Peroxisome Proliferator–Activated Receptors Modulate Proliferation and Angiogenesis in Human Endometrial Carcinoma

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Abstract
Peroxisome proliferator-activated receptors (PPAR) and retinoid X receptors (RXR) are implicated in the development of several obesity-related cancers. Little is known of either the expression or function of PPARs and RXRs in endometrial cancer although this increasingly common disease is highly associated with both obesity and insulin resistance. We investigated the expression of PPAR and RXR subtypes in human endometrial cancers and normal endometrium with immunoblotting and immunohistochemistry and subsequently showed PPAR/RXR binding preferences by coimmunoprecipitation. To determine the functions of PPARs within the endometrium, we investigated proliferation, apoptosis, PTEN expression, and secretion of vascular endothelial growth factor (VEGF) in endometrial cell lines after reducing the expression of PPARα and PPARγ with antisense RNA. The functional effects of PPAR ligands were also investigated in vitro. We identified differential expression of PPAR and RXR subtypes in endometrial cancers and discovered that PPARγ expression correlated with expression of PTEN. PPARα activation influences endometrial cell growth and VEGF secretion. PPARγ activation reduces proliferation of endometrial cells via regulation of PTEN and appears to reduce VEGF secretion. We conclude that the PPAR/RXR pathway contribute to endometrial carcinogenesis by control of PTEN expression and modulation of VEGF secretion. We propose that PPAR ligands should be considered for clinical investigation in early phase studies of women with endometrial cancer. Mol Cancer Res; 10(3); 441–53. ©2011 AACR.

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 and 2007 (1). Overall 5-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 postmenopausal women, EEC is believed to develop as a result of persistent unopposed estrogen stimulation on the endometrium resulting in increased risk of malignant transformation. In postmenopausal women, the main source of estrogens is adrenal androgens which are converted by aromatase enzymes to weak estrogens 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 heterodimerization 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 recognized, 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 tumors. 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 by in situ hybridization and immunohistochemistry (7). Microarray analysis of endometrial cancers also identified increased transcription of PPARα when compared with atrophic endometrium (8). There is conflicting evidence about the expression of PPARγ in solid tumors however. Expression of PPARγ seems to be reduced in gastric (9) and breast carcinomas (10), whereas expression in ovarian carcinomas is increased when compared with benign ovarian tumors (11). Immunohistochemical studies suggest that expression of PPARγ may be reduced in endometrial cancers although quantitative protein analysis was not conducted on human endometrial tissues (12). In situ hybridization and immunohistochemistry have been used to investigate expression of PPARβδ in endometrial cancers and concluded that expression was increased when compared with controls (13). Activating ligands for PPARγ increase transcriptional activation of the tumor 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; ref. 15). VEGF is a potent mitogen and angiogenic growth factor which is important for tumor growth and metastasis (16, 17). Data about the role of PPARs and their potential as a factor which is important for tumor growth and metastasis in prostate cancer however, activation of PPARs led to increased enzyme analysis and DNA profiling. Cells were incubated at 37°C in 5% CO2. PPARγ and PPARα expression was reduced by an siRNA method. QIAGEN FlexTube Gene-Solution (QIAGEN) was used which comprises a gene-specific package of 4 preselected siRNAs for the target gene. siRNA was first diluted in Opti-MEM (Invitrogen) and then Lipofectamine 2000 reagent (Invitrogen). 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 trypsinized, washed in medium, and harvested for protein extraction.

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 5 benign, endometrial samples and 5 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 gynecologic histopathologist examined all samples and selected areas of viable tissue for the study. Clinical information and histopathologic reports were obtained for all samples. A separate cohort of archived, paraffin-embedded endometrial tissues was identified from histopathology archives (5 benign postmenopausal endometrial samples, 5 severe atypical hyperplasias, 5 FIGO G1, and 5 FIGO G3 EECs).

