Peroxisome proliferator-activated Receptor $\gamma$ Ligands Are Potent Inhibitors of Angiogenesis in Vitro and in Vivo*

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Xiaohua Xin, Suya Yang, Joe Kowalski, and Mary E. Gerritsen‡

From the Department of Cardiovascular Research, Genentech, Inc., South San Francisco, California 94080

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‡ To whom correspondence should be addressed: Dept. of Cardiovascular Research, MS 42, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080. Tel.: 650-225-6870; Fax: 650-225-6327; E-mail: meg@gene.com.

Angiogenesis, the formation of new blood vessels, is not only critically involved in a number of normal physiological processes such as embryonic development, ovulation, and wound healing, but is also a critical step in many pathological conditions including solid tumor growth, diabetic retinopathy, and age-related macular degeneration (1). The complex steps involved in new vessel formation have been well characterized in recent years and involve the degradation of the basement membrane by cellular proteases, the penetration and migration of endothelial cells into the extracellular matrix, endothelial proliferation, and the resultant formation of patent, interconnected vascular networks (2, 3). It is now well established that the progression of solid tumor growth and metastasis is dependent on angiogenesis, with the resultant global effort by many laboratories to identify new angiostatic therapies for the treatment of cancer.

Peroxisome proliferator-activated receptors (PPARs)¹ are members of the steroid receptor superfamily and, as such, are ligand-activated transcription factors (4, 5). Three subtypes of PPAR, $\alpha$, $\beta$ (also known as $\delta$ or NUCI), and $\gamma$ have been identified and cloned. Like other members of this superfamily, PPARs mediate transcriptional regulation by their central DNA binding domain that recognizes response elements in the promoters of specific target genes (6, 7). Activation of PPAR$\gamma$ has been linked to adipocyte differentiation and regulation of glucose homeostasis in rodents and humans (8). Recent evidence also shows that the natural receptor ligand for PPAR$\gamma$, 15d-PGJ$_2$ (9, 10), and synthetic antidiabetic thiazolidinedione drugs (e.g. BRL49653 and ciglitzone), inhibit macrophage and monocyte activation (11, 12) and suppress tumor cell growth (14-16). PPAR$\gamma$ can heterodimerize with at least one other member of the steroid receptor superfamily, retinoid acid receptor (RXR) (6, 17). Specific ligands for the PPAR$\gamma$ and RXR have been shown to act synergistically to induce terminal differentiation of human liposarcoma cells (18) in vitro and to enhance insulin sensitivity in diabetic animals (19). Activation of PPAR$\alpha$ has been demonstrated to mediate lipid catabolism. Treatment of animals with PPAR$\alpha$ activators results in the proliferation of peroxisomes and the induction of hepatic genes involved in the $\beta$-oxidation of fatty acids (20). Mice that lack functional PPAR$\alpha$ accumulate lipid droplets in their livers (21). Several selective PPAR$\alpha$ activators have been described including WW 14643 and clofibrate (22). Inhibition of human aortic smooth muscle cell activation by PPAR$\alpha$ ligands has also been reported recently (13). Although PPAR$\beta$ has been found to be ubiquitously expressed, specific functions for this receptor are not known at the present time.

In this study we report on the potent and novel inhibitory activity of PPAR$\gamma$ ligands on HUVEC differentiation into tube-like structures and proliferation in vitro, and the inhibition of VEGF elicited angiogenesis in vivo. These studies demonstrate that PPAR$\gamma$ is an important molecular target for the development of small-molecule inhibitors of angiogenesis, which may be useful therapeutic agents in the treatment of cancer and other vasculoproliferative disorders.

