PROSTAGLANDINS AND THE REGULATION OF TUMOUR CELL GROWTH

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Increased expression of inducible cyclo-oxygenase (COX-2) is associated with a wide variety of tumours. In addition inhibitors of COX have shown a great deal of promise *in vitro* and in animal models as potential anti-tumour therapies. COX enzymes utilise the substrate arachidonic acid to produce prostaglandin (PG)H_2, the precursor to all the prostanoids. Therefore the release of individual prostanoids depends on the abundance and functional coupling to individual PG synthase isoenzymes. Colony stimulating factors (CSFs) are also potential anti-tumour agents via their ability to augment the immune response. When COX-2 is expressed, the CSF, granulocyte macrophage (GM)-CSF is exquisitely sensitive to inhibition by the endogenous PGs produced as a consequence. In addition, the ability of COX-2 to suppress GM-CSF release is mediated via traditional IP/EP prostanoid receptors linked to cAMP-dependant pathways. Therefore inhibition of COX-2 in tumours may have an important side effect of enhancing the immune response. Recently novel signalling pathways for PG derivatives have been discovered; in particular the PGD_2 dehydration product 15-deoxy-$\Delta^{12,14}$ (15d) PGJ_2, was identified as a ligand for the nuclear receptor/ transcription factor PPARγ. PPARγ is present at high levels in a number of tumours, and is also present in endothelial cells. 15d-PGJ_2 as well as other non-prostanoid PPARγ ligands are anti-tumour, and anti-angiogenic, by causing both dramatically inhibit the growth of tumour cells, and endothelial cells by either causing terminal differentiation, and/ or by induction of apoptosis. We have recently found that, in addition to IP and EP ligands generated by COX-2, PPARγ ligands similarly inhibit GM-CSF release. Effecting individual prostanoid pathways at the level of COX expression, profile of PG products produced or selective PG receptor activation may produce novel therapies, either dependent or independent of CSF release, to target cancers.

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Endothelial Cell Apoptosis Induced by the Peroxisome Proliferator-activated Receptor (PPAR) Ligand 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2*

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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is a bioactive prostanoid produced by dehydration and isomerization of PGD_2, a cyclooxygenase product. It was recently shown to activate the nuclear peroxisome proliferator-activated receptor γ (PPARγ), a critical transcription factor involved in adipocyte and monocyte differentiation. In this report, we show that 15d-PGJ_2 is a potent inducer of caspase-mediated endothelial cell apoptosis. PPARα, -δ, and -γ were expressed by endothelial cells, which, when treated with 15d-PGJ_2, induced receptor translocation into the nucleus, and an increase in PPAR response element-driven reporter gene expression. Ciglitizone, a selective activator of PPARγ, also induced transcriptional activation and endothelial cell apoptosis. Endothelial apoptosis induced by 15d-PGJ_2 was inhibited by treatment of cells with an oligonucleotide decoy to a consensus PPAR response element sequence. Furthermore, overexpression of the PPARγ isotype induced endothelial cell apoptosis, which was further potentiated by 15d-PGJ_2 treatment. We conclude that 15d-PGJ_2 induces endothelial cell apoptosis via a PPAR-dependent pathway. The PPAR pathway may be a therapeutic target for numerous pathologies in which excessive angiogenesis is implicated.

Peroxisome proliferator-activated receptors (PPAR)[1] are a family of at least three nuclear receptors (α, δ (also referred to as NUC1), and γ), which heterodimerize with the retinoid X receptors (1). PPARα is found predominantly in the liver, heart, kidney, brown adipose, and stomach mucosa, and PPARγ is found primarily in adipose tissue, where it plays a critical role in the differentiation of pre-adipocytes into adipocytes; PPARδ is almost ubiquitously expressed, but its function is relatively unknown (2, 3). Recently, PPARα and -γ have been suggested to be important immunomodulatory mediators. PPARα knockout mice have exacerbated inflammatory responses (4), whereas activation of PPARγ in monocyte/macrophages inhibits inflammatory mediator and cytokine production (5, 6). PPAR receptors can be activated by a number of ligands (7), including docosahexaenoic acid, linoleic acid, WY-14643 (selective for PPARα), the antidiabetic thiazoldinediones, and a number of eicosanoids, including 5,8,11,14-eicosatetraynoic acid and the prostanoids PGA_1, PGA_2, PGI_2, and PGD_2. Interestingly, the PGD_2 dehydration product 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) is the most potent endogenous ligand for PPARγ yet discovered (8, 9).

All the commonly occurring prostanoids are formed from the cyclooxygenase (COX; prostaglandin G/H synthase) product PGH_2 (10). COX is known to exist in at least two isoforms, a constitutively expressed (COX-1), and mitogen/cytokine-inducible isoform (COX-2). Prostaglandins of the A and J series in particular cause tumor cell apoptosis (11) and can also regulate endothelial cell function by inducing heat shock proteins (12). The mechanism by which PGA and PGJ cause these effects is presently unclear but appear to be associated with nuclear localization (13). Whether 15d-PGJ_2 shares the same properties as other J series PGs tested is unknown. Likewise, it is also not known whether any of the previously known responses of J series PGs are mediated through the PPAR pathway. Interestingly, aberrant PPARγ expression is often found in colon cancer, where COX-2 is known to be elevated (14).

In the present study we demonstrate that 15d-PGJ_2 induces endothelial cell apoptosis. Furthermore, using a decoy oligonucleotide approach against the PPAR response element (PPRE) to inhibit receptor function and overexpression of the PPAR receptors, we provide evidence that the mechanism of action by which 15d-PGJ_2 causes apoptosis is through a PPAR-dependent pathway.

