Peroxisome Proliferator-activated Receptor α Is an Androgen-responsive Gene in Human Prostate and Is Highly Expressed in Prostatic Adenocarcinoma

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ABSTRACT

Peroxisome proliferator-activated receptor (PPAR) α is a member of the nuclear receptor superfamily of ligand-activated transcription factors. PPARα is activated by peroxisome proliferators and fatty acids and has been shown to be involved in the transcriptional regulation of genes involved in fatty acid metabolism. In rodents, the PPARα-mediated change in such genes results in peroxisome proliferation and can lead to the induction of hepatocarcinogenesis. Using the mRNA differential display technique and Northern blot analysis, we have shown that chronic exposure of the prostate cancer epithelial cell line LNCaP to the synthetic androgen mibolerone results in the down-regulation of PPARα mRNA. Levels of PPARα mRNA are reduced to approximately 40% of control levels in LNCaP cells exposed to 10 nM mibolerone for 96 h. PPARα-responsive reporter plasmids derived from human ApoA-II and muscle carnitine palmitoyl-transferase I genes were stimulated by the PPARα-activating ligand Wy-14,643 in LNCaP cells. In situ hybridization and immunohistochemical analyses showed that PPARα expression in prostate is confined to epithelial cells. In benign prostatic tissue, PPARα mRNA was either absent or only weakly expressed in the basal epithelial cells. In 11 of 18 (61%) poorly differentiated (Gleason score, 8–10) prostatic carcinoma specimens, there was strong expression of PPARα compared with 4 of 12 Gleason score 7 tumors and 2 of 11 Gleason score 3–6 tumors (P < 0.01). These results suggest that PPARα is found and functional in human prostate and is down-regulated by androgens. The role of PPARα may be to integrate dietary fatty acid and steroid hormone signaling pathways, and its overexpression in advanced prostate cancer may indicate a role in tumor progression with the potential involvement of dietary factors.

INTRODUCTION

Peroxisome proliferators are a group of structurally diverse compounds that includes hypolipidemic drugs, herbicides, and industrial plasticisers (1–4). Administration of these compounds to rats and mice results in an increase in the size and number of peroxisomes, accompanied by increases in peroxisomal fatty acid β-oxidation and microsomal ω-hydroxylation (5). Long-term exposure leads to the induction of hepatomegaly and hepatocellular carcinoma (6). These effects of peroxisome proliferators are predominantly mediated via PPARα, a member of the nuclear receptor superfamily of ligand-activated transcription factors. This receptor and other members of its subfamily, PPARβ and PPARγ, regulate the transcription of target genes by binding to PPREs as a heterodimer with retinoid X receptor (7, 8). However, PPARβ and PPARγ show greatly reduced sensitivity to peroxisome proliferators compared with PPARα (9).

In addition to peroxisome proliferators, PPARα is also activated by fatty acids and fatty acid metabolites, leading to the induction of several genes involved in lipid metabolism as well as those not related to lipid metabolism such as transthyretin and α2u-globulin (10). The role of PPARα in fatty acid metabolism has been confirmed by the generation of a PPARα-null mouse (11). These animals are phenotypically normal and have normal basal levels of hepatic peroxisomes but are nonresponsive to peroxisome proliferation and do not display induction of target genes. Furthermore, these mice show altered constitutive expression of mitochondrial fatty acid-metabolizing enzymes (12) and changes in apolipoprotein and high-density lipoprotein metabolism (13).

In humans, PPARα is most highly expressed in the liver, heart, and kidney and is expressed at lower levels in other tissues (14, 15). However, hepatic levels of the receptor in humans are significantly lower than those in rats and mice, and it has been proposed that this accounts for the human liver being refractory to the pathological effects of peroxisome proliferator exposure (16). Nevertheless, it is clear that PPARα has a functional role in humans because the hypolipidemic effects of fibrate drugs in humans are mediated through the receptor (17).