EXPERIMENTAL PROCEDURES

Materials—PGA$_1$, PGA$_2$, PGB$_1$, PGB$_2$, PGD$_1$, PGD$_2$, PGE$_1$, PGE$_2$, ciglitzone, WW 14643, clofibrate, and 15d-PGJ$_2$ were from Cayman

¹ The abbreviations used are: PPARs, peroxisome proliferator-activated receptors; HUVEC, human umbilical vein endothelial cells; 15d-PGJ$_2$, 15-deoxy-$\Delta_{12,14}$-prostaglandin J$_2$; RXR, retinoid acid receptor; VEGF, vascular endothelial growth factor; PMA, phorbol myristate acetate; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BrdUrd, 5'-bromo-2-deoxyuridine; FBS, fetal bovine serum; bFGF, basic fibroblast growth factor; ITS, insulin, transferrin, and selenium A; uPA, urokinase-type plasminogen activator.
Chemical (Ann Arbor, MI). BRL49653 was kindly synthesized at Genentech by Dr. Jim Marsters (South San Francisco, CA). HUVEC were purchased from Clonetics (San Diego, CA) and maintained in Clonetics EGM medium supplemented with 10% fetal bovine serum (FBS) and endothelial cell growth supplements provided by the company. Type I rat tail collagen was purchased from Collaborative Research (Becton Dickinson Labware, Bedford, MA). Recombinant VEGF was from Genentech. 10× medium 199 (M199) and PMA were purchased from Sigma. FBS was from Hyclone (Logan, Utah). ITS (insulin, transferrin, and selenium A), trypsin, and versene were from Life Technologies, Inc. The mouse monoclonal antibody to PPARγ (E5-8, SC-7273) and anti-PPARα (N-19, SC-885) and PPARβ (C-20, SC-883) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Magnetic protein A beads (Dynabeads) were from Dynal (Lake Success, NY).

Endothelial Tube Assay—Collagen gels were formed by mixing together ice-cold gelation solution (10× M199, H2O, 0.53 mM NaHCO3, 200 mM t-glutamine, type 1 collagen, 0.1 mg NaOH, 100:27:2.5:10:750:62.5 by volume) and cells in 1× basal medium (see below) at a concentration of 3×105 cells/ml at a ratio of 4 volumes of gelation solution:1 volume of cells. After gelation at 37°C for 30 min, the gels were overlaid with 1× basal medium consisting of M199 supplemented with 1% FBS, 1× ITS, 2 mM t-glutamine, 50 μg/ml ascorbic acid, 26.5 mM NaHCO3, 100 units/ml penicillin, and 100 units/ml streptomycin supplemented with 40 ng/ml VEGF, 40 ng/ml VEGF, and 80 nM PMA. All drugs were added to the 1× basal medium at the time the gels were formed and pelleted by centrifugation. To quantitate tube formation, the number of tubes per high power (20×) field was determined at 48 h after addition of the basal medium. A tube was defined as an elongated structure comprised of one or more endothelial cells that exceeded 100 μm in length (long axis). Five independent fields separated by 100 μm optical sections were assessed for each well, and the average number of tubes/20X field determined. Cytokinesis was assessed using cell proliferation kit II from Boehringer Mannheim.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assays—Synthesis of complementary DNA (cDNA) was performed using 6 μg of total RNA extracted from human liver (positive control) or HUVEC cultured on three-dimensional collagen gels for 24 h by random primer according to the manufacturer’s instructions (Stratagene Co., San Diego, CA). Subsequent amplifications of the partial cDNA encoding PPARα, β, and γ were performed using different amounts of reverse transcribed mixture (4 μl for liver α, β, and γ and 5 μl for HUVEC α and β and 6 μl for HUVEC γ) as templates with specific oligonucleotide primers as follows: PPARα sense 5′-CCAGATTTAGGAACGGTCGTC-3′ and antisense 5′-AAATGTTCAATGAGCTTGCTG-3′; PPARβ sense 5′-AATCTGACAGTTGGGCGCTG-3′ and antisense 5′-TTCTGTTGATGCA-3′; PPARγ sense 5′-5′ and antisense 5′-CCATTAGAGACATCCCCAC-3′. The expected sizes of PCR products for PPARα, β, and γ were 492, 484, and 474 base pairs, respectively. Negative controls for reverse transcription and PCR amplifications were included. The PCR mixtures were subjected to 35 cycles of amplification by denaturation (30 s at 94°C), hybridization (55 s at 55°C), and elongation (55 s at 72°C). The PCR products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide.

