Peroxisome Proliferator-Activated Receptor-γ Suppresses Cyclooxygenase-2 Expression in Human Prostate Cells

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Abstract

Recent studies have found that cyclooxygenase-2 (COX-2) protein expression was low and inducible with cytokines in prostate cancer cells (in the absence of serum) and that, in contrast, COX-2 expression was high in normal prostate epithelial cells (EC). Peroxisome proliferator-activated receptor-γ (PPAR-γ) was expressed at high levels in the prostate cancer cell line PC-3 but not in ECs. In contrast to previous findings by others, PPAR-γ ligands did not induce PPAR-γ expression in EC or PC-3. The present study examined the relationship between PPAR-γ and COX-2 expression patterns in EC and PC-3 in the presence and absence of serum and/or the PPAR-γ agonist 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2). We also evaluated the effects that the forced expression of PPAR-γ1 and PPAR-γ2 had on COX-2 in ECs. We found that expression of PPAR-γ and COX-2 protein was inversely correlated in ECs and PC-3. Low COX-2 expression in PC-3 was up-regulated by serum, and 15d-PGJ_2 blocked serum-induced COX-2 expression and activity in a dose-dependent manner. 15d-PGJ_2 had no effect on COX-2 expression in ECs or PPAR-γ expression in either cell type. However, forced expression of PPAR-γ1 or PPAR-γ2 in ECs suppressed the high level of endogenous COX-2. This effect was not isoform specific and was augmented by 15d-PGJ_2. The present study showed that PPAR-γ activation can be an important regulator of COX-2 in prostate cells and may be an important target for prostate cancer chemoprevention. (Cancer Epidemiol Biomarkers Prev 2004;13(11):1704–9)

Introduction

Cyclooxygenase-2 (COX-2) and peroxisome proliferator-activated receptor-γ (PPAR-γ) are important regulators of cell growth and apoptosis (1-6). In previous *in vitro* studies we conducted without serum, COX-2 expression was low and inducible with cytokines in prostate cancer cells and high in normal prostate epithelial cells (EC; ref. 7). We showed recently that, in the prostate cancer cell line PC-3, PPAR-γ1 is highly expressed and the PPAR-γ agonist 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2) induces cell death.[2] In contrast, ECs, which do not constitutively express PPAR-γ, are sensitive to 15d-PGJ_2 only after undergoing transfection and forced expression of PPAR-γ1 or PPAR-γ.[3]

Studies in non-prostate-derived cells have suggested an interrelationship between PPAR-γ2 and COX-2 (8, 9). COX-2 catalyzes the rate-limiting step in the conversion of arachidonic acid to prostaglandin H_2 (1-6), a precursor of many prostaglandins, including 15d-PGJ_2, which binds with high specificity to and activates PPAR-γ (10-14). Once activated, PPAR-γ can modulate gene expression. Studies of breast and colon cancer cells in culture have shown that ligand activation of PPAR-γ down-regulates COX-2 expression (8, 9). In the present study, we extended our previous work[4] (7) to examine the relationship between PPAR-γ and COX-2 in normal and malignant prostate cells.

Materials and Methods

Tissue Culture. Primary cultures of EC (Clonetics, San Diego, CA) were maintained in prostate EC culture medium according to the manufacturer's instructions as described previously (7). Cells were passaged using 0.025% trypsin and 0.01% EDTA in HEPES-buffered saline followed by trypsin-neutralizing solution (Clonetics). Human prostate carcinoma cell line PC-3 was obtained from American Type Culture Collection (Manassas, VA) and was maintained in 1:1 mixture of DMEM and F-12 (Life Technologies, Inc., Bethesda, MD) supplemented with 10% fetal bovine serum.

