Expression and Function of PPARγ in Rat and Human Vascular Smooth Muscle Cells

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Background—Peroxisome proliferator–activated receptor-γ (PPARγ) is activated by fatty acids, eicosanoids, and insulin-sensitizing thiazolidinediones (TZDs). The TZD troglitazone (TRO) inhibits vascular smooth muscle cell (VSMC) proliferation and migration in vitro and in postinjury intimal hyperplasia.

Methods and Results—Rat and human VSMCs express mRNA and nuclear receptors for PPARγ1. Three PPARγ ligands, the TZDs TRO and rosiglitazone and the prostanoid 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), all inhibited VSMC proliferation and migration. PPARγ is upregulated in rat neointima at 7 days and 14 days after balloon injury and is also present in early human atheroma and precursor lesions.

Conclusions—Pharmacological activation of PPARγ expressed in VSMCs inhibits their proliferation and migration, potentially limiting restenosis and atherosclerosis. These receptors are upregulated during vascular injury. (Circulation. 2000;101:1311-1318.)

Key Words: atherosclerosis ■ restenosis ■ growth substances ■ migration ■ thiazolidinediones

Peroxisome proliferator–activated receptor-γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors. PPARγ expression is abundant in adipose tissue, where it promotes adipocyte differentiation and regulates expression of genes involved in fatty acid metabolism.1 Various fatty acids and eicosanoids are likely physiological ligands for PPARγ.2–4 Thiazolidinediones (TZDs) are oral antidiabetic compounds that enhance sensitivity to the metabolic effects of insulin and that bind with high affinity to PPARγ.5 In humans and animals with insulin resistance and type 2 diabetes, TZDs ameliorate hyperglycemia, hyperinsulinemia, and hypertriglyceridemia.6–8

We previously reported that troglitazone (TRO) suppressed neointima formation in rat aorta after endothelial injury, most likely as a result of direct vascular action to inhibit vascular smooth muscle cell (VSMC) growth and migration.9 However, our initial studies did not address whether the vascular effects of TRO were mediated through PPARγ, which at that time was thought to be highly restricted to adipose tissue. Recent studies have identified PPARγ in a variety of nonadipose tissues: skeletal muscle,10,11 heart,11 kidney proximal tubules,12 colon,13 bone marrow stromal cells,14 neutrophils,14 macrophages,15–20 and breast carcinoma,21 which implicate novel functions for this receptor distinct from its well-characterized metabolic activity. TRO, however, is distinguishable from other TZD PPARγ ligands because it also contains a vitamin E moiety, which is also known to inhibit VSMC growth and intimal hyperplasia.22 The vascular effects of TRO, therefore, could be independent of PPARγ.

The expression and function of PPARγ in VSMCs is somewhat controversial. In human VSMCs, Staels et al23 observed faint expression of PPARγ that was not involved in the negative regulation of cytokine-induced interleukin-6 and cyclooxygenase-2 expression, this effect being mediated by PPARα. In contrast, a recent study reported that human VSMCs express PPARγ, which inhibited matrix metalloproteinase expression and cell migration.24 Therefore, we examined the expression and function of PPARγ in rat and human VSMCs, focusing on VSMC growth and migration.

Results

PPARγ Ligands Inhibit bFGF-Induced DNA Synthesis in Rat and Human VSMCs

The TZD PPARγ ligands TRO and RSG, and 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), a non-TZD PPARγ ligand, all inhibited basic fibroblast growth factor (bFGF)-induced DNA synthesis in rat VSMCs (Figure 1). At concentrations of 5 μmol/L, rosiglitazone (RSG) and TRO inhibited DNA synthesis by ≈65% and 58%, respectively.
15d-PGJ2 was a far more potent inhibitor of VSMC DNA synthesis, reducing \(^{3}H\)thymidine incorporation by 64.2±7% and 95±3.4% at 0.1 and 1 \(\mu\)mol/L, respectively.

In human coronary artery VSMCs (CASMCs), all tested PPAR\(\gamma\) ligands inhibited bFGF-stimulated DNA synthesis (Figure 2). RSG and 15d-PGJ2 were more potent than TRO. Even at 0.1 \(\mu\)mol/L, RSG and 15d-PGJ2 caused a statistically significant inhibition of CASMC proliferation (25.5±5.5% and 42.8±5.8%, respectively). Inhibition of \(\geq 50\%\) was observed at concentrations \(>0.5 \mu\)mol/L for 15d-PGJ2 or \(>1 \mu\)mol/L for RSG. TRO exhibited weaker antiproliferative activity with a maximum effect of 45.5±4.7% inhibition observed at 10 \(\mu\)mol/L.

