Peroxisome Proliferator-Activated Receptors Increase Human Sebum Production

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Sebum production is key in the pathophysiology of acne, an extremely common condition, which when severe, may require treatment with isotretinoin, a known teratogen. Apart from isotretinoin and hormonal therapy, no agents are available to reduce sebum. Increasing our understanding of the regulation of sebum production is a milestone in identifying alternative therapeutic targets. Studies in sebocytes and human sebaceous glands indicate that agonists of peroxisome proliferator-activated receptors (PPARs) alter sebaceous lipid production. The goal of this study is to verify the expression and activity of PPARs in human skin and SEB-1 sebocytes and to assess the effects of PPAR ligands on sebum production in patients. To investigate the contribution of each receptor subtype to sebum production, lipogenesis assays were performed in SEB-1 sebocytes that were treated with PPAR ligands and isotretinoin. Isotretinoin significantly decreased lipogenesis, while the PPARα agonist-GW7647, PPARδ agonist-GW0742, PPARα/δ agonist-GW2433, PPARγ agonist rosiglitazone, and the pan-agonist-GW4148, increased lipogenesis. Patients treated with thiazolidinediones or fibrates had significant increases in sebum production (37 and 77%, respectively) when compared to age-, disease-, and sex-matched controls. These data indicate that PPARs play a role in regulating sebum production and that selective modulation of their activity may represent a novel therapeutic strategy for the treatment of acne.

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INTRODUCTION

Acne is a common condition affecting millions of patients worldwide. Although not life-threatening, it can lead to feelings of low self-esteem and permanent facial scarring. The pathophysiology of acne centers on the interplay of follicular hyperkeratinization, inflammation induced by *Propionibacterium acnes* and the production of sebum that serves as a nutrient source for *P. acnes* (Zouboulis, 2001, 2004; Zouboulis and Degitz, 2004). Sebum production is essential to the development of acne. Only isotretinoin (13-*cis* retinoic acid (13-*cis* RA)) and hormonal therapy reduce sebum production. With increasing concerns regarding teratogenicity, there is a clear need for therapeutic alternatives to isotretinoin in the treatment of acne.

A series of key experiments demonstrated that co-administration of androgens with ligands of peroxisome proliferator-activated receptors (PPARs) augments the intracellular accumulation of lipids in sebaceous gland-like cells of rat preputial gland (Rosenfield *et al.*, 1999). This observation coupled with the many similarities that exist between adipogenesis and sebaceous lipogenesis led to the hypothesis that PPARs may be important in the regulation of human sebum production and the development of acne (Rosenfield *et al.*, 2000; Kim *et al.*, 2001; Zouboulis *et al.*, 2005). PPARs are members of the nuclear hormone receptor family that form heterodimers with retinoid X receptors and act as transcriptional regulators of a variety of genes including those involved in lipid metabolism in adipose tissue, liver and skin (Rosen and Spiegelman, 2001; Ferre, 2004; Knouff and Auwerx, 2004). As with retinoid receptors, there are three subclasses of PPARs: α, δ, and γ, which differ in their tissue localization and in their transcriptional activities (Berger and Moller, 2002; Desvergne *et al.*, 2004; van Raalte *et al.*, 2004). PPARα receptors are expressed in numerous tissues in humans including liver, kidney, heart, skeletal muscle, fat, and endothelial cells (Braissant *et al.*, 1996). PPARα has been shown to play a critical role in the regulation of cellular uptake, activation and β-oxidation of fatty acids. The most commonly used exogenous PPARα agonists are the fibrates

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Abbreviations: PPARS, peroxisome proliferator-activated receptor; 13-cis RA, 13-cis retinoic acid

