

Selective Activation of PPAR α and PPAR γ Induces Neoangiogenesis through a VEGF-dependent Mechanism

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ABSTRACT

Objective: Peroxisome proliferator-activated receptors (PPARs) are therapeutic targets for fibrates and thiazolidinediones, which are commonly used to ameliorate hyperlipidemia and hyperglycemia in type 2 diabetes mellitus (T2DM). In this study, we evaluated whether activation of PPAR α and PPAR γ stimulates neoangiogenesis.

Research design and Methods: We used selective synthetic PPAR α and PPAR γ agonists and investigated their angiogenic potentials *in vitro* and *in vivo*.

Results: Activation of PPAR α and PPAR γ leads to endothelial tube formation in an endothelial/interstitial cell co-culture assay. This effect is associated with increased production of the angiogenic cytokine Vascular Endothelial Growth Factor (VEGF). Neovascularization also occurs *in vivo*, when PPAR α and PPAR γ agonists are used in the murine corneal angiogenic model. No vascular growth is detectable when PPAR α and PPAR γ agonists are respectively used in PPAR α knock-out mice and mice treated with a specific PPAR γ inhibitor, demonstrating that this angiogenic response is PPAR-mediated. PPAR α - and PPAR γ -induced angiogenesis is associated with local VEGF production and does not differ in extent and morphology from that induced by VEGF. In addition, PPAR α - and PPAR γ -induced *in vitro* and *in vivo* angiogenesis may be significantly decreased by inhibiting VEGF activity. Finally, in corneas treated with PPAR α and PPAR γ agonists, there is increased phosphorylation of eNOS and Akt.

Conclusions: These findings demonstrate that PPAR α and PPAR γ activation stimulates neoangiogenesis through a VEGF-dependent mechanism. Neoangiogenesis is a crucial pathologic event in T2DM. The ability of PPAR α and PPAR γ agonists to induce neoangiogenesis might have important implications for the clinical and therapeutic management of T2DM.

Peroxisome proliferator-activated receptors (PPARs) are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily (1). The clinical importance of PPARs originates with fibrates and thiazolidinediones (TZDs), which respectively act on PPAR α and PPAR γ and are used to ameliorate hyperlipidemia and hyperglycemia in subjects with type 2 diabetes mellitus (T2DM). Fibrates (gemfibrozil, clofibrate, fenofibrate, and bezofibrate) are drugs that effectively reduce triglycerides (TG) and free fatty acids (FFA) and increase high density lipoproteins-cholesterol (2-5). Fibrates also improve glucose tolerance in T2DM patients, although this activity might be attributable to the fact that some of these compounds also have potential PPAR γ activity (6). TZDs (such as rosiglitazone, troglitazone, pioglitazone, and ciglitazone) are insulin-sensitizing drugs and have constituted a major advance in the recent therapeutic management of T2DM (7-9). In addition to improve insulin sensitivity, TZDs have also effects on TG, FFA, and ketone body level in several animal models of T2DM. Recently, PPAR α/γ dual agonists have also been produced, hypothesizing that the simultaneous activation of these nuclear receptors might provide better glucose and lipid control than single subtype selective agents (10-13).

In recent years, there has been increasing appreciation of the fact that PPAR α and PPAR γ might be involved in the molecular mechanisms that regulate neoangiogenesis, defined as the growth of new blood vessels from pre-existing vascular networks, through the action of growth factors and cytokines that stimulate migration, proliferation, and survival of endothelial cells (ECs). Neoangiogenesis plays a dual role in T2DM. On one hand, it is involved in the pathogenesis of diabetic retinopathy and, according to some studies, contributes to

rupture of atherosclerotic plaques in coronary and carotid arteries. On the other hand, neoangiogenesis is important to promote revascularization and contrast ischemia in tissues affected by diabetic microangiopathy. Therefore, the effects of PPAR α and PPAR γ in angiogenesis merit careful evaluation. Several studies have analyzed the angiogenic properties of PPAR α and PPAR γ by using fibrates and TZDs, respectively. However, the results of these investigations have been very controversial (14-22). In this regard, it is important to point out that both fibrates and TZDs do not selectively act on PPARs, but have pleiotropic activities that occur through PPAR-independent pathways. For instance, fibrates activate other nuclear and superficial receptors (23,24) and also stimulate pathways that do not depend on PPAR α (25, 26). Similarly, TZDs act on intracellular mechanisms regulated by pERK, p38 MAP-kinase, PAI-1, MMP-2, Cyclin D1, Bcl-xL/Bcl-2, and TNF α , in a PPAR γ -independent way (27-33). In addition, TZDs are not PPAR γ -specific, since high concentrations of these compounds may also activate PPAR α and PPAR δ (34). A clear demonstration of the PPAR γ -independent properties of TZDs is provided by the fact that these molecules are able to inhibit the release of pro-inflammatory mediators in cells that lack the PPAR γ gene (35).