Transfection and PPAR-γ Agonist Treatment. EC or PC-3 cells were transfected with 500 μg of empty expression vector PSG5 or vector containing human PPAR-γ1 (hPPAR-γ1) or human PPAR-γ2 (hPPAR-γ2) cDNA. Transfection was done in factor-free prostate EC culture medium or DMEM/F-12 medium for EC and PC-3, respectively, using 3:1 Fugene 6/DNA ratio (Roche-BMB, Indianapolis, IN). After a 30-minute incubation, the

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[4] V. Subbarayan et al., this issue.

medium was replaced with the appropriate complete growth medium containing 10% fetal bovine serum and the cells were incubated for 24 hours. Transfected cells were replated at a concentration of 2×10^5 cells/well in six-well plates. For stimulation studies, transfected EC or PC-3 cells were starved in factor-free medium for 24 hours prior to a 1-hour incubation with 2.5, 5.0, or 10 μmol/L 15d-PGJ_2 (Biomol, Plymouth Meeting, PA) and then harvested for analyses at 18 hours unless otherwise indicated.

Analysis of PPAR-γ and COX-2 by Immunofluorescence. Cells were grown on laminin-coated coverslips and then incubated for 24 hours in serum or serum-free conditions. The cells were then fixed with 1% paraformaldehyde and permeabilized using 1% Triton X-100. Specimens were blocked with Superblock (Pierce Chemical Co., Rockford, IL) or 5% bovine serum albumin solution in TBS-Tween 20. Primary mouse monoclonal antibodies selective for PPAR-γ (sc-7273, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or COX-2 (160112, Cayman Chemical Co., Ann Arbor, MI) were diluted to 2.5 μg/mL in Superblock or 5% bovine serum albumin solution. After overnight incubation at 4°C with primary antibody, the specimens were rinsed twice with PBS and incubated overnight with Alexa 488 goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) in TBS solution. Specimens were washed three times with PBS, rinsed with distilled water, and mounted on glass slides using Prolong anti-fade solution (Molecular Probes). Slides were then examined by epifluorescence microscopy and data were acquired using digital image analysis using a Quantix camera driven by IP Labs software (Scanalytics, Inc., Fairfax, VA) and attached to an IX70 inverted light microscope equipped with epi-illumination objectives (Olympus America, Inc., Lake Success, NY).

Immunoblot Analysis. Whole cell lysates were made as follows. Cells were washed twice with cold PBS containing Complete protease inhibitor cocktail (Roche-BMB), harvested by scraping, and lysed with 100 μL of lysis buffer containing 1% NP40, 1 mmol/L $NaMoO_4$, 30 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L $NaVO_4$, 30 mmol/L Tris (pH 8.3), 100 mmol/L NaCl, and Complete protease inhibitor cocktail. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). Protein (100 μg) per lane was loaded and electrophoresed through a 7.5% SDS-PAGE gel. Protein was transferred to a Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and blocked overnight in TBS-Tween 20 with 0.1% Tween 20 containing 3% bovine serum albumin, fraction V (U.S. Biochemical, Life Science, Swampscott, MA). Primary mouse monoclonal antibody to COX-2 (Cayman Chemical) or polyclonal anti-PPAR-γ (sc-7196, Santa Cruz Biotechnology) was used at a dilution of 1:1,000 and incubated at 4°C overnight. Secondary antibody (IgG, The Jackson Laboratory, West Grove, PA) diluted to 1:5,000 in TBS-Tween 20 was added at room temperature for 2 hours. Chemiluminescent detection was done using SuperSignal chemistry (Pierce Chemical). Anti-actin antibody (Pharmacia, Uppsala, Sweden) was used to determine the loading control.

Prostaglandin E_2 Enzyme Immunoassay. EC or PC-3 cells transfected with the expression vector containing hPPAR-γ1 or hPPAR-γ2 were replated at a concentration of 6×10^3 cells/well in 96-well tissue culture plates in complete medium and allowed to become established as monolayers. After 24 hours, the supernatant was collected and centrifuged to remove particulate material. Two aliquots of supernatant per sample were assayed for prostaglandin E_2 (PGE_2) by enzyme immunoassay according to the manufacturer's instructions (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The concentration of PGE_2 was determined for competitive binding enzyme immunoassay using Assay Zap software (Biosoft, Ferguson, MO). After removal of the supernatant, cells were harvested using trypsin and EDTA and counted using a hemocytometer. PGE_2 concentrations were normalized to cell number per well and reported as PGE_2 production per cell. Data were analyzed using Statview software (SAS Institute, Inc., Cary, NC).