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The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; BPH, benign prostatic hyperplasia; DCC, dextran-coated charcoal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PSA, prostate-specific antigen; ISH, in situ hybridization.
PPARα has been shown to be regulated by glucocorticoids (18), insulin (19), and tumor necrosis factor α (20). In this study, we identify PPARα as an androgen-regulated gene in human prostatic cells and describe its expression in human prostatic carcinoma.

PATIENTS AND METHODS

Patients. Forty-nine patients were investigated. Forty-one men had newly diagnosed and previously untreated prostate cancer, and eight men had BPH. Approval for the use of tissue was obtained from the local ethical committee. Prostate cancer specimens were further classified into three groups according to histological grade: (a) Gleason score 8–10 (18 tumors); (b) Gleason 7 (12 tumors); and (c) Gleason score 3–6 (11 tumors). Seventeen tumors were locally advanced (clinical T3 (9 tumors) or T4 (8 tumors)), and the remaining tumors were either T2 (8) or found incidentally after transurethral prostatectomy (pT1a, 5 tumors; pT1b, 11 tumors).

Cell Culture. Primary epithelial cell cultures were prepared from tissue obtained from patients undergoing transurethral prostatectomy for BPH as described previously (21). Epithelial cells were cultured in WAJC 404 medium supplemented with HEPES (pH 7.6; 25 mM), sodium hydrogen carbonate (15 mM), zinc-stabilized insulin (2.5 μg/mL), cholera toxin (10 ng/mL), dexamethasone (1 μM), epidermal growth factor (10 ng/mL), 0.5% bovine pituitary extract, heparin (4 units/mL), sodium selenite (10 ng/mL), transferrin (10 μg/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL). All primary cultures were used at the second passage and demonstrated to be responsive to androgens in proliferation assays using radiolabeled mibolerone (22) and demonstrated to be responsive to androgens in proliferation assays using radiolabeled mibolerone (22).

Differential Display. Differential display was performed as described previously (22). Primers T12 VG and 1019 (GGTACTCCAC) were employed in 30 cycles of PCR with the appropriate primers and cloned into the pCR1 vector (Invitrogen) according to the manufacturer’s protocol. The DNA sequences of the isolated clones were determined using the Thermo-Sequence cycle sequencing kit (Amersham Life Sciences), and homologies to known genes were determined using the GenBank database.

Northern Analysis. Total RNA samples were electrophoresed as described previously (24). Briefly, RNA (5 μg) was fractionated on an agarose gel, transferred to a nylon membrane (Hybond N+; Amersham), fixed by heating at 80°C for 2 h, and stained with methylene blue to assess the integrity of the RNA. Probes were generated from cloned cDNAs by restriction endonuclease digestion and radiolabeled with [α-32P]dATP using random-primed labeling mixture according to the manufacturer’s protocol (Boehringer Mannheim). Hybridization and washing were carried out as described previously (25). Blots were analyzed after exposure to a phosphor storage screen using a PhosphorImager (Molecular Dynamics) and subsequently re-probed with radiolabeled GAPDH cDNA as a control for RNA loading.

Western Analysis. Lysates were prepared by standard methods. Approximately equal amounts of protein (10 μg/lane) were resolved on 12% polyacrylamide gels. Proteins were blotted onto Hybond C extra membrane (Amersham). Filters were blocked in 5% nonfat milk in PBS and 0.05% Tween 20 at room temperature for 60 min. Primary and secondary antibody incubations were performed in 1% nonfat milk in PBS and 0.05% Tween 20 for 60 min. Washings were performed as recommended by the manufacturers, and signals were detected by enhanced chemiluminescence (Amersham).

Plasmids and DNA Transfections. The J1.TKpGL3 plasmid containing three copies of the human ApoA-II gene PPRE-containing J site cloned upstream of the thymidine kinase promoter in the pGL3 luciferase expression vector was obtained from Dr. Bart Staels (Institut Pasteur de Lille, Lille, France). pMCPTLuc.781 containing a single PPRE, its derivative pMCPTLuc.781ml containing a mutated inactive PPRE, and murine PPARα-expressing plasmid pPPARα (26) were obtained from Dr. Daniel Kelly (Washington University, St. Louis, MO). pCMVβGal was purchased from Clontech.