Real Time RT-PCR (Taquin) Assay—This technique has been used to quantitatively monitor mRNA expression and has been described in detail previously (20, 27). Total RNA was extracted from HUVEC cultured on three-dimensional collagen gels or on collagen-coated flasks for various times in 1× basal medium consisting of M199 supplemented with 1% FBS, 1× ITS, 2 mM t-glutamine, 50 μg/ml ascorbic acid, 26.5 mM NaHCO3, 100 units/ml penicillin, and 100 units/ml streptomycin supplemented with 40 ng/ml VEGF, 40 ng/ml VEGF, and 80 nM PMA. A gene-specific PCR oligonucleotide primer pair and an oligonucleotide probe labeled with a reporter fluorescent dye at the 5′ end were designed using the Oligo 4.0 software (National Bioscience, Plymouth, MN) following guidelines suggested in the Taqman Model 7700 sequence detection instrument manual (PE Applied Biosystem). The primers and probes used were as follows: human PPARα gene forward primer 5′-GGACCTGTCTTCCGG-3′, reverse primer 5′-CCATTAGAGACATCCCCAC-3′; probe 5′-CACCGGACGACGGACGCACGCATG-3′; human PPARβ gene forward primer 5′-TGAGTTGAGGACGACGG-3′, reverse primer 5′-CCATTAGAGACATCCCCAC-3′, probe 5′-ACAGAGTTGAGGACGACGGACGCATG-3′ and probe 5′-CCATTAGAGACATCCCCAC-3′; human PPARγ gene forward primer 5′-GGACCTGTCTTCCGG-3′, reverse primer 5′-CCATTAGAGACATCCCCAC-3′, and probe 5′-TGACGATCTGACATCCCGATATG-3′; human Flk/KDR gene forward primer 5′-CACCGGACGACGGACGCACGCATG-3′, reverse primer 5′-CCATTAGAGACATCCCCAC-3′, and probe 5′-TGACGATCTGACATCCCGATATG-3′; human Flt-1 gene forward primer 5′-ACCAGATAGGTCCCTGTTGG-3′, reverse primer 5′-CCATTAGAGACATCCCCAC-3′, and probe 5′-CCATTAGAGACATCCCCAC-3′; human urokinase-type plasminogen activator (uPA) gene forward primer 5′-CCATTAGAGACATCCCCAC-3′, reverse primer 5′-CCATTAGAGACATCCCCAC-3′, and probe 5′-CCATTAGAGACATCCCCAC-3′; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene forward primer 5′-GAAGGGGATTTC-3′, reverse primer 5′-GAAGGGGATTTC-3′, and probe 5′-GAAGGGGATTTC-3′.

To determine differences between groups. A p value <0.05 was accepted as significant.

RESULTS AND DISCUSSION

Endothelial Tube Formation in Three-dimensional Collagen Gels—In this study we used a model of in vitro angiogenesis
significantly different from vehicle treated control (Bonferroni modified t

deleted). Inhibitory effects of 15d-PGJ$_2$ on tube formation. HUVEC were grown in three-dimensional collagen gels in the presence of VEGF, bFGF, and PMA at 4, 24 or 48 h of incubation with 15d-PGJ$_2$ or vehicle in HUVEC cultured in three-dimensional collagen gels or on collagen-coated surfaces (data not shown). The expression of PPAR$\gamma$ protein in HUVEC was confirmed by Western blot analysis (Fig. 2C), and PPAR$\gamma$ protein levels were not significantly altered by the growth factor mixture when HUVEC were cultured on collagen-coated surfaces (Fig. 2C). Using commercially available antibodies, we were unable to demonstrate protein expression of PPAR$\alpha$ and -$\beta$ in HUVEC (data not shown). The expression of PPAR$\alpha$ mRNA in HUVEC has been previously reported (28); however, this study demonstrates for the first time the expression of PPAR$\gamma$ mRNA and protein and PPAR$\gamma$ mRNA by HUVEC.