Results

Immunofluorescence Analysis of COX-2 and PPAR-γ Expression in EC and PC-3 Cells in the Presence of PPAR-γ Ligand: 15d-PGJ_2 Suppresses COX-2 in PC-3 Cells. COX-2 and PPAR-γ protein expression was determined in normal prostate ECs and in the prostate cancer cell line PC-3 by immunofluorescence (in the presence of serum). The prostate-derived normal ECs expressed high levels of COX-2 and essentially undetectable levels of PPAR-γ protein. An inverse pattern of expression was seen in PC-3 cells: weak COX-2 and strong PPAR-γ expression (Fig. 1A, *top*). Addition of 15d-PGJ_2 did not influence COX-2 or PPAR-γ expression in EC cells as detected by immunofluorescence (Fig. 1A). Subcellular distribution of COX-2 and PPAR-γ proteins was also evaluated by immunofluorescence analysis. COX-2 protein in ECs was concentrated in the perinuclear region, whereas in PC-3 it was more diffusely distributed in the cytoplasm. The distribution of PPAR-γ staining was nuclear in PC-3. The subcellular distribution of COX-2 or PPAR-γ proteins was not altered with addition of PPAR-γ agonist (Fig. 1A). Cells were also incubated in the presence of increasing concentrations of PPAR-γ ligand 15d-PGJ_2 (0, 2.5, 5, and 10 mmol/L) at 6, 18, and 96 hours and analyzed by Western blot for PPAR-γ expression (Fig. 1B). COS-1 cells were used as a control to determine anticipated location of PPAR-γ1 and PPAR-γ2. Quantitation of multiple Western blot analyses showed no significant induction of PPAR-γ1 or PPAR-γ2 protein in EC or PC-3 under these conditions ($P \geq 0.11$, the lowest significance value observed of any dose range examined for both cell lines). COX-2 protein expression in these cell lines was also confirmed by immunoblot analysis (data not shown). However, PPAR-γ-rich PC-3 cells responded to PPAR-γ agonist (2.5 μmol/L) with a marked reduction in COX-2 expression. There was no detectable effect of 15d-PGJ_2 on PPAR-γ expression in PC-3 by immunocytochemistry (Fig. 1) or by Western blot analysis (Fig. 1B).

Effects of PPAR-γ-Specific Ligand 15d-PGJ_2 on COX-2 Expression: PPAR-γ Agonist 15d-PGJ_2 Suppresses Serum-Induced COX-2 Expression in PC-3 Cells. To further assess COX-2 protein expression in response to PPAR-γ ligand in PC-3 and EC, immunoblot analysis