TRO and RSG had no effect on VSMC viability at 10 \(\mu\)mol/L, but 5 \(\mu\)mol/L 15d-PGJ2 for 48 hours induced significant cell death.

**PPAR\(\gamma\) Ligands Inhibit PDGF-Directed Migration in Rat and Human VSMCs**

Platelet-derived growth factor (PDGF) is one of the most potent in vitro chemoattractants for VSMCs. TRO, RSG, and 15d-PGJ2 all blocked PDGF-directed VSMC migration (Figure 3). PDGF induced a 5.6-fold increase in the number of rat VSMCs that migrated through the gelatin-coated membrane. TRO and RSG inhibited PDGF-directed migration in a dose-dependent manner at concentrations of 0.1 to 10 \(\mu\)mol/L. A modest but statistically significant effect was observed at 0.1 \(\mu\)mol/L: At \(\geq 1 \mu\)mol/L for RSG and at \(\geq 5 \mu\)mol/L for TRO, migration was inhibited by \(>50\%\). At 10 \(\mu\)mol/L, RSG totally abolished PDGF-directed migration.

In contrast to its strong antiproliferative activity, 15d-PGJ2 was only a slightly more potent inhibitor of PDGF-directed migration than TRO or RSG. The concentrations of TRO, RSG, and 15d-PGJ2 required to inhibit PDGF-directed migration by 50% were 2.4 \(\mu\)mol/L, 0.3 \(\mu\)mol/L, and 0.2 \(\mu\)mol/L, respectively. TRO, RSG, and 15d-PGJ2 also inhibited PDGF-directed migration of human CASMCs with very similar dose-response curves (Figure 4).

**Rat and Human VSMCs Express PPAR\(\gamma\) mRNA**

The PPAR\(\gamma\) gene produces 2 major mRNA species through alternative promoter usage. Adipose tissues express both isoforms, but PPAR\(\gamma1\) expression is much higher than PPAR\(\gamma2\) in nonadipose tissues. Using a sensitive RNase protection assay (RPA) (Figure 5), we observed only faint expression of PPAR\(\gamma1\) mRNA in mouse 3T3-L1 preadipocyte cells, whereas significant upregulation of both PPAR\(\gamma1\) and -\(\gamma2\) mRNAs occurred during their in vitro differentiation to adipocytes. VSMCs from rat aorta and human VSMCs from umbilical artery, coronary artery, and aorta expressed PPAR\(\gamma1\) mRNA exclusively, as evidenced by the single protected band of 258 (human) or 185 (rat) bases. Although rat aortic tissue contained PPAR\(\gamma1\) and -\(\gamma2\), the presence of PPAR\(\gamma2\) mRNA in aorta and its absence in cultured VSMCs...
are most likely due to contaminating adventitial fat. Human umbilical vein endothelial cells also prominently expressed PPARγ1 but not -γ2 mRNA.

Expression and Subcellular Localization of PPARγ in Rat and Human VSMCs

To detect PPARγ protein in VSMCs, we performed Western immunoblotting using a murine monoclonal antibody to human recombinant PPARγ (Glaxo Wellcome) previously shown to recognize 2 bands of ~56 and 52 kDa, corresponding to PPARγ2 and -γ1, respectively, in 3T3-L1 adipocyte nuclear extracts (Figure 6). Receptor levels were low in nuclear extracts of undifferentiated 3T3-L1 preadipocytes. Cultured aortic and human coronary artery VSMCs expressed only PPARγ1, which was present almost exclusively in the nuclear fraction (Figure 6). Nuclear extracts from rat and human coronary artery VSMCs contained a protein with a molecular weight greater than that of PPARγ2 that is probably not PPARγ2, because these cells do not express detectable mRNA for this isoform by RPA (see Figure 5).

Whole-tissue extracts from normal rat aortas contained PPARγ1 and -γ2 protein, consistent with the pattern of PPARγ mRNA expression detected by RPA (Figure 5).

PPARγ Expression in Human Vascular Lesions and Rat Neointima

In human atherosclerotic lesions, PPARγ is expressed in macrophages and to a lesser extent in VSMCs. To validate the quality of PPARγ antibodies used for immunohistochemical analysis, we first examined human coronary arteries for receptor expression. Immunoreactive PPARγ colocalizes with macrophages visualized by staining of parallel sections of a type II atherosclerotic lesion (Figure 7) with the macrophage-specific antibody anti-CD68. In a type I lesion exhibiting adaptive intimal thickening, faint expression of PPARγ is seen both in neointimal regions devoid of CD68-positive cells and in the underlying media in VSMCs, as demonstrated in serial sections stained with antibody against α-smooth muscle actin. Similar results were obtained.
with either of the 2 commercial antibodies to stain 2 additional type I and type II lesions from separate biopsies.