In the present study, we used selective PPAR α and PPAR γ synthetic agonists and tested their potential ability to stimulate neoangiogenesis in well-established *in vitro* and *in vivo* assays. We found that specific and selective activation of PPAR α and PPAR γ leads to increased production of vascular endothelial growth factor (VEGF), a prototypical angiogenic agent, and formation of endothelial tubules when ECs are co-cultured with interstitial cells. *In vivo*, PPAR α and PPAR γ synthetic agonists stimulate angiogenesis in the mouse corneal

neovascularization assay, while fibrates and TZDs are unable to induce angiogenesis in the same experimental setting. PPAR α - and PPAR γ -angiogenic process is associated with increased expression of VEGF and increased phosphorylation of eNOS and Akt. Finally, it may be inhibited by blocking VEGF activity.

MATERIALS AND METHODS

PPAR selective agonists. The WY14643 and the GW1929 compounds were used to specifically activate PPAR α and PPAR γ , respectively. GW9662 was used to inhibit PPAR γ . VEGF was used as positive control. All molecules were from Sigma.

EC migration and proliferation assays. Human umbilical vein ECs (HUVECs) and human dermal microvascular ECs (HDMVECs) (Cambrex) were cultured in EGM2. Migration of ECs was determined using a monolayer denudation assay. 80-90% confluent ECs were scraped with a 1000 μ l pipette tip. Cultures were washed three times with HBSS and incubated with EBM2/0.5% FBS. Control cultures received media alone, while experimental groups received PPAR agonists at concentrations of 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μ mol/L, 5 μ mol/L, 10, and 20 μ mol/L. VEGF was used as positive control at a concentration of 10 nmol/L. The rate of wound closure was determined by photographing cells at 3 pre-marked areas at time 0 and at 6 and 12 hours. Using a digital imaging system, the rate of cell migration was determined by calculating the difference of the wound area after 6 and 12 hours, divided by two. For cell proliferation experiments, ECs were plated into 96-well plates at 1.0×10^5 cells/well or 0.5×10^5 and allowed to attach overnight. Cells were incubated in EBM2/0.5%FBS with PPAR agonists at concentrations of 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μ mol/L, 5 μ mol/L, 10, and 20 μ mol/L. VEGF was used as positive control at a concentration of 10 nmol/L. After 24, 36, and 48 hours, the amount of proliferation was

compared by an MTT assay performed according to manufacturer's instructions (ATCC). We also evaluated whether WY14643 and GW1929 were able to increase VEGF levels in ECs, by using a commercially available ELISA kit (R&D Systems). All the experiments were performed in triplicate.

EC/interstitial cell co-culture assay. We used a commercially available assay (TCS CellWorks), in which proliferating early passage normal human ECs are co-cultured with early passage normal human interstitial cells in 24-well plates, in a specially formulated culture medium. In this assay, cells spontaneously proliferate and then enter a migratory phase, during which they move through the matrix to form, after 11 days in culture, a network of capillary-like tubules (36). This degree of spontaneous angiogenic activity was evaluated and used as control to quantify the angiogenic effects induced by the addition of PPAR agonists. The following concentrations of WY14643 and GW1929 were used: 1 nmol/L, 10 nmol/L, 100 nmol/L, and 1 μ mol/L. VEGF and suramin were respectively used as positive and negative controls, at the concentration suggested by the manufacturer (TCS CellWorks). On day 11, cells were fixed with ice cold 70% ethanol and tubule formation was visualized by immunostaining for the EC marker CD31 and quantified by image analysis (TCS AngioSys software), as previously described (37,38). Number of tubules, total tubule length, and number of junctions were calculated in untreated cells and in cells stimulated with PPAR selective agonists, VEGF, and suramin. The ability of WY14643 and GW1929 to increase VEGF levels was evaluated by using a commercially available ELISA kit (R&D Systems). Activity of VEGF was inhibited by adding to the culture medium a goat anti-human VEGF neutralizing antibody (R&D Systems) at a concentration of 500 ng/ml, as previously described (39). All the experiments were performed in triplicate.