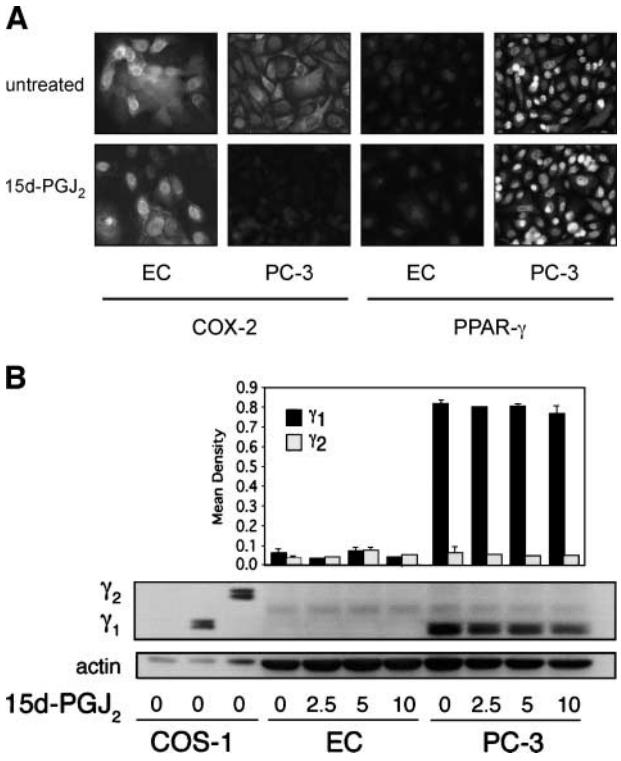

Figure 1. A. Immunofluorescent staining of COX-2 protein in untreated cells growing in serum revealed well-organized perinuclear distribution in EC but poorly organized distribution in PC-3. PPAR-γ expression was nuclear in both EC and PC-3 but poorly expressed in EC cells. PC-3 cells express high levels of PPAR-γ, whereas PPAR-γ expression in EC cells is negligible. Expression of COX-2 is high in EC and low in PC-3. After treatment with 2.5 μmol/L 15d-PGJ_2, COX-2 expression was reduced in PC-3 but unchanged in EC. **B.** Western analysis of EC and PC-3 cells incubated with increasing concentrations of 15d-PGJ_2 (0, 2.5, 5, and 10 μmol/L) was done to determine if PPAR-γ protein expression was induced. COS-1 cells transfected with empty vector or vector containing hPPAR-γ1 and hPPAR-γ2 cDNA were used as a controls for protein migration to accurately determine location of protein. Mean density and SD represent the quantitation of two blots. Actin serves as the normalization control.

was done in the absence and presence of serum after exposure to increasing concentrations of 15d-PGJ_2. In the absence of serum and ligand, constitutive COX-2 protein expression was high in EC and negligible in PC-3 cells (Fig. 2, *top*). Addition of serum to the medium did not affect the already high expression of COX-2 in ECs but substantially induced COX-2 protein expression in PC-3 cells (Fig. 2). Serum-induced COX-2 expression in PPAR-γ-bearing PC-3 cells was blocked in a dose-dependent manner by increasing concentrations of PPAR-γ agonist (2.5-5 μmol/L). An agonist effect on COX-2 expression was not apparent in either cell type in the absence of serum. Additionally, 15d-PGJ_2 did not affect the expression of COX-2 protein in EC cells in the presence of serum (Figs. 1 and 2).

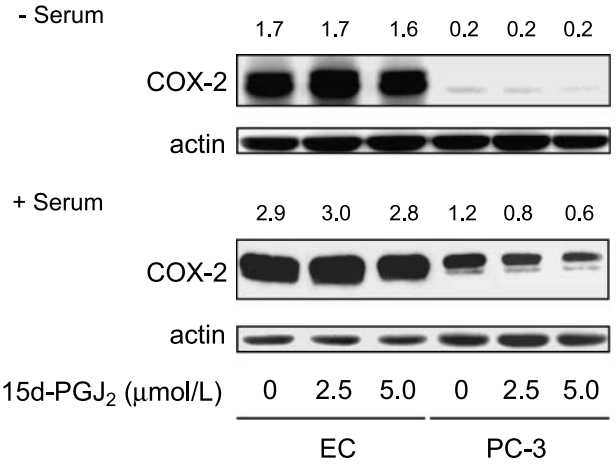

Figure 2. Immunoblot analysis of 15d-PGJ_2-treated EC and PC-3 in the absence or presence of serum. Under serum-free conditions, constitutive COX-2 expression was high in EC and very low in PC-3. COX-2 expression level was not affected by 15d-PGJ_2 in either cell line. In the presence of serum, 15d-PGJ_2 treatment decreased the induced COX-2 expression in PC-3 in a dose-dependent manner but did not affect expression of COX-2 in ECs. Mean density of COX-2 protein levels were normalized to actin loading control.

