survival *in vitro*.

MATERIALS AND METHODS

RT-PCR analysis

Chemical. Molecular weight markers (M): a 50–2,000 bp ladder was purchased from Bio-Rad (Hercules, CA, USA).

Adipocytes. Human adipocytes and adipocyte culture medium were purchased from Stratagene (La Jolla, CA, USA).

Plucked hair follicles. Five anagen hairs were gently plucked from the vertex of volunteers and immediately observed under a microscope to check for the presence of an intact outer root sheath (ORS) as well as for the absence of dermal papilla and sebaceous gland, before being immersed in 400 µl of guanidinium thiocyanate. As previously reported (28), plucked hair consists exclusively of the upper two-thirds of the hair follicle (i.e. mainly ORS and inner root sheath [IRS] keratinocytes without their perifollicular external connective layer).

Isolation and culture of dermal papilla cells. Dermal papillae were microdissected from individually isolated hair follicles as previously described>29, 30), and cultured in medium 199 containing 2 mM L-glutamine, 1% antibiotic/antimycotic solution and 10% fetal calf serum (Gibco BRL, Bethesda, MD, USA). Cells were incubated at 37°C in a water-saturated atmosphere of 5% CO$_2$/95% air.

Culture of fibroblasts from connective tissue sheath. Fibroblasts from human hair follicle connective tissue sheath (CTS) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-

glutamine (2 mM), 1 mM sodium pyruvate and antibiotics (penicillin-G 100 U/ml; streptomycin-S 100 μg/ml; amphotericin 250 ng/ml) in the presence of 10% fetal calf serum.

Dermal fibroblasts. Dermal fibroblasts from human skin were cultured in DMEM supplemented with 10% fetal calf serum at 37°C in a water-saturated atmosphere of 5% CO_2/95% air, as previously described (31).

mRNA expression. After incubation, cells (5×10^6 cells per 100 mm diameter dish) were washed twice with phosphate-buffered saline (PBS), scraped off with a rubber policeman and dissolved in 400 μl of guanidium thiocyanate. Messenger RNA, obtained either from 5 plucked hairs (see above) or from 5×10^6 specific type cells, was purified using the QuickPrep® mRNA preparation kit (Pharmacia Biotech, Uppsala, Sweden). Poly-A+ mRNA(s) were then reverse transcribed using the first strand cDNA synthesis kit from Pharmacia and PCR was performed using the Taq polymerase and 10X buffer marketed by Amersham (Les Ulis, France) as previously described (1, 2), with the following primers purchased from Genset (Paris, France): *h β actin (1067 bp)*: (FP) 5′-ATG GAT GAT GAT ATC GCC GCG CT-3′; (RP) 5′-CGG ACT CGT CAT ACT CCT GCT GCT TG-3′. *hPPARα (492 bp)*: (FP) 5′-CCA GTA TTT AGG AAG CTG TCC-3′; (RP) 5′-AAG TTC TTC AAG TAG GCC TCG-3′. *hPPARβ (484 bp)* and *hPPARγ (474 bp)* primers have been described by others (32). For PCR 1/15 of the cDNA was used as described (1, 2). After one first cycle at 95°C for 4 min, PCR was carried out for 30 cycles, including 25 s of denaturation at 95°C, 1 min of primer annealing at 57°C and 1 min of extension at 72°C. Each amplified fragment was then migrated on 2% agarose gel containing 0.5 μg/ml ethidium bromide. The gels obtained were irradiated under a UV transilluminator and a video camera (Vilbert Lourmat, Marne-la-Vallée, France) captured the image.

Restriction enzyme mapping. The identity of PPAR isoform amplimers was checked by restriction enzyme digestion in CTS cells. The cDNA was digested with Alu 1, Hinf 1 or Bam H1 restriction enzymes (Boehringer Mannheim GmbH, Germany) for 3 h before gel analysis. The specific endonuclease cleavage sites were obtained from published sequences (12–15).