LNCaP cells were transfected at 40–50% confluence in Corning 6-well plates using Superfect reagent (Qiagen) according to the manufacturer’s instructions. Plasmid DNA totaled 0.6 μg/well, comprising 0.25 μg of luciferase reporter plasmid, 0.1 μg of PPARα expression plasmid (where indicated), and 0.25 μg of pCMVβGal internal control plasmid. After transfections, cells were washed with PBS and transferred to RPMI 1640 containing 100 μM Wy-14,643 (Calbiochem) or vehicle (Me3SO4) and incubated for an additional 72 h. Luciferase and β-galactosidase activities were determined on cell extracts using reporter lysis buffer (Promega). Transfections were performed at least three times, in quadruplicate. Results, which were expressed as relative luciferase activity, were corrected for differ-
ences in transfection frequency by standardizing against β-galactosidase activities.

**ISH.** Digoxigenin-labeled antisense and sense PPARα probes were generated using a 621-bp PPARα cDNA cloned into the pCRII vector as template. Linearization of the plasmid was carried out using the appropriate restriction endonuclease, and probes were then synthesized using the digoxigenin labeling kit (Boehringer Mannheim) according to the manufacturer’s protocol. Deparaffinized, rehydrated prostatic tissue sections (5 μm) on silane-coated microscope slides were permeabilized by incubation in proteinase 75 (20 μg/ml) for 30 min at 37°C and acetylated in PBS containing 0.25% acetic anhydride and 0.1 M triethanolamine for 10 min at room temperature. Prehybridization was carried out in 50% formamide, 4× SSC, 1× Denhardt’s solution, 125 μg/ml tRNA, and 100 μg/ml freshly de-denatured salmon sperm DNA for 30 min at 42°C. Hybridization was performed using prehybridization solution containing denatured PPARα antisense or sense probes (50 ng/slide) for 16 h at 42°C. Washes were then carried out at 52°C (2× SSC and 50% formamide, 30 min; 1× SSC and 50% formamide, 30 min; 0.5× SSC and 50% formamide, 30 min). The slides were then washed in buffer 1 containing 5% BSA and 0.3% Triton X-100 for 30 min at room temperature. The sections were incubated in alkaline phosphatase-conjugated antidigoxigenin antibody diluted 1:500 in buffer 1 containing 5% BSA and 0.3% Triton X-100 for 2 h at room temperature. After washing in buffer 1, the slides were briefly immersed in buffer 2 containing 0.34 mg/ml nitroblue tetrazolium and 0.18 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in the dark for 16 h at 4°C. The slides were then immersed in 10 mM Tris-HCl, 1 mM EDTA buffer (pH 8.0), rinsed in water, counterstained with Mayer’s hematoxylin, and mounted in Glycergel mounting medium.

**Immunohistochemistry.** For immunohistochemical studies, rabbit antiserum raised against the full-length recombinant PPARα was kindly provided by Dr. David R. Bell (University of Nottingham, Nottingham, United Kingdom). Formalin-fixed paraffin-embedded prostatic tissue sections were dewaxed, rehydrated, and trypsinized at 37°C for 15 min. Endogenous peroxidase was blocked with hydrogen peroxide in methanol, and nonspecific staining was blocked by incubation with normal goat serum. Sections were incubated with the rabbit primary antibody diluted 1:50 for 1 h and then incubated with a biotinylated goat antirabbit antibody diluted 1:500 for 30 min. The detection of the antibody reaction was carried out using a standard streptavidin-biotin complex (DAKO) combined with 3,3′-diaminobenzidine tetrahydrochloride. As a negative control, incubation with the primary antibody was omitted.

**Statistical Analysis.** Comparison of PPARα expression was carried out by means of the χ² test with Yates’ correction for continuity.

