Peroxisome proliferator-activated receptors modulate proliferation and angiogenesis in human endometrial carcinoma

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INTRODUCTION

Endometrial cancer is common in the United Kingdom. Recent figures indicate that in the most frequently affected age group (60-79 years) incidence of endometrial cancer almost doubled between 1975 – 2007[1]. Overall five-year survival is approximately 75% reflecting early presentation in a majority of cases. Nonetheless approximately 25% of women have a poorer prognosis which may be due to more advanced disease at presentation or more aggressive disease. Surgery remains the main primary treatment modality but there is a need to develop both preventative strategies and improved treatments for advanced disease in the face of this increasing health problem.

The most common variant of endometrial cancer is endometrioid type adenocarcinoma (EEC) which is strongly associated with obesity[2]. Most commonly seen in post-menopausal women, EEC is believed to develop as a result of persistent unopposed oestrogen stimulation on the endometrium resulting in increased risk of malignant transformation. In post-menopausal women, the main source of oestrogens is adrenal androgens which are converted by aromatase enzymes to weak oestrogens in peripheral fat deposits[3]. Adipose tissue is no longer considered an inert tissue but an active “organ” that may influence cancer risk in other ways. Genes regulated by the peroxisome proliferator-activated receptor (PPAR) pathway are also abnormally expressed in obese individuals[4]. PPARs are ligand-activated transcription factors that belong to the nuclear hormone receptor family and are primarily concerned with lipid and carbohydrate metabolism[5]. PPAR-mediated transcription occurs following heterodimerisation with a member of the retinoid X receptor (RXR) family. Subsequent binding to a peroxisome proliferator response element (PPRE) within the regulatory region of target genes leads to transcriptional activation or repression[6]. Three PPAR subtypes are recognised, PPARα, PPARγ and PPARβ/δ. In health expression of these isotypes is tissue dependent although both PPARα and PPARγ are abundant in adipose tissues[5].
An increasing body of evidence suggests that PPARs contribute to the development of some solid tumours. A number of studies show differential expression of PPARs in malignant tissues. For example PPARα is highly expressed in prostate cancer compared with benign prostate tissue when assessed using in situ hybridisation and immunohistochemistry[7]. Microarray analysis of endometrial cancers also identified increased transcription of PPARα when compared to atrophic endometrium[8]. There is conflicting evidence regarding the expression of PPARγ in solid tumours however. Expression of PPARγ appears to be reduced in gastric[9]and breast carcinomas[10] whilst expression in ovarian carcinomas is increased when compared to benign ovarian tumours[11]. Immunohistochemical studies suggest that expression of PPARγ may be reduced in endometrial cancers although quantitative protein analysis was not performed on human endometrial tissues[12]. In situ hybridisation and immunohistochemistry have been used to investigate expression of PPARβ/δ in endometrial cancers and concluded that expression was increased when compared to controls[13]. Activating ligands for PPARγ increase transcriptional activation of the tumour suppressor phosphatase and tensin homolog (PTEN) in breast cancer cell lines suggesting that PPARγ activation could be a novel approach for breast cancer treatment[14]. In a bladder cancer model however, activation of PPARs led to increased secretion of vascular endothelial growth factor (VEGF)[15]. VEGF is a potent mitogen and angiogenic growth factor which is important for tumour growth and metastasis[16, 17]. Data regarding the role of PPARs and their potential as therapeutic targets in cancer treatment is therefore conflicting and it is possible that their actions are tissue-specific. EEC is an obesity-related malignancy[2] with loss of functional PTEN implicated early in its development[18]. The aim of this study was to determine the potential role of PPARs, particularly α and γ isotypes, in the development of endometrial cancer. We investigated the expression of PPAR and RXR isotypes in endometrial cancers and determined the effect of reducing cellular PPARγ and PPARα expression on the growth of endometrial cancer cells and the expression of PTEN in vitro. In addition we investigated the effects of PPARγ and PPARα activating ligands on cellular growth and secretion of VEGF.
MATERIALS AND METHODS

Tissues

Twenty fresh endometrial tissue samples were obtained with ethical approval (North Manchester Research Ethics committee no.06/Q1406/29) from women undergoing hysterectomy for benign indications or known endometrial malignancy. These samples comprised five benign, endometrial samples and five each of the different grades of EEC adenocarcinoma (G1, n=5; G2, n=5; G3, n=5) as classified by FIGO (International Federation of Obstetrics and Gynaecology). A specialist gynaecological histopathologist examined all samples and selected areas of viable tissue for the study. Clinical information and histopathological reports were obtained for all samples. A separate cohort of archived, paraffin-embedded endometrial tissues was identified from histopathology archives (five benign post-menopausal endometrial samples, five severe atypical hyperplasias, five FIGO G1 and five FIGO G3 EECs).