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that are clinically used for the treatment of hyperlipidemia (Fazio and Linton, 2004). Fibrates effectively reduce plasma triglycerides in patients by mediating increased lipid uptake and transcriptional modulation of numerous genes that regulate these processes (Motojima *et al.*, 1998). PPARα agonists have been shown to amplify the expression of lipoprotein lipase and inhibit apolipoprotein C-III in the liver (Schoonjans *et al.*, 1996). However, the exact action of PPARα agonists on lipid metabolism is relatively complex and has been shown to vary in different cellular systems. PPARδ is widely expressed in a wide range of tissues and cells with relatively higher levels found in the brain, adipose tissue, and skin. Recently, compounds that interact with PPARδ receptors have been shown to increase differentiation and lipogenesis in epidermal keratinocytes (Westergaard *et al.*, 2001; Schmuth *et al.*, 2004). PPARγ is predominantly located in adipose tissue. There is strong evidence that PPARγ is key in promoting adipocyte differentiation. Treatment of fibroblasts with ligands of PPARγ induces adipogenesis and increases lipid production; effects resulting from the induction of adipogenic target genes (Sandouk *et al.*, 1993; Mao-Qiang *et al.*, 2004). PPARs are also expressed in an immortalized human sebocyte cell line (SZ95) and treatment of these cells with linoleic acid, a ligand for PPARδ and PPARγ, also increases the intracellular content of lipids (Chen *et al.*, 2003). In contrast, treatment of isolated human sebaceous glands with some ligands of PPARα and PPARγ decreases lipogenesis (Downie *et al.*, 2004). Studies to date have not investigated the effects of PPAR ligands on human sebum production, which is key in the pathophysiology of acne. The goals of this study were: (1) to confirm the localization of PPAR subtypes in human skin, as currently shown (Alestas *et al.*, 2006), and to determine their presence and localization in SEB-1 sebocytes; (2) to compare the effects of a panel of potent and isoform-specific PPAR agonists and antagonist with the known inhibitory effects of 13-*cis* RA on lipogenesis in SEB-1 cells and (3) to test the hypothesis that PPAR ligands (thiazolidinediones and fibrates) alter sebum production in patients treated for diabetes or hyperlipidemia.

RESULTS

PPARs are expressed in human skin and SEB-1 cells

The role of PPAR receptors and the effects of their subtype-specific ligands in regulating the lipid metabolism in the liver and adipose tissue have been well documented. Since our study was aimed at examining the effects of subtype specific PPAR ligands on human sebum production, we initially examined the presence and pattern of distribution of each of these receptor subtypes in normal human skin as well as in our SEB-1 sebocyte cell line. Immunohistochemistry of sections of whole skin revealed expression of each of the PPAR subtypes in epidermis, hair follicles, sebaceous ducts and sebaceous glands (Figure 1, representative of data from five subjects). Reactivity within the epidermis was most prominent with PPARα and PPARγ (Figure 1b and d). In examining expression within the sebaceous gland, each antibody reacted with basal sebocytes (Figure 1b–d, insets).

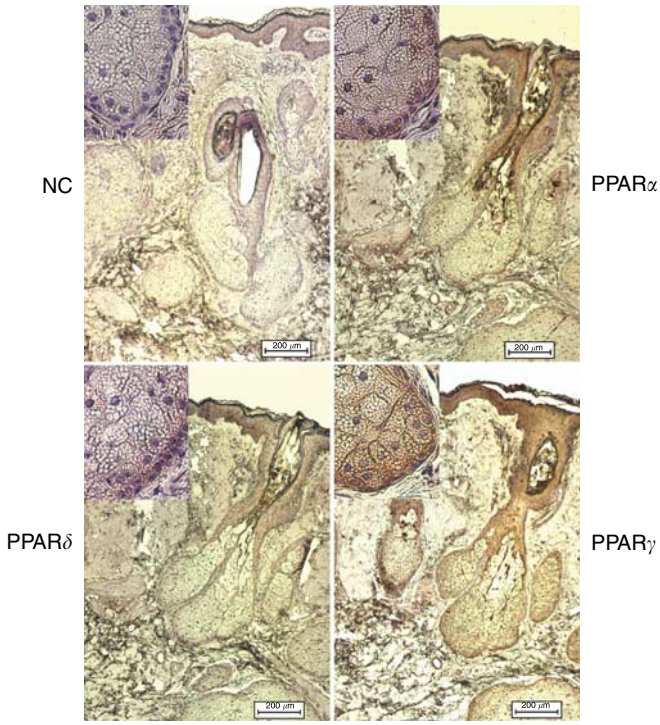

Figure 1. PPAR isoforms localize to epithelia in normal human skin. (**b–d**, respectively) Immunoreactivity to PPARα, δ, and γ subtypes is noted in epidermis, hair follicle, and sebaceous gland as a brown to red color. Insets demonstrate reactivity in basal cells of the sebaceous gland with expression of PPARγ extending to differentiated sebaceous cells. (**a**) Negative controls were treated with nonimmune serum in place of primary antibody. (**a–d**) Bar = 200 μm.

PPARγ expression however was also noted within differentiated sebocytes. PPAR expression was also examined in undifferentiated SEB-1 sebocytes (30% confluent) and in SEB-1 sebocytes that were differentiated by treating with insulin and growing to confluence (Figure 2). Under both conditions, PPARα immunoreactivity was perinuclear. In the undifferentiated state, PPARδ reactivity was perinuclear but distinctly nuclear in the differentiated state. PPARγ immunoreactivity was perinuclear in undifferentiated cells with more nuclear reactivity noted in differentiated cells.