In neointima formed after balloon injury of rat aortas, faint expression of PPARγ is observed in the media of uninjured vessels (Figure 8). Neointima that developed at 7 and 14 days after balloon injury displayed intense staining for immunoreactive PPARγ, which suggests that this receptor is upregulated in response to vascular injury. VSMCs were the major cell type present in rat neointima, as shown by its strong positive staining for α-smooth muscle actin and the absence of staining for the macrophage marker ED1. Immunoreactive PPARγ did not localize specifically to the nucleus of neointimal or medial VSMCs, because staining of the cytoplasm was observed. We do not know whether VSMCs in arterial vessels actually contain PPARγ in their cytoplasm or whether this is an artifact of tissue fixation.

To confirm that the immunoreactive signal detected in rat neointima was bona fide PPARγ, we used nuclear extracts from differentiated 3T3-L1 adipocytes to preabsorb PPARγ antibodies before their use in immunostaining. Addition of 50 μg of nuclear extracts of differentiated 3T3-L1 adipocytes, which contain high levels of PPARγ1 and -γ2 compared with undifferentiated 3T3-L1 cells (see Figure 6), markedly attenuated staining in both the neointima and media (Figure 9), whereas extracts from undifferentiated 3T3-L1 preadipocytes had little effect. Thus, the immunoreactivity observed in these tissues corresponds to PPARγ protein.

Discussion

PPARγ mRNA and protein have previously been identified in rat aortic and human saphenous vein VSMCs. In human aortic VSMCs, Staels et al found extremely low levels of PPARγ mRNA relative to PPARα message detected by RPA. However, the pattern of PPARγ isoform expression was not described. Using an RPA that permits the detection of both isoforms, we found that PPARγ1 mRNA is easily detectable in cultured rat aortic VSMCs, in whole aortas from uninjured animals, and in human VSMCs. PPARγ1 expression was substantially higher in VSMCs from human coronary artery and umbilical artery than in cells from aortas. None of these cells expressed detectable PPARγ2 mRNA.

Cultured VSMCs from rat aortas and human coronary arteries expressed only PPARγ1 protein, consistent with their pattern of mRNA isoform expression. Rat aortic tissue contained receptors for both PPARγ1 and -γ2, consistent with the presence of both mRNA species in that tissue. Levels of PPARγ1 protein in rat aortic and human coronary arterial VSMCs appeared to be substantial, because they express levels similar to those of differentiated 3T3-L1 adipocytes, a major in vitro model for studying PPARγ function. Our data are also consistent with previous studies showing that PPARγ is present in rat aortic and human saphenous vein VSMCs.

Inhibition of VSMC growth and migration in vitro occurred at low micromolar concentrations of PPARγ ligands, which are achievable in the circulation of humans or animals given TRO for insulin sensitization. TRO and RSG had comparable activities to inhibit VSMC growth and migration. This finding is somewhat surprising, because other studies have shown RSG to be 5- to 20-fold more efficacious than TRO in binding to PPARγ and in increasing transcriptional activity of PPARγ; stimulating insulin-mediated glucose transport, lowering hyperglycemia in ob/ob diabetic mice, and inducing adipocyte differentiation. The roughly equal potencies between TRO and RSG for inhibiting VSMC proliferation and migration may be the result of TRO being a bifunctional molecule having both a TZD and α-tocopherol (vitamin E) moiety. Tocopherol inhibits VSMC proliferation and macrophage migration. TRO has also recently been shown to inhibit atheroma in ob/ob diabetic mice.
to inhibit cholesterol synthesis through a mechanism independent of its vitamin E or PPARγ ligand properties. The vascular effects of TRO, therefore, may be complex, with its activity mediated partially through α-tocopherol and/or other PPARγ-independent mechanisms and partially through PPARγ. RSG lacks α-tocopherol and is a more “pure” PPARγ ligand. Its vascular effects are likely to be mediated exclusively through PPARγ. The non-TZD PPARγ ligand 15d-PGJ2 displayed the strongest antiproliferative and antimigration activity in VSMCs. RSG is 20-fold more potent than 15d-PGJ2 in activating PPARγ as a transcription factor in transient transfection experiments and in inducing differentiation of 3T3-L1 cells into adipocytes. The biological effects of 15d-PGJ2, however, are complex because of its potential to activate prostaglandin receptors. RSG, therefore, may provide the clearest evidence for PPARγ-mediated effects. The vascular effects of RSG we observed importantly distinguish this study from that of Marx et al., which used only TRO and 15d-PGJ2 to inhibit human VSMC migration.