Immunohistochemistry

PPARα rabbit polyclonal antibody (Affinity BioReagents, USA) and PPARδ or -ψ goat polyclonal antibodies (Santa Cruz Biotechnology, Inc., CA, USA) were used to immunohistochemically characterize PPAR expression in the different hair follicle compartments. Longitudinal frozen sections (5 μm thick) of hair follicles were prepared as described by Commo & Bernard (33). Sections were fixed in 3.7% paraformaldehyde (PFA) at room temperature for 10 min followed by several washes in PBS and then fixed again in acetone at – 20°C for 10 min followed by several washes in PBS. Immunolabelling was performed as described previously (33). Primary antibodies were diluted 1:50 (anti-PPARα) or 1:20 (anti-PPARδ and anti-PPARψ in 0.05% PBS–Tween containing 10% of normal serum corresponding to the conjugated secondary antibody species.

Human hair follicle in vitro

Chemical. 2-(*p*-Chlorophenoxy)-2-methyl-propionic acid ethyl ester (clofibrate) was purchased from Sigma (St Louis, MO, USA). Clofibrate was dissolved in ethanol as a 10^{-3} M solution.

Human hair follicle isolation and culture. Individual human terminal scalp hair follicles from facelift surgery (exclusively from female patients) were isolated as previously described (34). Each isolated hair follicle with its perifollicular external connective tissue layer was then

incubated at 37°C in a water-saturated atmosphere of 5% CO_2/95% air. Hair follicles were maintained free floating (but not at the air/medium interface) in William's E culture medium supplemented with 2 mM L-glutamine, 10 μg/ml insulin, 10 ng/ml hydrocortisone and antibiotics (Gibco BRL). Twelve hair follicles were used for each experiment. Clofibrate was added to the culture medium at final concentrations ranging from 10^{-6} M to 10^{-12} M, the medium being changed every 2 days. The integrity of each hair follicle was checked and its length measured every day under binoculars equipped with a micrometer. A follicle was scored as intact and healthy when no keratinocyte outgrowth was observed and growth of hair was maintained. The number and percentage of surviving follicles were evaluated every day under binoculars. Two independent experiments were performed using biopsies obtained from 2 different subjects.

Statistical analysis

Follicle survival was determined using the Kaplan–Meier method. Differences between untreated and treated follicles were evaluated by the log-rank test. All tests were two-sided. Statistical significance was detected at the 5% significance level while statistical trends were detected at a 10% significance level.

RESULTS

The major structural components of anagen follicles include the dermal papilla, CTS, ORS, IRS, epithelial hair matrix and the hair shaft. In the present report, we studied dermal papilla cells, ORS-derived keratinocytes in culture and CTS cells as isolation and culture of human hair matrix cells is not technically possible. Cells between passages 2 and 5 were used (1, 2). In addition, we studied freshly plucked hair follicles which exactly reflect the *in vivo* situation of the upper two-thirds of the IRS and ORS keratinocytes at the time of plucking.

PPAR gene expression in the pilosebaceous unit compartments

PPARα, -δ and -ψ were found to be actively transcribed in primary cultures of dermal papilla and CTS, as well as in human skin-cultured fibroblasts, as reflected by the detection of amplimers of expected size: 492 bp (PPARα), 484 bp (PPARδ) and 474 bp (PPARψ) (Fig 1A, 1B and 1D, respectively). Freshly plucked hairs also expressed the 3 PPAR subtypes (Fig. 1C). The identity of PPARα, -δ and -ψ amplimers was checked by restriction enzyme digestion in CTS cells and restriction fragments of the expected sizes were obtained. Five (229, 176, 47, 26 and 12 bp), 2 (305 and 177 bp) and 2 fragments (394 and 79) were respectively obtained for PPARα, -δ and -ψ amplimers after digestion with Alu I, Hinf 1 or Bam H1 (Fig. 1E). As a control, the expression of PPARs was checked in adipocyte cells and, as previously reported (32), only PPARψ was strongly detected (Fig. 1F). As PPARα, -δ and -ψ transcripts were widely expressed with variable expression levels in all compartments of the human hair follicle, we decided to characterize PPAR protein distribution in the human hair follicle by immuno-histochemistry.

