The Effect of Ligand-Activated PPARβ on Breast and Liver Cancer Cell Proliferation

Renee L. Killins, McNair Scholar, Penn State University

Jeffrey M. Peters, Ph.D
Associate Professor or Environmental Toxicology
Veterinary Science Department
Penn State University

Abstract

There is considerable controversy regarding the effects of ligand activation of peroxisome proliferator-activated receptor-β (PPARβ) on cell growth. Some studies show that ligand activation of PPARβ leads to a decrease in cell proliferation and promotes differentiation. However, other work shows that ligand activation of PPARβ increases proliferation of breast and colon cancer cells. The purpose of this study was to determine the effects of a PPARβ-specific ligand, GW0742, on the proliferation of breast and liver cancer cell lines, and to examine possible pathways through which ligand-activated PPARβ causes the observed effects. In contrast to some previous reports, results from these studies show that ligand activation of PPARβ caused inhibition of cell growth in both breast and liver cancer cell lines. We examined if changes in extracellular signal-regulated kinase (ERK) phosphorylation contributed to the decrease in cell proliferation, as ERK can be a central regulator of all growth. However, no change in ERK phosphorylation was observed in response to GW0742. Combined results from the studies show that ligand activation of PPARβ inhibits, but does not potentiate, cell growth of breast and liver cancer cells. Thus, PPARβ may be a target that could be used to inhibit liver cancer.

Introduction

The history of peroxisome proliferator-activated receptors began in 1990 when scientists cloned PPARα from rodent liver [1]. Shortly thereafter, the other two members of the PPAR family, PPARγ and PPARβ/δ, were identified [2]. PPARs are ligand-activated transcription factors belonging to the nuclear-hormone-receptor-receptor family (NHR) [3]. While the three receptors have similar structures, the expression and function of the three PPAR isoforms can vary greatly.

PPARα is found in liver, heart and skeletal muscle [4]. This receptor was found to function in lipid homeostasis, inflammation, and liver carcinogenesis in rodents. PPARγ is mainly found in adipose tissue. It functions in adipocyte differentiation, fatty acid storage and inflammation [4]. PPARβ/δ (from here on referred to as PPARβ) is ubiquitous with a high expression found in skin. Less is known about this isoform than what is known about PPARα and PPARγ. This is due, in part, to the fact that PPARα and PPARγ are targets of dyslipidemia and type 2 diabetes drugs [4]. Scientists have shown conclusively that PPARβ functions in keratinocyte

differentiation, apoptosis and cell proliferation [5]. However, the role of PPARβ in the differentiation, apoptosis and cell proliferation of other cell types remains controversial.

Xenografts from PPARβ-null colon cancer cells formed fewer tumors than the xenografts for wild-type colon cancer cells [6]. This suggests that PPARβ promotes cell growth of colon cancer cells. The pro-proliferative role of PPARβ has also been reported in response to ligand activation of this receptor in breast, prostate, and liver cancer cell lines ([7], [8]). In contrast, more evidence exists supporting an anti-proliferative role for PPARβ. In the first experiment to demonstrate the in vivo effects of PPARβ on epidermal cell proliferation, results showed that PPARβ-null mice had a greater hyperplastic response to TPA than wild-type mice [9]. Also, results indicated that null mice treated with TPA had more inflammation than the controls, suggesting that not only does PPARβ have anti-proliferative properties, but also anti-inflammatory properties [9]. A similar study done in 2005 supported these findings when mice treated with TPA had a greater hyperplastic response in the epidermis than controls; scientists found that these effects were mediated by changes to the MAP kinase pathway caused by PPARβ [10]. Further studies on the effect of PPARβ activation on keratinocytes showed that treatment with GW0742 resulted in keratinocyte terminal differentiation as well as inhibition of keratinocyte proliferation [11]. Also, topical treatment of mice with PPARβ-specific ligand, GW501516, resulted in keratinocyte differentiation as well as anti-inflammatory effects [12]. Another study also showed that PPARβ increased human keratinocyte differentiation [13]. Scientists have also studied the effects of PPARβ in colon carcinogenesis. Recently, it was found that PPARβ is involved in the negative growth control of lung cancer cells [14]. A recent study showed that PPARβ is involved in the differentiation of colon cancer cells [15]. Ligand activation of PPARβ has also been found to inhibit colon carcinogenesis in mice [16].