Antibodies

Anti-PPARα and -PPARγ antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PPARβ, -RXRα, -RXRβ, -RXRγ, -PTEN, -CD68 and -P-AKT were supplied by Abcam (Cambridge, UK). Anti-CD31, biotinylated and HRP-conjugated anti-goat, anti-mouse and anti-rabbit secondary antibodies were supplied by Dako (Dako Cytomation, Ely, UK). FITC-conjugated goat anti-mouse antibody was obtained from Sigma (Sigma-Aldrich, Dorset, UK) and Alexo Fluor A555 and A488 antibodies from Invitrogen (Invitrogen Ltd, Paisley, UK).

Cell culture and gene silencing

The Ishikawa endometrial cell line was maintained in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, UK). The HEC-1A endometrial cell line was maintained in DMEM and Hams F12 medium (1:1) (Invitrogen, Paisley, UK) supplemented with 10% FBS. Cell lines were obtained from the European Collection of Cell Cultures and American Type Culture Collection respectively. Both were authenticated at source using isoenzyme analysis and DNA profiling. Cells were incubated at 37°C in 5% CO₂.
and PPARα expression was reduced using a short interfering RNA (siRNA) method. Qiagen Flexitube GeneSolution (Qiagen, West Sussex, UK) was used which comprises a gene-specific package of four preselected siRNAs for the target gene. siRNA was first diluted in Opti-MEM® (Invitrogen, Paisley, UK) and then Lipofectamine 2000 reagent (Invitrogen, Paisley, UK). Transfection of cells was achieved by incubation with the lipofectamine/siRNA mixture for 24 hours at 37°C and in 5% CO2. After 24 hours, cells were trypsinised, washed in medium and harvested for protein extraction.

**Protein extraction and Western blotting**

Nuclear protein was extracted from snap-frozen endometrial tissues and lysed cultured cells using the NE-PER® nuclear and cytoplasmic extraction kit (PIERCE Biotechnology, Rockford) according to the manufacturer’s instructions. Protein content of the nuclear extract in the supernatant obtained was determined using a Bio-Rad protein assay according to manufacturers’ instructions (Bio-Rad Laboratories, Hertfordshire, UK). Proteins (60μg) were mixed 1:1 with loading buffer (22% glycerol, 139 mM Tris-HCl, 154 mM SDS, 4.4 M urea, 0.002% bromophenol blue, and 10% vol/vol 2-mercaptoethanol) and heat reduced for 5 min at 95°C prior to separation on a 7% SDS-PAGE gel and electrophoretic transfer onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, UK). Non-specific binding was blocked by incubating the nitrocellulose blots with 3% skimmed milk powder in TBS with 0.05% Tween. Blots were incubated with primary antibody (1:200 in TBS/0.05% Tween) overnight at 4°C. Negative controls comprised diluent only. Blots were washed with TBS/0.05% Tween and incubated with the appropriate secondary antibody (diluted 1:2000 with TBS/0.05% Tween) conjugated to horseradish peroxidase (DAKO, Cambridgeshire, UK). Membranes were washed with TBS/0.05% Tween and protein bands visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, UK). The molecular mass of each visualized band was interpolated from a plot of log molecular mass versus distance migrated using kaleidoscope-pre-stained standards (Bio-Rad Laboratories, Hertfordshire, UK). Image J software
was used to analyse band density. Band densities were calculated relative to the density of β-actin expression.

**Co-immunoprecipitation**

Co-immunoprecipitation (co-IP) uses an antibody directed against a protein of interest to immobilize the target protein on a solid support on which either Protein A or Protein G has also been immobilized. Interacting proteins bind to the target and are "co-precipitated" and proteins not immobilized can be washed away. Three tubes containing 250µg of protein from endometrial samples were prepared from fresh frozen benign endometrium as described above using the NE-PER® nuclear and cytoplasmic extraction kit (PIERCE Biotechnology, Rockford) according to manufacturer's instructions. The lysates were mixed with lysis buffer and anti-RXRα, anti-RXRβ and anti-RXRγ each at a dilution of 1:200. The resulting mix was incubated at 4°C on rollers for 2hrs before addition to sepharose G beads (Amersham Life Sciences Ltd, UK) which were pre-washed with buffer (50 mM Tris pH7.5, 150 mM NaCl, 1% NP40, 1 mM DTT, 2 mM NaOH, 5 mM EDTA, 0.2% protease inhibitor cocktail mix III [Sigma-Aldrich Co. Ltd, Dorset, UK], 10 mM NaF). Following further incubation for 1.5 h, the supernatant was removed by centrifugation. Following repeated washes and centrifugation, loading buffer was added to the beads and the mixture was heated at 95°C for 5 minutes. The mixture was spun to pellet the beads and the supernatant loaded onto a 7% SDS-PAGE gel. Western blotting was performed as described above using antibodies against PPARs-α, -γ and –β/δ.