EXPERIMENTAL PROCEDURES

Materials—pCMX-PPARα, pCMX-PPARδ, and pCMX-PPARγ, were a gift from Drs. Ronald Evans (Salk Institute) and Christopher Glass (University of California, San Diego, CA). h6/29 PPARα, and pACO.gLuc. (15, 16) were gifts from Dr. Ruth Roberts, Zeneca Pharmaceuticals (Macclesfield, UK). pGL2 and pSV-β-galactosidase were from Promega. Antisera against PPARα, -δ, and -γ were from Santa Cruz, and fluorescein isothiocyanate-conjugated rabbit anti-goat antibody was from Cappel. Prostaglandins were from Cayman Chemical Co. Ciglitizone, WY-14643, and anti-poly(A)DP-ribose polymerase (PARP) antibody was from Biomol. [^{32}P]dCTP was from *NEN* Life Science Products. Benzyloxocarbonyl-Val-Ala-Asp(OCH_3)-CH_2F (ZVAD-fmk) was from Calbiochem. Immortalized human endothelial cells (ECV-304) were from ATCC, and human umbilical vein endothelial cells (HUVEC) and brain bovine microvascular endothelial cells (BMEC-b) were from Cell Systems. Matrigel basement membrane matrix was from Becton Dickinson. Lipofectamine and Lipofectin were from Life Technologies, Inc. Novafector was from Venn-Nova. Fastrack 2.0 poly(A)$^+$ RNA isolation

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[1] The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PG, prostaglandin; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; HUVEC, human umbilical vein endothelial cells; BMEC-b, bovine brain microvascular endothelial cells; PPRE, PPAR response element; COX, cyclooxygenase; ZVAD-fmk, benzyloxocarbonyl-Val-Ala-Asp(OCH_3)-CH_2F; ACO, acyl-CoA oxidase; PARP, poly(A)DP-ribose polymerase; GFP, green fluorescent protein; FBS, fetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; kb, kilobase(s); PBS, phosphate-buffered saline; BSA, bovine serum albumin; HEK, human embryonic kidney.

kit was from Invitrogen. Luciferin and coenzyme A were from Roche Molecular Biochemicals. Cell culture reagents were from Fisher. Crude fibroblast growth factor was purified from sheep brain (17). Unless stated, all other reagents were from Sigma.

Cell Culture—HUVEC and ECV were cultured as described previously (18). BMEC-b were cultured in medium 199 containing 10% FBS, 0.15 mg/ml crude fibroblast growth factor, 5 units/ml heparin antibiotics, and antimycotic mix (Life Technologies) on gelatin (0.1%)-coated plates.

Viability and Apoptosis Assays—Cell viability was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (19) and presented as the % of control culture conditions. Nuclear morphology was assessed using Hoechst 33258 staining (20). Apoptotic cells were distinguished by their characteristic patterns of nuclear condensation, cytoplasmic rounding, and membrane blebbing. Cells were imaged by either confocal (Zeiss CLSM410 laser-scanning confocal microscope at the Center for Biomedical Imaging at the University of Connecticut Health Center; ×1000 magnification) or a Zeiss TV100 inverted fluorescence microscope. The % of apoptotic cells in 2–5 fields (×100 magnification) per data point were counted. Apoptosis was also demonstrated by Western blot for PARP cleavage. Nonadherent cells were pelleted (800 × *g* for 5 min), and protein was extracted from pooled adherent and floating cells using a gel-loading buffer consisting of 62.5 mM Tris (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.0012% bromphenol blue, and 5% β-mercaptoethanol. Extracts were freeze thawed (−80 °C), sonicated, denatured by boiling, then separated by SDS-polyacrylamide gel electrophoresis. Protein was blotted onto nitrocellulose, and PARP was detected using a monoclonal anti-PARP and secondary goat-anti-mouse peroxidase-conjugated antibody (Cappel) using the ECL (Amersham Pharmacia Biotech) chemiluminescent visualization system. Apoptosis is characterized by the caspase-dependent appearance of an 83-kDa fragment.

Endothelial Cell Differentiation Assay —HUVEC were seeded on Matrigel in 24-well plates, and network formation was stimulated by the addition of FBS (21). Cells were treated for 24 h in the presence or absence of 15d-PGJ_2, and cell death was assessed morphologically.

Northern Blot Analysis for PPAR Isoforms—Poly-$(A)^+$ RNA was prepared from ECV using Fastrack 2.0 RNA isolated kit, according to the manufacturer's protocol. 3 μg of poly-$(A)^+$ RNA were size-fractionated on a 1% agarose/formaldehyde gel and transferred onto Zeta Probe (Bio-Rad) filters, and UV cross-linked. Probes corresponding to the full-length open reading frames of PPAR isoforms were labeled to high specific activity (>10^8 cpm/μg) using the Random primer labeling kit (Roche Molecular Biochemicals). The probe for PPARα was the 1.8-kb *Xho*I fragment of h6/29 PPARα, a dominant negative isoform isolated from human liver (16), which differs from wild type hPPARα by only 4 point mutations. The probe for PPARδ was the 1.3-kb *Hin*dIII-*Bam*HI fragment from pCMX-PPARδ, and the probe for PPARγ was the 1.6-kb *Kpn*I-*Nhe*I fragment of pCMX-PPARγ. Filters were hybridized in 0.5 M sodium phosphate (pH 7.2) containing 7% SDS, 0.5% BSA, 40 mM EDTA, and 20% formamide for 16–20 h at 55 °C, and then washed (each 2 × 15 min at 55 °C) in (i) 40 mM sodium phosphate containing 1 mM EDTA, 5% SDS, 0.5% BSA; (ii) 40 mM sodium phosphate containing 1 mM EDTA and 1% SDS, and (iii) 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) containing 0.5% SDS. To control for differences in loading or transfer of the RNA, the filters were hybridized with cDNA from human glyceraldehyde-3-phosphate dehydrogenase (from ATCC; 1kb).

