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Research Paper

PPARα/γ Expression and Activity in Mouse and Human Melanocytes and Melanoma Cells

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Purpose. We examined the expression of PPARs and the effects of PPARα and PPARγ agonists on growth of mouse and human melanocytes and melanoma cells.
Methods. PPARα,β, and PPARγ mRNA qualitative expression in melan-a mouse melanocytes, B16 mouse melanoma, human melanocytes, and A375 and SK-mel28 human melanoma cells was determined by RT-PCR, while quantitative PPARα mRNA levels were determined by QuantiGene assay. PPARα and PPARγ protein was assessed by Western blotting. The effect of natural and synthetic PPAR ligands on cell growth was determined by either hemocytometer counting or crystal violet assay. PPAR transcriptional activity was determined by a PPRE-reporter gene assay, while knockdown of PPARα expression was achieved by transient transfection of siRNA.
Results. Both mouse and human melanoma cells produced more PPARα and PPARγ protein compared to melanocytes. PPARα mRNA levels were elevated in human melanoma cells, but not in mouse melanoma cells relative to melanocytes. Silencing of PPARα in human melanoma cells did not alter cell proliferation or morphology. PPARγ-selective agonists inhibited the growth of both mouse and human melanoma cells, while PPARα-selective agonists had limited effects.
Conclusion. Increased expression of PPARα in melanoma relative to melanocytes may be a common occurrence, however its biologic significance remains to be determined. PPARγ agonists may be useful for arresting the growth of some melanomas.

KEY WORDS: growth inhibition; melanocytes; melanoma; PPAR expression.

INTRODUCTION

PPARs are members of the nuclear receptor superfamily of ligand-activated transcription factors. There are three genes that give rise to the three subtypes—PPARα, PPARβ/δ and PPARγ. Endogenous fatty acids and synthetic agonists activate these receptors (1–3). Upon activation, PPARs regulate genes involved in lipid and glucose homeostasis (1–3). These receptors also regulate normal and disease related processes, including embryo implantation, inflammation, cell cycle, differentiation and cancer (3).

Activation of PPARs has been reported to decrease cell growth and induce differentiation in many cancer cell lines (4–6). However, there is little information on the PPAR subtypes and relative levels of PPAR expressed in melanocytes and melanoma cells. We therefore characterized the types of PPARs, level of PPARα and PPARγ mRNA and protein, and the ability of PPAR-selective agonists to inhibit proliferation in mouse and human melanocytes and melanoma cells.

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ABBREVIATIONS: CDK, cyclin depdendent protein kinase; GAPDH, glyceraldehyde phosphate dehydrogenase; MCAD, medium chain acyl CoA dehydrogenase; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator response element.

MATERIALS AND METHODS

PPAR Agonists

Leukotriene B_4 was obtained from Sigma Chemical Co. (St. Louis, MO). Cigliazone, PGJ_2, and WY14643 were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Troglitazone was obtained from Axxora, LLC (San Diego, CA).

Cell Culture

B16 mouse melanoma, as well as SK-mel28 and A375 human melanoma cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Invitrogen, Carlsbad, CA), as described previously (7). Melan-a cells (Dr. D. Bennett, Department of Anatomy and Developmental Biology, St. George's Hospital Medical School, London, England) were growth in RPMI 1640 medium as previously described (7). HEMn-LP human melanocytes cells were derived from human foreskin (Cascade Biologics, Portland, OR) and maintained in Medium 254 supplemented with 50 ml HMGS (Cascade Biologics) and 1 ml PSA solution (Cascade). Cells