**Immunohistochemistry**

Immunohistochemistry was performed on 6µM thick formalin-fixed paraffin embedded tissue sections. Murine kidney and myocardium were positive controls for PPARα and PPARβ respectively. Human fetal omentum was the positive control for PPARγ, RXRα, RXRβ and RXRγ. Rat brain was the positive control for PTEN. Sections were dewaxed in xylene and rehydrated in graduated alcohols. Following heat-induced epitope retrieval in citrate buffer (pH 6.0) endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Non-specific binding
sites were blocked with 20% blocking serum in TBS/0.1% Tween. Sections were incubated in primary antibody diluted in non-immune blocking serum, in a humidified chamber overnight at 4°C. PPARα, PPARγ, PPARβ/δ and RXRγ antibodies were used at 1:100 dilution. RXRα, RXRβ, CD31 and CD68 antibodies were used at 1:200 dilution. The antibody for PTEN was used at 1:500 dilution. The appropriate biotinylated secondary antibody (diluted 1:200) was added to each section for 1 hour at room temperature. Antibody localisation was performed the Vectastain Elite ABC kit (Vector Laboratories, Peterborough) followed by incubation with 3,3-diaminobenzidine (DAB). Nuclei were stained using Mayer’s haemotoxylin. As a negative control in all cases, primary antibody was replaced with normal IgG from the same species.

Immunofluorescent detection was used to compare the localization of PPARα with vascular endothelium (CD31 antibody) and PPARγ with tissue macrophages (CD68). Briefly, after dewaxing and rehydrating, sections were incubated with both primary antibodies diluted in 10% relevant blocking serum, 2% human serum and TBS/0.1% Tween for one hour at 37°C. After washing, the appropriate secondary antibodies were diluted in 10% blocking serum, 2% human serum and TBS/0.1% Tween before incubating for 1 hour at room temperature in the dark. The slides were washed as before, the nuclei counterstained with DAPI and sections mounted using Vectashield (Vector Laboratories). Sections incubated with normal IgG’s provided negative controls. Images were viewed on an Olympus BX51 upright microscope using a 10x/ 0.30 Plan Fln objective and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Images were processed and analysed using the Image J program (http://rsb.info.nih.gov/ij).

**Proliferation assays**

Ishikawa and HEC-1A cells were seeded into 96-well culture plates. Cells were cultured for 24 hours at 37°C and 5% CO₂ in the presence of varying doses of an agonist PPARα ligand fenofibrate, with or without a PPARα antagonist GW6471 or an agonist PPARγ ligand ciglitazone, with or without the PPARγ, GW9662. The RXR agonist, all-trans retinoic acid (ATRA) was also
tested. All drugs tested were obtained from Sigma-Aldrich, Dorset, UK. Control cells were treated with vehicle only. A minimum of 5 replicates were used at each dose. After 24 hours incubation, cell proliferation was assessed by the uptake of 5-bromo-2'-deoxy-uridine (BrdU) using the BrdU labeling and detection kit III (Roche-Diagnostics, West Sussex, UK) according to the manufacturer's instructions. Absorbance values for cultures in each well were measured on a microtitre plate reader (Anthos Labtech Instruments GMbH, Austria) at 405nm with a reference wavelength at 490nm. Results were recorded as optical density measurements (OD) at 405nm/490nm.

**Apoptosis assays**

Cells were grown in a 96-well culture. After incubation at 37°C and 5% CO₂ for 24 hours, the medium was replaced with fresh complete culture medium and varying doses of experimental drug or control medium as described above. Apoptosis was detected using the APOPercentage™ Dye labelling assay (Biocolor UK, County Antrim, UK) (5μL) according to manufacturers instructions. Dye absorbance values for each well were measured at 550nm with a reference wavelength at 620nm.

**Enzyme-linked Immunosorbant assay (ELISA) for VEGF**

VEGF concentration in conditioned cell culture medium was measured using the QuantiGlo VEGF ELISA solid phase ELISA system (R&D systems, Abingdon, UK) according to manufacturers instructions. The relative light unit (RLU) of each microplate well was determined using a luminometer. VEGF was quantified by interpolating the results of the experimental samples from a standard curve generated using serial dilutions of the sample standard supplied with the kit.

**Statistical Analysis**
All statistical analyses were performed using Prism version 4. (GraphPad Software, San Antonio, CA). Non-parametric analysis of variance was performed incorporating Kruskal-Wallis statistic and Dunn’s multiple comparison tests. Statistical significance was accepted at p<0.05.

RESULTS

PPARs, RXRs and PTEN are differentially expressed in benign and malignant endometrium

The expression of each of the PPAR and RXR subtypes was determined in fresh endometrial tissues using Western blotting. Nuclear PPARα was least abundant in benign endometrium with increasing expression in malignant endometrium, being most highly expressed in grade 3 tumours (p<0.05) (figure 1a). This pattern of expression was the same for PPARβ. In marked contrast, nuclear PPARγ was most abundant in benign endometrium with a sequential decrease in expression from grade 1 through grade 3 tumours (p,0.01) (figure 1c). RXRα was expressed at lower levels in benign endometrium although expression increased in malignancy with grade 3 tumours demonstrating highest expression (p<0.05) (figure 1d). Conversely, expression of both RXRβ and RXRγ was higher in benign compared with malignant endometrium (figure 1e and 1f). In both cases there appeared to be an inverse relationship between expression and tumour grade (p<0.05). Expression of PTEN was highest in benign endometrium and declined with tumour grade with lowest expression seen in high grade (G3) tumours (p<0.05) (figure 1g). A concurrent increase in nuclear phosphorylated-AKT was noted (figure 1h). Co-immunoprecipitation studies using cell lysates from fresh benign endometrium, showed that both PPARα and PPARγ bind preferentially to RXRα compared to other RXR subtypes (figure 1i). PPARβ/δ demonstrated no clear preferential binding for any of the RXR subtypes.