Immunofluorescence of PPAR Isoforms—Cells cultured on coverslips were fixed in methanol:acetone (1:1, −20 °C). Cells were then washed with PBS and blocked for 30 min with 1% BSA in PBS. Cells were then incubated with primary antibody (1:250 dilution; 0.1% BSA in PBS) at 4 °C overnight, followed by 2 washes with PBS. Secondary fluorescein isothiocyanate-conjugated antibody (1:500, in 0.1% BSA in PBS) was then added for 30 min, after which the cells were further washed 3 times with PBS and viewed by fluorescence microscopy. In some experiments, primary antibody was excluded, or primary antibody was preincubated for 1 h with a 50-fold excess of blocking peptide before the combination being added to the cells.

Reporter Assay for PPAR Activation—All transient transfections were performed using Lipofectamine (Life Technologies) or Novafector (Venn-Nova, Pompano Beach, FL) according to the manufacturer's recommended protocol. ECV cells were used throughout for transfections because of the low transfection efficiency of nonimmortalized endothelial cell strains. To measure activation of PPARs, the well characterized PPAR-responsive promoter region for acyl-CoA oxidase (−581 to −471) fused to the minimal β-globin promoter upstream of a luciferase reporter (pGL2 Basic; Promega), termed pACO.gLuc (15, 16), was used. pACO.gLuc, and pSV-β-galactosidase in the presence or absence of pCMX-PPARα, pCMX-PPARδ, or pCMX-PPARγ were co-transfected into ECV grown in 6-well plates. As a negative control, the promoterless pGL2 Basic (Promega) was utilized. The total amount of DNA transfected (1–2 μg) was normalized with a carrier DNA (pcDNA3.1; Invitrogen). After 24 h, cells were stimulated with test drugs for a further 24 h. Luciferase activity was normalized to β-galactosidase content. Both activities were measured according to the manufacturer's protocol (Promega).

Morphology of Cells Transiently Transfected with PPARγ—The effect of expression of PPARγ in living cells was measured indirectly by co-transfected green fluorescent protein (GFP). ECV were co-transfected with pCMX-PPARγ (2 μg) or carrier DNA alone in the presence of the GFP expression vector (0.3 μg; p-EGFPN-1; CLONTECH). After 24 h, the morphology of cells expressing GFP was analyzed by fluorescence microscopy.

Decoy Experiments—PPRE decoy Zd5 was selected on the basis of binding of PPARα to PPREs as reported by Palmer (22). Decoy (ACT TGA TCC CGT TTC AAC TC) or scrambled (TTA GGG AAT CAG CAA GAG GT) oligonucleotides were annealed to their respective complementary sequence in the buffer containing 20 mM Tris·HCl (pH 8.4), 150 mM NaCl, 50 mM KCl, and 1.5 mM $MgCl_2$. ECV cells cultured on 24-well plates were transfected with either decoy (0.1–1 μM) or scrambled (1 μM) oligonucleotides using 5 μg/ml Lipofectin for 4 h. The medium was then replaced, and cells were either left untreated or supplemented with 10 μM 15d-PGJ_2 for 24 h. The % of apoptosis was measured morphologically by Hoechst staining (as above). To test the efficacy of the oligonucleotide decoy at blocking responses through PPRE, we used the human embryonic kidney (HEK) 293 cell line. HEK293 contained low level messages for PPARα, -δ, and -γ as measured by reverse transcription-polymerase chain reaction.[2] HEK293 were transfected with pACO.gLuc and pSV-β-galactosidase in the presence or absence of decoy (0.1–1 μM) or scrambled (1 μM) oligonucleotide. After 24 h, the medium was replaced with medium without FBS, and decoy (0.1–1 μM) or scrambled (1 μM) oligonucleotide added again to the cell for a further 24 h. Luciferase activity was normalized to β-galactosidase content. Both activities were measured according to the manufacturer's protocol (Promega).

RESULTS

Effect of PPAR Agonists on Endothelial Cell Apoptosis—Immortalized human endothelial cells (ECV-304) were treated with various prostanoids, and cell viability was determined. As shown in Fig. 1*a*, PGD_2, 15d-PGJ_2, and PGA_1 significantly reduced endothelial cell viability as determined by the MTT assay. Other prostanoids such as carbaprost (a stable prostacyclin analog), PGE_2, $PGF_{2\alpha}$, and U46619 (a thromboxane mimetic) did not induce endothelial cell toxicity. Because 15d-PGJ_2 is a dehydration product of PGD_2 and 15d-PGJ_2 is a potent activator of PPARγ, these data are consistent with the involvement of this pathway in the reduction of endothelial cell viability. This effect was partially reversed by 10% FBS, consistent with the survival-promoting activity of the serum-borne polypeptide growth factors and lipids. Dose-response analysis of 15d-PGJ_2 was conducted on HUVEC and BMEC-b, two nonimmortalized endothelial cell strains, as well as the ECV-304. As shown in Fig. 1*b*, 15d-PGJ_2 induced the reduction of endothelial cell viability with an IC_{50} in the range of 2–10 μM. This is consistent with the effective dose-range for PPARγ activation (8, 9).

The reduction in endothelial cell viability induced by 15d-PGJ_2 is associated with a dramatic increase in apoptosis. As shown in Fig. 2, treated ECV-304 cells grown on plastic exhibit cytoplasmic rounding, nuclear condensation, and fragmentation into "apoptotic" bodies. Quantitative analysis of apoptosis was done by counting apoptotic nuclei after staining with the Hoechst 33258 dye. Within 20 h after treatment with 10 μM 15d-PGJ_2, 33 ± 5% of cells possessed apoptotic nuclei. In contrast, similar treatment with vehicle or the inactive prostanoid PGE_2 resulted in 1 ± 0.5% and 2 ± 0.5% of apoptotic nuclei,

[2] D. Bishop-Bailey and T. Hla, unpublished observations.