Results were expressed as ratio between the angiogenic effect induced by the above-mentioned compounds and that observed in untreated cells. All quantifications were performed in a blinded fashion, by two independent operators.

Mouse cornea neovascularization assay.

Male 8-12-week-old C57BL/6J mice were used for these experiments. Mice were anesthetized with an intramuscular injection of ketamine and sacrificed with an overdose of ketamine. A single corneal pocket was created and pellets containing 0.3 µg of WY14643 or GW1929 were prepared and placed in each corneal pocket, as previously described (40). Pellets containing 0.3 µg of VEGF were used as positive controls. We also implanted pellets containing 0.03 µg, 0.3 µg, and 3 µg of gemfibrozil (Teva Pharma Italia) or rosiglitazone (GlaxoSmithKline), in order to perform a direct comparative analysis between the angiogenic properties of these two classical PPAR agonists and those of WY14643 and GW1929. Doses were calculated on the basis of the established ability of gemfibrozil and rosiglitazone to respectively bind PPAR α and PPAR γ *in vitro*, as compared to WY14643 and GW1929 (41,42). Pellets containing control buffer were used as negative controls. Eight to ten eyes were studied in each group. The investigation was in accordance with the A. Gemelli University Hospital Institutional Animal Care and Use Committee. Corneal neovascularization was quantified 6 days after pellet implantation, as established and described in previous reports (40). Vessel length, circumferential extent of neovascularity, number of vessels per cross section, and lumen diameter were evaluated. by using fluorescence microscopy. For these analyses, mice received an intracardiac injection of 500 µg of the EC marker BS-1 lectin, conjugated to FITC (Vector Laboratories). Thirty minutes later, mice were sacrificed and each eye was enucleated and fixed in 1% paraformaldehyde

solution for 1 hour. Corneal hemispheres were prepared under the dissecting microscope, placed on glass slides, and analyzed by using a fluorescent microscope. To verify that the neoangiogenic effects of WY14643 and GW1929 were specifically mediated by PPAR α and PPAR γ , respectively, the experiments were repeated in mice lacking the PPAR α gene (PPAR α -/- mice, homozygous for the *Ppara^{tm1Gonz}* gene) (Jackson Laboratories) and in mice in which PPAR γ activity was blocked by the specific inhibitor GW9662 (Sigma) (43,44). In addition, the ability of PPAR α -/- mice and mice treated with the PPAR γ inhibitor GW9662 to respond normally to a prototypical angiogenic stimulus were tested by implanting pellets containing VEGF. All quantifications were performed in a blinded fashion, by two independent operators.

VEGF expression in mouse cornea. We analyzed whether PPAR α - and PPAR γ -induced corneal neovascularization occurred in association with production of VEGF. Levels of VEGF were studied at the RNA level by RT-PCR and at the protein level by ELISA and immunohistochemistry. For this set of experiments, the right corneas were implanted with pellets containing WY14643 or GW1929, while the left corneas of the same animals were implanted with pellets containing control buffer and served as internal control. For RT-PCR analyses, corneas were harvested 4 days after pellet implantation and RNA was extracted, as previously established (45). Complementary DNA (cDNA) was obtained and amplified using the SuperScript preamplification system (Gibco-BRL). Signals were normalized to 18S rRNA using optimal 18S primer/competimer ratios as determined for the target gene following the manufacturer's recommendations (Ambion). The following primer pairs and PCR conditions were used: 5'-CACATCTGCAAGTACGTTTCGTTT-3'

and 5'-GTTTCAGAGCGGAGAAAGCATTG-3' with 30 cycles of 94 (30 s), 62 (1 min), and 72 °C (1 min). Results were expressed as mRNA fold increase, calculated as the ratio between VEGF mRNA expression in right and left (control) corneas. For ELISA, corneas were harvested 6 days after pellet implantation. VEGF₁₆₅ levels were measured by using a commercially available kit (R&D Systems), as previously described (45). Results were expressed as protein fold increase, calculated as the ratio between VEGF protein levels in right and left (control) corneas. For immunohistochemical staining, eyes treated with pellets containing PPAR α and PPAR γ synthetic agonists were excised 6 days after pellet implantation and fixed in 1% paraformaldehyde solution for 1 hour. After fixation, corneas were embedded in paraffin and cut in cross sections. VEGF immunostaining was performed using a rabbit polyclonal anti-mouse VEGF antibody (Santa Cruz Biotechnology) followed by a biotinylated goat anti-rabbit immunoglobulin secondary antibody (Signet Labs). Negative control slides were prepared by substituting preimmune rat serum.