Forced Expression of hPPAR-γ1 and hPPAR-γ2 in EC: hPPAR-γ Isoforms Suppress COX-2 Expression. To assist in determining if reduction of COX-2 expression by PPAR-γ agonist was affected through PPAR-γ, COX-2 expression was assessed in ECs transiently transfected with hPPAR-γ1 or hPPAR-γ2. Forced expression of both hPPAR-γ isoforms reduced the level of COX-2 expression in EC compared with the parental cells transfected with vector alone in serum-containing medium (Fig. 3). Treatment with 2.5 μmol/L 15d-PGJ_2 further reduced COX-2 expression in hPPAR-γ1- and hPPAR-γ2-transfected ECs compared with the untreated cells. PPAR-γ

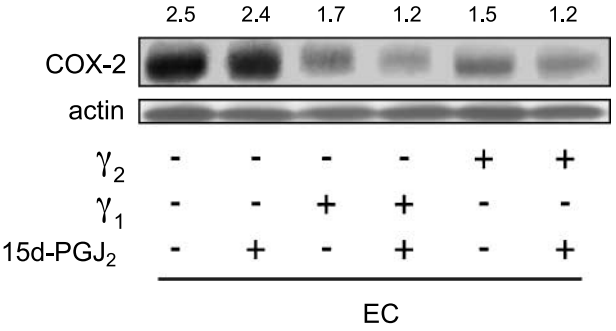

Figure 3. Transfection of hPPAR-γ1 or hPPAR-γ2 with 500 μg/well suppressed COX-2 expression in EC shown by Western analysis. COX-2 suppression by hPPAR-γ1 or hPPAR-γ2 transfection into EC was enhanced after treating these cells with 15d-PGJ_2 (2.5 μmol/L). Mean density of COX-2 protein levels were normalized to actin.

ligand did not alter COX-2 expression in vector alone–transfected control cells. These findings support the hypothesis that endogenous or exogenous PPAR-γ ligand modulates COX-2 expression at least in part via PPAR-γ receptor.

Forced Expression of hPPAR-γ1 and hPPAR-γ2 in EC Cells Suppresses PGE_2 Production. To determine if the modulation of COX-2 protein expression by PPAR-γ agonist was correlated with COX-2 activity, we assessed production of PGE_2, a downstream mediator of COX-2, in hPPAR-γ-transfected EC and PC-3 under serum-free conditions. Forced expression of hPPAR-γ1 or hPPAR-γ2 in EC correlated with reduction of COX-2 expression (Fig. 4A) and PGE_2 production (Fig. 4B) compared with control-transfected EC cells. PGE_2 levels were significantly lower in ECs transfected with hPPAR-γ1 (P = 0.012) and hPPAR-γ2 (P = 0.049) compared with control. Under these conditions, very low PGE_2 levels produced by PC-3 cells correlated with the negligible COX-2 protein expression. PGE_2 expression in PC3 cells was ~10-fold lower than produced by ECs, roughly corresponding to the lower endogenous baseline level of COX-2 expression in PC-3 compared with EC cells (Fig. 4A).