Patients receiving fibrates (PPARα ligands) for hyperlipidemia or thiazolidinediones (PPARγ ligands) for diabetes have increased sebum secretion compared to age-, sex-, and disease-matched controls

To analyze the effects of subtype-specific PPAR ligands on sebum secretion we studied a sample of patients receiving fibrates (PPARα ligands) for hyperlipidemia or thiazolidinediones (PPARγ ligands) for diabetes. Interim analyses of the data were performed after 1 year of subject recruitment and the study was terminated. A total of 45 patients receiving thiazolidinediones and 45 age-, sex-, and disease-matched controls were enrolled and analyzed (Table 1). Twenty-seven patients in the treatment group were receiving pioglitazone and 18 were receiving rosiglitazone. Most patients in both the thiazolidinedione and control groups were receiving

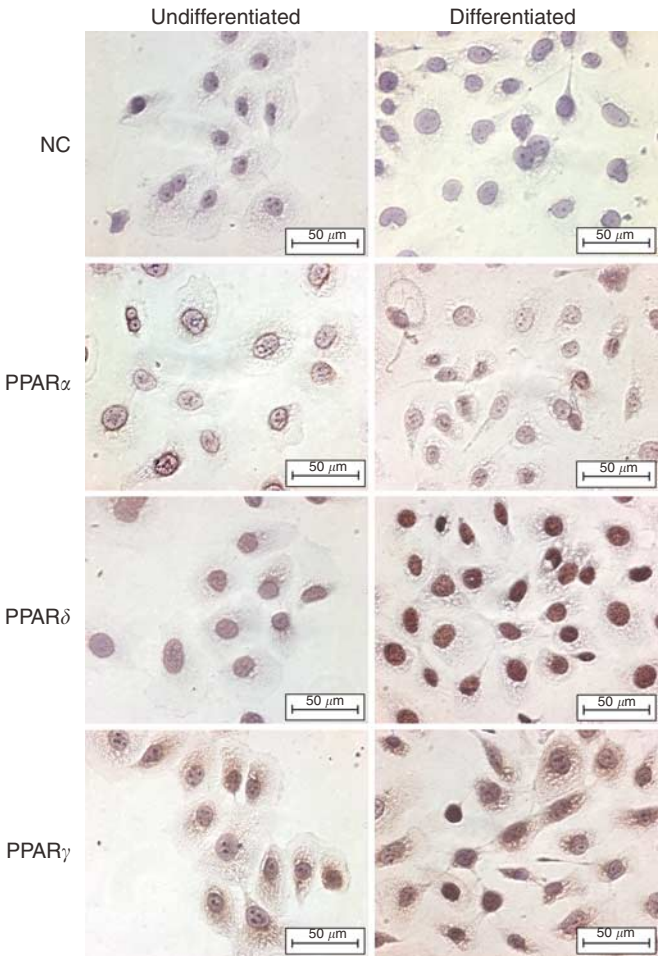

Figure 2. PPAR subtypes are expressed in SEB-1 sebocytes. SEB-1 sebocytes were grown to 30% confluence (undifferentiated) or were treated with 1 μM insulin and grown to confluence (differentiated). PPARα immunoreactivity is perinuclear under both conditions. PPARδ reactivity is perinuclear in the undifferentiated cells and nuclear in differentiated cells. PPARγ reactivity was perinuclear and cytoplasmic under both condition. Negative control sections incubated with nonimmune serum in place of primary antibody. Bar = 50 μm.

additional treatments including insulin, oral hypoglycemic drugs, ACE inhibitors, and statins. There were no statistical differences between treatment and control groups in terms of renal function, presence of vascular disease, or hemoglobin A1C. Patients in the treatment group had significantly greater body mass index ($P<0.0001$) reflecting a predominance of type II diabetes. Patients in the control group reported a longer duration of diabetes ($P=0.05$). Subjects taking thiazolidinediones for at least 3 months had an approximate 37% increase in mean sebum secretion as compared to controls ($P=0.017$). A total of 14 subjects receiving treatment with fibrates and 14 age-, sex-, and disease-matched controls were enrolled. Seven patients were receiving fenofibrate and seven were receiving gemfibrizol. The majority of patients in both the treatment group and the control group were also receiving treatment with drugs of the statin class, beta-blockers, and ACE inhibitors. There were no differences between treatment and control groups in terms of duration of hyperlipidemia or total cholesterol. Triglycerides were significantly higher in the treatment group ($P=0.004$), as might be anticipated in a group requiring fibrates. The subjects taking fibrates had an approximate 77% increase in mean sebum secretion compared with controls ($P=0.023$).