Western Blotting for phosphorylated eNOS and Akt. For immunoblotting, homogenates of corneal tissues were analyzed. Proteins (40 μ g per lane) were separated in 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were incubated with antibodies against eNOS (1:1000) (BD Biosciences Pharmingen), phospho-eNOS (Ser1177, 1:1000) (Cell Signaling Technology Company), phospho-Akt (Ser473, 1:500) (Cell Signaling Technology Company), and Akt (1:1000) (New England Biolabs). Antibody binding was detected with horseradish peroxidase-conjugated secondary antibodies (1:2000) (Chemicon) and enhanced chemiluminescence system (GE Healthcare Bioscience). Then, the blots were reprobated

with total eNOS (1:1000) (Transduction Laboratories), Akt (1:5000) (Sigma), or actin (1:5000) (Sigma).

In vivo inhibition of VEGF activity. Activity of VEGF was systemically inhibited *in vivo* by transfection of mice thigh muscles with a plasmid DNA encoding a soluble form of the murine VEGF receptor Flt-1, as previously described (45). The plasmid was kindly provided by Dr. Kensuke Egashira. Soluble Flt-1 (sFlt-1) can suppress VEGF activity both by sequestering VEGF and by functioning as a dominant-negative inhibitor of VEGF receptors. Control mice received an equal amount of empty plasmid with an intramuscular injection on the same time schedule. Eight eyes were analyzed in each group.

Statistical analysis. All results are expressed as mean \pm SEM. Differences were analyzed by t-student test and considered statistically significant at $P < 0.05$.

RESULTS

Effects of PPAR α and PPAR γ selective agonists on EC migration and proliferation.

We first tested whether PPAR α and PPAR γ activation stimulates EC migration or proliferation, two events that are crucial for angiogenesis. No increased migration was seen in HUVECs treated with PPAR agonists (**Fig. 1a**). Likewise, PPAR agonists were unable to induce proliferation of HUVECs, compared to control (**Fig. 1b**). Similar results were obtained by using a microvascular cell line (HDMVECs) (**Fig. 1c and d**). PPAR α and PPAR γ agonists were also unable to increase VEGF production, measured by ELISA, in ECs (data not shown).

Effects of PPAR α and PPAR γ selective agonists in an EC/interstitial cell co-culture assay.

For these experiments, we used a co-culture system containing both ECs and interstitial cells (TCS CellWorks). In this assay, ECs spontaneously migrate and proliferate to form tubule-like CD31-positive

structures (**Fig. 2a**). Treatment with the anti-angiogenic molecule suramin resulted in abolishment of tubule formation (**Fig. 2b**). In contrast, treatment with the angiogenic cytokine VEGF led to significant increase of tubule formation (**Fig. 2c**). Interestingly, both PPAR α and PPAR γ agonists exhibited the ability to strongly increase tubule formation (**Fig. 2d** and **e**). The neoangiogenic properties of these compounds were quantified in terms of number of tubules, tubule length, and number of junctions and compared to those displayed by untreated cells and VEGF-treated cells (**Fig. 2f**). PPAR α and PPAR γ agonists induced significant increase of tubule number, tubule length, and number of junctions, with the maximum effect being observed at the dose of 100 nM and 10 nM, respectively. Protein analyses also demonstrated that, in culture media of cells treated with PPAR α and PPAR γ agonists, VEGF levels were significantly higher than in untreated cells (data not shown).