Immunohistochemical detection of PPARs

Polyclonal antibodies directed against either PPARα, -δ or -ψ clearly reacted with the matrix hair cells as well as with the dermal
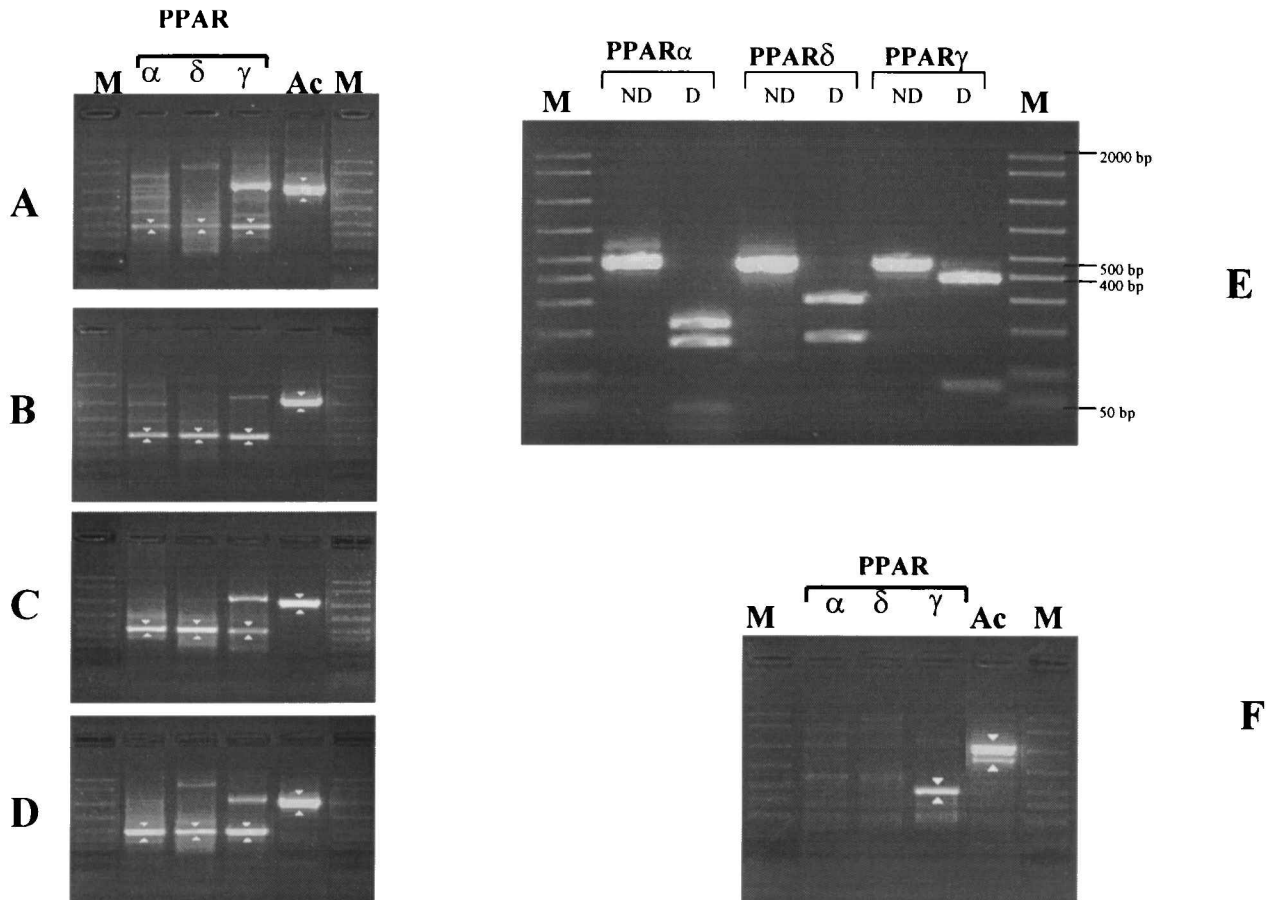
Fig. 1. Semi-quantitative RT-PCR analysis of PPARs in (A) primary culture of dermal papilla cells, (B) primary culture of CTS fibroblasts, (C) culture of freshly plucked hairs and (D) culture of dermal fibroblast cells. Predicted PPAR amplimers of 492 bp (PPARα), 484 bp (PPARδ) and 474 bp (PPARψ) were obtained. Note that other bands detected are not specific. In (E), the identity of PPAR amplimers was checked by restriction enzyme digestion in CTS cells. As expected from the sequences of PPARα, -δ and -ψ, new products of expected sizes were obtained. ND, non-digested; D, digested. As a control in (F), PPARψ was the predominant subtype expressed in adipocytes whereas PPARα and -δ were faintly expressed. Actin (house-keeping gene) was used as internal standard. In all experiments (except restriction enzyme digestion), cDNA was diluted 50-fold for amplification of actin (Ac).