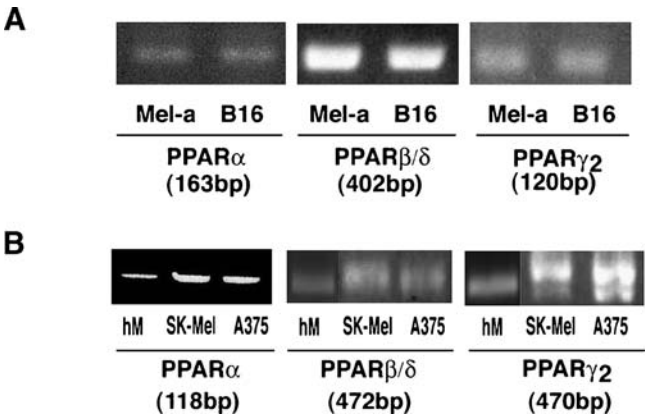

Fig. 1. Expression of PPAR subtype mRNA in **A** mouse and **B** human melanocytes and melanoma. RNA was isolated, purified and reverse transcribed using the Advantage RT-for PCR kit (Clontech, Palo Alto, CA). PCR analysis was performed using the specific primers for each subtype listed in the "Materials and Methods".

were maintained in a 10% CO_2/90% air humidified atmosphere. Both the B16 and melan-a cell lines were derived from the C57/BL6 mouse. SK-mel28 and A375 were obtained from the ATCC (Manassas, VA).

Western Blots and Antibodies

Equal amounts of protein (50 μg) were separated using 7.5% SDS-PAGE gels and transferred to blotting membranes. The blots were blocked with 5% non-fat dry milk overnight at 4°C and probed with the following antibodies: monoclonal mouse PPARα for mouse melanocytes and melanoma (Affinity Bioreagents, Golden, CO) 1:250; and polyclonal PPARγ2 1:500 (Cell Signaling Technology, Beverly, MA). For the human melanocytes and melanoma cells, PPARα antibody (Panomics, Freemont, CA) was used according to the manufacturer's protocol. PPARγ (Cell Signaling Beverly, MA) 1:1,000; MCAD antibody (Cayman Chemicals Ann Arbor, MI) 1:2,000, and β-actin antibody (Sigma) 1:5,000. Secondary antibodies were polyclonal HRP-linked anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) and monoclonal anti-mouse IgG (Amersham, Chicago, IL) used at 1:3,000. Blots were developed with ECL reagent (Amersham, Chicago, IL), and the chemiluminescent signal quantitated with a Chemi-doc Imager (Biorad).

Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted using Tri-Reagent (Sigma Chemical Co.), and reverse transcribed using the RT for PCR kit (Clontech, Palo Alto, CA). PCR was performed using the following primers: mouse; PPARα forward 5′-AGCTGGTG TAGCAAGTGT-3′, reverse 5′-TCTGCTTTCAGTTTTGC TTT-3′ product is 163 bp; PPARβ/δ forward 5′-CCCGGGAA GAGGAGAAAGAG-3′, reverse 5′-AAAGCGGATAGC GTTGTGC-3′ product 402 bp; and PPARγ2 forward 5′-CCAGTGTGAATTACAGAAATCTCTGTTTTATGCTG-3′ reverse 5′-AGAACGTGATTTCTCAGCC-3′ product 120 bp. Human; PPARα forward 5′-GGCAAGACAAGCT CAGAAC-3′, reverse 5′TTATCTATGAAGCAGGAAGC AC-3′ product 118 bp; PPARβ/δ forward 5′-ATGGAG CAGCCACAGGAG-3′, reverse 5′-CCACCAGCTTCCT CTTCTCA-3′ product 472 bp; PPARγ2 forward 5′-GGGT GAAACTCTGGGAGATTCTC-3′, reverse 5′-CCCTTGCA TCCTTCACAAGCATG-3′ product 470 bp. The PCR was performed with the AccuPrime *Taq* DNA Polymerase System (Invitrogen) with the following conditions: PPARα and PPARγ, 40 (35 human) cycles of 94°C, 30 s (1°min human); 94°C, 30 s; 68°C, 1 min: PPARβ/δ, 40 cycles of: 94°C, 30 s; 65° C, 30 s, 68°C, 1 min. PCR products were electrophoresed through 1% agarose gels and visualized with ethidium bromide.

