Endothelial Cell Apoptosis Induced by the Peroxisome Proliferator-activated Receptor (PPAR) Ligand 15-Deoxy-Δ12,14-prostaglandin J2*

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15-Deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) is a bioactive prostanoid produced by dehydration and isomerization of PGD2, 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-PGJ2 is a potent inducer of caspase-mediated endothelial cell apoptosis. PPARα, -δ, and -γ were expressed by endothelial cells, which, when treated with 15d-PGJ2, 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-PGJ2 was inhibited by treatment of cells with an oligonucleotide decoy to a consensus PPAR response element sequence. PPARγ translocation into the nucleus, and an increase in PPAR response element-driven reporter gene expression. In this report, we show that 15d-PGJ2 is a potent inducer of caspase-mediated endothelial cell apoptosis. PPARα, -δ, and -γ were expressed by endothelial cells, which, when treated with 15d-PGJ2, 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-PGJ2 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-PGJ2 treatment. We conclude that 15d-PGJ2 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.

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The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PG, prostaglandin; 15d-PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; HUVEC, human umbilical vein endothelial cells; BMEC-b, bovine brain microvascular endothelial cells; MUGE-b, human umbilical vein endothelial cells; PPRE, PPAR response element; COX, cyclooxygenase; ZVAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp(OCH3)-CH2F; ACO, acyl-CoA oxidase; PARP, poly(ADP-ribose) polymerase; GFP, green fluorescent protein; PBS, 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.

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 monocoyte/macrophages inhibits its 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 anti-diabetic thiazolidinediones, and a number of eicosanoids, including 5,8,11,14-eicosatetraynoic acid and the prostanoids PGA1, PGJ2, PGH2, and PGD2. Interestingly, the PGJ2 dehydroxy product 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) 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 PGH2 (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 appears to be associated with nuclear localization (13). Whether 15d-PGJ2 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-PGJ2 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-PGJ2 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-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. Ciglitizone, WY-14643, and anti-poly(ADP-ribose) polymerase (PARP) antibody was from Biomer. [3H]DCTP was from NEN Life Science Products. Benzoyloxycarbonyl-Val-Ala-Asp(OCH3)-CH2F (ZVAD-fmk) was from Calbiochem. Immortalized human endothelial cells (ECV-304) were from ATCC, and human umbilical vein endothelial cells (HUVVEC) 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 system was from Stratagene. 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.

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

Cell Culture—HUVEC and ECV cells were cultured as described previously (18). BMEC-b cells 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. MTT was detected using a monoclonal anti-PARP and secondary goat-anti-mouse peroxidase-conjugated antibody (Cappel) using the ECL (Amer sham) system. 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, Burlington, VT) 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% urea, 10% glycerol, 2% SDS, 0.0012% bromophenol blue, and 0.01% SDS-m-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 (Amer sham 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-PGJ2, and cell death was assessed morphologically.

Northern Blot Analysis for PPAR Isoforms—Poly-(A)+ RNA was prepared from ECV using Fastrack 2.0 RNA isolation kit, according to the manufacturer’s protocol. 5 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 human embryonic kidney (HEK) 293 cell line. HEK293 contained PGD 2 and 15d-PGJ 2 significantly induced cell viability. This effect was partially reversed by 10% FBS, and 15d-PGJ2 was shown to be 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-PGJ2 is a dehydration product of PGD 2 and 15d-PGJ2 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-PGJ2 was conducted on HUVEC and BMEC-b, two non-immortalized endothelial cell strains, as well as the ECV-304. As shown in Fig. 1b, 15d-PGJ2 induced the reduction of endothelial cell viability with an IC50 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-PGJ2 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-PGJ2, 33 ± 5% of cells possessed apoptotic nuclei. Contrast, similar treatment with vehicle or the inactive prostaglandin PGE2 resulted in 1 ± 0.5% and 2 ± 0.5% of apoptotic nuclei.