Immunohistochemistry using paraffin-embedded tissue sections from a separate cohort of women confirmed staining for PPARα in glandular epithelial cells within benign endometrium (figure 2b) atypical hyperplasias (image not shown) and malignant endometrial cells (figure 2d). Double immunofluorescent staining with anti-CD31 also showed staining of vascular endothelium (figure 3a-h). In benign endometrium, very weak staining for PPARα was seen in glands and stroma.
The distribution of staining for PPARβ/δ was similar to that seen for PPARα with staining in epithelial tumour cells (figure 2j) and epithelial cells within atypical hyperplasia (image not shown). Benign endometrium stained strongly for PPARγ with staining localised to endometrial glandular cells (figure 2f). The same pattern of staining was seen in atypical hyperplasia (image not shown). Some weak stromal staining was noted. In malignant endometrium, staining of tumour cells was weak or absent (figure 2h). Double immunofluorescent staining with anti-CD38 also demonstrated immunostaining for PPARγ in macrophages (figure 4e-h). Immunostaining for RXRα in benign endometrium appeared weak with staining in both stroma and epithelial cells (figure 3b). In FIGO G1 cancer, intense RXRα staining was seen mainly in the tumour cells (figure 3d). Staining was mainly cytoplasmic in benign endometrial glands with both nuclear and cytoplasmic staining evident in malignant glands. RXRβ and RXRγ immunostaining was seen in both glands and stroma in benign endometrium and FIGO G1 cancers (figures 3f, 3h, 3j and 3l).

**PPAR-α and PPAR-γ exert growth effects on endometrial cancer cells**

In order to determine the whether or not PPAR-α and PPAR-γ function to control growth of endometrial cancer cells, cells were transfected with siRNA directed against either PPAR-α or PPAR-γ respectively. Western blotting confirmed reduced expression of both PPAR-α and PPAR-γ following transfection (figure 5a). PPARα and PPARγ expression in Ishikawa cells was reduced by 70% and 80% respectively whilst in HEC-1A cells 75% and 90% reduction of PPARα and PPARγ expression was achieved respectively (figure 5b and c). In vitro, reduced PPARα expression was associated with reduced cellular proliferation (figure 5d) and a small increase in apoptosis (figure 5e). Data for HEC-1A cells is shown only. Data for Ishikawa cells is shown as supplementary information. In untransfected cells, these effects were potentiated by incubating cells with a PPARα ligand, fenofibrate at doses of 5μM and above (p<0.01)(figure 5d and 5e). The effect of treating siRNA-transfected cells was less marked with higher doses of 20μM and above required to effect any significant difference. Reduced PPARγ expression in itself had no clear effect on proliferation or apoptosis (figures 5f and g). However, addition of the PPARγ agonist ciglitazone at doses as low as 5μM were sufficient to reduce cellular proliferation in both
untransfected and transfected cells (p<0.01)(figure 5f). Similarly, apoptosis was increased following incubation with ciglitazone although effects on apoptosis were only seen with doses of 10μM or higher (p<0.01)(figure 5g). Results for Ishikawa cells were very similar although higher doses of ciglitazone were required to effect any significant difference in either proliferation or apoptosis. Western blotting was used to investigate PTEN and phosphorylated-AKT (P-AKT) expression in both endometrial cell lines. Ishikawa cells were found to have no expression of PTEN and to express P-AKT only (figure 6a) although PTEN expression was confirmed in HEC-1A cells (figure 6b). HEC-1A cells with reduced expression of PPARγ were found to have diminished expression of PTEN when compared with “un-silenced” cells (figure 6c). A concurrent increase in expression of P-AKT was also seen (figure 6d). When HEC-1A cells were treated with ciglitazone, PTEN expression increased (p<0.01) and P-AKT levels diminished (p<0.05). These effects were reversed by the addition of a PPARγ antagonist, GW9662 (figure 6e).

**PPAR-α and PPAR-γ modulate VEGF secretion in endometrial cancer cells in vitro**

Secreted VEGF levels in both cell lines were lower when cells were treated with PPARα siRNA compared to controls (p<0.05)(figure 7a). Conversely VEGF levels were slightly increased in cells with reduced expression of PPARγ compared to control cells (p<0.05)(figure 7b). Neither of these effects was reversed with the addition of a PPAR-specific ligand and VEGF secretion was unaltered following incubation with either fenofibrate or ciglitazone in cells transfected with antisense RNA for PPARα and PPARγ respectively (figure 7c and 7d). Treatment of untransfected cells with the PPARα agonist fenofibrate reduced VEGF concentration in both Ishikawa and HEC-1A cells (P<0.05)(figure 7e and 7f). No significant effect following ciglitazone treatment was noted in either cell line (figure 7e and 7f). The reduction in VEGF in HEC-1A cells treated with fenofibrate was potentiated by co-incubation with the RXR ligand, All-Trans Retinoic Acid (ATRA) (figure 7f) but the same effect was not noted in Ishikawa cells (figure 7e). Interestingly, the effects of both fenofibrate and ATRA in HEC-1A cells, were almost fully reversed by co-incubation with the PPARα antagonist GW6471 (figure 7f) although no significant effect was seen in Ishikawa cells.
Discussion