QuantiGene Assay

RNA from cells was extracted with Tri-Reagent. QuantiGene assays (Panomics, Inc., Freemont, CA) were performed according to the manufacturer's protocol. Briefly, 2 μg/10 μl RNA was loaded into each well of a 96 well plate and incubated with PPARα, β-actin or GAPDH target probes, in conjunction with dendrimer DNA amplifier and labeled probes at 56°C overnight. Following several washes with supplied buffer, chemiluminescent substrate was added to the wells and incubated at 56°C for 30 min, then read on a Centro LB 960 luminometric plate reader (W. Nuhsbaum Inc., Mc Henry, IL).

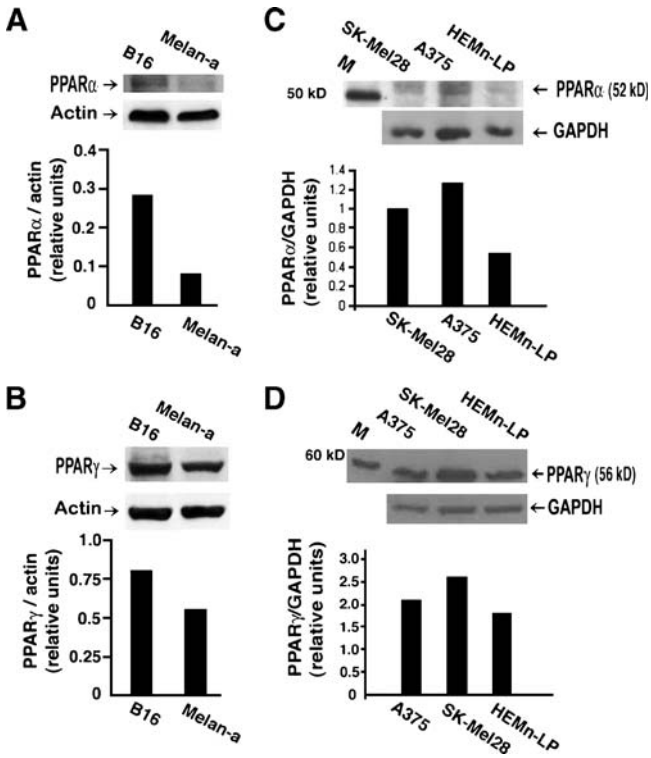

Fig. 2. Expression of PPAR subtype protein in mouse and human melanoma cells. Proteins were separated and PPARs detected by selected antibodies as outlined in "Materials and Methods". Relative amounts of PPARα and PPARγ were determined by densitometry and corrected for the amount of actin or GAPDH in each sample. **A** and **B** PPARα and PPARγ respectively in mouse melan-a melanocytes and B16 melanoma. **C** and **D** PPARα and PPARγ in human melanocytes (HEMn-LP) and Sk-mel28 and A375 human melanoma cells. A representative analysis is shown. The experiments were repeated three additional times with similar qualitative results.