There is good evidence that PPARβ ligands may be useful for treating type 2 diabetes [17]. A study done in 2000 demonstrated the therapeutic value of PPARβ when the activation of PPARβ in insulin resistant db/db mice resulted in an increase of total plasma cholesterol concentrations which were associated with an increase in high density lipoproteins (HDL), the good cholesterol [18]. This experiment was repeated in insulin-resistant middle-aged obese rhesus monkeys and resulted in an increase in HDL levels as well [20]. In a more recent study, scientists treated macrophages with a PPARβ-specific ligand. This treatment resulted in an increase in the catabolism of fatty acids [19], thus further confirming the possibility of PPARβ ligands being used therapeutically. Furthermore, treatment of mice and rats with PPARβ-specific ligand, GW501516 was found to promote fatty acid oxidation in skeletal muscles as well as decrease plasma glucose and blood insulin levels in ob/ob mice [20]. Another similar study found that PPARβ is involved in the regulation of *ucp-2* gene expression, a gene involved in the regulation of ATP synthesis as well as the regulation of fatty acid oxidation [21]. However, before this receptor can be used as a pharmacological agent, it is important that the toxicological effects of its activation are fully known. The purpose of the present study was to determine the effects of ligand activation of PPARβ on cell proliferation of breast cancer (MCF7) and liver cancer (HEPG2) cell lines. The dose-dependent response of cell proliferation was studied using a PPARβ-specific ligand, GW0742. It was hypothesized that ligand activation of PPARβ would result in a dose-dependent decrease in cell proliferation in both cell lines.

Materials and Methods

Materials. DMEM was obtained from Sigma-Aldrich. MEM was obtained from Gibco. Sodium bicarbonate was purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was obtained from Gemini Bio Products.

Cell culture and proliferation. The MCF-7 cell line was purchased from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, penicillin (100 U/mL) and streptomycin sulfate (100 µg/mL). Both cell lines were grown in an incubator at 37° C with 5% CO2 and 95%O2. The HepG2 cell line was purchased from the American Type Culture Collection (ATCC) and grown in modified Eagle's medium (MEM) with 10% FBS, penicillin (100 U/mL) and streptomycin sulfate (100 µg/mL). Both cell lines were plated at 100,000 cells/well on 6-well plates. For the 14 day experiments, MCF-7 cells were treated with 2 µL of DMSO, and 100 nM 100 nM—5 µM GW0742 three days after being plated. Cells were counted on days 0, 7 and 14 using a Coulter counter. For the 7 day experiments, HepG2 cells were treated with 2 µL of DMSO, and 100 nM—5 µM GW0742 three days after being plated. Cells were counted daily using a Coulter Counter.

Protein Analysis. Nuclear extracts from HepG2 cells were collected from cells grown to 80% confluency using cell lysis buffer. Protein was quantified using a BCA assay. Following separation via SDS-PAGE and proteins were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane by electroblotting in standard Tris-glycine buffer. Immunodetection was performed using primary antibodies: αLDH, phosphorylated extracellular signal-regulated kinase (ERK/MAPK) and ERK/MAPK. Membranes were incubated in the primary antibodies at 4° C over night. Membranes were then incubated in biotinylated 2° antibodies: anti-goat for α LDH and anti-rabbit for phosphorylated ERK/MAPK and ERK/MAPK for one hour at room temperature and then washed in TBST. Membranes were then incubated in ^{125}I-strepavidin for 40 minutes and washed. Radiolabeled membranes were exposed to phosphorimager plates and the hybridization signal was quantified after normalization to LDH using a Cyclone Phosphorimager and Image Quant software.

Quantitative Real Time PCR. Cells were grown to 80% confluency and then treated in triplicate with 10 µL of either 0.1%DMSO or 500 nM GW0742. Cells were then harvested from cells using 750 µL of Trizol per dish. RNA was then purified using 0.25 mL of cold chloroform per tube and then centrifuged at 12,000 rpm for 15 minutes. Next, RNA was precipitated using isopropanol. Samples were centrifuged at 12,000 rpm in a refrigerated (4°) Eppendorf centrifuge for 15 minutes. The supernatant was removed, and samples were air-dried to remove any excess supernatant. RNA was then resuspended in 0.4 mL of DEPC water and vortexed. Then 0.4 mL of cold phenol was added and samples were again centrifuged at 12,000 rpm for 15 minutes. Finally, the upper phase was removed and placed in a fresh autoclaved tube and then spun down again. The supernatant was removed and the pellet was resuspended in DEPC water. The quantity of the RNA was then measured in a spectrophotometer at 260/280 nm. cDNA was generated using 2.5 µg total RNA with MultiScribe Reverse Transcriptase kit (Applied Biosystems). The GenBank accession numbers for the forward and reverse primers used to