These results indicate that activation of PPARα by endogenous ligands in vivo could affect the growth of EEC. We have shown that reduced cellular expression of PPARα leads to reduced proliferation in endometrial cancer cells indicating that PPARα by either direct or indirect mechanisms, has a role in endometrial cell proliferation. Because we demonstrated expression of PPARα in vascular endothelial cells as well as endometrial glandular and tumour cells, we postulated that PPARα may influence secretion of VEGF. Endometrial cancer cells express VEGF[19] which may influence cancer growth by autocrine action on endometrial cells or indirectly by encouraging endothelial vessel sprouting and angiogenesis. Contrary to our expectations we demonstrated that treatment of endometrial cells with a PPARα agonist leads to reduced rather than increased secretion of VEGF in addition to reduced proliferation. The potentiation of this effect by the pan-RXR agonist, ATRA and the blunting of this effect by the use of a PPARα antagonist in HEC-1A cells, indicates that these effects were likely PPARα dependent. No effects on VEGF secretion occurred when siRNA-PPARα-transfected cells were treated with a PPARα antagonist. Although, total knock down of PPARα was not achieved (70-75% knockdown), these results strongly suggest that the effects of PPARα agonist on VEGF secretion seen here, are dependent on abundant levels of PPARα. The differential effect on VEGF secretion from HEC-1A cells and Ishikawa cells following treatment with a PPARα may be explained by differing expression of steroid hormone receptors. HEC-1A cells do not express estrogen receptor alpha (ERα) in contrast to Ishikawa cells which constitutively express this. Evidence indicates that ERα can regulate cellular PPARα levels[20]. Therefore it is plausible that in Ishikawa cells the presence of ERα may ensure sufficient PPARα levels to maintain normal PPARα binding with naturally occurring ligands, even in the presence of a PPAR agonist. It seems counterintuitive that reducing the expression of PPARα within endometrial cells reduces VEGF secretion yet enhancing PPARα activity with an agonist ligand also reduces VEGF secretion. One explanation for this could be a cytotoxic effect of fenofibrate at the dose used (50μM) with subsequent reduction in VEGF secretion. This seems unlikely given that doses of 50μM of fenofibrate have minimal cytotoxic effects on hepatic cells in culture up to 96 hours[21].
Whilst PPARα-independent actions of fenofibrate are reported, this is unlikely to be the explanation for our findings because after knocking-down the expression of PPARα by 75-90%, no effect of fenofibrate on VEGF secretion was seen. The paradoxical action of PPARα described here may be explained by a biphasic or U-shaped dose-response curve to PPARα activity. That is, very low expression or activity of PPARα and very high activity of PPARα result in the same outcome, in this case reduced VEGF secretion. This type of response could be a natural mechanism for more tightly controlling PPARα activity with respect to VEGF secretion. Biphasic dose responses have also been observed with other PPAR agonists and biphasic responses are known for other classes of drug[22]. Overall therefore, PPARα can affect both proliferation and VEGF secretion although it is not possible to attribute the reduced proliferation of endometrial cells to the reduced levels of VEGF following PPARα knock-down as there may be effects on other cellular pathways.

PPAR/RXR heterodimers bind to estrogen response elements (EREs) in the promoter region of target genes[23] and the VEGF promoter contains an ERE[24]. Fenofibrate suppresses endothelial cell proliferation and inhibits neovascularisation in mouse cornea[25] which is relevant since we have shown that vascular endothelial cells within endometrial cancers express PPARα. Suppression of angiogenesis using fenofibrate is also reported in mouse tumours derived from a number of different human tumour cell lines although none of these were endometrial in origin[25]. We suggest therefore that fenofibrate has the potential to suppress angiogenesis within endometrial cancers and thus reduce growth and/or metastasis either by directly targeting endometrial tumour cells or the vascular endothelial cells within the tumour.

In addition to fibrate drugs, PPARα can be activated by a large variety of naturally occurring ligands, principally saturated and unsaturated fatty acids (including arachidonic acid) as well as eicosanoids[26]. Fatty acid derivatives involved with inflammatory processes (e.g. prostaglandins and leukotrienes) are intimately involved with tumour growth and progression and can act directly on malignant cells by autocrine or paracrine action[27]. Our expression data for PPARβ/δ support
the findings of Tong et al. who demonstrated PPARβ/δ in endometrial tumour cells and also found that PPARβ/δ was co-localised with cyclo-oxygenase 2 (COX-2) expression[13]. COX-2, overexpressed in many cancers including endometrial cancers is a central regulator of inflammation as well as a key regulator of angiogenesis and metastasis[28]. Prostaglandins derived from COX-2 are naturally occurring ligands for PPARβ/δ. Our results are therefore in accord with the evidence supporting fatty acid derivatives as potent mediators of cancer growth[29].