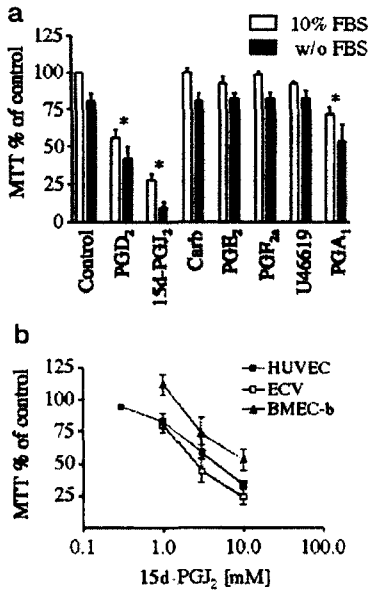

FIG. 1. **Effect of 15d-PGJ$_2$ on endothelial cell viability.** *a*, ECV-304 cells were treated with various prostanoids (10 μM) or vehicle for 20 h, and cell viability was measured by the MTT assay and expressed as % of control culture conditions. These data represent n = 9–23 incubations from 4–9 separate experiments. * denotes significant difference ($p < 0.05$) by one sample t test between viability under control and drug induced conditions. *Carb*, carbaprostacyclin. *b* shows the dose-response analysis of endothelial cell viability, measured by MTT assay of HUVEC, ECV-304, and BMEC-b cells treated with 15d-PGJ$_2$ (20 h; 0 –10 μM). Data represent the mean ±S.E. for n = 9–14 replications from 5 separate experiments.

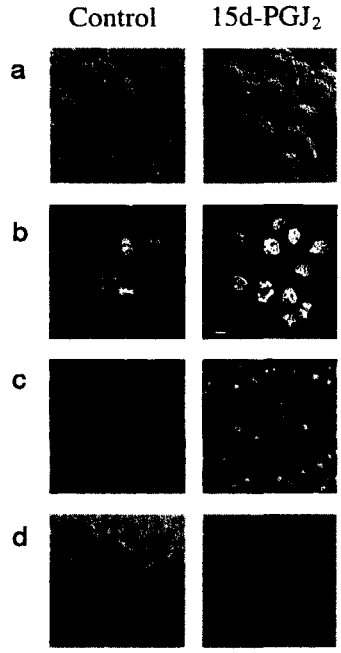

FIG. 2. **Endothelial cell apoptosis induced by 15d-PGJ$_2$.** Differential interference contrast (Normaski) microscopic image (*a*) and corresponding Hoechst staining of nuclei (*b*) is shown of ECV-304 cells treated or not with 10 μM 15d-PGJ$_2$ (20 h; ×1000 magnification) and a low power field of Hoechst-stained ECV-304 cells (*c*), indicating the widespread occurrence of apoptotic nuclei after treatment (20 h; ×200 magnification). The *scale bar* (*b, right hand panel*) represents a distance of 10 μm (for *a* and *b*). 15d-PGJ$_2$ induces the characteristic cytoplasmic rounding and blebbing (*a*), nuclear condensation and fragmentation (*b* and *c*) associated with the apoptotic process, viewed by Zeiss CLSM410 laser-scanning confocal or Zeiss TV100 inverted microscopy. These pictures are representative of n = 6 separate experiments, with at least 3 random fields taken per experiment. *d* shows the cell death induced by 15d-PGJ$_2$ (20 h; 10 μM) of HUVEC plated on Matrigel (×100 magnification).

respectively. In addition, 15d-PGJ$_2$ potently induced apoptosis to differentiating HUVEC grown on the three-dimensional matrix Matrigel (Fig. 2). These data suggest that 15d-PGJ$_2$ reduced endothelial cell viability by inducing apoptosis.

To further characterize the endothelial cell apoptosis induced by 15d-PGJ$_2$, ECV-304 cells were treated with the inhibitor ZVAD-fmk, which potently inhibit the caspase enzymes, the common executors of cell death (24). As shown in Fig. 3*a*, 10–30 μM ZVAD-fmk reversed the ability of 10 μM 15d-PGJ$_2$ to induce endothelial cell apoptosis. Caspase activation results in the specific cleavage of cellular substrates such as PARP (24). As shown in Fig. 3*b*, 15d-PGJ$_2$ induced a characteristic cleavage pattern of a PARP immunoreactive band of 83 kDa, which was inhibited by preincubation with 30 μM ZVAD-fmk. These data strongly suggest that 15d-PGJ$_2$ induces caspase-dependent apoptosis in endothelial cells.

Expression and Activation of PPAR Isoforms in Vascular Endothelial Cells—To determine whether endothelial cells express PPAR isoforms, poly(A)$^+$ RNA from ECV-304 cells were analyzed by a Northern blot analysis with cDNA probes for PPARα, -δ, and -γ isoforms. As shown in Fig. 4*a*, transcripts of approximately 9.5, 4, and 2 kb were detected by PPARα, -δ, and -γ probes, respectively. To determine whether PPAR polypeptides were expressed, we stained the endothelial cells with subtype-specific antisera for the PPAR isotypes in the indirect immunofluorescence assay. As shown in Fig. 4*b*, specific signals for PPARα, -δ, and -γ receptor immunoreactivity were detected. Immunoreactivity was localized in the cytoplasmic, perinuclear region of the endothelial cells. However, the PPARγ receptor immunoreactivity was the strongest and exhibited a punctate perinuclear reticular pattern. Treatment with 10 μM 15d-PGJ$_2$ for 20 h resulted in the nuclear translocation of all three receptor isoforms. Treatment with other prostanoids PGE$_2$ or carbaprost did not alter the subcellular localization of any of the PPAR isoforms (data not shown). These data suggest that 15d-PGJ$_2$ interacts with all three PPAR isoforms in endothelial cells.

To determine whether the PPAR isoforms are active in endothelial cells, we transfected the ECV-304 endothelial cells with the PPRE reporter construct (pACO.gLuc) and measured the luciferase activity. As shown in Fig. 5, pACO.gLuc construct alone exhibited 4-fold enhanced luciferase activity over the control pGL2 basic vector. Treatment with 15d-PGJ$_2$ induced the PPRE-driven luciferase activity in a dose-dependent manner. At higher doses of 15d-PGJ$_2$, significant cell death was observed, and thus, activation of transcription was reduced. These data strongly suggest 15d-PGJ$_2$ activates the PPAR-dependent transcriptional responses in endothelial cells.