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respectively. In addition, 15d-PGJ₂ potent ly induced apoptosis to differentiating HUVEC grown on the three-dimensional matrix Matrigel (Fig. 2). These data suggest that 15d-PGJ₂ reduced endothelial cell viability by inducing apoptosis.

To further characterize the endothelial cell apoptosis induced by 15d-PGJ₂, ECV-304 cells were treated with the inhibitor ZVAD-fmk, which potent ly inhibits the caspase enzymes, the common executors of cell death (24). As shown in Fig. 3a, 10–30 μM ZVAD-fmk reversed the ability of 10 μM 15d-PGJ₂ to induce endothelial cell apoptosis. Caspase activation results in the specific cleavage of cellular substrates such as PARP (24). As shown in Fig. 3b, 15d-PGJ₂ 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-PGJ₂ 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. 4a, 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 isoforms in the indirect immunofluorescence assay. As shown in Fig. 4b, 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-PGJ₂ for 20 h resulted in the nuclear translocation of all three receptor isoforms. Treatment with other prostanoids PGE₂ or carbaprost did not alter the subcellular localization of any of the PPAR isoforms (data not shown). These data suggest that 15d-PGJ₂ interacts with all three PPAR isoforms in endothelial cells.

PPAR Regulation of Endothelial Cell Apoptosis—We next determined if activation of PPAR receptors is responsible for the 15d-PGJ₂-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

![Image](https://example.com/image1.png)

**Fig. 1.** Effect of 15d-PGJ₂ 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 differences from 5 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-PGJ₂ (20 h; 0–10 μM). Data represent the mean ±S.E. for n = 9–14 replications from 5 separate experiments.

![Image](https://example.com/image2.png)

**Fig. 2.** Endothelial cell apoptosis induced by 15d-PGJ₂. Differential interference contrast (Nomarski) microscopic image (a) and corresponding Hoechst staining of nuclei (b) is shown of ECV-304 cells treated or not with 10 μM 15d-PGJ₂ (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-PGJ₂ 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-PGJ₂ (20 h; 10 μM) of HUVEC plated on Matrigel (×100 magnification).
the absence of serum. These data provide pharmacological evidence that activation of the PPAR \( g \) 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 \( \beta \)-galactosidase activity in HEK293 cells, whereas we obtained only up to 100 relative light units/unit of \( \beta \)-galactosidase activity of luciferase activity in ECV-304 cells. As shown in Fig. 7a, Lipofectin-mediated loading of HEK293 cells with the PPRE decoy oligonucleotide (Zd5) attenuated 10 \( \mu \)M 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 HEK293 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. 7b). These data suggest that PPAR-dependent transcriptional responses are required, at least in part, for the

FIG. 3. Endothelial cell apoptosis induced by 15d-PGJ\(_2\) is mediated via caspase activation. a shows the inhibition of 15d-PGJ\(_2\) (10 \( \mu \)M)-induced cell viability in ECV by increasing concentrations of the selective caspase-inhibitor ZVAD-fmk (1–100 \( \mu \)M). 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 \( \mu \)M; 2nd lane) causes the characteristic cleavage of PARP, leaving a detectable fragment at approximately 83 kDa. When ZVAD-fmk (30 \( \mu \)M; 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 PPAR\( \alpha \), PPAR\( \delta \), and PPAR\( \gamma \) in ECV-304. Poly(A)\(^+\) RNA (3 \( \mu \)g/lane) was separated on a 1% formaldehyde-agarose gel, transferred to a nylon membrane, and probed with the radiolabeled open reading frames of human 6/29PPAR\( \alpha \) or murine PPAR\( \delta \) or PPAR\( \gamma \) (see “Experimental Procedures”). GADPH, glyceraldehyde-3-phosphate dehydrogenase. b shows immunofluorescence micrographs of PPAR\( \alpha \), PPAR\( \delta \), and PPAR\( \gamma \), in ECV-304 under control culture conditions or treated with 15d-PGJ\(_2\) (20 h; 10 \( \mu \)M). 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 \( \mu \)m.