We have shown that reduced expression or loss of PPARγ is a feature of EEC endometrial carcinoma and parallels the loss of PTEN frequently seen in these tumours[18]. Although upregulation of PPARγ is reported in some human cancers e.g. ovarian carcinoma[11], loss of PPARγ is a feature of a number of solid tumours including oesophageal[30] and ureteric tumours[31]. We conclude that loss of PPARγ in EEC in our study is a result of reduced gene transcription as transcriptome analysis of EEC has previously identified reduced messenger RNA encoding PPARγ[8]. In human colorectal carcinoma, methylation of the PPARγ promoter with loss of expression was identified in 30% of clinical samples studied and correlated with poor prognosis[32]. We have also demonstrated that PPARγ activation suppresses proliferation of endometrial cancer cells via a mechanism which involves regulation of PTEN. Addition of a PPARγ agonist, ciglitazone, increased PTEN expression and reduced the expression of phosphorylated-AKT (P-AKT). PPARγ can also upregulate the transcription of PTEN in breast cancer[14, 33]. PTEN acts to reduce P-AKT indirectly by inhibiting phosphoinositide 3-kinase (PI3K) with the net effect of reducing cellular proliferation[34]. Two response elements (PPREs) have been identified within the PTEN promoter[35] and in the context of our results, this suggests that PPARγ can function within the endometrium to regulate PTEN expression. It is of interest that in a PTEN-null endometrial cell line (Ishikawa), reducing PPARγ expression leads to decreased expression of P-AKT. Therefore in the endometrium, PI3K may be directly regulated by PPARγ. Although the regulation of PI3K is incompletely understood, our study, to our knowledge, is the first to indicate a direct effect on PI3K by reducing PPARγ expression. In addition, treatment of PPARγ-
expressing PTEN-null cells with ciglitazone also reduces P-AKT. This has potential implications for the possible use of PPARγ agonists as it suggests that beneficial effects may be expected in patients both with and without PTEN-expressing tumours. In endometrial cancer cells, PPAR agonists exert dose-dependent effects on both cellular proliferation and apoptosis. It seems likely that the PPAR agonists act at least partially via PPARγ as the addition of an antagonist abrogates these effects. In addition, although we were able to reduce expression of PPARα or PPARγ by 70-90% in our cell lines using siRNA, we were unable to fully silence their expression and the effects of subsequent treatment with exogenous ligand were very similar to those seen in non-transfected cells. Hence it seems likely that the ligands were acting upon the residual transcription factors present.

We have demonstrated that endometrial cancer cells can be stimulated to increase VEGF secretion by reducing cellular expression of PPARγ. This indicates that PPARγ can negatively regulate tumour angiogenesis. Similar to the anti-angiogenic actions of PPARα agonists we conclude that PPARγ is also necessary for anti-angiogenic actions of PPARγ agonists. Ciglitazone reduced VEGF secretion in both endometrial cell lines at doses equivalent to those in clinical use and the dependence of this effect on PPARγ is strengthened by our observation that these effects were largely reversed by treatment with a PPARγ antagonist. Tumour cells are influenced by secretions from and contact with other cell types within the tumour microenvironment such as tumour-associated macrophages. We have shown that PPARγ is expressed in endometrial tumour macrophages and this is consistent with a role for PPARs in macrophage differentiation and regulation of inflammatory activities[36, 37]. Interestingly, PPARγ appears to be most associated with M2 macrophages, that is macrophages with an anti-inflammatory phenotype[37]. We have not further characterized the macrophages within the endometrial cancers studied here and therefore are unable to determine whether the PPARγ-expressing macrophages are of the M1 (pro-inflammatory tumour-promoting) variety or the M2 phenotype. This warrants further investigation if PPARγ-ligands are to be considered within clinical trials.
In order to function, each PPAR subtype must bind with a member of the RXR family of receptors, forming a heterodimer which then binds to a specific response element within the promoter region of target genes\[6\]. To our knowledge, ours is the first study to demonstrate preferential binding of endometrial PPAR\(\alpha\) and PPAR\(\gamma\) with RXR\(\alpha\), as compared to other RXR subtypes. Our finding of reduced RXR\(\beta\) expression in ECC is consistent with quantitative RNA analysis\[8\]. Nuclear RXR\(\gamma\) expression is also diminished but interestingly nuclear RXR\(\alpha\) in endometrial cancers is more highly expressed compared to benign endometrium. This suggests that actions of PPAR/RXR complexes within the endometrium may be modulated by availability of one or both heterodimer partners. This is supported by studies that demonstrate nuclear-cytoplasmic shuttling of PPAR\(\gamma\) and a nuclear localisation signal within PPAR\(\alpha\)[38, 39]. We demonstrated RXR\(\alpha\) and RXR\(\gamma\) in the nuclei of epithelial cells within EEC using immunohistochemistry both in benign and malignant endometrium which is consistent with other published studies.