PPAR Regulation of Endothelial Cell Apoptosis—We next determined if activation of PPAR receptors is responsible for the 15d-PGJ$_2$-induced apoptosis of vascular endothelial cells. First, we tested the known PPARγ agonist ciglitizone. As shown in Fig. 6, ciglitizone induced PPRE-luciferase activity in a dose-dependent manner in vascular endothelial cells. The effect of ciglitizone is most pronounced in the absence of serum. Similarly, it induced endothelial cell apoptosis most potently in

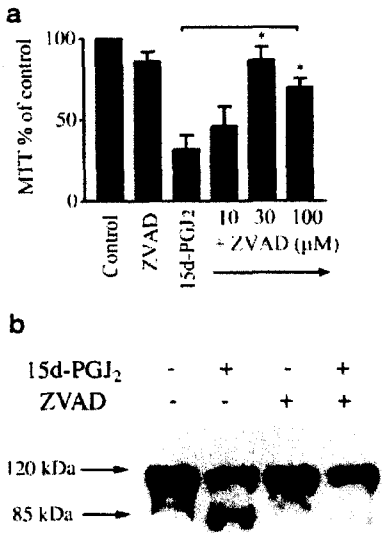

FIG. 3. **Endothelial cell apoptosis induced by 15d-PGJ$_2$ is mediated via caspase activation.** *a* shows the inhibition of 15d-PGJ$_2$ (10 μM)-induced cell viability in ECV by increasing concentrations of the selective caspase-inhibitor ZVAD-fmk (1–100 μM). Data represent the mean ±S.E. for $n = 6$ replications from 3 separate experiments. *b* shows a Western blot for PARP (112 kDa) in ECV. Compared with control (first *lane*) 15d-PGJ$_2$ (10 μM; 2nd *lane*) causes the characteristic cleavage of PARP, leaving a detectable fragment at approximately 83 kDa. When ZVAD-fmk (30 μM; ZVAD-fmk alone, 3rd *lane*) was included in the incubations, 15d-PGJ$_2$-induced PARP cleavage was abolished (4th *lane*).

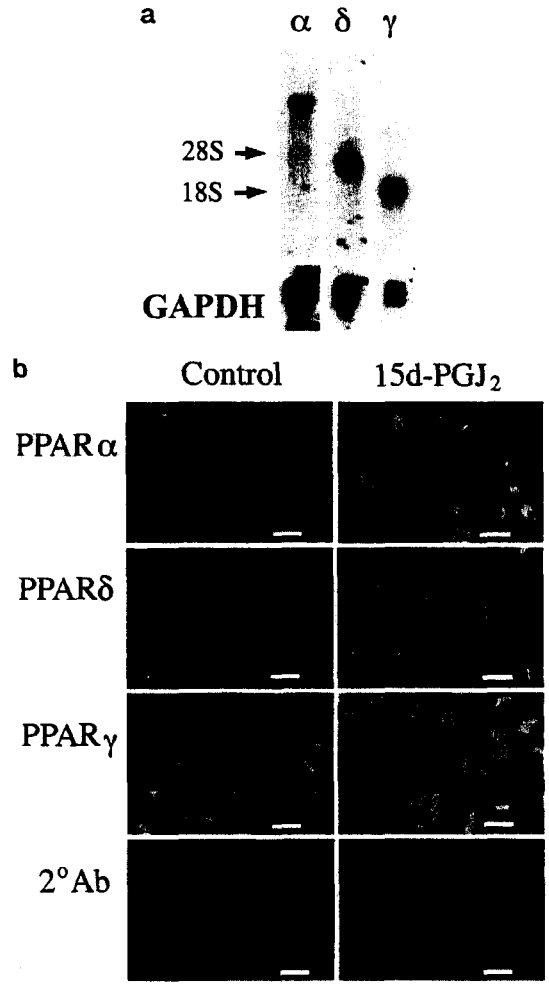

FIG. 4. **PPAR expression and activation in ECV.** *a* shows mRNA expression by Northern blot analysis for PPARα, PPARδ, and PPARγ in ECV-304. Poly(A)$^+$ RNA (3 μg/*lane*) was separated on a 1% formaldehyde-agarose gel, transferred to a nylon membrane, and probed with the radiolabeled open reading frames of human 6/29PPARα or murine PPARδ or PPARγ (see "Experimental Procedures"). *GADPH*, glyceraldehyde-3-phosphate dehydrogenase. *b* shows immunofluorescence micrographs of PPARα, PPARδ, and PPARγ, in ECV-304 under control culture conditions or treated with 15d-PGJ$_2$ (20 h; 10 μM). The figure shows PPARs stained using specific antibodies or in the absence of a primary antibody (*Ab*, 2° antibody). The figure is representative of four separate experiments. The *scale bar* represents a distance of 11 μm.

the absence of serum. These data provide pharmacological evidence that activation of the PPARγ pathway induces endothelial cell apoptosis.