**RESULTS**

**Identification of PPARα As an Androgen-responsive Gene.** Differential display was carried out on polyadenylated RNA from untreated primary epithelial cells and cells exposed to 10 nM mibolerone for 12–48 h. Whereas band patterns obtained from cells undergoing different treatments were similar, several bands were shown to be reproducibly differentially expressed, one of which was down-regulated after exposure to androgen compared with untreated control cells. This band was excised from the gel, cloned into the pCRII vector, and fully sequenced. The clone contained a 362-bp insert with the correct flanking primer sequences (T12, V and 1019) and showed a 93% homology with the 3′ end of the human PPARα gene.

**Effect of Androgen on PPARα Levels in LNCaP Cells.** Attempts to confirm the putative androgen-regulated expression of PPARα in primary epithelial cells by Northern analysis using the 362-bp cDNA fragment obtained from differential display as a probe were unsuccessful because PPARα mRNA was undetectable using this technique, reflecting the decreased sensitivity of Northern analysis compared with the PCR-based differential display technique. However, PPARα mRNA was detectable and shown to be androgen regulated in the androgen-responsive human prostate cancer cell line LNCaP. A 1.8-kb transcript was detected that was identical to the previously reported size of PPARα mRNA (27). In each of five separate experiments, PPARα was shown to be down-regulated to between 41% and 54% of control levels in response to treatment with 10 nM...
A comparable down-regulation of PPARα was observed using 0.1 and 1 nM mibolerone. Treatment of cells with 10 μM casodex, a nonsteroidal antiandrogen, resulted in the complete abolition of the androgen-induced down-regulation of PPARα mRNA (data not shown).

**PPARα Is Functional in LNCaP Cells.** On Western analysis, a single band of approximately M<sub>r</sub> 55,000 was apparent at low levels in LNCaP cells and high levels in two advanced prostate cancers (data not shown). To determine whether PPARα expressed in LNCaP cells was able to mediate ligand-induced effects, we performed luciferase reporter assays using plasmid constructs containing multiple PPREs derived from the PPARα-responsive human ApoA-II and muscle carnitine palmityltransferase I genes combined with the PPARα ligand Wy-14,643. Control experiments using a mutated PPRE reporter plasmid derived from the muscle carnitine palmityltransferase I gene failed to exhibit a response to Wy-14,643 in LNCaP cells (data not shown). Further transfection with a PPARα-expressing cDNA enhanced the Wy-14,643 ligand-induced response approximately 3-fold (Fig. 2). These results indicate that PPARα is functional in LNCaP cells.

**ISH.** Hybridization of digoxigenin-labeled PPARα riboprobes to representative sections of human benign prostatic tissue and prostatic adenocarcinoma is shown in Fig. 3. mRNA for PPARα was detected exclusively in the cytoplasm of prostatic epithelial cells, with no signals in the prostatic stroma. BPH samples and histologically benign areas of prostatic tissue present in sections of prostate cancer were negative or exhibited low levels of patchy staining in the basal epithelial cells (Fig. 3A). In prostatic adenocarcinoma, PPARα mRNA expression was related to the histological grade of the tumor and to the tumor stage. Table 1 demonstrates that in 11 of 18 (61%) poorly differentiated (Gleason score, 8–10) prostatic carcinoma specimens, there was strong expression of PPARα (Fig. 3C) compared with 4 of 12 Gleason score 7 tumors and 2 of 11 Gleason score 3–6 (Fig. 3B) tumors (P < 0.01). Table 2 shows that 11 of 17 advanced tumors (T3/T4) displayed strong expression of PPARα compared with 6 of 24 of the remaining samples (P < 0.05). Sections hybridized with the corresponding sense probe showed no detectable signal (Fig. 3D).