papilla cells (Fig. 2A). This expression was highly specific of the hair bulb since it was sparse in the upper part of the follicle and in the epidermis (Fig. 2C). As previously observed by others, staining was both cytoplasmic and nuclear (35). PPARα, -δ and -ψ positive cells were detected in the keratogenous zone of the hair shaft as well as in the hair shaft cuticle (Fig. 2B). As shown in Fig. 2B, PPARs were also detected in both basal ORS layer cells and CTS cells. However, only PPARδ and -ψ antibodies reacted with IRS cells (Fig. 2B). Although the staining intensity of cells was generally heterogeneous—some cells being strongly stained, others weakly—these results showed that all dermal and epidermal hair follicle compartments express PPARα, -δ and -ψ.

Effects of clofibrate on in vitro culture of human hair follicles

We investigated the effects of clofibrate, a PPARα ligand, on the survival of freshly dissected human hair follicles grown *in vitro*. As shown in Fig. 3, we found that the survival of follicles was enhanced by clofibrate dose-dependently up to 10^{-8} M, after which a decrease in survival time was observed. Survival of clofibrate-treated follicles was similar to that of untreated control hair follicles during the first 3 days of culture but, at day 11, none of the 12 untreated control hair

follicles were still growing whilst hair growth could still be detected in the presence of 10^{-10} M and 10^{-8} M clofibrate. A statistically significant positive effect on survival was observed at 10^{-8} M (p= 0.095). However, a higher concentration of clofibrate (10^{-6} M) induced cessation of follicle growth compared with the control conditions. Indeed, although the survival percentage of 10^{-6} M clofibrate-treated follicles was similar to that of untreated control hair follicles during the first 3 days of culture, at day 4 the survival rate fell to 75% in treated hair follicles vs 92% in the control group and, at day 8, none of the treated hair follicles were still growing, whilst 17% of control untreated hair follicles were still growing. Similar results were obtained in 2 independent experiments, with follicles obtained from 2 different donors.