PPAR agonists and 13-*cis* RA have differential effects on sebaceous gland lipogenesis and each alter the pattern of sebaceous lipids produced

In order to observe the differences in lipid production following treatment of SEB-1 sebocytes with the novel PPAR subtype-specific ligands and 13-*cis* RA, lipogenesis assays were performed. The effects of PPAR ligands and 13-*cis* RA on the overall incorporation of ^{14}C-acetate into sebaceous lipids are depicted in Table 2. Significant increases in the total production of neutral lipids were noted with the PPARα agonist GW7647, PPARδ agonist GW0742, PPARα/δ agonist GW2433, PPARγ agonist rosiglitazone, and the PPARα, δ, and γ agonist GW4148 (Figure 3). However no significant differences were seen following treatment with PPARγ

Table 1. Increased sebum secretion in patients treated with the PPAR ligands: thiazolidinediones and fibrates

	TZD, *N*=45	TZD: control group, *N*=45	Fibrate, *N*=14	Fibrate: control group, *N*=14
Mean age±SD	51.2±9	49±12	54±7.7	55±6.7
Male	25	25	10	10
Female	20	20	4	4
Mean sebum secretion (μg/cm^2±SD)	92±57	67±37	131±55	74±37
P-value, unpaired *t*-test, α=0.05	0.017		0.023	
Fold increase in sebum drug treated versus control	1.37		1.77	

TZD, thiazolidinediones.
Levels of the skin surface sebum in patients treated with TZD class of drugs (PPARγ agonists – rosiglitazone and pioglitazone) or the fibrate class of drugs (PPARα agonists – gemfibrizol and fenofibrate) when compared to age-, sex-, and disease-matched control patients. Sebum levels were measured using a Sebumeter SM 810® from three different sites on the forehead of each patient. Statistical analysis was performed using an unpaired *t*-test.

Table 2. Effect of various PPAR ligands on total lipid production in SEB-1 cells

	Total lipid production (CPM/10^6 cells/hour ± SE)		
Compound	**Untreated**	**Treated**	***P*-value (ANOVA)**
GW7647 (α agonist) 100 nM	1,237 ± 51	1,525 ± 55	0.005
Fenofibrate (α agonist) 1 μM	1,170 ± 39	1,201 ± 34	NS
GW0742 (α/δ agonist) 100 nM	1,303 ± 44	1,453 ± 40	0.0470
GW2433 (α/δ agonist) 100 nM	1,670 ± 59	1,917 ± 41	0.0270
Rosiglitazone (γ agonist) 10 μM	1,625 ± 35	3,252 ± 42	0.0002
Pioglitazone (γ agonist) 10 μM	1,625 ± 35	1,381 ± 18	NS
GW4148 (α/δ/γ pan agonist) 1 μM	1,460 ± 67	1,730 ± 73	0.01
13-*cis* RA 1 μM	1,084 ± 64	830 ± 50	0.0012

ANOVA, analysis of variance; 13-*cis* RA, 13-*cis* retinoic acid.
The amount of lipid produced following 6 days of incubation with the various PPAR compounds was determined by performing the lipogenesis assay and the average of three separate experiments was used to quantify the differences between ligand-treated cells versus vehicle-treated controls. Significant increases in the total production of neutral lipids were noted with the PPARα agonist GW7647 (100 nM), PPARδ agonist GW0742 (100 nM), PPARα agonist GW2433 (100 nM), PPARγ agonist rosiglitazone (10 μM), and the PPARα/δ/γ agonist GW4 148 (1 μM). An inverse relationship was seen following treatment of SEB-1 cells with 13-*cis* RA (1 μM), which showed a significant decrease in total lipid produced at 72 hours.

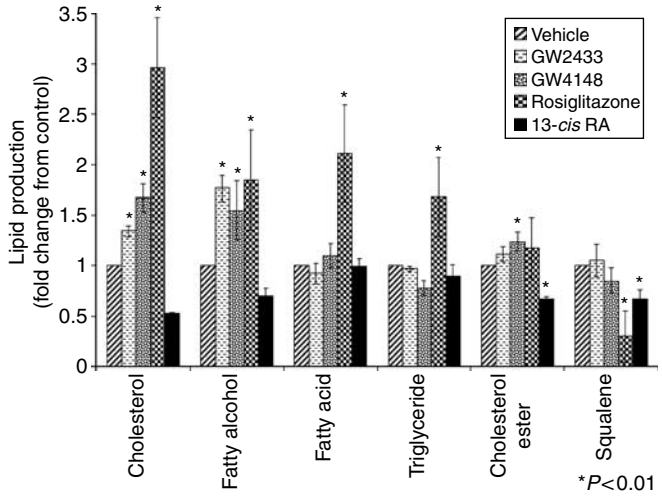

Figure 3. PPAR agonists and 13-*cis* RA alter the pattern of lipid production in SEB-1 sebocytes. SEB-1 sebocytes were grown in SEB-1 medium until they reached 90% confluence then treated with PPARα/δ agonist GW2433 (100 nM), PPARα/δ/γ agonist GW4148 (1 μM), Rosiglitazone (10 μM) every 48 hours until the 6th day to study the differential pattern of lipid production. Cells were treated with 13-*cis* RA (1 μM) for 72 hours. Statistically significant increases were noted in the production of cholesterol and fatty acid following treatment with PPAR agonists (GW2433, GW4148, and rosiglitazone) when compared to vehicle-treated controls ($P<0.01$); whereas treatment with 13-*cis* RA showed a statistically significant decrease in the production of cholesterol, fatty alcohol, cholesterol ester, and squalene ($P<0.01$). Values represented are means ± SE.