Antibodies

Anti-PPARα and anti-PPARγ antibodies were supplied by Santa Cruz Biotechnology. Anti-PPARβδ, anti-RXRα, anti-RXRβ, anti-RXRγ, anti-PTEN, anti-CD68, and anti–P-AKT were supplied by Abcam. Anti-CD31, biotinylated and hors eradish peroxidase (HRP)-conjugated anti-goat, anti-mouse, and anti-rabbit secondary antibodies were supplied by Dako (DakoCytomation). Fluorescein isothiocyanate–conjugated goat anti-mouse antibody was obtained from Sigma (Sigma-Aldrich) and Alexa Fluor A555 and A488 antibodies from Invitrogen (Invitrogen Ltd.).

Cell culture and gene silencing

The Ishikawa endometrial cell line was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS (Gibco BRL). The HEC-1A endometrial cell line was maintained in DMEM and Hams F12 medium (1:1; Invitrogen) 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 by isoenzyme analysis and DNA profiling. Cells were incubated at 37°C in 5% CO2. PPARγ and PPARα expression was reduced by an siRNA method. QIAGEN FlexTube Gene-Solution (QIAGEN) was used which comprises a genespecific package of 4 preselected siRNAs for the target gene. siRNA was first diluted in Opti-MEM (Invitrogen) and then Lipofectamine 2000 reagent (Invitrogen). 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 trypsinized, 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) according to the manufacturer’s instructions. Protein content of the nuclear extract in the supernatant obtained was determined by a Bio-Rad protein assay according to manufacturers’ instructions (Bio-Rad Laboratories). Proteins (60 μg) were mixed 1:1 with loading buffer (2% glycerol, 139 mmol/L Tris-HCl, 154 mmol/L SDS, 4.4 mol/L urea, 0.002% bromophenol blue, and 10% vol/vol 2-mercaptoethanol) and heat reduced for 5 minutes at 95°C prior to separation on a 7% SDS-PAGE gel and electrophoretic transfer onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Nonspecific 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 1:2,000 with TBS/0.05% Tween) conjugated to HRP (Dako). Membranes were washed with TBS/0.05% Tween and protein bands visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). The molecular mass of each visualized band was determined using a plot of log molecular mass versus distance migrated using kaleidoscope-prestained standards (Bio-Rad Laboratories). Image J software (http://rsb.info.nih.gov/ij) was used to analyze 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 “coprecipitated” and proteins not immobilized can be washed away. Three tubes containing 250 μg 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) according to the manufacturer’s instructions. The lysates were mixed with lysis buffer and anti-PPARα, anti-PPARβ, and anti-PPARγ each at a dilution of 1:200. The resulting mix was incubated at 4°C on rollers for 2 hours before addition to sepharose G beads (Amersham Life Sciences Ltd.) which were prewashed with buffer [50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1% NP40, 1 mmol/L DTT, 2 mmol/L NaOH, 5 mmol/L EDTA, 0.2% protease inhibitor cocktail mix III (Sigma-Aldrich Co.), 10 mmol/L NaF]. Following further incubation for 1.5 hours, 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 conducted as described above with antibodies against PPARα, PPARγ, and PPAR-β/δ.

Immunohistochemistry

Immunohistochemistry was conducted on 6 μmol/L 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 with 3% hydrogen peroxide. Nonspecific binding sites were blocked with 20% blocking serum in TBS/0.1% Tween. Sections were incubated in primary antibody diluted in nonimmune 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 localization was carried out with the Vectastain Elite ABC Kit (Vector Laboratories) followed by incubation with 3,3-diaminobenzidine. Nuclei were stained with Mayer’s hematoxylin. As a negative control in all cases, primary antibody was replaced with normal immunoglobulin G (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 1 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 IgGs provided negative controls. Images were viewed on an Olympus BX51 upright microscope using a 10×/0.30 Plan Fln objective and captured with a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Images were processed and analyzed by 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 PPARβ 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. 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) according to the manufacturer’s instructions. Absorbance values for cultures in each well were measured on a microtiter plate reader (Anthos Labtech Instruments GmbH) at 405 nm with a reference wavelength at 490 nm. Results were recorded as optical density measurements at 405 nm/490 nm.