quantify mRNAs were hADRP (NM_007408): forward, 5-CTGCTCTTCGCCTTTCGCT-3', and reverse, 5'-ACCACCCGAGTCACCACACT-3'. All mRNAs examined were normalized to the gene encoding glyceraldehyde-3-phosphate dehydrogenase (hGAPDH; BC013310) using the following primers: forward, 5'-TGCACCACCACCTGCTTAGC-3', and reverse, 5'-GGCATGGACTGTGGTCATGAG-3'. Real-time PCR reactions were carried out using SYBR green PCR master mix (Finnzymes, Espoo, Finland) in the PTC-200 DNA Engine Cycler and detected using the CFD-3200 Opticon Detector (MJ Research, Waltham, MA). The reactions were run at 95°C for 15 seconds, 94°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. This was repeated for 45 cycles. The PCR had a no-template control reaction in order to control for contamination. Relative expression levels of mRNA were normalized to GAPDH and analyzed for statistical significance using one-way ANOVA (Prism 4.0).

Results

Effect of GW0742 in the MCF-7 cell line. To investigate the effects of activated PPARβ on breast cancer cells, MCF-7 cells were treated with 0.1% DMSO, 100 nM or 500 nM GW0742 over the course of 14 days and counted every seven days. These results showed no significant differences between the treated groups and the control group (figure 1A).

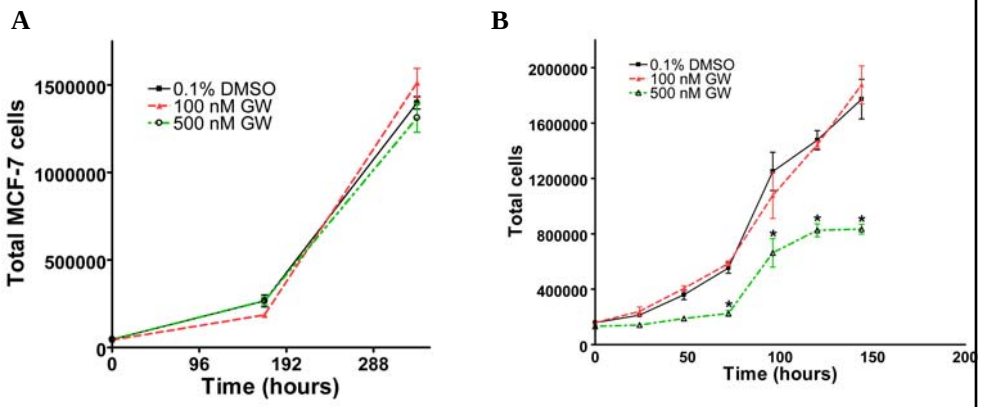

Figure 1. **Effect of GW0742 on cell proliferation.** Both cell lines were treated with 0.1% DMSO and 500 nM GW0742 for fourteen days. **A.** Results showed no significant difference between the treated MCF-7 cells and control cells. Data points are means ± S.E, averaged from 3 separate wells counted in triplicate. **B.** There was a significant decrease in HepG2 cell proliferation on days 3, 4, 5 and 6. Data points are means ± S.E, averaged from 3 separate wells counted in triplicate. *, values are significantly different from the control 0.1% DMSO

Effect of GW0742 in the HepG2 cell line. Liver cancer cells, HepG2, were treated with 0.1% DMSO, 100 nM or 500nM GW0742 over the course of 6 days and counted every day. Results showed that at 100 nM GW0742, there was no significant effect on cell growth, while at 500 nM, there was a significant decrease in cell proliferation (Figure 1B).

Activation of PPARβ via GW0742. In order to confirm that PPARβ was activated by the ligand treatments, a real time polymerase chain reaction (rtPCR) was performed to quantify the levels of adipose differentiation regulated protein (ADRP), a gene known to be up-regulated by PPARβ. Results showed that ADRP levels found in the treated MCF7 cells were not statistically different from those found in the control MCF7 cells (figure 2A). This finding may explain why there was no