agonist pioglitazone. In contrast, treatment of SEB-1 cells with 13-*cis* RA for 24 or 72 hours resulted in significant decreases in lipid production compared to untreated cells (Table 2) and a decrease in intracellular lipid as detected with Oil Red O staining (Figure 4). We also tested the effect of an antagonist of PPARγ GW0072 on total lipid production, however, no statistically significant changes in total lipid production were seen (data not shown).

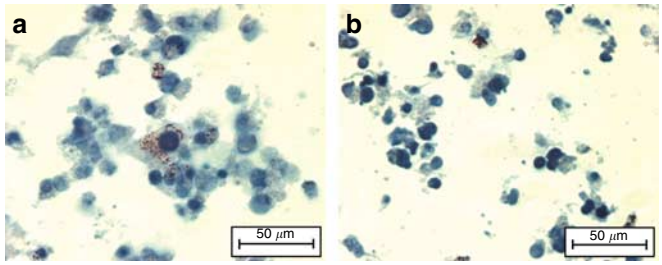

Figure 4. Oil Red O staining of SEB-1 sebocytes indicates decreased intracellular lipidsin SEB-1 sebocytes treated with 13-*cis* RA. SEB-1 sebocytes were treated with 0.1 μM (**a**) 13-*cis* RA or (**b**) vehicle for 72 hours. Note decrease in intracellular lipids with cells treated with 13-*cis* RA. Bar = 50 μm.

In addition to analyzing changes in total lipid production, changes in each individual lipid were also analyzed. Results for PPARα/δ agonist GW2433, PPARα/δ/γ agonist GW4148, PPARγ agonist rosiglitazone, and 13-*cis* RA are shown in Figure 3. The PPARα/δ agonist GW2433, PPARα/δ/γ agonist GW4148, and the PPARγ agonist rosiglitazone caused a statistically significant increase in the incorporation of acetate into cholesterol and fatty alcohols, indicating an effect on both the cholesterol and fatty acid biosynthetic pathways. In addition the PPARγ agonist rosiglitazone, caused a statistically significant increase in the levels of triglyceride (68%) when compared to vehicle treated control. In contrast, 13-*cis* RA decreased acetate incorporation into lipids derived from both the cholesterol and fatty acid biosynthetic pathways.

DISCUSSION

Acne is the most common skin condition affecting young people. Acne treatment is limited by the lack of effective agents that reduce sebum apart from isotretinoin and from hormonal therapy in women. With concerns regarding issues of teratogenicity with isotretinoin, alternative means of reducing sebum production are clearly needed. Developments in

this area are dependent upon advances in our understanding of the factors that regulate lipid production in human skin and sebaceous glands.

Pivotal studies in rat preputial cells, SZ95 immortalized human sebocytes, and isolated human sebaceous glands have raised the interesting possibility that compounds acting at PPARs can alter sebaceous lipids and, as such, may be useful in the treatment of acne (Rosenfield *et al.*, 1999; Chen *et al.*, 2003; Downie *et al.*, 2004). To date, only initial clinical studies with zileuton, an inhibitor of leukotriene B4 synthesis, where leukotriene B4 is a potent natural ligand of PPARα, exist, which revealed a significant reduction of acne lesions and sebaceous lipid synthesis (Zouboulis *et al.*, 2003; Zouboulis Ch *et al.*, 2005). In this study, we confirm that PPARs are expressed in human skin as well as sebocytes and we demonstrate that PPAR agonists increase sebum production in adults with diabetes and hyperlipidemia.

Each of the PPAR subtypes are expressed in rat preputial sebocytes, SZ95 immortalized human sebocytes, human sebaceous glands, hair follicles, and cultured human keratinocytes (Rivier *et al.*, 1998; Rosenfield *et al.*, 1999; Billoni *et al.*, 2000; Chen *et al.*, 2003; Downie *et al.*, 2004). Localization studies in whole specimens of human skin have been completed, showing that all PPAR subtypes are present in sebaceous glands and the pilosebaceous ducts of both healthy and acne-involved skin (Chen *et al.*, 2003; Alestas *et al.*, 2006). PPAR expression was verified in SEB-1 using immunohistochemistry. Our data confirm that each of the PPAR subtypes is expressed in human skin where they localize to epidermis, sebaceous glands, and hair follicles. Each subtype is expressed in the basal layer of the sebaceous gland with PPARγ expression noted in differentiated cells within the gland. These findings are in agreement with those of Alestas *et al.* (2006), wherein each of the PPAR subtypes was expressed within the sebaceous gland of healthy skin and in acne-involved and uninvolved skin of subjects with acne.