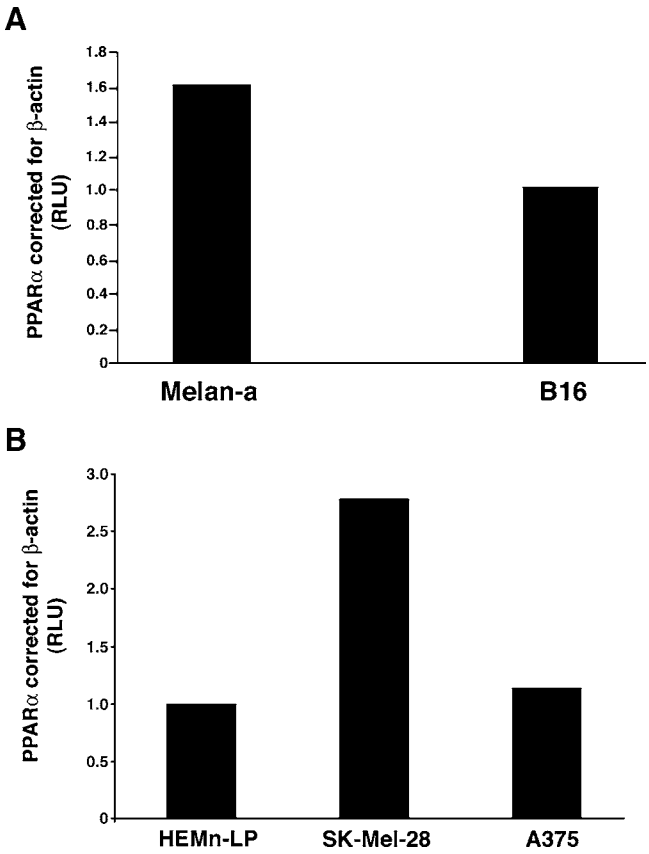

Fig. 3. Quantitation of PPARα mRNA in mouse and human melanocytes and melanoma cells. The QuantiGene assay system was used to determine relative amounts of PPARα mRNA. Cell lysates were loaded into wells of a QuantiGene capture plate and hybridized to probes specific for mouse or human PPARα mRNA. Relative light units were measured on a luminometer. The ratio of PPARα expression, corrected for β-actin is shown. **A** Mouse melanocytes (melan-a) and melanoma (B16). **B** Human melanocytes (HEMn-LP) and melanoma (SK-mel28 and A375).

Knockdown of PPARα

SK-mel28 cells were seeded at 1.5×10^5 cells/well in a six-well plate. At 24 h after seeding, cells were treated with 100 nM control or PPARα siRNA (Dharmacon, Lafayette, CO), using the RNAifect kit (Qiagen, Inc., Valencia, CA). At 48, 72, and 96 h after transfection, total RNA was isolated using Tri-Reagent (Molecular Res, Cincinnati, OH) and the amount of PPARα RNA determined by QuantiGene assay. The amount of MCAD protein was determined by Western blotting using 50 μg of total cell protein.

Reporter Gene Assays

B16 cells were seeded at a density of 3×10^5 per 60 mm tissue culture dish. Each assay was performed in triplicate. B16 cells were transfected with β-galactosidase (β-galactosidase enzyme assay system, Promega Corp., Madison, WI); pGL2–2×-PPRE–Luciferase reporter plasmid (Dr. John Capone, Department of Biochemistry, McMaster University Hamilton, ON, Canada); pSV sport–PPARγ expression vector (Dr. Bruce Spiegelman, Dana-Farber Cancer Institute, Boston, MA). The plasmid DNA ratio was 1:10:10 respectively. Lipofectamine plus reagent kit was used to transfect the cells (Invitrogen). After transfection, media, serum and appropriate agonists were added to the cells. Cells were harvested after 48 h, and β-galactosidase and luciferase assayed using kits from Promega Corp. Chemiluminescence was determined using a Lumat LB 9501 Luminometer. Transfection efficiency was corrected by measuring the amount of β-galactosidase activity.

RESULTS

Expression of PPAR Subtypes

The expression of PPAR subtypes in mouse and human melanocytes, as well as in mouse and human melanoma cells was examined using RT-PCR. Figure 1A and B shows that both mouse (A) and human (B) melanocytes and melanoma cells express mRNA for PPARα, PPARβ/δ, and PPARγ2.

Since RT-PCR provides qualitative results, we measured the relative amount of PPARα and PPARγ protein and PPAR α mRNA in these cells using Western blotting and QuantiGene assays respectively. Mouse and human melanoma cells express higher levels of PPARα (52 kD) and PPARγ (56 kD) protein compared to their non-malignant counterparts (Fig. 2). Densitometry of the immunoblots revealed that B16 mouse melanoma cells have threefold more PPARα and 40% more PPARγ protein than melan-a mouse melanocytes (Fig. 2A and B). SK-mel28 and A375 human melanoma cells