Our expression data and in vitro studies support a role for PPAR/RXR complexes in the development of endometrial cancer. However, the mechanisms by which this is achieved appear complex. Our data suggest that PPARs directly affect growth of endometrial cancer cells by affecting intracellular growth pathways and also by altering availability of growth factors such as VEGF. The counterintuitive effects of PPAR agonist ligands indicate the possibility of a tightly regulated therapeutic range for PPAR activity. The PPAR\(\gamma\) agonist rosiglitazone has been shown to inhibit proliferation and induce apoptosis in a mouse model of endometrial hyperplasia suggesting a potential for chemoprevention[40] and our results also suggest that fenofibrate has the potential for antiangiogenic treatment. There has previously been a paucity of data regarding the PPAR/RXR system in human endometrial cancer compared to other tumour types. It seems likely that the actions of PPARs and RXRS in respect of carcinogenesis differ between tissues. We believe our results together with data from mouse models provide a strong rationale for conducting early phase II studies of PPAR and RXR-specific ligands in women with EEC.
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REFERENCES

7. Collett GP, Betts AM, Johnson MI, Pulimood AB, Cook S, Neal DE, Robson CN: Peroxisome proliferator-activated receptor alpha is an androgen-responsive gene


15. Fauconnet S, Lascombe I, Chabannes E, Adessi GL, Desvergne B, Wahl W, Bittard H:
Differential regulation of vascular endothelial growth factor expression by
peroxisome proliferator-activated receptors in bladder cancer cells. Journal Of


17. Fidler IJ, Ellis LM: The Implications Of Angiogenesis For The Biology And Therapy

Altered PTEN expression as a diagnostic marker for the earliest endometrial

genes in endometrial atypical hyperplasia and endometrial cancer. British Journal Of

20. Faddy HM, Robinson JA, Lee WJ, Holman NA, Monteith GR, Roberts-Thomson SJ:
Peroxisome proliferator-activated receptor alpha expression is regulated by
estrogen receptor alpha and modulates the response of MCF-7 cells to sodium

HepG2 hepatoma cell line and relevant mechanisms. Toxicology And Applied

22. Panigrahy D, Singer S, Shen LQ, Butterfield CE, Freedman DA, Chen EJ, Moses MA,
Kilroy S, Duensing S, Fletcher C, et al: PPAR gamma ligands inhibit primary tumor
growth and metastasis by inhibiting angiogenesis. Journal Of Clinical Investigation

receptor and peroxisome proliferator-activated receptor activate an estrogen


34. Maehama T, Dixon JE: **The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate.** *Journal Of Biological Chemistry* 1998, **273**:13375-13378.

35. Patel L, Pass I, Coxon P, Downes CP, Smith SA, Macphee CH: **Tumor suppressor and anti-inflammatory actions of PPAR gamma agonists are mediated via upregulation of PTEN.** *Current Biology* 2001, **11**:764-768.


40. Wu W, Celestino J, Milam MR, Schmeler KM, Broaddus RR, Ellenson LH, Lu KH:

Primary chemoprevention of endometrial hyperplasia with the peroxisome
proliferator-activated receptor gamma agonist rosiglitazone in the PTEN
heterozygote murine model. *International Journal Of Gynecological Cancer* 2008,
18:329-338.
Figure legends

**Figure 1. Nuclear expression of PPARs, Retinoid X receptors, PTEN and phosphorylated-AKT in benign and malignant endometrium.** Representative protein blots for benign and malignant endometrial tissues are displayed (a- h) above densitometry measurements shown as box and whisker plots. Boxes extend from the 25th to 75th percentiles and whiskers mark the range. Medians are displayed as horizontal lines within the boxes. N=5 for all densitometry plots. *p<0.05, **p<0.01 (Dunns’ multiple comparison test). Figure 1i is a representative protein blot for each PPAR sub-type following co-immunoprecipitation of RXRα, RXRβ and RXRγ in benign endometrial tissue. 250μg protein was loaded in each experiment.

**Figure 2. Immunohistochemical staining for individual PPAR subtypes and and PTEN within benign endometrium and endometrioid endometrial carcinoma.** a. Negative control for PPARα in benign endometrium; b. PPARα expression in benign endometrium; c. negative control for PPARα in G1 cancer; d. PPARα expression in FIGO grade 1 cancer; e. negative control for PPARγ in benign endometrium; f. PPARγ expression in benign endometrium; g. negative control for PPARγ in G3 cancer; h. PPARγ expression in FIGO grade 3 cancer; i. negative control for PPARβ/δ in G1 cancer; j. PPARβ/δ expression in FIGO G1 cancer; k. negative control for PTEN expression in benign endometrium; l. PTEN expression in benign endometrium. For each negative control, sections were incubated with non-immune IgG instead of primary antibody. Scale bar = 100μM.

**Figure 3. Immunohistochemical staining for Retinoid X receptor subtypes within benign and endometrioid endometrial carcinoma.**

a. Negative control for RXRα in benign endometrium; b. RXRα expression in benign endometrium; c. negative control for RXRα in G1 endometrial cancer; d. RXRα expression in G1 endometrial cancer; e. negative control for RXRβ in benign endometrium; f. RXRβ expression in benign endometrium; g. negative control for RXRβ in G3 endometrial cancer; h. RXRβ expression in G3 endometrial cancer; i. negative control for RXRγ in benign endometrium;
j. RXRγ expression in benign endometrium; k. negative control for RXRγ in G1 endometrial cancer; l. RXRγ expression in G1 endometrial cancer. For each negative control, sections were incubated with non-immune IgG instead of primary antibody. Scale bar = 100μM.