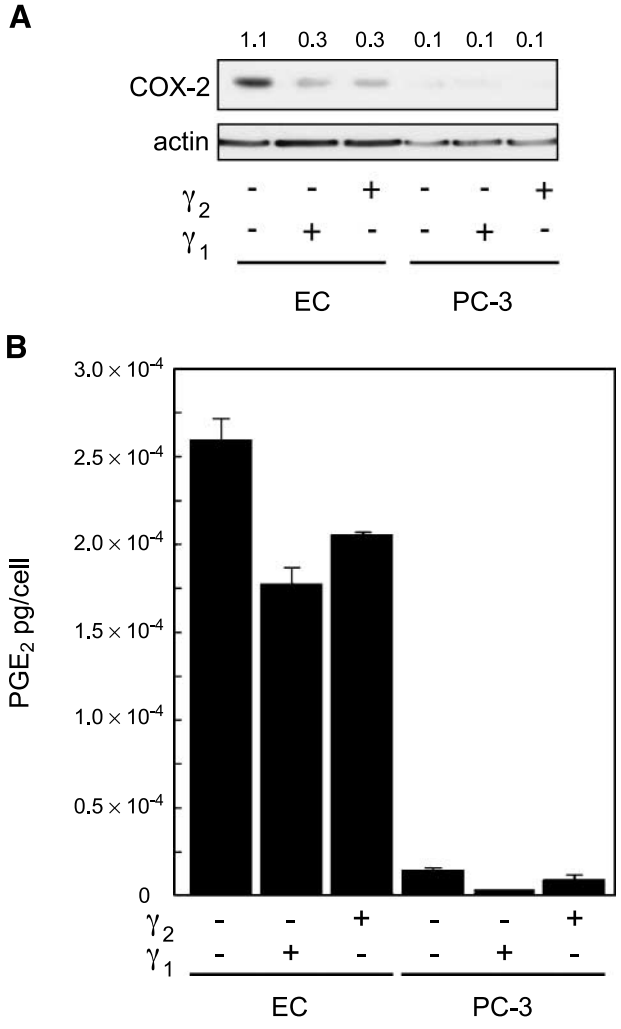

Figure 4. A. COX-2 protein expression decreased in EC when transfected with hPPAR-γ1 or hPPAR-γ2 in the absence of serum as shown by immunoblot analysis. No discernable changes were observed in COX-2 protein expression in PC-3 following transfection. Actin serves as the loading control. Mean density of COX-2 protein levels were normalized to actin. **B.** PGE_2 production was suppressed by forced expression of hPPAR-γ1 or hPPAR-γ2 in EC, whereas only slight changes were observed in PGE_2 production by PC-3. Levels of PGE_2 correlated with COX-2 expression levels in both cell lines.

Discussion

We showed previously that COX-2 expression was elevated in ECs and low in PC-3 and other prostate cancer cell lines in the absence of serum (7). We observed recently that the pattern of PPAR-γ expression is the inverse of that of COX-2 expression in ECs and PC-3 (i.e., PPAR-γ is elevated in PC-3 and low in ECs).[5] Recent studies in non-prostate-derived cells indicate that PPAR-γ ligands can down-regulate COX-2. These findings prompted us to investigate the interrelationship between PPAR-γ and COX-2 in normal and malignant prostate cells.

In the present study, the PPAR-γ ligand 15d-PGJ_2 blocked serum-induced COX-2 expression and activity in PC-3 cells in a dose-dependent manner. The 15d-PGJ_2 concentrations that suppressed PC-3 cell COX-2 are comparable with that required for PPAR-γ activation (15). These findings, along with other work confirming the transactivation of the direct repeat-1 promoter in ECs in response to ligand,[6] suggest that COX-2 suppression is regulated at least in part through the ligand-activated PPAR-γ pathway in prostate cells. We found that the forced expression of hPPAR-γ1 or hPPAR-γ2 in PPAR-γ-deficient ECs reduced COX-2 expression and activity even without PPAR-γ ligand treatment. There was no apparent difference in activity between isoforms. These findings of reduced COX-2 after forced expression of PPAR-γ in the absence of an exogenous PPAR-γ ligand are not unexpected in view of the recently reported findings that benign prostate ECs contain endogenous PPAR-γ ligands 15d-PGJ_2 (16) and 15-*S*-hydroxyeicosatetraenoic acid (17). The endogenous PPAR-γ ligand expression (15-*S*-hydroxyeicosatetraenoic acid) is lost in PC-3 (17).