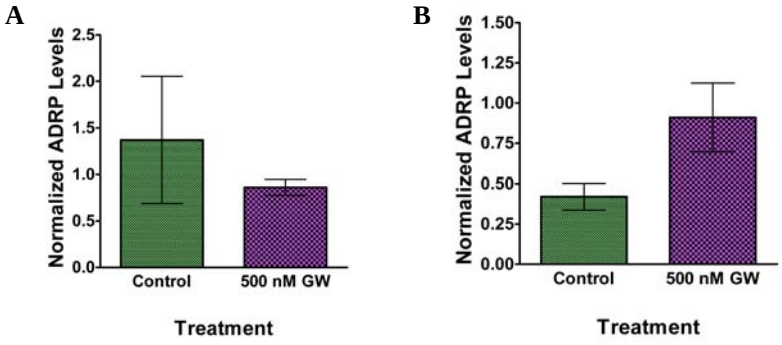

Figure 2. **Effect of GW0742 on ADRP gene expression**. Both cell lines were treated with 0.1% DMSO and 500 nM GW0742 and harvested at 6 hours. A real time PCR was performed to quantify the levels of ADRP, a gene known to be up-regulated by PPARβ. **A.** Results showed that PPARβ was not activated in MCF-7 cells by ligand treatment **B.** Results showed a clear increase in ADRP expression with GW0742 treatment in HepG2 cells.

significant change in the proliferation of MCF-7 cells when treated with GW0742. However, there was a clear increase in ADRP levels was found in the ligand-treated HepG2 cells, demonstrating that PPARβ was activated (Figure 2B).

Effect of GW0742 on HepG2 ERK and phospho-ERK levels. To determine the means by which ligand-activated PPARβ causes the observed effect of decreasing cellular proliferation, HepG2 cells were grown in 10 cm dishes and grown to 80% confluency. Once the cells reached the desired confluency, they were treated in triplicate with either 10 µL of 0.1% DMSO or 500 nM GW0742. Cells were harvested at 12 hour and 24 hour time points and a western blot was performed to determine the protein levels of ERK and phoshporylated ERK. Results indicated that treatment of cells with GW0742 does not significantly change the levels of ERK (Figure 3). There appeared to be no measurable levels of phosphorylated ERK in neither the control nor the treated cells (Figure 3).

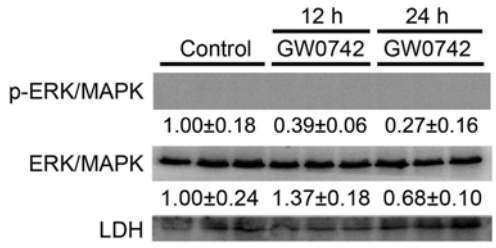

Figure 3. **Western Blot of HepG2 cells.** There was no significant change in the levels of ERK in treated cells. There was no appreciable amount of phosphorylated ERK in the HepG2 cell line

Conclusion

Results from this study, show that ligand-activated PPARβ has no significant effect on MCF-7 cell growth (a breast cancer cell line). These findings conflict with previous research showing that ligand activated PPARβ increased MCF-7 cell proliferation [7]. This could be due to differences in the methods of the experiments. For example, in the previous experiment, researchers used the PPARβ-specific ligand, compound F rather than the ligand, GW0742, which was used in our research. Findings from that study showed that under hormonal deprivation, PPARβ activation stimulated cell proliferation in MCF-7 cells. This was done by stripping the serum, however, in our study, the serum was not stripped. Furthermore, the previous study used MCF-7 Tet-On cells to ensure that there was enough PPARβ in the cells to be activated. The difference in the findings could be due to the fact that there was not enough PPARβ expressed in our MCF-7 cells to cause an effect on cell proliferation or it could be because the cells were not grown under hormonal deprivation. Further research should be done to determine the levels of PPARβ expressed in the control as well as the ligand treated MCF-7 cells.

From this research, it was determined that GW0742 ligand activation of PPARβ significantly decreases cell proliferation in HepG2 cells (a liver cancer cell line). These findings are consistent with many of the previous studies suggesting an anti-proliferative role of PPARβ and thus the possibility for a therapeutic use of PPARβ-specific ligands in treatments such as Type II diabetes or liver cancer treatment [11, 17, 18]. We believe that these effects are caused by PPARβ dependent changes in a cell cycle pathway. From these results, it can be concluded that this decrease in cell proliferation is not caused by PPARβ dependent changes in the ERK pathway. Further research should be done to determine the specific pathway, such as the p70S6K1 or the Jak-STAT pathways that PPARβ uses to cause the observed effects.

Collectively, results from these studies are inconsistent with previous work by others suggesting that PPARβ potentiates cell growth and are more consistent with the greater body of evidence suggesting that PPARβ inhibits cell growth. Results from this study suggest that the PPARβ-specific ligand, GW0742 could be a safe and useful pharmaceutical. Further research should be done to determine the in vivo effects of ligand-activated PPARβ on other cell types and to determine the specific mechanisms underlying these effects. Also, it is imperative to confirm that the effects found are not due to cytotoxicity to the cell line.