Ligands of PPARδ increase lipid accumulation in murine keratinocytes (Schmuth *et al.*, 2004). There have been conflicting reports, however, with regard to the effects of PPAR ligands on sebaceous lipid production. Studies carried out in rat preputial cells indicate that PPAR ligands such as fibrates, linoleic acid, and thiazolidinediones increase intracellular lipid droplets (Rosenfield *et al.*, 1999; Chen *et al.*, 2003). This effect is more pronounced when cells are simultaneously treated with dihydrotestosterone or in the presence of an retinoid X receptor agonist (Rosenfield *et al.*, 1998, 1999; Kim *et al.*, 2001). Studies carried out in SZ95 sebocytes indicate that linoleic acid, but not other ligands of PPARα or PPARγ, increase lipid production (Chen *et al.*, 2003). However, studies in isolated human sebaceous glands indicate that PPAR ligands such as linoleic acid decrease lipogenesis (Downie *et al.*, 2004).

Since 13-*cis* RA is the most potent known agent for reducing sebum production, it was used as a comparator in the *in vitro* studies of the activity of PPAR ligands on sebaceous gland lipogenesis. 13-*cis* RA markedly decreased acetate incorporation into lipids, which is in agreement with data generated in primary cultures of human sebocytes (Zouboulis *et al.*, 1991). This was accompanied by a decrease in intracellular lipids as seen with Oil Red O staining. In contrast, PPAR ligands such as the PPARα agonist GW7647, PPARδ agonist GW0742, the PPARα, δ agonist GW2433, PPARγ agonist rosiglitazone, and the PPAR pan-agonist GW4148 increased lipogenesis. The pattern of lipid synthesis was altered by many of these compounds to favor the incorporation of acetate into cholesterol, fatty acids, and fatty alcohols, effects opposite to that of 13-*cis* RA. Rosiglitazone induced a 68% increase in acetate incorporation into triglycerides which supports the recent findings of increased triglycerides in hamster sebaceous glands treated with a similar PPARγ agonist, troglitazone (Iwata *et al.*, 2005). These findings in cell culture are in agreement with the known effects of PPAR ligands in upregulating many of the genes involved in fatty acid and cholesterol biosynthesis. Apart from possible differences in the lipogenic response to PPARδ and PPARγ agonists, no biological differences were noted between SEB-1 and SZ-95 sebocytes (Chen *et al.*, 2003).

A similar trend in sebum production was noted in patients treated with PPARα and γ agonists. Sebum production was significantly greater in patients with hyperlipidemia receiving fibrates (PPARα agonists) and in patients receiving thiazolidinediones (PPARγ agonists) for type II diabetes compared to age-, sex-, and disease-matched controls. No ligands of PPARδ are approved for use in humans to allow testing of their effects on sebum production. Although sebum is increased, no increase in the incidence of acne has been reported in patients who have been treated with fibrates or thiazolidinediones. This is not surprising as these patients are older than those affected by acne. Many older patients have seborrhea but do not have acne. It is important to appreciate that these data were obtained in patients with underlying medical problems and that by virtue of their disease, differences in body mass index, and triglyceride levels were noted. Definitive studies in otherwise healthy subjects, perhaps in a younger age group would be required to most accurately assess the effects of PPAR ligands on sebum production.

Although rosiglitazone increased lipid production in SEB-1 cells, the levels were relatively unchanged after treatment with another PPARγ agonist, pioglitazone. These data agree with reports that show that while rosiglitazone and pioglitazone have similar effects in lowering glucose, they exert significantly different effects on triglyceride, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol particle size, and concentration (Boyle *et al.*, 2002; Khan *et al.*, 2002; Goldberg *et al.*, 2005). Of interest is the lack of inhibition of sebocyte lipogenesis by a PPARγ antagonist. The compound GW0072 though labeled as a PPAR antagonist by some is actually one of the first partial agonists of PPAR activity (Oberfield *et al.*, 1999; Berger and Moller, 2002; Rival *et al.*, 2004). While earlier studies described this compound as a PPARγ antagonist because of its ability to inhibit thiazolidinedione-induced adipocyte differentiation, more recent studies have focused on the analysis of the

co-crystal structure of this compound with the PPARγ ligand-binding domain and highlighted a more complex mechanism of action and termed the compound as a partial agonist (Oberfield *et al.*, 1999). Further studies examining the role of PPARγ partial agonists in the presence of PPARγ agonists may help to bring to light the true antagonistic properties of these compounds.