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 by the APOPercentage Dye labelling assay (Bisocol UR; 5 μL) according to manufacturer’s instructions. Dye absorbance values for each well were measured at 550 nm with a reference wavelength at 620 nm.
ELISA for VEGF

VEGF concentration in conditioned cell culture medium was measured with the QuantiGlo VEGF ELISA solid phase ELISA system (R&D systems) according to manufacturer’s instructions. The relative light unit of each microplate well was determined by 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 conducted with Prism (version 4; GraphPad Software). Nonparametric analysis of variance was conducted incorporating Kruskal–Wallis statistic and Dunn 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 PPAR\( \alpha \) was least abundant in benign endometrium with increasing expression in malignant endometrium, being most highly expressed in grade 3 tumors \( (P < 0.05) \); Fig. 1A). This pattern of expression was the same for PPAR\( \beta \). In marked contrast, nuclear PPAR\( \gamma \) was most abundant in benign endometrium with a sequential decrease in expression from grade 1 through grade 3 tumors \( (P < 0.01) \); Fig. 1C). RXR\( \alpha \) was expressed at lower levels in benign endometrium although expression increased in malignancy with grade 3 tumors showing highest expression \( (P < 0.05) \); Fig. 1D). Conversely, expression of both RXR\( \beta \) and RXR\( \gamma \) was higher in benign compared with malignant endometrium (Fig. 1E and F). In both cases, there seemed to be an inverse relationship between expression and tumor grade \( (P < 0.05) \). Expression of PTEN was highest in benign endometrium and declined with tumor grade with lowest expression seen in high grade (G3) tumors \( (P < 0.05) \); Fig. 1G). A concurrent increase in nuclear phosphorylated-AKT (P-AKT) was noted (Fig. 1H).

In vitro studies with cell lysates from fresh benign endometrium showed that both PPAR\( \alpha \) and PPAR\( \gamma \) bind preferentially to RXR\( \alpha \) compared with other RXR subtypes (Fig. 1I). PPAR\( \beta/\delta \) showed 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 PPAR\( \alpha \) in glandular epithelial cells within benign endometrium (Fig. 2B) atypical hyperplasias (image not shown), and malignant endometrial cells (Fig. 2D). Double immunofluorescent staining with anti-CD31 also showed staining of vascular endothelium (Fig. 3A–H). In benign endometrium, very weak staining for PPAR\( \alpha \) was seen in glands and stroma. The distribution of staining for PPAR\( \beta/\delta \) was similar to that seen for PPAR\( \alpha \) with staining in epithelial tumor cells (Fig. 2J) and epithelial cells within atypical hyperplasia (image not shown). Benign endometrium stained strongly for PPAR\( \gamma \) with staining localized to endometrial glandular cells (Fig. 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 tumor cells was weak or absent (Fig. 2H). Double immunofluorescent staining with anti-CD38 also showed immunostaining for PPAR\( \gamma \) in macrophages (Fig. 4E–H). Immunostaining for RXR\( \alpha \) in benign endometrium appeared weak with staining in both stroma and epithelial cells (Fig. 3B). In FIGO G1 cancer, intense RXR\( \alpha \) staining was seen mainly in the tumor cells (Fig. 3D). Staining was mainly cytoplasmic in benign endometrial glands with both nuclear and cytoplasmic staining evident in malignant glands. RXR\( \beta \) and RXR\( \gamma \) immunostaining was seen in both glands and stroma in benign endometrium and FIGO G1 cancers (Figs. 3F, H, J and I).

PPAR-\( \alpha \) and PPAR-\( \gamma \) exert growth effects on endometrial cancer cells