**PPAR$\gamma$ Mediates the Inhibitory Effect on HUVEC Tube Formation and Proliferation**—To determine which PPAR isoform(s) mediated the inhibition of HUVEC tube formation, the effect of specific PPAR ligands was assessed. As shown in Fig. 3A, treatment of HUVEC with the specific PPAR ligands, 15d-PGJ$_2$, BRL49653, and ciglitzone, dose dependently inhibited tube formation with half-maximal inhibition concentrations (IC$_{50}$) of 2.8, 6.2, and 2.7 $\mu$m, respectively. In contrast, the selective agonists for PPAR$\alpha$ (PW 14463 and clofibric acid) and PPAR$\beta$ (erucic acid) (29) did not significantly inhibit tube formation (Fig. 3B). These observations demonstrated that activation of PPAR$\gamma$ results in inhibition of endothelial cell differentiation into tube-like structures in vitro. We further investigated whether PPAR$\gamma$ ligands also regulate the proliferative response of HUVEC to growth factors. BrdUrd incorporation assays were performed to detect proliferation of HUVEC cultured on type I collagen-coated surface in medium containing VEGF, bFGF, and PMA in the presence of vehicle or PPAR$\gamma$ ligands. As shown in Fig. 3C, 15d-PGJ$_2$ and BRL49653 dose

![Fig. 1. Effect of eicosanoid derivatives on HUVEC tube formation in three-dimensional collagen gels. Panel A, representative photomicrograph documenting the formation of network of tube-like structures containing lumens in HUVEC grown in three-dimensional collagen gels for 48 h in the presence of 40 ng/ml VEGF, 40 ng/ml bFGF, and 80 nM PMA; panel B, inhibitory effects of 15d-PGJ$_2$ on tube formation. HUVEC were grown in three-dimensional collagen gels in the presence of VEGF, bFGF, and PMA as well as 10 $\mu$m 15d-PGJ$_2$ for 48 h. Note that most cells in the gel remain rounded and devoid of vacuoles or lumen-like structures. Parallel experiments using the vehicle and growth factors were similar to panel A. Panel C, effects of different eicosanoids on tube formation of HUVEC in three-dimensional collagen gels. Drugs were tested at 1, 10, and 100 $\mu$m. Data are expressed as percent inhibition compared with controls incubated with growth factors and the vehicle and are shown as mean $\pm$ S.E. of $n \geq 3$, *p<0.05 significantly different from vehicle treated control (Bonferroni modified Student’s t test for multiple comparisons).

initially described by Davis et al. (23). In this model, endothelial cells suspended in three-dimensional collagen lattices comprised of type I collagen in the presence of VEGF, bFGF, and PMA undergo rapid morphogenesis. Numerous vacuoles are observed in the majority of endothelial cells within 4 h; the formation of tube-like structures can be observed at 24 h, and the majority of cells in the gels remained rounded and devoid of vacuoles or lumen-like structures. Vehicle-treated control groups did not show any significant inhibitory effect (data not shown). We extended our observations to a variety of metabolites of 20:4 and 20:3 fatty acids for their ability to inhibit tube formation in three-dimensional collagen gels (Fig. 1C). PGJ$_2$ and PGB$_2$ and their derivatives/analogs demonstrated dose-dependent inhibition of tube formation over the concentration range of 1 to 100 $\mu$m. PGB$_1$ and PGB$_2$ possessed only weak inhibitory effects. In contrast, two prostanoids known to activate cell surface G-protein-coupled receptors, namely PGE$_1$ and PGE$_2$, had no significant effect over this concentration range. Among the tested drugs, 15d-PGJ$_2$ was the most potent inhibitor of HUVEC tube formation. Data from parallel cytotoxicity assays indicated that none of the tested prostanoids exhibited significant toxicity over the time course (48 h) of the experiment (data not shown). Previous studies (24, 25) have demonstrated that PGD$_2$ can spontaneously convert to PGJ$_2$, and in the presence of serum or albumin, to 15d-PGJ$_2$, a natural selective ligand for PPAR$\gamma$. Therefore, activation of PPAR$\gamma$ may modulate endothelial morphogenesis.