To provide further evidence that the PPAR pathway is required for 15d-PGJ$_2$-induced endothelial cell apoptosis, we developed a double-stranded decoy oligonucleotide that corresponds to the PPAR response element (22). The rationale for this approach is that the decoy will compete for the activated receptor binding to the promoter and thus block PPAR-dependent transcriptional responses. Double-stranded decoy oligonucleotide or a scrambled control was introduced into cells by a Lipofectin-mediated technique (23). To determine the efficacy of this approach in inhibiting PPAR-dependent transcription, HEK293 cells were used because higher efficiency of transfection was obtained. Under optimal conditions, we obtained up to 2800 relative light units/unit of β-galactosidase activity in HEK293 cells, whereas we obtained only up to 100 relative light units/unit of β-galactosidase activity of luciferase activity in ECV-304 cells. As shown in Fig. 7*a*, Lipofectin-mediated loading of HEK293 cells with the PPRE decoy oligonucleotide (Zd5) attenuated 10 μM 15d-PGJ$_2$-induced PPAR-dependent transcription. Neither Lipofectin alone nor the scrambled oligonucleotide inhibited transcriptional activity. Introduction of the decoy and the scrambled counterpart into ECV-304 cells also had a similar effect on the 15d-PGJ$_2$-induced PPRE-dependent transcriptional responses (data not shown). These data suggest that the decoy oligonucleotide is capable of blocking PPRE-dependent transcriptional responses. Introduction of the PPRE decoy (Zd5) oligonucleotide but not the scrambled counterpart, inhibited in part 15d-PGJ$_2$-induced ECV-304 cell apoptosis (Fig. 7*b*). These data suggest that PPAR-dependent transcriptional responses are required, at least in part, for the 15d-PGJ$_2$-induced endothelial cell apoptosis. Similarly, the PPRE decoy oligonucleotide but not the scrambled counterpart inhibited the ciglitizone-induced apoptosis of ECV-304 cells (Fig. 7*c*), suggesting that PPARγ-induced transcription is required for 15d-PGJ$_2$-induced endothelial cell death.

We next determined if overexpression of PPAR receptors modulated endothelial cell apoptosis. ECV-304 cells were transiently transfected with the PPARγ expression vector along with the GFP expression vector plasmid. Cells expressing transfected plasmids can be readily observed by GFP autofluorescence in this assay. As shown in Fig. 8*a*, ECV-304 cells transfected with vector and GFP plasmids expressed GFP predominantly in flattened, healthy cells. In contrast, cells trans-

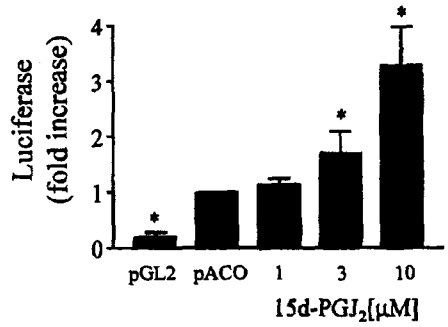

FIG. 5. **Activation of PPAR mediated transcription by 15d-PGJ_2.** ECV-304 cells were transiently transfixed with the PPAR response element reporter gene (pACO.gLuc) or the promoter-less control vector pGL2 basic, and the reporter activity was quantitated as described. To control for transfection efficiency, a β-galactosidase expression plasmid was also co-transfected. 15d-PGJ_2 was added 24 h after the start of transfection and dose-dependently activated the reporter gene. Normalized luciferase activity was represented as fold increase over control (pACO) conditions. These data represent the mean ±S.E. of 3 separate experiments. * denotes $p < 0.05$ (one sample t test) between pACO.gLuc and 15d-PGJ_2.

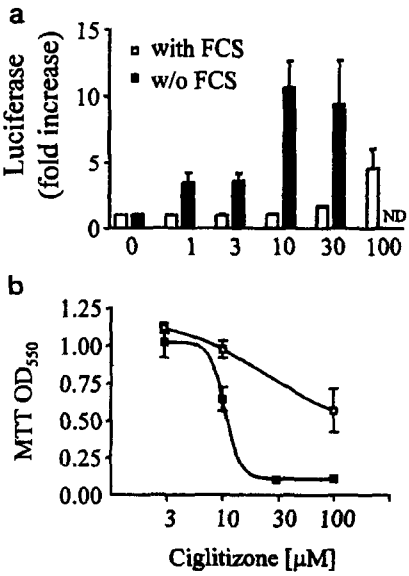

FIG. 6. **Ciglitizone induction of PPAR activation and endothelial cell death.** *a* shows the activation of PPRE by ciglitizone. ECV-304 cells were transiently transfected with the PPAR response element reporter gene (pACO.gLuc). 24 h after the start of transfection, the medium was changed so that ECV were incubated in the presence (*open bars*) or absence (*closed bars*) of FBS (*FCS*, 10%) and ciglitizone (24 h; 1–100 μM) added. *Luciferase activity is represented as the fold increase* of luciferase (normalized to β-galactosidase content) compared with control culture incubations. *ND* indicates that luciferase could not be determined because of high levels of cell death. These data represent the mean ±S.E. of four separate experiments. *b* shows the decrease in endothelial cell viability, measured by MTT assay of ECV-304 cells incubated in the presence (*open squares*) or absence (*closed squares*) of FBS (10%) treated with ciglitizone (20 h; 0 –100 μM). The data represent the mean ±S.E. for n = 9–14 replications from 5 separate experiments.

fected with PPARγ expression vector and GFP exhibited rounded and fragmented morphology, characteristic of apoptotic cells. Quantitative analysis of transfected cells indicated that PPARγ transfection resulted in ~3.5-fold increase in apoptotic cells in ECV-304 cells. This was further enhanced by treatment with 15d-PGJ_2 (Fig. 8*b*). These data indicate that 15d-PGJ_2 activation of PPARγ induces endothelial apoptosis.

DISCUSSION

In this report, we demonstrate that 15d-PGJ_2, PGD_2, and PGA_1 induced endothelial apoptosis. Prostanoids that act via plasma membrane receptors, such as PGE_2, carbaprost, and U46619 did not induce endothelial cell apoptosis. Removal of serum increased the potency of all these agonists, with the exception of PGD_2, suggesting that mitogens and survival factors present in serum counteract cell death. The lack of increased potency observed with PGD_2 in serum-free medium may be because of the requirement for dehydration and isomerization of PGD_2 to 15d-PGJ_2 by the enzymes and carriers present in serum. Δ^{12}-PGJ_2, another nuclear-acting prostanoid, was shown to be an inducer of tumor cell apoptosis (11). These data suggest that nuclear-acting prostanoids have distinct effects on endothelial cell behavior from their counterparts, which signal via plasma membrane receptors.