The specific identity of the endogenous ligands that act on PPARs in the skin is unknown. Potential ligands include fatty acids, eicosanoids, and other lipids found in the skin and sebaceous glands. This hypothesis is supported by pilot observations of decreased sebum production and acne in patients treated with zileuton, an inhibitor of lipoxygenase enzymes that are known to result in the formation of eicosanoids that interact with PPARs (Zouboulis *et al.*, 2003). Clearly additional clinical studies in varying populations of patients affected by acne will help determine whether agonism or antagonism of specific PPAR subtypes reduces sebum production and improves acne.

MATERIALS AND METHODS

Cell culture

The SEB-1 sebocyte cell line was produced from human sebocytes that were transformed with SV 40 large T antigen (Thiboutot *et al.*, 2003). Passage 21–23 SEB-1 immortalized human sebocytes were used in all experiments. They were cultured in standard sebocyte medium consisting of DMEM containing 5.5 mM glucose supplemented with Ham's F-12 (3:1), fetal bovine serum (2.5%), adenine (1.8×10^{-4} M), hydrocortisone (0.4 μg/ml), insulin (10 ng/ml), epidermal growth factor (3 ng/ml) and cholera toxin (1.2×10^{-10} M) for immunohistochemistry and assays of lipogenesis. Serum-free sebocyte medium used in lipogenesis experiments consisted of DMEM containing 5.5 mM glucose and antibiotics without any other additives.

Antibodies

For immunohistochemistry in SEB-1 sebocytes and in formalin-fixed, paraffin-embedded sections of the human skin, polyclonal rabbit anti-human antibodies to PPARα, PPARδ, and PPARγ were purchased from Cayman Chemical Company (Ann Arbor, MI) and used at dilutions of 1:500, 1:250, and 1:200, respectively. Biotinylated goat anti-rabbit secondary antibody was purchased from Vector Laboratories Inc. (Burlingame, CA) and used at 1:200 dilution.

Immunohistochemistry

Immunohistochemistry was performed on SEB-1 sebocytes and formalin-fixed, paraffin-embedded human skin sections using the avidin–biotin complex method and AEC development (ABC kit and AEC Substrate Kit for Peroxidase, Vector Laboratories Inc.; Burlingame, CA). Briefly, SEB-1 sebocytes were grown until 30–50% confluent or confluent in standard medium on chamber slides before the addition of 1 μM insulin for 24 hours to correlate immunoreactivity with confluency and the differentiation state of the cells. Cells were fixed with 100% methanol. Sections of normal facial skin were subjected to deparaffinization, rehydration, and antigen retrival prior to immunohistochemistry. Antigen retrival was preformed using TRILOGY buffer (Cell Marque, Hot Springs, AR).

Oil red O staining

SEB-1 (p23) sebocytes were treated with 0.1 μM 13-*cis* RA or vehicle (DMSO) to determine whether these cells decrease their lipid content in response to 13-*cis* RA. Cells were grown in standard medium and were then treated with 13-*cis* RA or vehicle in serum-free medium for an additional 3 days. Oil Red O staining was performed using standard techniques and slides were counterstained with hematoxylin.

Clinical protocols for sebum assessment in subjects treated with PPAR ligands

The clinical protocol for the assessment of sebum in subjects treated with PPAR ligands was approved by the Institutional Review Board of the Pennsylvania State University College of Medicine and was conducted in accordance with the guidelines set forth in the Declaration of Helsinki Principles. All subjects signed informed consent. Subjects were recruited from the Diabetes clinic or Cardiology clinic at the Milton S. Hershey Medical Center. Since drugs of the fibrate and thiazolidinedione class are in use for the treatment of hyperlipidemia and diabetes, we sought to study the effects of these drugs on sebum production in patients receiving these medications compared to age-, sex-, and disease-matched controls in order to provide a potential rationale for the future study of these drugs in acne. Inclusion criteria included men and women aged 18–65 years with diabetes who have been taking drugs of the thiazolidinedione class (pioglitazone or rosiglitazone), which are PPARγ agonists, or patients with hyperlipidemia who had been taking a fibrate (gemfibrizol and fenofibrate) which are PPARα agonists. Patients had received treatment with these agents for a minimum of 3 months. Age- and disease-matched controls were recruited from both clinics. Subjects were excluded from the study if they were on any form of hormonal therapy, were pregnant or breast feeding, were previously diagnosed with hyperandrogenism or polycystic ovary disease, or who were within 6 months of treatment with isotretinoin as these factors could influence sebum production. Following informed consent, subjects gave their medical history including concomitant medications and had three sebum readings taken from left, center, and right sides of the forehead. For diabetic patients receiving thiazolidinediones and their matched controls data were collected regarding duration of diabetes, hemoglobin A1C, serum creatinine, urine albumin, body mass index, and presence of vascular disease. For the patients in the fibrate group and their matched controls, data on total cholesterol, triglyceride, and duration of hyperlipidemia were collected. These clinical parameters were examined for differences between the treatment and control groups using an unpaired *t*-test, $\alpha = 0.05$.