In vivo neoangiogenic properties of PPAR α and PPAR γ selective agonists. The murine corneal model of angiogenesis was used for these experiments. Pellets containing selective PPAR agonists were implanted in the corneas of mice and the resulting neoangiogenic response was analyzed 6 days later. BS1-lectin fluorescent staining demonstrated an important neoangiogenic response in eyes treated with pellets containing 0.3 μ g of WY14643 or GW1929 (**Fig. 3a** and **b**). In contrast, no angiogenic response was observed by using pellets containing gemfibrozil or rosiglitazone (**Fig. 3c** and **d**). As expected, VEGF pellets induced strong neoangiogenic growth (**Fig. 3e**), while no angiogenic response was obtained in corneas implanted with pellets containing control buffer (**Fig. 3f**). To confirm that the neoangiogenic effect of WY14643 was mediated by PPAR α , WY14643-containing pellets were implanted in the corneas of PPAR α $-/-$ mice. No angiogenic response was

detected in these animals (**Fig. 3g**). Similarly, the PPAR γ -specific activity of GW1929 was confirmed by implanting GW1929-containing pellets in the corneas of mice that had been pre-treated with the specific PPAR γ inhibitor GW9662. No angiogenic response was detected in these animals (**Fig. 3h**). Importantly, both PPAR α $-/-$ mice and mice treated with the specific PPAR γ antibody responded normally to stimulation with pellets containing VEGF (**Fig. 3i** and **l**), demonstrating that these animals do not have an endogenous impairment of angiogenesis.

In order to quantify the extent of PPAR α - and PPAR γ -induced corneal angiogenesis, measurements of vessel length, circumferential neovascularity, number of vessels per cross section, and lumen diameter were carried out. These analyses demonstrated that stimulation of the PPAR α and PPAR γ pathways resulted in a neoangiogenic process that was statistically similar, in terms of morphological characteristics, to that induced by VEGF (**Fig. 4**). The average length of neovessels was 0.73 ± 0.11 mm in PPAR α -induced neovascularization, 0.71 ± 0.12 mm in PPAR γ -induced angiogenesis, and 0.75 ± 0.09 mm in the VEGF group. Mean lumen diameter was 8.02 ± 1.20 μ m in PPAR α -induced neovascularization, 7.73 ± 1.10 μ m in the PPAR γ group, and 8.33 ± 1.10 μ m in the VEGF group. Circumferential extent of corneal neovascularity was 102.30 ± 7.50 degrees in PPAR α -induced neovascularization, 99.10 ± 3.90 degrees in the PPAR γ group, and 107.20 ± 4.40 degrees in the VEGF group. The number of vessels per cross section was 121.30 ± 6.90 in PPAR α -induced neovascularization, 112.30 ± 7.20 in PPAR γ -induced angiogenesis, and 131.21 ± 7.40 in the VEGF group.

PPAR α - and PPAR γ -induced neoangiogenesis occurs in association with VEGF production and increased phosphorylation of eNOS and Akt. First, we

evaluated whether VEGF is expressed in association with PPAR α - and PPAR γ -induced neovascularization. RT-PCR and ELISA analyses demonstrated that VEGF RNA and protein levels are significantly increased in corneas treated with WY14643 and GW1929 (**Fig. 5a** and **b**). Second, we performed immunohistochemical analysis that showed that corneas treated with PPAR agonists were strikingly immunopositive for VEGF (**Fig. 5c** and **d**). VEGF-producing cells during corneal neovascularization were immunopositive for vimentin (data not shown), a fibroblast mesenchymal marker, suggesting that PPAR agonists stimulate expression of endogenous VEGF in interstitial fibroblasts within the neovascular foci. No VEGF-positive staining was seen in corneas implanted with control pellets or where control staining was performed (**Fig. 5e** and **f**). We also investigated the potential impact of PPAR activation on other important angiogenic players, such as eNOS and Akt. We found that, in corneas implanted with pellets containing WY14643 and GW1929, there is a significant upregulation of eNOS phosphorylation at Ser-1177 and Akt phosphorylation at Ser-473 (**Fig. 6**). Taken together, these findings indicate that a mechanism involving VEGF, eNOS and Akt contributes to PPAR α - and PPAR γ -induced angiogenesis *in vivo*.

PPAR α - and PPAR γ -induced neoangiogenesis is VEGF-dependent.

Following the observation that PPAR α - and PPAR γ -induced endothelial tube formation *in vitro* and neoangiogenesis *in vivo* occurs in association with VEGF production, we tested the hypothesis that the angiogenic properties of PPAR agonists might depend on VEGF activity. By adding an anti-human VEGF neutralizing antibody to the medium of the EC/interstitial cell co-culture assay, we found that inhibition of VEGF activity was sufficient to significantly decrease PPAR α - and PPAR γ -induced tubulogenesis (**Fig. 7a**