Works Cited

1. Issemann, I. and S. Green, *Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators.* Nature, 1990. **347**(6294): p. 645-50.
2. Burk, P.G., et al., *Xeroderma pigmentosum and D. N. A. repair.* Lancet, 1971. **1**(7699): p. 601.
3. Michalik, L., B. Desvergne, and W. Wahli, *Peroxisome-proliferator-activated receptors and cancers: complex stories.* Nat Rev Cancer, 2004. **4**(1): p. 61-70.
4. Staels, B. and J.C. Fruchart, *Therapeutic roles of peroxisome proliferator-activated receptor agonists.* Diabetes, 2005. **54**(8): p. 2460-70.
5. Burdick, A.D., et al., *The role of peroxisome proliferator-activated receptor-beta/delta in epithelial cell growth and differentiation.* Cell Signal, 2006. **18**(1): p. 9-20.
6. Park, B.H., B. Vogelstein, and K.W. Kinzler, *Genetic disruption of PPARδ decreases the tumorigenicity of human colon cancer cells.* Proc Natl Acad Sci U S A, 2001. **98**(5): p. 2598-2603.
7. Stephen, R.L., et al., *Activation of peroxisome proliferator-activated receptor delta stimulates the proliferation of human breast and prostate cancer cell lines.* Cancer Res, 2004. **64**(9): p. 3162-70.
8. Glinghammar, B., et al., *PPARdelta activation induces COX-2 gene expression and cell proliferation in human hepatocellular carcinoma cells.* Biochem Biophys Res Commun, 2003. **308**(2): p. 361-8.
9. Peters, J.M., et al., *Growth, adipose, brain and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor β(δ).* Molecular and Cellular Biology, 2000. **20**: p. 5119-5128.
10. Kim, D.J., et al., *PPARbeta/delta selectively induces differentiation and inhibits cell proliferation.* Cell Death Differ, 2006. **13**(1): p. 53-60.
11. Kim, D.J., et al., *Peroxisome proliferator-activated receptor-beta/delta inhibits epidermal cell proliferation by down-regulation of kinase activity.* J Biol Chem, 2005. **280**(10): p. 9519-27.
12. Schmuth, M., et al., *Peroxisome proliferator-activated receptor (PPAR)-beta/delta stimulates differentiation and lipid accumulation in keratinocytes.* J Invest Dermatol, 2004. **122**(4): p. 971-83.
13. Westergaard, M., et al., *Modulation of keratinocyte gene expression and differentiation by PPAR-selective ligands and tetradecylthioacetic acid.* J Invest Dermatol, 2001. **116**(5): p. 702-12.
14. Fukumoto, K., et al., *Peroxisome proliferator-activated receptor delta as a molecular target to regulate lung cancer cell growth.* FEBS Lett, 2005. **579**(17): p. 3829-36.
15. Aung, C.S., et al., *Isoform specific changes in PPAR alpha and beta in colon and breast cancer with differentiation.* Biochem Biophys Res Commun, 2006. **340**(2): p. 656-60.
16. Marin, H.E., et al., *Ligand activation of peroxisome proliferator-activated receptor beta inhibits colon carcinogenesis.* Cancer Res, 2006. **66**(8): p. 4394-401.
17. Luquet, S., et al., *Roles of PPAR delta in lipid absorption and metabolism: a new target for the treatment of type 2 diabetes.* Biochim Biophys Acta, 2005. **1740**(2): p. 313-7.
18. Leibowitz, M.D., et al., *Activation of PPARdelta alters lipid metabolism in db/db mice.* FEBS Lett, 2000. **473**(3): p. 333-6.

19. Lee, C.H., et al., *Peroxisome proliferator-activated receptor delta promotes very low-density lipoprotein-derived fatty acid catabolism in the macrophage.* Proc Natl Acad Sci U S A, 2006. **103**(7): p. 2434-9.
20. Tanaka, T., et al., *Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle and attenuates metabolic syndrome.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15924-9.
21. Chevillotte, E., et al., *The regulation of uncoupling protein-2 gene expression by omega-6 polyunsaturated fatty acids in human skeletal muscle cells involves multiple pathways, including the nuclear receptor peroxisome proliferator-activated receptor beta.* J Biol Chem, 2001. **276**(14): p. 10853-60.