**PPAR$\gamma$ Is Expressed in HUVEC—RT-PCR was used to demonstrate the expression of all three PPAR ($\alpha$, $\beta$, and $\gamma$) mRNAs in HUVEC cultured in the three-dimensional collagen gels. In two independent experiments conducted with different HUVEC populations derived from different donors, the mRNA expression of three isoforms of PPAR were demonstrated (Fig. 2A). By using real time quantitative RT-PCR (Taqman) (26, 27), the relative mRNA expression levels of each isoform were determined to be PPAR$\beta$ > PPAR$\alpha$ > PPAR$\gamma$ (Fig. 2B). Preliminary observations also suggested that expression of PPAR$\gamma$ mRNA was not altered in medium containing VEGF, bFGF and PMA at 4, 24 or 48 h of incubation with 15d-PGJ$_2$ or vehicle in HUVEC cultured in three-dimensional collagen gels or on collagen-coated surfaces (data not shown). The expression of PPAR$\gamma$ protein in HUVEC was confirmed by Western blot analysis (Fig. 2C), and PPAR$\gamma$ protein levels were not significantly altered by the growth factor mixture when HUVEC were cultured on collagen-coated surfaces (Fig. 2C). Using commercially available antibodies, we were unable to demonstrate protein expression of PPAR$\alpha$ and -$\beta$ in HUVEC (data not shown). The expression of PPAR$\alpha$ mRNA in HUVEC has been previously reported (28); however, this study demonstrates for the first time the expression of PPAR$\gamma$ mRNA and protein and PPAR$\gamma$ mRNA by HUVEC.

**Effects of Eicosanoids on Tube Formation by HUVEC**—It has recently been shown that a number of eicosanoid metabolites can serve as PPAR ligands (8–10, 22), and activation of PPARs has been linked to numerous functions in a number of cell types (8, 11–16). To determine whether PPARs could affect HUVEC differentiation, we investigated the effect of 15d-PGJ$_2$, a naturally occurring endogenous ligand of PPAR$\gamma$ on HUVEC tube formation. HUVEC were grown in three-dimensional collagen gels in medium containing VEGF, bFGF and PMA with addi-
dependently inhibited HUVEC proliferation with IC$\text{_{50}}$ of 2.4 and 15.7 $\mu$M, respectively. In contrast, PGE1 and PGE2 did not affect HUVEC proliferation (data not shown). These results demonstrate that PPAR$\gamma$ ligands also repress growth factor-induced HUVEC proliferation in vitro.

15d-PGJ$_2$ Regulates Gene Expression Events Associated with Angiogenesis—Flk/KDR and Flt-1 are two structurally related endothelial cell tyrosine kinase receptors for VEGF. The importance of these two receptors during angiogenesis has been clearly demonstrated by the findings that KDR functions as a
transducer to signal endothelial cell proliferation and differentiation and that Flt-1 is a critical survival factor involved in endothelial cell morphogenesis (30–32). We examined whether activation of PPARγ alters Flt-1 and KDR gene expression using real time quantitative RT-PCR. Both KDR and Flt-1 mRNA were up-regulated by the mixture of growth factors in...
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15d-PGJ2 demonstrates anti-angiogenic activity in vivo—To determine the potential anti-angiogenic effect of PPARγ ligand 15d-PGJ2 in vivo, hydron pellets containing 200 μg of recombinant VEGF with or without 10 μg of 15d-PGJ2 were implanted into the corneas of Sprague-Dawley rats. Summary data from the experiment show that pellets containing the combination of 15d-PGJ2 and VEGF demonstrated a significant reduction in vessel area compared with the VEGF only positive controls (Fig. 5, A and B). Results from this experiment were repeated in a second independent experiment. These in vivo data strongly support our in vitro observations that activation of PPARγ results in marked inhibition of angiogenesis.

Recent evidence has demonstrated that PPARγ is highly expressed in most colon cancer cells and breast cancer cells (14, 16). Activation of the PPARγ resulted in growth arrest and induction of differentiation of cancer cells and reduction of tumor cell growth rate, suggesting potential implications of PPARγ as a target for treatment of human cancers. In this study we have documented, for the first time, the expression of PPARγ in human endothelial cells. Activation of PPARγ with either naturally occurring ligand or synthetic selective ligands results in potent inhibition of endothelial differentiation into tube-like structures and proliferation in vitro and suppression of VEGF-induced angiogenesis in vivo. PPARγ activation also inhibits the expression of at least three important genes in the angiogenic process, the VEGF receptors Flk/KDR, Flt-1, and the protease uPA. This study thus provides a new insight into the mechanism of PPARγ ligands as inhibitors of solid tumor growth and suggests that such drugs may also provide novel means to control other angiogenic disorders.

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