15d-PGJ\(_2\)-induced endothelial cell apoptosis. Similarly, the PPRE decoy oligonucleotide but not the scrambled counterpart inhibited the cigitizone-induced apoptosis of ECV-304 cells (Fig. 7c), suggesting that PPAR\( \gamma \)-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 PPAR\( \gamma \) 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. 8a, ECV-304 cells transfected with vector and GFP plasmids expressed GFP predominantly in flattened, healthy cells. In contrast, cells trans-
that PPARγ transfection resulted in ~3.5-fold increase in apoptotic cells in ECV-304 cells. This was further enhanced by treatment with 15d-PGJ2 (Fig. 8b). These data indicate that 15d-PGJ2 activation of PPARγ induces endothelial apoptosis.

DISCUSSION

In this report, we demonstrate that 15d-PGJ2, PGD2, and PGA1 induced endothelial apoptosis. Prostanoids that act via plasma membrane receptors, such as PGE2, carbaprost, and U46619 did not induce endothelial cell apoptosis. Removal of serum increased the potency of all these agonists, with the exception of PGD2, suggesting that mitogens and survival factors present in serum counteract cell death. The lack of increased potency observed with PGD2 in serum-free medium may be because of the requirement for dehydration and isomerization of PGD2 to 15d-PGJ2 by the enzymes and carriers present in serum. Δ15d-PGJ2, 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-PGJ2-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-PGJ2 on cell viability, morphology, and the cleavage of the PARP protein. 15d-PGJ2 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-PGJ2 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-PGJ2 are potent activators of the PPARγ receptor isofrom (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 PPARs 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 isoforms were localized predominantly in the peri-nuclear region and the cytoplasm. Treatment of endothelial cells with 15d-PGJ2 caused all three PPAR receptors (9, 27, and 28). We present evidence that nuclear-acting prostanoids have distinct effects on endothelial cell behavior from their counterparts, which signal via plasma membrane receptors.

Fig. 5. Activation of PPAR mediated transcription by 15d-PGJ2. ECV-304 cells were transiently transfected 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-PGJ2 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-PGJ2.

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.
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-PGJ2 was added for 20 more h in serum-free medium, and luciferase activity normalized to 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 % rounded morphology observed in cells transiently transfected with pCMX-PPARγ. The scale bar represents 10 μm. b shows % rounded morphology observed in cells transfected with pEGFPN-1 (Control) or pCMX-PPARγ. The scale bar represents 10 μm. * denotes significance p < 0.05 (one way ANOVA) between control and PPARγ-transfected 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-PGJ2 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 % 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 nuclei from 4 separate experiments, each experimental n being the mean of 200 magnification fields. * denotes significance p < 0.05 (one unpaired t test) between control and PPARγ-expressed cells.

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-responsive induction of endothelial cell death.

We next provided evidence that the activation of PPARγ is a critical event in 15d-PGJ2-induced endothelial cell death. First, ciglitizone induced PPARγ 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 inducer 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 PPARγ transcriptional responses and 15d-PGJ2-induced apoptosis. Third, in experiments where PPARγ was overexpressed, cell viability was concomitantly reduced. These data strongly suggest that 15d-PGJ2 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.
PPAR-induced Endothelial Cell Apoptosis

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-PGJ2 to activate apoptotic pathways through the different PPRE-containing genes. Nevertheless, 15d-PGJ2 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 PGE2, 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 prostanooid production to PGD2, causing an autocrine apoptosis of the capillary network. Nonetheless, the use of PPAR ligands as anti-tumor, 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 monocye/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, PGD2 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 PGD2, through the conversion to 15d-PGJ2, 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-PGJ2 results in nuclear localization and transcriptional responses, and (iii) PPAR signaling in endothelial cells is a critical event in 15d-PGJ2-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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