DISCUSSION

We used RT-PCR and immunohistochemical analysis to detect and characterize PPAR expression in the human pilosebaceous unit. The expression profiles of PPARα, -δ and -ψ were first analyzed by RT-PCR in primary culture of dermal papilla, CTS cells and freshly plucked hairs (mainly ORS keratinocytes). Although it cannot be discounted
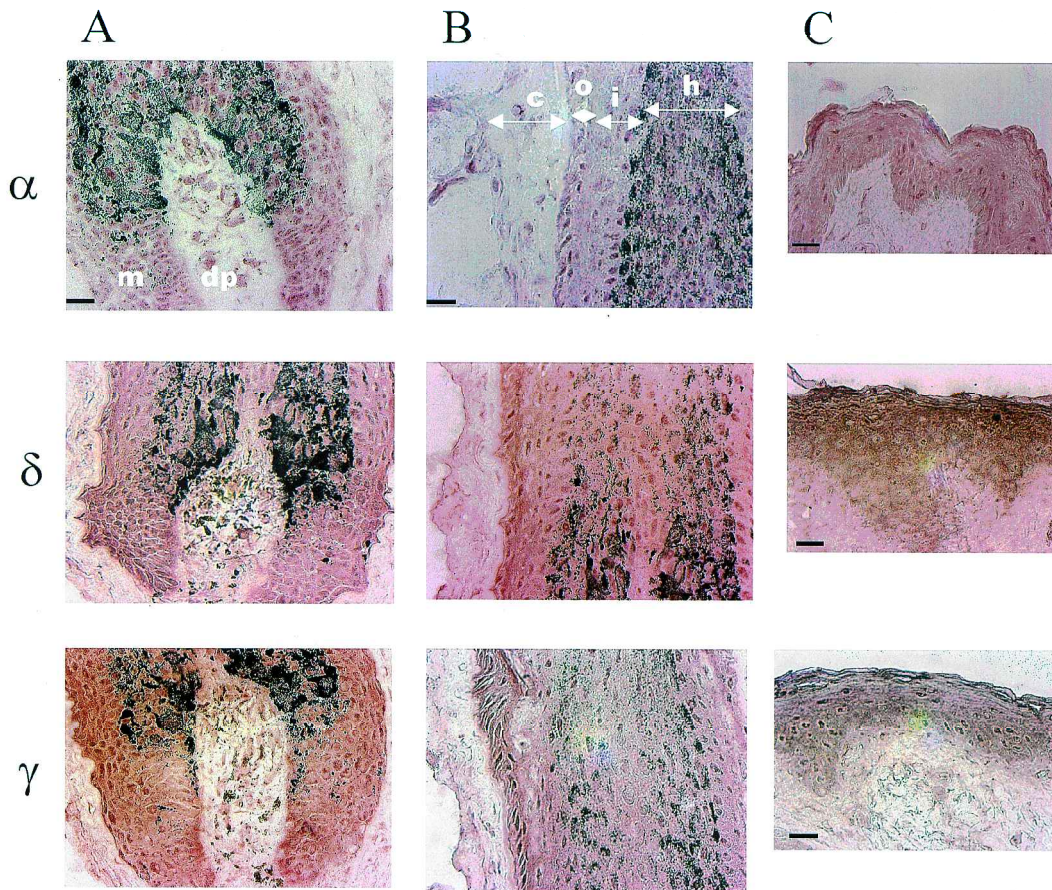
Fig. 2. Immunohistochemical localization of PPARs in longitudinal frozen sections of human hair follicle and skin. Cryostat sections were reacted with (α) PPARα, (δ) PPARδ and (ψ) PPARψ antibodies in (A) dermal papilla (dp) and matrix cells (m); (B) outer root sheath (o), inner root sheath (i), connective tissue sheath (c) and hair shaft (h); and (C) epidermis and dermis. Bars represent 20 µm.
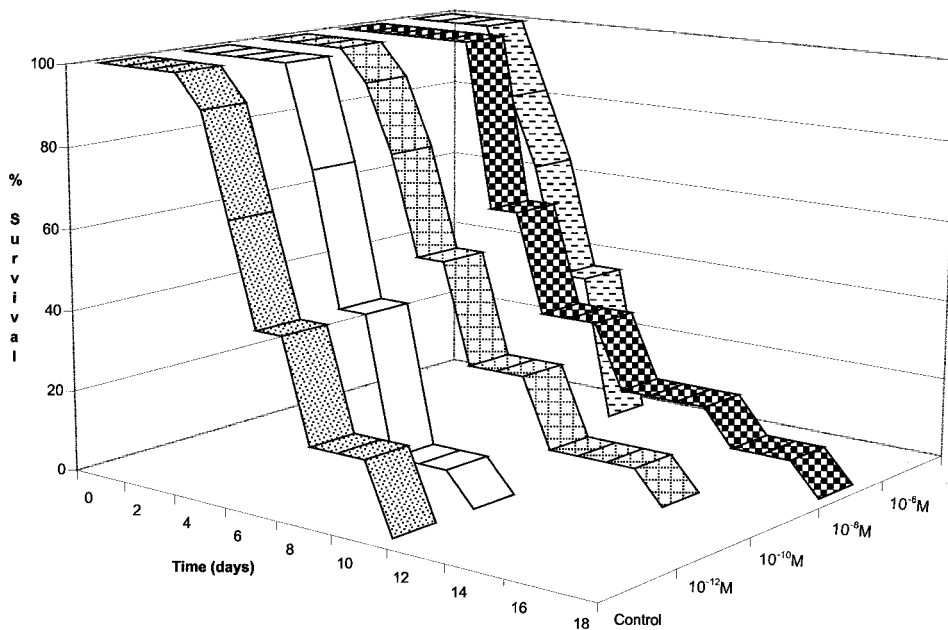