**Immunohistochemistry.** In malignant prostatic tissue, PPARα protein was detected exclusively in the cytoplasm of the epithelial cells, with no staining in the stromal compartment (Fig. 3E). In benign prostatic tissue or in areas of benign epithelium adjacent to tumor, PPARα protein was either absent or weakly expressed. This pattern of expression was confirmed in an additional 12 samples.

**DISCUSSION**

Prostate cancer is the second most common male malignancy in Europe, with over 85,000 cases registered every year (28). In the United States it is the most common malignancy in males, with an estimated 317,000 new cases diagnosed in 1996 (29). Androgens are intimately involved in the regulation of normal growth and differentiation of the prostate; therefore, androgen-regulated genes are likely to contribute to the development and progression of prostate cancer. Androgen-responsive genes such as PSA (30) and prostate specific membrane antigen (31) have been characterized, and PSA is commonly used as a clinical marker for prostate cancer (32). We have previously used the differential display technique to identify androgen-responsive genes in human prostatic tissues (33, 34). In this study, we have identified PPARα as an androgen-responsive gene. Direct exposure of LNCaP cells to 10 nM mibolerone resulted in a down-regulation of PPARα to approximately 40% of control levels after 96 h. This effect was abolished by the treatment of cells with the nonsteroidal antiandrogen casodex, demonstrating that the androgen receptor is directly involved in this process. The kinetics of down-regulation of PPARα by androgens in LNCaP cells is similar to those of other steroid receptors, such as the androgen receptor and the orphan receptor TR2. All three receptors show maximal down-regulation between 48 and 96 h of androgen treatment to between 40% and 60% of control levels (35, 36). The relatively long androgen exposure time required to cause down-regulation suggests that this response may be a secondary effect of androgen exposure. This hypothesis is supported by our finding that the androgen-induced down-regulation of PPARα is completely abolished by the treatment of cells with the protein synthesis inhibitor cycloheximide (data not shown). In the case of the human androgen receptor, the lack of an androgen response element in the promoter region, combined with the presence of cAMP and AP-1 binding sites, indicates that regulation may occur via interaction with other transcription factors (37). Alternatively, androgen may regulate the release of autocrine/paracrine factors that directly down-regulate PPARα expression. Previous studies in which male rats have been shown to display a greater response to peroxisome...
proliferators than females (38, 39) and the adrenal androgen dehydroepiandrosterone has been shown to be able to elicit a peroxisome proliferative response in rodents (40) have supported the possibility of a link between androgens and PPARs. However, these instances would more likely involve an androgen-mediated increase in PPARα as opposed to the down-regulation that we have found in our study.

Western analysis and transient transfection studies demonstrated that PPARα is present at low levels in LNCaP cells and is responsive to the highly specific ligand Wy-14,643. Cross-talk between the signaling pathways for androgen receptor and PPARα likely occurs through common coactivator proteins (41). Preliminary transfection studies suggest that androgens decrease PPARα-mediated transcriptional activity.

Fig. 3 Photomicrographs of ISH (A–D) and immunohistochemical (E and F) studies on prostatic tissue sections; magnification, approximately ×200. A, section of BPH showing patchy staining in basal epithelial cells with antisense PPARα riboprobe. B, section of moderately differentiated (Gleason score = 6) prostatic adenocarcinoma showing weak staining in tumor cells with antisense PPARα riboprobe. C, section of high-grade (Gleason score = 9) prostatic adenocarcinoma showing strong staining of tumor cells with antisense PPARα riboprobe. D, section of high-grade prostatic adenocarcinoma showing negative staining with sense PPARα riboprobe. E, section of high-grade (Gleason score = 10) prostatic adenocarcinoma showing strong staining of tumor cells with anti-PPARα antibody. F, section of high-grade prostatic adenocarcinoma showing negative staining with omission of primary antibody.
and, furthermore, that PPARα-activating ligands stimulate transcription of the androgen-responsive PSA gene through the androgen receptor. 4