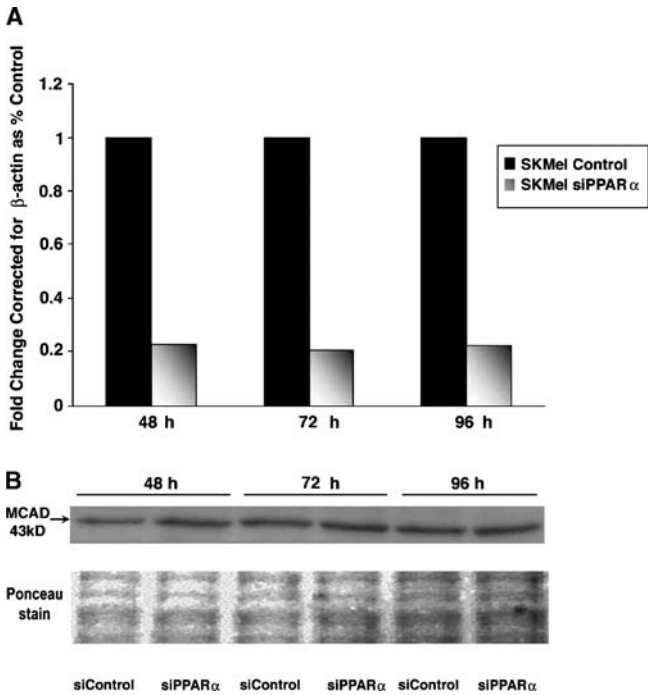

Fig. 4. A QuantiGene assessment of PPARα knockdown and expression of MCAD. SK-mel28 human melanoma cells were transfected with control or PPARα specific siRNA (Dharmacom). At the indicated time, cells were harvested and assayed for the relative amount of PPARα by QuantiGene analysis as described in Fig. 3. **B** The amount of the PPARα target gene, MCAD was determined by immunoblotting using 1:2,000 MCAD antibody (Cayman Chemicals, Ann Arbor). In these experiments, total protein on the blot, as determined by Ponceau staining, was used as a loading controll.

have PPARα protein levels 47 and 20% higher, respectively, than the normal human melanocytes (Fig. 2C). PPARγ protein levels are 40–50% greater in both human melanoma cell lines relative to normal human melanocytes (Fig. 2D). QuantiGene assays (Panomics) revealed that mouse B16 melanoma cells had 60% less PPARα mRNA compared to melan-a melanocytes (Fig. 3A). This is in stark contrast to the level of PPARα protein, which is threefold higher in B16 relative to melan-a cells. PPARα mRNA was nearly threefold higher in SK-mel28 human melanoma cells relative to normal human melanocytes, while A375 melanoma cells had only a slight increase in this mRNA relative to normal human melanocytes (Fig. 3B). Although not as drastically different as the PPARα protein *vs* RNA for B16 cells, there is still not a strong correlation between mRNA and protein levels for this receptor in the human melanoma cells. Unfortunately, we could not quantitate PPARγ mRNA levels since there are no capture probes available for this RNA species.

Knockdown of PPARα Expression in Human Melanoma Cells

To determine if increased PPARα expression in human melanoma cells contributes to their *in vitro* transformed properties, we decreased its expression by transfecting SK-mel28 cells withPPARα selective siRNA. The effectiveness of the PPARα siRNA was assessed by QuantiGene assay. Transfection of these siRNAs into SK-mel28 cells resulted in >80% decrease in PPARα relative to cells transfected with control siRNA. The decrease was maintained for at least 96 h