To determine whether or not PPAR-\( \alpha \) and PPAR-\( \gamma \) function to control growth of endometrial cancer cells, cells were transfected with siRNA directed against either PPAR-\( \alpha \) or PPAR-\( \gamma \), respectively. Western blotting confirmed reduced expression of both PPAR-\( \alpha \) and PPAR-\( \gamma \) following transfection (Fig. 5A). PPAR\( \alpha \) and PPAR\( \gamma \) expression in Ishikawa cells was reduced by 70% and 80%, respectively, whereas in HEC-1A cells, 75% and 90% reduction of PPAR\( \alpha \) and PPAR\( \gamma \) expression was achieved, respectively (Fig. 5B and C). In vitro, reduced PPAR\( \alpha \) expression was associated with reduced cellular proliferation (Fig. 5D) and a small increase in apoptosis (Fig. 5E). Data for HEC-1A cells are shown only. Data for Ishikawa cells are shown as Supplementary Information. In untransfected cells, these effects were potentiated by incubating cells with a PPAR\( \alpha \) ligand, fenofibrate at doses of 5 \( \mu \text{mol/L} \) and above \( (P < 0.01) \); Fig. 5D and E). The effect of treating siRNA-transfected cells was less marked with higher doses of 20 \( \mu \text{mol/L} \) and above required to affect any significant difference. Reduced PPAR\( \gamma \) expression in itself had no clear effect on proliferation or apoptosis (Figs. 5F and G). However, addition of the PPAR\( \gamma \) agonist ciglitazone at doses as low as 5 \( \mu \text{mol/L} \) was sufficient to reduce cellular proliferation in both untransfected and transfected cells \( (P < 0.01) \); Fig. 5F). Similarly, apoptosis was increased following incubation with ciglitazone although effects on apoptosis were only seen with doses of 10 \( \mu \text{mol/L} \) or higher \( (P < 0.01) \); Fig. 5G). Results for Ishikawa cells were very similar although higher doses of ciglitazone were required to affect any significant difference in either proliferation or apoptosis. Western blotting was used to investigate PTEN and P-AKT expression in both endometrial cell lines. Ishikawa cells were found to have no expression of PTEN and to express P-AKT only (Fig. 6A) although PTEN expression was confirmed in HEC-1A cells (Fig. 6B). HEC-1A cells with reduced
Figure 1. Nuclear expression of PPARs, RXRs, PTEN, and P-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 (Dunn’s multiple comparison test). I, representative protein blot for each PPAR subtype following co-IP of RXRα, RXRβ, and RXRγ in benign endometrial tissue. Protein (250 μg) was loaded in each experiment.
expression of PPARγ were found to have diminished expression of PTEN when compared with "unsilenced" cells (Fig. 6C). A concurrent increase in expression of P-AKT was also seen (Fig. 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 (Fig. 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 with controls (P < 0.05; Fig. 7A). Conversely VEGF levels were slightly increased in cells with reduced expression of PPARγ compared with control cells (P < 0.05; Fig. 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 cigitazone in cells transfected with antisense RNA for PPARα and PPARγ, respectively (Fig. 7C and D). Treatment of untransfected cells with the PPARα agonist fenofibrate reduced VEGF concentration in both Ishikawa and HEC-1A cells (P < 0.05; Fig. 7E and F). No significant effect following ciglitazone treatment was noted in either cell line (Fig. 7E and F). The reduction in VEGF in HEC-1A cells treated with fenofibrate was potentiated by coincubation with the RXR ligand, ATRA (Fig. 7F) but the same effect was not noted in Ishikawa cells (Fig. 7E). Interestingly, the effects of both fenofibrate and ATRA in HEC-1A cells, were almost fully reversed by coincubation with the PPARα antagonist GW6471 (Fig. 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
showed expression of PPARα in vascular endothelial cells as well as endometrial glandular and tumor 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 showed 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 knockdown 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 α (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 counter-intuitive that reducing the expression of PPARα within endometrial cells reduces VEGF secretion yet enhancing PPARα activity with an agonist ligand also reducing VEGF secretion. One explanation for this could be a cytotoxic effect of fenofibrate at the dose used (50 μmol/L) with subsequent reduction in VEGF secretion. This seems unlikely given that doses of 50 μmol/L of fenofibrate have minimal cytotoxic effects on hepatic cells in culture up to 96 hours (21). Although 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% to 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 knockdown as there may be effects on other cellular pathways.

PPAR/RXR heterodimers bind to estrogen response elements (ERE) in the promoter region of target genes (23) and the VEGF promoter contains an ERE (24). Fenofibrate suppresses endothelial cell proliferation and inhibits neovascularization in mouse cornea (25) which is relevant because we have shown that vascular endothelial cells within endometrial cancers express PPARα. Suppression of angiogenesis using fenofibrate is also reported in mouse tumors (25) and is relevant because we have shown that vascular endothelial cells within endometrial cancers express PPARα.