Fig. 3. Effect of clofibrate ($10^{-12} - 10^{-6}$ M) on the survival of freshly dissected human hair follicles in culture (1 representative experiment). The y-axis reflects the percentage of follicles still growing at a given time.

that some changes in mRNA expression levels may occur during culture of cells *in vitro*, our data indicate that these cells expressed, at comparable levels, the 3 PPARs. In adipocytes, PPARψ was expressed predominantly as previously reported (32).

As a second step, we investigated, by immunohistochemistry, PPARα, -δ and -ψ protein distributions in human anagen hair follicles. We found that dermal (dermal papilla and CTS cells) as well as epidermal (ORS and matrix hair cells) compartments were PPARα, -δ and -ψ positive with the exception of the IRS, which was found to be only PPARδ and -ψ positive. Immunohistochemical data were thus consistent with the PPARα, -δ, and -ψ transcript patterns observed in the various compartments of the human hair follicle. To our knowledge, this is the first time that the expression of these 3 PPAR isotypes has been documented in human hair follicles.

Additionally, we showed that human anagen hair follicle responded *in vitro* to clofibrate, a PPARα ligand, suggesting that the receptor corresponding to the detected mRNA, as well as to the protein staining, was functional in the human hair follicle. However, 2 effects were observed in the whole-organ culture system: while high clofibrate concentration (10^{-6} M) led to cessation of hair follicle growth, low clofibrate concentrations (10^{-10} M – 10^{-8} M) enhanced the *in vitro* survival of human hair follicles. This effect on survival suggests that clofibrate may have a beneficial effect on hair growth, albeit within a narrow concentration window. The cause of hair growth cessation observed with 10^{-6} M clofibrate remains obscure but probably cannot be accounted for by a toxic effect because (i) a 300 µM clofibrate concentration was previously shown to stimulate keratinocyte differentiation (24, 25); and (ii) serum clofibrate concentration was reported to be as high as 0.5 mM in treated patients (36). Interestingly enough, similar narrow concentration windows were observed for vitamin D_3 and T_3, which exhibited a beneficial effect on *in vitro* hair growth only at 1–10 nM and 1–10 pM, respectively (1, 2, 4).

Our present observations *in vitro* may provide some clues to explain why clofibrate has been reported to alter scalp hair growth *in vivo* by occasionally causing hair loss (37, 38): high clofibrate concentrations are detrimental to hair growth and are thus not appropriate for sustaining follicular homeostasis. As well as hypolipidemic drugs such as clofibrate, other modulators of lipid metabolism, indomethacin, an inhibitor of prostaglandin synthesis and a PPAR ligand have been reported to induce alopecia (37). Although these effects on human hair have been known for a long time, the putative direct effect of these agents on the hair cycle remains to be demonstrated. Taken together these clinical studies clearly demonstrate that an unbalanced lipid metabolism can lead to an alteration of the human hair cycle. We can hypothesize from our results that this alteration in the hair cycle might be at least partially due to altered PPAR-controlled pathways. This could be confirmed by using ligands other than clofibrate which are specific for the 2 other PPARs (-δ and -ψ) expressed in the human hair follicle.

ACKNOWLEDGEMENTS

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