The mechanism of PPAR-γ-mediated down-regulation of COX-2 in prostate cells is unclear. Recent studies in other cell systems have shown that PPAR-γ agonists can affect COX-2 expression via transcriptional regulation of the *COX-2* gene (18) and that PPARs cross-talk with transcription factors nuclear factor-κB (19, 20), activator protein-1 (AP-1; refs. 9, 20), and cyclic AMP–responsive element binding protein binding protein (CBP)/p300 (9). c-Jun and CBP/p300 proteins are important factors for optimal AP-1-mediated transcription (21). The activation of COX-2 transcription by a variety of stimuli seems to involve AP-1 (10, 22-25). Subbaramaiah et al. (9) showed

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that PPAR-γ ligands inhibited COX-2 induction by antagonizing AP-1-mediated transcription. These investigators found that ligand-induced inhibition of *COX-2* gene expression may involve multiple mechanisms including reduced expression of c-Jun, a primary component of the AP-1 transcription factor complex, and increased interaction between PPAR-γ and CBP/p300 (26, 27), which limits the availability of CBP/p300 coactivator protein (9). It is possible that some of these effects might also be due to paracrine effects of prostaglandins (28).

The relationship and interaction we report here between PPAR-γ and COX-2 in PC-3 and ECs are similar to those reported in a recent study (29) of PPAR-γ effects on macrophage-like differentiated U937 cells, which express PPAR-γ, and endothelial cells, which do not express the receptor. That study showed that 15d-PGJ_2 suppressed the lipopolysaccharide-induced expression of COX-2 in PPAR-γ-expressing cells but not in the endothelial cells. After transfection of a PPAR-γ expression vector into the endothelial cells, however, 15d-PGJ_2 suppressed the induction of COX-2. Based on these results, the authors proposed that the expression of COX-2 is mediated through PPAR-γ.

Our findings reported on the interrelationship between PPAR-γ and COX-2 are also similar to those of a recent study of retinoic acid receptor-β and COX-2 (30). Retinoic acid receptor-β (like PPAR-γ) is a member of the steroid receptor superfamily (31). Li et al. (30) showed that retinoic acid receptor-β expression is low and COX-2 expression is high (inversely correlated) in esophageal cells and that transfecting retinoic acid receptor-β into these cells suppressed COX-2 expression. A recently reported study in mammary cells showed that retinoic acid suppressed the activation of COX-2 transcription by a phorbol ester (23). An evaluation of the mechanism involved in this suppression suggested that retinoic acid antagonized AP-1-mediated activation of COX-2 transcription by limiting the availability of CBP/p300 (23).

PPAR-γ has been implicated in the pathogenesis of human malignancy, and hemizygous deletions of PPAR-γ are commonly found in prostate cancer, suggesting that loss of function may contribute to malignant phenotype (32). PPAR-γ expression has been examined, but data on constitutive expression of PPAR-γ in prostate tissue and cell lines are contradictory. PPAR-γ has been constitutively expressed in most of the prostate cancer cell lines studied (32-34). However, immunohistochemical analysis (156 tumors, 12 normal) showed strong expression of PPAR-γ in tumors but negligible or very low expression in the normal tissue (35). Our immunocytochemistry findings are consistent with these in that higher expression is seen in tumor cell lines compared with normal prostate ECs. We did not see induction of PPAR-γ after incubation with ligand at 2.5 μmol/L by immunocytochemistry or Western analysis.[7] In contrast, Butler et al. (34) reported expression of the PPAR-γ1 but not the PPAR-γ2 isoform in benign and malignant human prostate tissues and up-regulation of PPAR-γ2 in prostate cancer cells lines in response to 2.5 μmol/L 15d-PGJ_2. Nwankwo and Robbins (33) also found that PPAR-γ was not constitutively expressed in EC but was inducible after incubation with γ-linolenic acid, whereas PC-3 constitutively expressed the receptor and was not significantly changed with ligand. Reasons for this discrepancy are not clear.

The present study details the interaction between PPAR-γ isoforms and COX-2, including the effects of a PPAR-γ agonist on this interaction, in normal and malignant cells. This work supports the potential activity of combination chemoprevention with a PPAR-γ agonist to suppress COX-2 synthesis and a COX-2 enzyme inhibitor (e.g., a nonsteroidal anti-inflammatory drug). Inhibition of the important molecular target COX-2 by suppressing synthesis and inhibiting the enzymatic activity may provide a basis for an effective strategy for combination chemoprevention.

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