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 tumor cells or the vascular endothelial cells within the tumor.

In addition to fibrates, 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 tumor 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 and colleagues who showed PPARβ/δ in endometrial tumor cells and also found that PPARβ/δ was colocalized with 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 tumors (18). Although upregulation of PPARγ is reported in some human cancers, for example, ovarian carcinoma (11), loss of PPARγ is a feature of a number of solid tumors including esophageal (30) and ureteric tumors (31). We conclude that loss of PPARγ in EEC in our study is a result of reduced gene transcription as transcription analysis of EEC has previously identified reduced mRNA 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 shown 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 P-AKT. PPARγ can also upregulate the transcription of PTEN in breast cancer (14, 33). PTEN...
Figure 5. Effects of PPARα and PPARγ and their respective agonist ligands on the growth of endometrial cancer cells in vitro. A–C, the effect of transfection of endometrial cancer cells with PPARα or PPARγ siRNA is illustrated. A, Western blot showing effect of antisense RNA for PPARα and PPARγ on Ishikawa cells (ISH) and HEC-1A cells in culture compared with 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 antisense RNA. The "whiskers" show the range with a horizontal line representing the median (n = 6). D and E, cellular proliferation and apoptosis, respectively, following treatment of HEC-1A cells with the PPARα agonist, fenofibrate are shown. F and G, the effects of the PPARγ ciglitazone on cellular proliferation and apoptosis, respectively, in HEC-1A cells are shown. A–G, light colored bars represent untransfected cells and bars with darker shading represent cells that have been transfected with antisense RNA against PPARα (D and E) and PPARγ (F and G); error bars represent interquartile ranges; 6 replicates were carried out for each experimental condition and each experiment repeated 3 times. These experiments were also conducted in Ishikawa cells. The data from the latter are provided as Supplementary Figures.
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 tumors. 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 PPARγ or PPARγ by 70% to 90% in our cell lines with 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 nontransfected cells. Hence, it seems likely that the ligands were acting upon the residual transcription factors present.

We have shown that endometrial cancer cells can be stimulated to increase VEGF secretion by reducing cellular expression of PPARγ. This indicates that PPARγ can

Figure 6. Effects of PPARγ expression and PPARγ ligands on expression of the tumor suppressor PTEN in endometrial cells in vitro. A and B, protein blots showing the effect of downregulating PPARγ on expression of PTEN and P-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 are shown as box and whisker plots. Each box extends from the 25th to 75th percentiles and whiskers show the range with the median shown 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 P-AKT in wild-type HEC-1A cells. The effect of treatment with increasing drug concentrations is shown with doses ranging from 5 to 50 μmol/L. Cells treated with drug vehicle only were used as negative controls.
negatively regulate tumor angiogenesis. Similar to the anti-angiogenic actions of PPARα agonists, we conclude that PPARγ is also necessary for antiangiogenic 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. Tumor cells are...
influenced by secretions from and contact with other cell types within the tumor microenvironment such as tumor-associated macrophages. We have shown that PPARγ is expressed in endometrial tumor macrophages and this is consistent with a role for PPARs in macrophage differentiation and regulation of inflammatory activities (36, 37). Interestingly, PPARγ seems 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 (proinflammatory tumor promoting) variety or the M2 phenotype. This warrants further investigation if PPARγ ligands are to be considered within clinical trials.

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 show preferential binding of endometrial PPARα and PPARγ with RXRα, as compared with other RXR subtypes. Our finding of reduced RXRβ expression in ECC is consistent with quantitative RNA analysis (8). Nuclear RXRγ expression is also diminished but interestingly nuclear RXRα in endometrial cancers is more highly expressed compared with 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 show nuclear-cytoplasmic shuttling of PPARγ and a nuclear localization signal within PPARα (38, 39). We showed RXRα and RXRγ in the nuclei of epithelial cells within ECC 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 PPARγ 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 about the PPAR/RXR system in human endometrial cancer compared with other tumor 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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