15d-PGJ_2-induced reduction in endothelial cell viability was characterized morphologically by rounded cells, condensed nuclei, and by the cleavage of the caspase-3 substrate PARP (24). These are the hallmarks of apoptosis, and indeed, the caspase inhibitor ZVAD-fmk reversed the effects of 15d-PGJ_2 on loss of cell viability, morphology, and the cleavage of the PARP protein. 15d-PGJ_2 also induces apoptosis of endothelial tubular networks in three-dimensional gels, suggesting that *in vivo* anti-tumor activity of PGJ may not only be because of an effect on the tumor cells themselves but may also be because of an anti-angiogenic affect on tumor capillaries. Apoptosis was induced by 15d-PGJ_2 in the nonimmortalized endothelial cell strain HUVEC, the immortalized human endothelial cell line ECV-304, and in microvascular endothelial cells BMEC-b, a consistent effect between different species and endothelial subtypes.

Recent studies have shown that nuclear-acting prostanoids including 15d-PGJ_2 are potent activators of the PPARγ receptor isoform (1, 2). Indeed various eicosanoids were shown to be activators of PPAR isoforms (4, 8, 9). We present evidence that transcripts for PPARα, -δ, and -γ are present in endothelial cell poly(A)$^+$ mRNA preparations, and the proteins were detected by immunofluorescence. These results are consistent with the recent finding using reverse transcription-polymerase chain reaction, that PPARα is expressed in human endothelial cells (25). Northern blot analysis using total RNA preparations did not yield a detectable signal for any PPAR isoform, suggesting that endothelial cells have low expression of PPARs. Although we could not detect PPAR isoforms in ECV cells using immunoblot methodology, a highly sensitive immunofluorescence method was capable of detecting specific PPAR isoforms. In unstimulated cells, PPAR isotypes were localized predominantly in the peri-nuclear region and the cytoplasm. Treatment of endothelial cells with 15d-PGJ_2 caused all three PPAR receptor isoforms to become nuclear-localized. Often visible also in stimulated cells was the appearance of cytoplasmic vacuolar compartments, which may be indicative of cytoplasmic breakdown. These results differ from differentiated macrophages, where PPARα was found predominantly in the cytoplasm, but PPARγ was found predominantly the nucleus (26). This ability of 15d-PGJ_2 to induce nuclear localization of all three isoforms may be an initial step in PPAR activation. These data agree with the data in the literature that, although 15d-PGJ_2 is considered primarily a PPARγ activator, it can activate all PPAR receptors (9, 27, and 28). Using a transient reporter gene assay utilizing the rat acyl-CoA oxidase PPRE linked to a

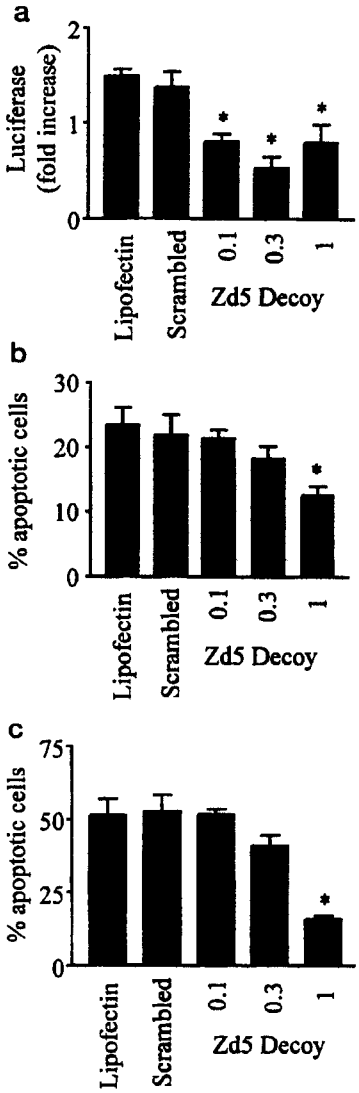

FIG. 7. **Effect of PPRE decoy oligonucleotide on endothelial cell transcriptional responses and apoptosis.** *a* shows the inhibition of PPRE activation by administration of a decoy oligonucleotide (Zd5) to a consensus PPRE sequence. Decoy (0.1–1 μM) or scrambled oligonucleotides (1 μM) together with pACO.gLuc were transfected to HEK293 cells (4 h), after which time fresh medium was added to recover the cells for 20 h. Cells were incubated with serum-free medium and decoy (0.1–1 μM) or scrambled oligonucleotides (1 μM) for a further 4 h. At that time 10 μM 15d-PGJ_2 was added for 20 more h in serum-free medium, and luciferase activity normalized to β-galactosidase was quantitated. Reporter gene activity is represented as fold activation over control levels. The absolute values of normalized luciferase activity in HEK293 cells under control conditions are 415 ± 39 (n = 3) relative light units/units of β-galactosidase activity. These data represent the mean ±S.E. of four separate experiments. * denotes significance $p <$ 0.05 (unpaired t test) between scrambled transfected cells and Zd5 decoy transfected cells. *b* shows the inhibition of 10 μM 15d-PGJ_2-induced endothelial cell apoptosis (% apoptotic nuclei as determined by Hoechst staining) by addition of the decoy but not the scrambled oligonucleotide Zd5 (0.1–1 μM) to a consensus PPRE. These results represent the mean ±S.E. % apoptotic cells from 4 separate experiments, each experimental n being the mean of 4–5 random (×100) fields. * denotes significance $p < 0.05$ (one way ANOVA) between 15d-PGJ_2 and decoy or scrambled oligonucleotide-treated cells. *c* shows the inhibition of ciglitizone-induced endothelial cell apoptosis (% apoptotic nuclei as determined by Hoechst staining) by the addition of the decoy but not the scrambled oligonucleotide Zd5 (0.1–1 μM) to a consensus PPRE. Ciglitizone (30 μM) was incubated with the ECV-304 cells in the absence of serum. These results represent the mean ±S.E. % apoptotic cells from 3 separate experiments, each experimental n being the mean of 4 random (×100) fields. * denotes significance $p < 0.05$ (one way ANOVA) between ciglitizone-treated and decoy or scrambled oligonucleotide-treated cells.