and **b**). In addition, we suppressed VEGF activity *in vivo* and evaluated whether pellets containing WY14643 or GW1929 were still able to induce corneal angiogenesis. *In vivo* inhibition of VEGF was accomplished by using the sFlt1 plasmid, which suppresses VEGF activity both by sequestering VEGF and by functioning as a dominant-negative inhibitor of VEGF receptors (45). Mice transfected with the empty plasmid were used as controls. A dramatic reduction of PPAR α - and PPAR γ -induced corneal neoangiogenesis was observed when VEGF activity was suppressed (**Fig. 7c-f**). Quantification analyses demonstrated that inhibition of VEGF activity resulted in statistically significant reduction of PPAR α - and PPAR γ -induced neoangiogenesis, measured in terms of vessel length, circumferential extent of neovascularity, and number of vessels per cross section (**Fig. 7g**). These findings demonstrate that selective PPAR α and PPAR γ agonists induce angiogenesis via a VEGF-dependent mechanism.

DISCUSSION

In recent years, the angiogenic potentials of TZDs have been intensively investigated. Some studies have reported that ciglitazone, troglitazone, and rosiglitazone suppress migration, proliferation, and differentiation of ECs and inhibit angiogenesis in a number of experimental models (17-21). Other studies have instead provided evidence that the same molecules may have important pro-angiogenic effects. In particular, it has been shown that TZDs are able to increase VEGF expression in smooth muscle vascular cells and macrophages (14,15), that rosiglitazone increases number and migratory activity of endothelial progenitor cells (16) and promotes angiogenesis after focal cerebral ischemia in rats (22), and that troglitazone induces the expression of VEGF and its receptors in cultured cardiac myofibroblasts (35). In all these studies, the Authors have assumed that

the effects induced by TZDs were mediated by the activation of PPAR γ , without considering that TZDs are non-selective and non-specific ligands of this nuclear receptor, since they are able to stimulate several PPAR γ -independent pathways that are potentially important in angiogenesis (27-33,46). Similarly, the role of PPAR α in angiogenesis has been investigated by using fibrates, that not only activate PPAR α , but also bind other nuclear and superficial receptors and regulate important PPAR α -independent pathways (23-26). Therefore, in order to conclude that the effects of TZDs and fibrates on angiogenesis specifically depend on PPAR, all the potential activities that these molecules exert on pathways that are PPAR-independent should be excluded.

In this study, we used selective PPAR α and PPAR γ synthetic agonists and found that they were able to enhance endothelial tube formation *in vitro* and induce neovascularization *in vivo*. We also demonstrated that these effects were specifically PPAR-mediated, by showing that the PPAR α activator WY14643 was unable to induce angiogenesis in mice lacking the PPAR α gene and that the PPAR γ activator GW1929 did not stimulate angiogenesis in mice treated with a PPAR γ specific inhibitor. Importantly, PPAR α -/- mice and mice treated with the PPAR γ inhibitor showed a normal response upon stimulation with VEGF, demonstrating that these animals do not have an endogenous impairment of angiogenesis and providing additional confirmation that WY14643 and GW1929 inability to induce angiogenesis in these mice depends on PPAR lack of function. An other interesting finding was that gemfibrozil and rosiglitazone, two prototypical fibrates and TZDs that are generally viewed as classical PPAR agonists and have been widely used to respectively test the biological effects of PPAR α and PPAR γ , were unable to induce angiogenesis in our experimental setting. This finding might help

to understand the controversial results in the literature about the angiogenic properties of PPARs. Under this respect, it is important to point out that this is the first time that the angiogenic effects of TZDs and fibrates are directly compared to those of synthetic PPAR specific agonists, such as WY14643 and GW1929. These data clearly eliminate the possibility that our observations differ from those of other research groups as a result of different experimental settings and/or surgical procedures. They instead support the concept that fibrates and TZDs cannot be considered specific and selective PPAR agonists and that not all their biological activities can be attributed to activation of PPARs.