Sebum assessment in human subjects

An estimate of the quantity of skin surface sebum in subjects treated with oral fibrates or oral thiazolidinediones was performed using Sebumeter SM 810® (Courage + Khazaka Electronic, Köln, Germany) that uses a photometric method of analysis of skin surface sebum. The skin was cleansed with a 70% isopropyl alcohol swab. A cassette containing a 0.1 mm thickness of synthetic tape is applied to the skin for 30 seconds. The cassette head exposes a 64 mm^2 measuring section of the tape. For the determination of the amount of sebum, the measuring head is inserted into the aperture of the

device, where a photocell measures the transparency of the tape. The light transmission is representative of the sebum content on the surface of the measuring area. A microprocessor calculates the μg sebum/cm^2 of the skin. Three measurements were taken from each test area on the forehead. An average of the three test sites was calculated and a Student's *t*-test was performed to determine differences between patients treated with thiazolidinediones versus controls and those treated with fibrates versus controls.

PPAR ligands

GlaxoSmithKline (Research Triangle Park, NC) generously supplied a series of subtype-specific PPAR ligands GW7647 (α agonist), GW0742 (δ agonist), GW2433 (α/δ agonist), GW4148 ($\alpha/\delta/\gamma$ pan agonist), and GW0072 (antagonist). 13-*cis* RA was obtained from Sigma (St Louis, MO) and the PPAR agonists rosiglitazone and pioglitazone were obtained from Cayman Chemical Company (Ann Arbor, MI). All ligands were solubilized in DMSO.

Lipogenesis assay: ^{14}C-acetate incorporation into neutral lipids

SEB-1 sebocytes were treated with PPAR ligands and 13-*cis* RA at concentrations listed in Table 2 and were assayed for the incorporation of ^{14}C-acetate into neutral lipids. Preliminary dose–response studies were performed with three doses chosen around the reported K_m's for these compounds. The doses shown in Table 2 represent data obtained with the highest dose. Three experiments were performed at three different days for each ligand tested. SEB-1 cells were cultured in standard medium until confluent. Cells were then treated every 3 days over a 6-day period with either a PPAR ligand in serum free medium or serum free medium with 0.1% DMSO as a negative control. Cells were treated with 13-*cis* RA for 72 hours as marked cell death occurs with longer time points. Cells were then collected with trypsin and an aliquot taken for a cell count that was used to normalize the data. The remaining cells were suspended in a solution containing 1 μCi of ^{14}C-acetate in either DMEM alone (5.5 mM glucose) or DMEM (5.5 mM glucose) containing varying concentrations of the compound to be tested and incubated for 2 hours at 37°C. Lipids were extracted, separated with thin layer chromatography and subjected to liquid scintillation counting according to methods in place in our laboratory (Smith *et al.*, in press). Acetate incorporation into lipids was expressed as c.p.m. ^{14}C-acetate incorporated/10^6 cells/hour.

Statistical analyses

Sebum assessments in patients treated with topical fibrates and thiazolidinediones. Using a predetermined assessment of the variability in sebum measurements, a sample size determination was performed for the assessment of sebum production in patients treated with fibrates or thiazolidinediones. With 75 subjects in each group (control, thiazolidinedione, or fibrate), there would be a 90% power to detect a 20% change in sebum. Two-sided unpaired *t*-tests ($\alpha = 0.05$) were performed to look for differences in sebum excretion between: (1) thiazolidinedione and matched control group and (2) fibrate and matched control group. The effect size of 20% used to determine the sample size was chosen by conjecture and not based on historical data. Therefore, interim analyses were planned at fixed time intervals during study enrollment so that recruitment would be stopped if the accumulated data provided evidence that the true effect size was larger than 20%.

Lipogenesis assays in SEB-1 sebocytes treated with PPAR ligands and 13-cis RA. To test for the null hypothesis of equal lipid levels in control group and groups treated with compounds, repeated measures analysis of variance models with terms indicating date when the experiments were done, treatment and date by treatment interaction effects were used to fit the data. All analyses were done using computer package SAS version 8 (SAS Institute Inc., Cary, NC).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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