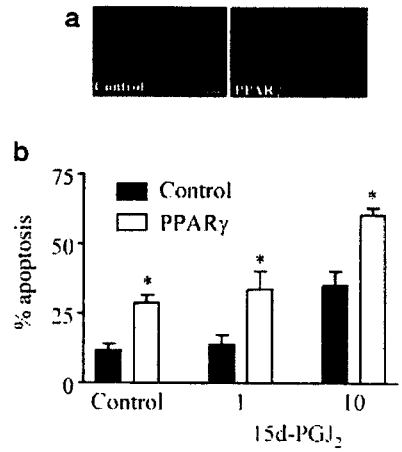

FIG. 8. **Effects of overexpression of PPARγ receptor on endothelial cell apoptosis.** *a*, ECV-304 cells were transfected with either vector (pCDNA) (*Control*) or pCMX-PPARγ in the presence of GFP expression vector (pEGFPN-1). At 24 h post-transfection, cells were treated with indicated doses of 15d-PGJ_2 for a further 24 h, and cells were observed for apoptotic morphology. *a* shows representative pictures of GFP expressing healthy flattened ECV cells (*Control*) and the rounded cell morphology observed in cells transiently transfected with pCMX-mPPARγ. The *scale bar* represents 10 μm. *b* shows % rounded ECV cells transfected with pEGFPN-1 (*Control*) or pCMX-PPARγ. These experiments represent the mean ±S.E. from 3 separate ×200 magnification fields, each from 4 separate transfections. * denotes significance $p < 0.05$ (one unpaired t test) between control and PPARγ-transfected cells.

luciferase reporter (16), we show that 15d-PGJ_2 and ciglitizone dose-dependently stimulate endogenous PPAR receptors. These data suggest that PPAR receptors are present and are functional in vascular endothelial cells.

We next provided evidence that the activation of PPARγ is a critical event in 15d-PGJ_2-induced endothelial cell death. First, ciglitizone induced PPAR transcriptional activation and endothelial cell death. Both effects were maximal in serum-free medium, probably because of either the inactivation of ciglitizone by serum factors or because of the survival-promoting actions of serum-borne factors. Because ciglitizone is a selective indicia of PPARγ, these data provide a pharmacological evidence that PPARγ is critical for endothelial cell apoptosis. Second, the double-stranded PPRE decoy, but not the scrambled counterpart, inhibited the PPAR transcriptional responses and 15d-PGJ_2-induced apoptosis. Third, in experiments where PPARγ was overexpressed, cell viability was concomitantly reduced. These data strongly suggest that 15d-PGJ_2 activates the PPARγ pathway in endothelial cells, and such activation is required, at least in part, for the induction of endothelial cell apoptosis.

The regulation of PPAR receptor activation is extremely complex, involving heterodimerization with retinoid X receptors, the presence of different co-activators/repressors, and the binding to different PPREs (7). The exogenous activation of retinoid X receptors does not appear to be required for PPAR

pathway activation. However, the presence of other nuclear binding partners for retinoid X receptors, different co-activators, or repressors for the individual receptors and the selectivity of different receptors for the PPREs involved in this apoptotic response is not known. Differences in these pathways may occur between cell types and reflect the responses of a particular ligand such as 15d-PGJ_2 to activate apoptotic pathways through the different PPRE-containing genes. Nevertheless, 15d-PGJ_2 activation of PPARγ pathway appears to be critical for endothelial cell apoptosis.

The up-regulation of COX-1 (29, 30) or COX-2 (30) in the proximity of endothelial cells may regulate angiogenesis, a critical event in tumor formation and chronic inflammatory diseases. Although, these may not be due exclusively to the products of the cyclooxygenase (as opposed to the peroxidase activity) of COX (29), PG synthesis, especially prostacyclin, and PGE_2, a known angiogenic mediator, are greatly elevated. The pattern of release of COX metabolites is governed by the presence of secondary metabolizing enzymes (31). It is therefore possible that a novel specific anti-angiogenic therapy may be utilized by targeting PGD synthase to the angiogenic site. The elevated endogenous COX activity would divert prostanoid production to PGD_2, causing an autocrine apoptosis of the capillary network. Nonetheless, the use of PPAR ligands as antitumor, anti-angiogenic therapies, may well have considerable potential for novel therapeutic intervention. Indeed, recently PPARγ ligands have been shown to inhibit tumor cell growth *in vitro* and *in vivo* (32). Moreover, endothelial damage or dysfunction is considered one of the primary causes of large vessel disease. Oxidized low density lipoproteins and associated lipid components (HODEs, hydroxyoctadecadienoic acids) were recently shown to be PPARγ ligands involved in monocyte/macrophage foam cell formation in atherosclerotic lesions (33, 34). Oxidized low density lipoprotein also causes endothelial cell apoptosis, in part via caspase activation and the generation of superoxides (35). Similarly, PGD_2 synthesis is elevated in human coronary artery disease via lipocalin-type prostaglandin D synthase (36). It is conceivable that oxidized low density lipoprotein or indeed PGD_2, through the conversion to 15d-PGJ_2, may induce endothelial cell death or dysfunction in the atherosclerotic lesion. Interestingly, activation of the PPARγ (also PPARα) pathway in differentiated macrophages was also shown to lead to their apoptosis (36).

In conclusion, our data demonstrate that (i) PPAR isotypes are expressed in endothelial cells, (ii) activation of these receptors by 15d-PGJ_2 results in nuclear localization and transcriptional responses, and (iii) PPAR signaling in endothelial cells is a critical event in 15d-PGJ_2-induced apoptosis. Modulation of this pathway may lead to important therapeutic interventions in the diverse pathological conditions where endothelial cells play such critical roles.

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