An other important finding of this study is the demonstration that PPAR α - and PPAR γ -induced angiogenesis does not occur through direct stimulation of EC migration or proliferation. In contrast, PPAR α and PPAR γ agonists stimulate angiogenesis indirectly, through upregulation of the angiogenic cytokine VEGF. Indeed, the *in vivo* neovascularization resulting from the activation of PPAR α and PPAR γ is morphologically similar to that induced by VEGF and is associated with local VEGF production. Most importantly, suppression of VEGF activity is sufficient to inhibit PPAR α - and PPAR γ -induced angiogenesis both *in vitro* and *in vivo*. However, it is important to note that VEGF inhibition does not completely suppress PPAR α - and PPAR γ -induced neovascularization. Therefore, it is possible to speculate that additional cytokines might contribute to the angiogenic process stimulated by PPARs. In this scenario, we also show that PPAR α - and PPAR γ -induced neovascularization occurs in association with enhanced phosphorylation of eNOS and Akt. The importance of this finding consists in the fact that nitric oxide and VEGF display reciprocal regulatory activities that are crucial for angiogenesis, with the fundamental contribution of Akt. On one hand, VEGF may

activate eNOS by activating Akt. On the other hand, NO can mediate VEGF expression by stimulating hypoxia-inducible factors and heme oxygenase-1 activity. Although the precise molecular mechanisms responsible for PPAR-induced angiogenesis still merits further investigation, our data provide new insights to understand the ability of PPARs to modulate the angiogenic process *in vivo*.

The discovery that both PPAR α and PPAR γ are able to induce angiogenesis also suggests the possible existence of a synergistic interaction between the two receptors, although we have no data regarding their possible relative roles in the activation of VEGF and/or other angiogenic molecules. This concept might be particularly important when considering the biological activities of PPAR α/γ dual agonists. Indeed, in addition to reduce glucose levels and improve lipid profile, these molecules also display beneficial activities on cardiac and endothelial function, oxidative stress and atherosclerosis (47). It is possible to hypothesize that these effects might partially result from the ability of PPAR α/γ dual agonists to induce angiogenesis and VEGF production. Similar mechanisms might be hypothesized to explain the potential carcinogenic effects of some PPAR α/γ dual agonists (48), as well as their ability to induce edema and precipitate heart failure (49,50).

Although our data need to be confirmed in other experimental models and in humans, the observation that neoangiogenesis is a potential biological result of PPAR activation might help to understand, at al least in part, some of the biological and clinical effects of TZDs and fibrates. For instance, PPAR γ -induced VEGF upregulation might be responsible for the recently reported ability of rosiglitazone to ameliorate endothelial

dysfunction in T2DM (51) and increase number and migratory activity of endothelial progenitor cells (16). Since VEGF augments vascular permeability (52), increased production of this cytokine might also contribute to edema, a common side effects of TZDs. It is also possible that PPAR γ -induced neoangiogenesis has deleterious effects in subjects with T2DM. Indeed, although angiogenic therapy has been widely regarded as an attractive approach both for treating ischemic heart disease and for enhancing arterioprotective functions of the endothelium, a variety of studies have also suggested that neovascularization contributes to the growth of atherosclerotic lesions and is a key factor in plaque destabilization and rupture (53). These negative effects might contribute to the recently reported potential adverse cardiovascular effects of rosiglitazone in T2DM (54). Similarly, some clinical activities of fibrates, such as preservation of renal function and amelioration of endothelial dysfunction (55,56), might be partially explained by PPAR α -induced angiogenesis and VEGF production.

In conclusion, we used selective synthetic agonists of PPAR α and PPAR γ and demonstrated that the stimulation of these nuclear receptors results in the activation of a strong neoangiogenic process *in vitro* and *in vivo*. This angiogenic response does not occur through direct stimulation of ECs, but is instead dependent on VEGF activity. These findings provide new information to understand the biological, clinical, and therapeutic effects of drugs that stimulate the activity of PPAR α and PPAR γ , with potentially important implications for the management of subjects affected by T2DM.

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FIGURE LEGENDS

Figure 1.

Inability of PPAR α and PPAR γ ligands to induce migration or proliferation of ECs. (a) Stimulation of HUVECs with different concentrations of PPAR α and PPAR γ agonists for 6 and 12 hours does not increase migration compared to untreated cells. In contrast, VEGF significantly increases migration compared to untreated and PPAR-stimulated cells. (b) Stimulation of HUVECs with different concentrations of PPAR α and PPAR γ agonists for 24, 36, and 48 hours does not result in increased proliferation, compared to untreated cells. VEGF stimulation induces significant increment of proliferation compared to untreated and PPAR-stimulated cells. (c and d) Similar results were obtained by using HDMVECs.

Figure 2.