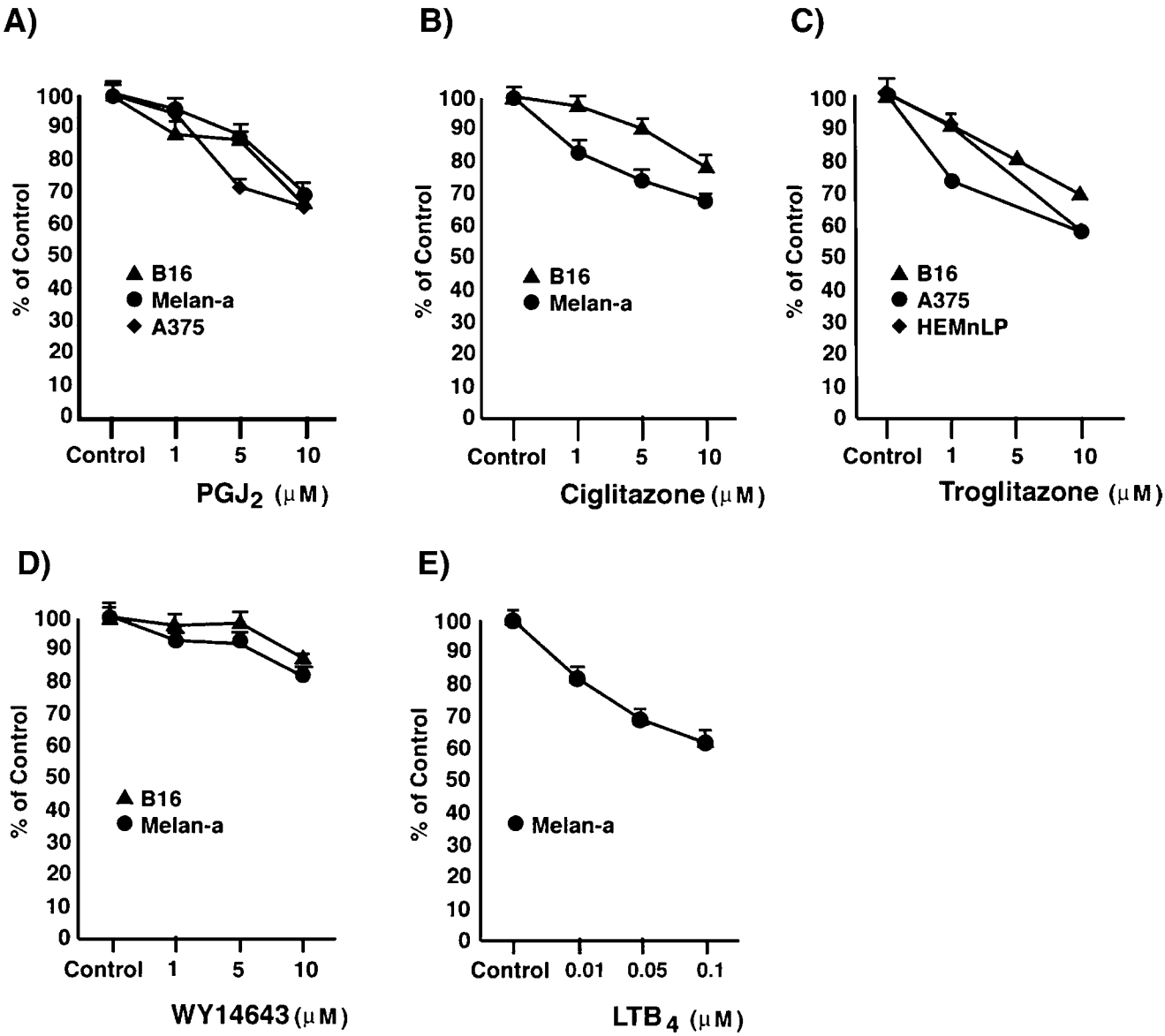

Fig. 5. Effect of PPAR agonists (**A** PGJ2, **B** ciglitazone, **C** troglitazone, **D** WY14643, and **E** LTB4) on mouse and human melanocyte and melanoma proliferation. Triplicate dishes of cells were treated with the indicated concentration of agonist for 48 h (previously determined to be the most effective treatment time), then harvested and cell number determined either by cell counts with a hemocytometer (mouse melanocytes and melanoma) or crystal violet absorbance at 570 nm. Data is presented as percent of growth relative to cells treated with the vehicle only. The *error bars* represent the SEM of triplicate dishes at each concentration of agonist tested. All experiments were repeated a minimum of three times with similar results.

(Fig. 4A). There was no change in cell growth, morphology, or viability in cells having decreased PPARα mRNA compared to cells having high levels of PPARα mRNA (data not shown). A known transcriptional target of PPARα, medium chain acyl CoA dehydrogenase (MCAD) protein was measured by Western blotting to determine whether the PPARα knockdown decreased the amount of this protein. There was no change in MCAD protein levels between SK-mel28 cells treated with PPARα specific siRNA and those treated with control siRNA. (Fig. 4B). These results suggest that PPARα may not control the basal expression of MCAD.