Figure 4. Co-localisation studies for PPARα and PPARγ using immunofluorescent immunohistochemistry

Panels a-d show immunostaining in a FIGO G1 endometrial cancer; a. DAPI staining identifies nuclei (blue); b. endothelial cells are identified by staining with CD31 (represented by red colour); c. PPARα staining is shown by green colour; d. co-localisation of antigens is seen as yellow colouration under fluorescent microscopy. Panels e-h show immunostaining in a section of benign endometrium; e. DAPI staining identifies nuclei (blue); f. macrophages are identified by staining with CD68 (represented by a red colour); g. PPARγ staining is shown by green colour; h. co-localisation of antigens is seen as yellow colouration under fluorescent microscopy.

Figure 5. Effects of PPARα and PPARγ and their respective agonist ligands on the growth of endometrial cancer cells in vitro. Panels a-c illustrate the effect of transfection of endometrial cancer cells with PPARα or PPARγ siRNA; a. Western blot showing effect of antisense RNA for PPARα and PPARγ on Ishikawa cells (ISH) and HEC-1A cells in culture compared to untreated cells. Protein extracted from murine kidney and human omentum provided positive controls for PPARα and PPARγ respectively; b and c. box and whisker plots of densitometry for PPAR expression in Ishikawa and HEC-1A cell lines following treatment with anti-sense RNA. The “whiskers” show the range with a horizontal line representing the median (n=6). Panels d and e show cellular proliferation and apoptosis respectively following treatment of HEC-1A cells with the PPARα agonist, fenofibrate. Panels f and g show the effects of the PPARγ ciglitazone on cellular proliferation and apoptosis respectively in HEC-1A cells. In all panels, light coloured bars represent un-transfected cells and bars with darker shading represent cells that have been transfected with anti-sense RNA against PPARα (panels d and e) and PPARγ (panels f and g); error bars represent interquartile ranges; six replicates were performed for each
experimental condition and each experiment repeated three times. These experiments were also conducted in Ishikawa cells. The data from the latter are provided as supplementary figures.

**Figure 6. Effects of PPARγ expression and PPARγ ligands on expression of the tumour suppressor PTEN in endometrial cells in vitro.** a and b. protein blots demonstrating the effect of down-regulating PPARγ on expression of PTEN and phosphorylated-AKT in Ishikawa cells and HEC-1A cells respectively. Comparison is made with untreated cells. β-actin was used as a loading control; c and d. densitometry measurements from protein blots for PTEN and phosphorylated AKT following treatment with PPARγ siRNA. Data is shown as box and whisker plots. Each box extends from the 25th to 75th percentiles and whiskers demonstrate the range with the median demonstrated as a horizontal line within each box (n=6). *p<0.05, **p<0.01 (Dunns’ multiple comparison test); e. protein blots showing the effect of treatment of ciglitazone, a PPARγ agonist and GW9662, a PPARγ antagonist, on the expression of PTEN and phosphorylated-AKT in wild type HEC-1A cells. The effect of treatment with increasing drug concentrations is shown with doses ranging from 5-50μM. Cells treated with drug vehicle only were used as negative controls.

**Figure 7. Secretion of VEGF following PPAR gene silencing and treatment with PPAR ligands.** VEGF secretion was measured by ELISA; pale coloured bars represent experiments conducted with the Ishikawa cell line and darker bars, with the HEC-1A cell line; error bars represent inter-quartile ranges; * p<0.05. a. VEGF secretion in cells treated with anti-sense RNA against PPARα. Untreated cells comprise the control groups; b. VEGF secretion in cells treated with anti-sense RNA against PPARγ; c. VEGF secretion in Ishikawa cells treated with anti-sense RNA against PPARα or PPARγ and their respective agonist ligands fenofibrate and ciglitazone (each at 50μM); d. VEGF secretion in HEC-1A cells treated with anti-sense RNA against PPARα or PPARγ and their ligands fenofibrate and ciglitazone (each at 50μM); e. the effects of PPARα and PPARγ agonists, antagonists and all-trans retinoic acid (each at 50μM) on VEGF secretion in...
“unsilenced” Ishikawa cells; f. the effects of PPARα and PPARγ agonists, antagonists and all-trans retinoic acid (each at 50μM) on VEGF secretion in “unsilenced” IHEC-1A cells.
Figure 1
Figure 2
Figure 3
a

+ve control untreated PPARα

+ve control untreated PPARγ

ISH

HEC-1A

B-actin

b

c

Relative expression of PPARα to β-actin (arbitrary units)

Relative expression of PPARγ to β-actin (arbitrary units)
Figure 5
Figure 6
Peroxisome proliferator-activated receptors modulate proliferation and angiogenesis in human endometrial carcinoma

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