PPAR α and PPAR γ ligands induce tube formation in an EC/interstitial cell co-culture assay. Immunostaining with the EC marker CD31 demonstrates the ability of untreated cells to spontaneously form tubules (a). In cells treated with suramin, tubule formation is greatly reduced (b). In contrast, tubule formation is importantly increased in cells stimulated with VEGF (c). Similarly, treatment of cells with WY14643 (100 nmol/L) (d) and GW1929 (10 nmol/L) (e) results in conspicuous enhancement of tubule formation. Quantification of tube formation shows that, compared to untreated cells, number of tubules, total tubule length, and number of junctions are significantly increased in cells treated with VEGF, WY14643, and GW1929 (f). The ratio between the number of tubules detectable in the VEGF group and that observed in untreated cells was 10.40 ± 0.52 ($P < 0.001$). The ratio between the total tubule length in the VEGF group and in untreated cells was 16.22 ± 0.81 ($P < 0.001$). The ratio between the number of junctions in the VEGF group and in untreated cells was 29.71 ± 1.48 ($P < 0.001$). Also cells stimulated with WY14643 showed significant increments of tubule number, tubule length, and junction number, compared to untreated cells. WY14643 exhibited its maximum pro-angiogenic effect at the concentration of 100 nM, with ratio of 18.75 ± 0.93 , 9.43 ± 0.47 , and 23.89 ± 1.19 for tubule number, tubule length, and number of junctions, respectively ($P < 0.001$). Likewise, cells stimulated with GW1929 showed significant increments of all the investigated parameters, compared to untreated cells. The maximum effect was observed at the concentration of 10 nM, with a tubule ratio of 21.28 ± 1.06 ($P < 0.001$), a length ratio of 16.00 ± 0.8 ($P < 0.001$), and a junction ratio of 70.00 ± 3.5 ($P < 0.001$).

Figure 3.

PPAR α and PPAR γ agonists induce angiogenesis *in vivo*. BS1 lectin whole mount staining in corneas where pellets containing 0.3 μ g of WY14643 (a), 0.3 μ g of GW1929 (b), 3 μ g of gemfibrozil (c), 3 μ g of rosiglitazone (d), 0.3 μ g of VEGF (e), or control buffer (f) were implanted. Pellets containing WY14643 are unable to induce corneal angiogenesis in PPAR α $-/-$ mice (g). Pellets containing GW1929 are unable to induce corneal angiogenesis in mice pre-treated with the PPAR γ inhibitor GW9662 (h). VEGF induces a normal angiogenic response in PPAR α $-/-$ mice (i) and in mice pre-treated with the PPAR γ inhibitor GW9662 (l).

Figure 4.

Quantification of corneal angiogenesis induced by PPAR agonists. Vessel length, lumen diameter, circumferential extent of neovascularity, and number of vessels per cross section are not statistically different between corneal angiogenesis induced by WY14643, GW1929, and VEGF.

Figure 5.

Angiogenesis induced by PPAR agonists is associated with local VEGF production. RT-PCR for mouse VEGF shows significantly increased RNA levels in corneas stimulated with pellets containing WY14643 and GW1929 (*a*). VEGF protein levels, measured by ELISA, are significantly increased in corneas stimulated with pellets containing WY14643 and GW1929 (*b*). Immunohistochemistry for mouse VEGF in 5 μ m sections shows VEGF positive staining (red staining) in the neovascular area of corneas stimulated with pellets containing WY14643 (*c*) and GW1929 (*d*). No staining is present in corneas where control pellets were implanted (*e*) and in sections where control staining was performed (*f*).

Figure 6.

Angiogenesis induced by PPAR agonists is associated with increased phosphorylation of eNOS and Akt. Western Blotting analysis shows increased phosphorylation of eNOS Ser1177 and Akt Ser473 in corneas implanted with pellets containing WY14643 and GW1929.

Figure 7.

Angiogenesis induced by PPAR agonists is inhibited by suppression of VEGF activity. CD31 immunostaining shows that treatment with a VEGF neutralizing antibody significantly reduces WY14643 (*a*) and GW1929 (*b*) -induced tubulogenesis in the EC/interstitial cell co-culture assay. BS-1 lectin fluorescent staining shows that, compared to mice transfected with empty plasmid (*c* and *d*), corneal angiogenesis induced by PPAR α and PPAR γ ligands is inhibited in mice transfected with sFlt-1 vector (*e* and *f*). Quantification of corneal vascularization in the two groups (*g*).

NOTE: The figures for this article can be found using the link entitled “Figures”. (Available at <http://dx.doi.org/10.2337/db07-0765>.)