Effect of PPARα and PPARγ Agonists on Cell Proliferation

We measured the effect of PPAR-selective agonists on melanocyte and melanoma cell proliferation. Agonist concentrations were chosen that would maximize receptor subtype specificity. The natural PPARγ agonist, PGJ_2 inhibited the growth of melan-a mouse melanocytes, B16 mouse melanoma cells, and A375 human melanoma cells (Fig. 5A). It had no effect, at the concentrations tested, on the proliferation of human melanocytes and SK-mel28 human melanoma cells (data not shown). The synthetic PPARγ agonist ciglitazone inhibited the growth of B16 mouse melanoma and melan-a mouse melanocytes, while troglitazone, another reportedly more powerful PPARγ agonist, also inhibited B16, while also inhibiting the growth of A375 human melanoma cells and normal human melanocytes (Fig. 5B and C), but not melan-a cells (data not shown).

The synthetic PPARα agonist, WY14643 had only slight effects on proliferation of melan-a and B16 cells, while the natural PPARα agonist, LTB_4 only inhibited the growth of the mouse melanocytes, melan-a (Fig. 5D and E). None of the PPARα agonists had an effect on the proliferation of normal human melanocytes or human melanoma cell lines.

PPAR Trans-Activation

PPAR agonists have been reported to have non-receptor-mediated effects. For example, PGJ2 has been shown to alter NF-κB activity (8). Therefore, we compared the concentration-dependent ability of the PPARγ agonists to stimulate PPRE reporter gene activity in B16 mouse melanoma cells and compared this effect to their ability to inhibit the growth of the same cell line. B16 cells were co-transfected with a PPARγ expression plasmid since endogenous PPRE reporter activity was too low to be accurately detected. The PPARγ agonists, PGJ_2 and ciglitazone stimulated PPRE reporter gene activity in a dose-dependent manner (Fig. 6). This correlated with their ability to inhibit B16 cell proliferation (Fig. 5A and B).

DISCUSSION

Aberrant expression of PPAR receptors has been found in several types of cancers such as colon, prostate, lung, skin, breast, and liposarcomas (9–14). Overexpression of PPARα might contribute to tumorigenesis through its ability to stimulate proliferation by enhancing expression of genes encoding CDK-1 and CDK-4 and c-myc (15,16). In contrast, expression and activation of PPARγ is reported to stimulate differentiation and inhibit cell proliferation of several carcinoma-derived cell lines (4,5,17). In the skin, PPARα is involved in maintaining epidermal barrier function as well as differentiation and wound healing (18,19). The types of PPAR expressed in normal melanocytes and whether their expression is altered in melanoma cells has not been well-studied. We found that all of the PPAR subtypes, including PPARβ/δ are expressed at the mRNA level by mouse and human melanocytes as well as all of the melanoma cell lines examined. Quantitative assay of mRNA levels revealed that melan-a mouse melanocytes had more PPARα mRNA than B16 mouse melanoma cells, while SK-mel28 human melanoma cells had much higher PPARα mRNA levels than normal human melanocytes or A375 melanoma cells. In contrast, mouse B16 melanoma cells had much higher amounts of PPARα protein relative to melan-a mouse melanocytes. Likewise, A375 human melanoma cells had high levels of PPARα protein relative to normal human melanocytes, despite having similar mRNA levels. These findings suggest a significant regulation of PPARα at the protein level. The amount of PPARγ protein was also higher in all of the melanoma cells relative to mouse or human melanocytes. Unfortunately, due to a lack of a PPARγ capture probe for the QuantiGene assay, we could not quantitatively compare the mRNA and proteins levels for this PPAR subtype to see whether it also might be subjected to regulation at the protein level. Kang *et al.* (20) also found that human melanocytes expressed all three PPAR subtypes. However, they did not compare levels of expression of these receptors between normal human melanocytes and human melanoma.