At the present time, we can only speculate on the downstream effects of regulation of PPARα expression by androgens. Genes regulated by PPARα include those involved in both intracellular and extracellular lipid metabolism (42, 43). Androgens have previously been shown to induce an accumulation of lipid droplets in LNCaP cells (44), and a key lipogenic enzyme, fatty acid synthase, is up-regulated by androgens in these cells (45). Furthermore, the finding that several other enzymes involved in fatty acid synthesis and in the biosynthesis of cholesterol are regulated by androgens in LNCaP cells has led to the proposal that androgens might not affect the expression of all these enzymes individually but instead may modulate the expression and/or activity of one or more common transcription factor(s) involved in the coordinate control of these lipogenic genes (46). It is possible therefore that PPARα may be one such factor. However, most enzymes involved in lipid metabolism that have been identified thus far as being regulated by PPARα are involved in catabolic pathways; therefore, it may be that down-regulation of these enzymes as a consequence of androgen-mediated reduction in PPARα expression results in a concomitant increase in fatty acid synthesis.

ISH was carried out to examine the distribution of PPARα mRNA in samples of human benign prostatic tissue and prostatic adenocarcinoma. The specificity of the riboprobes used was confirmed by Northern analysis, demonstrating a single transcript of 1.8 kb as described previously (27). This was significantly different from human PPARβ and PPARγ, which show a high degree of homology to PPARα but have transcript sizes of 4 and 2.1 kb, respectively (47, 48). Although ISH does not allow accurate quantification of PPARα mRNA expression, our findings clearly show an increase in expression of PPARαs in high-grade, poorly differentiated prostatic adenocarcinoma compared with benign epithelium or well-differentiated tumor. This pattern of expression was confirmed by our immunohistochemical results, which showed that PPARα protein was present at higher levels in tumor cells than in benign epithelium. The finding that PPARα was expressed in the cytoplasm is in agreement with a previous immunohistochemical study (49). To our knowledge, this is the first demonstration of PPARα expression in human prostate, and the pattern of expression that we have observed suggests that it may be associated with the development and/or progression of prostate cancer. The finding that PPARα is expressed in the basal epithelial cells of some benign glands is also of interest. Basal cells have been advocated as having specific properties in the progression of benign prostatic glands to the malignant phenotype; the majority of proliferating cells in atypical hyperplasias that progress to invasive carcinomas are localized in the basal cells (50). Therefore, the expression of proteins in these cells is likely to play a role in the behavior of developing tumors.

Activation of PPARα by peroxisome proliferators can ultimately lead to hepatocarcinogenesis in rats and mice. Although this process has not been observed in humans, PPARαs have been suggested to play a role in other human cancers, including breast (51, 52) and colon (53) cancer. Recently, it has been shown that PPARγ is expressed at prominent levels in prostate cancer cells, but normal prostatic tissue had very low-level expression of PPARγ (54). Furthermore, the ligand for PPARγ, troglitazone, showed potent antitumor effects against prostate cancer both in vitro and in vivo (54). The action of PPARα ligands, including Wy-14,643 and fatty acids, on prostate cell growth is now being studied. Interestingly, a high intake of dietary fat has been implicated in the progression of prostate cancer (55, 56); it is possible, therefore, that it may be mediated via the activation of PPARαs by fatty acids and their metabolites. Indeed, a study has shown that treatment of food-restricted rats with nafenopin, a peroxisome proliferator and potent tumor promoter, produced only half as many hepatocellular adenomas and carcinomas as seen in animals fed unrestrictedly (57). The possible involvement of PPARα-activating ligands in prostate tumor formation is currently being investigated. Mutations in the androgen receptor are frequently observed in advanced prostate cancer and may also effect the cross-talk between androgen receptor and PPARα signaling pathways, resulting in an up-regulation of PPARα.

In summary, we have shown that PPARα is an androgen-regulated gene in human prostate and is highly expressed in prostatic carcinoma. Further study on the downstream effects of this receptor will be required to assess any role that it may play in the development and progression of prostate cancer.

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4 G. P. Collett and C. N. Robson, unpublished data.
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REFERENCES