We investigated the biological significance of high PPARα expression in SK-mel28 cells by decreasing the level of its mRNA through transfection of siRNA. While we documented a >80% decrease in these cells relative to cells transfected with control siRNA, we could not detect any change in the cell phenotype (growth, morphology, viability). In this human melanoma cell line, there was a better correlation between PPARα mRNA and protein levels, thus lack of an effect of the knockdown on phenotype is probably not due to lack of knockdown of the protein, although if the PPARα protein has a very long half-life, then a strong

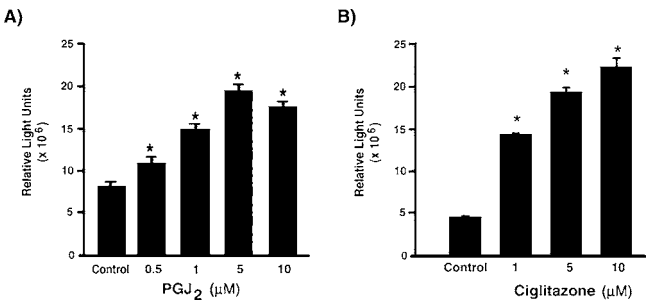

Fig. 6. Ability of PPARγ agonists to increase PPRE reporter gene expression. A β-galactosidase expression plasmid and a 2x PPRE pSG5–luciferase plasmid were transiently co-transfected into B16 cells. The transfection mixture also contained pSV-PPARγ, with a plasmid DNA ratio of 1:10:10 respectively. Cells were harvested and assayed for β-galactosidase and luciferase activity 48 h after agonist addition. The error bars represent the SEM of triplicate dishes of transfected cells. The experiment was repeated three times with similar results. *$p < 0.001$ relative to transfected cells without agonist. (**A**) PGJ2; (**B**) Ciglitazone.

knockdown of the corresponding RNA may not yield a large decrease in the protein over the time course of the experiment (96 h). Due to relatively low antibody reactivity with PPARα, we could not achieve quantitative protein comparisons between cells transiently transfected with control *vs* PPARα siRNA. It is possible that other PPARs can perform the functions of PPARα under the conditions used in our experiments.

Several groups have reported that PPARγ-selective ligands can inhibit the growth of human melanoma cells (21–23). However, in several of these reports, significant inhibition of proliferation did not occur until 20 μM or higher concentrations of ligand. Many natural and synthetic PPAR ligands loose their receptor selectivity at these concentrations (24). We therefore limited our ligand concentrations to a maximum of 10 μM. Growth of the human melanoma cell line SK-mel28 was not inhibited by any ligand tested, while human melanocytes were only affected by the PPARγ ligand troglitazone. The only cell line to be significantly affect by any PPARα ligands was melan-a mouse melanocytes. It is curious that mouse and human melanoma cells express high levels of both PPARα and γ relative to their non-transformed counterparts, yet are either not growth inhibited by receptor-selective ligands or their inhibition is similar to normal melanocytes. Since it has been reported that PPAR ligands can have receptor-independent effects (8,25), we compared their concentration-dependent ability to activate PPRE reporter gene activity with their concentration-dependent ability to inhibit the growth of B16 mouse melanoma cells. In general, we found a good correlation between these two activities in B16 cells. However, in these reporter gene assays, we cotransfected a plasmid encoding the PPARγ into the B16 cells, since it was difficult to detect endogenous ligand-activated reporter gene activity. Therefore, it is possible that the endogenous PPARα&γ receptors in the melanoma cells are defective in transcriptional activation despite being over-expressed relative to normal melanocytes.

CONCLUSION

In summary, we have found that mouse and human melanocytes and melanoma express PPARα, PPARβ/δ, and PPARγ2 receptors. The melanoma cells overexpress PPARα and PPARγ protein relative to melanocytes, however, there is a discordance between RNA and protein levels for these receptors. The ability of PPAR ligands, at concentrations where selectivity was maintained, to inhibit replication of these cells was variable and did not correlate with expression level of the relevant PPAR. We did not obtain convincing evidence that PPARs are involved in development or maintenance of the melanoma phenotype. However, PPARγ ligands at pharmacological concentrations might be useful in the treatment of certain melanoma lesions (26).

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