The molecular basis for the inhibition of VSMC growth and migration by PPARγ remains to be elucidated. PPARγ-mediated inhibition of transcription factor function (i.e., transrepression) critical for these processes is probably involved. We previously observed that TRO inhibited the activity of ELK-1, an ets-family transcription factor, after mitogenic stimulation of VSMCs by bFGF. Transrepression of ELK-1, and possibly other transcription factors, by TRO may be the underlying mechanism for its inhibition of VSMC growth and migration and hence intimal hyperplasia. This hypothesis is supported by studies in macrophages in which PPARγ also negatively regulates gene expression. Iijima et al. observed that TRO and 15d-PGJ2 poorly activate (<1-fold induction) endogenous PPARγ in rat VSMCs, a finding we reproduced (unpublished data). By comparison, PPARγ present in 3T3-L1 adipocytes or overexpressed by transfection in CV-1 renal fibroblasts show a 5- to 100-fold increase in transcription factor activity in response to RSG, TRO, or 15d-PGJ2. These data suggest that transcriptional activation by PPARγ may have a different pharmacology than

**Figure 7.** Expression of PPARγ in early-stage human atheroma (type II) (a) and a precursor lesion (d) with adaptive intimal thickening (type I). High-power views show immunoreactive PPARγ (detected with a polyclonal rabbit anti-human PPARγ antibody from Biomol) in medial and intimal VSMCs (M indicates intima; I, media). In type II (early atheroma) lesions, highest levels of immunoreactive PPARγ colocalized with macrophages detected in parallel sections stained with macrophage marker CD68 (b, e). Staining for α-smooth muscle actin was used as a marker for VSMCs (c, f). Data are representative of 3 type I and type II lesions examined.
transrepression by these receptors and is dependent on the cell type.

To date, only 2 previous studies have described the expression of PPARγ in normal or diseased vasculature. Immunohistochemical analysis of PPARγ human atherosclerotic lesions revealed strong expression in macrophages, with fainter expression observed in VSMCs. VSMCs in the underlying media of lesions or in unaffected areas of the coronary artery had nearly undetectable levels of PPARγ.19,20 In early human atheroma (type II), we found that the highest levels of PPARγ colocalized with macrophages in the neointima. VSMCs present in the neointima and the underlying media stained positively for PPARγ, but staining was less than in macrophages. We also observed significant staining for PPARγ in human VSMCs present in regions of adaptive intimal thickening in type I lesions that can be precursors to atheromas.

Our study also provides new insight concerning the in vivo expression of PPARγ in the injured vasculature. Neointimal VSMCs prominently upregulate PPARγ protein levels. Lesions that result from this model of vascular injury differ from atheromas in several important aspects. First, intimal hyperplasia after mechanical injury is a more acute response than atherosclerosis, which develops over a longer period of time. Second, VSMCs are the predominant cell type in balloon injury–induced neointimal lesions, where we find little infiltration of macrophages. In contrast, macrophages are abundant in atherosclerotic lesions, where we find little infiltration of macrophages. In contrast, macrophages are abundant in atherosclerotic lesions and play a major role in driving atherogenesis.31 Therefore, upregulation of PPARγ and its activation by physiological or pharmacological ligands in the damaged vasculature may be important in limiting lesions dependent on VSMC activity.

The present data are in stark contrast to a recent report emphasizing the role of PPARα and dismissing involvement of PPARγ in VSMC responses that promote restenosis and atherosclerosis. In that study, Staels et al.23 using a different antibody and not using nuclear extracts, did not detect significant levels of PPARγ in human aortic VSMCs. Using RPA, we find that human aortic VSMCs express much lower

Figure 8. Expression of PPARγ in intimal VSMCs after balloon injury of rat aortas. High-power view shows strong immunoreactive staining for PPARγ in neointima at 7 and 14 days after balloon injury (c, d) (I indicates intima; M, media). Faint staining for PPARγ is observed in media of an uninjured control aorta (a) and at 2 days after injury (b). Few macrophages were present in a 14-day lesion (e), because there was no staining for macrophage-specific marker ED1 (Serotec Laboratories). VSMCs in a 14-day lesion were detected by immunostaining for α-smooth muscle actin (f). Data are representative of 3 aortas examined per time point.
levels of PPARγ mRNA than human coronary VSMCs. Either or both of these differences may have resulted in our experimental approach being more sensitive for detecting PPARγ protein. We also found that PPARγ ligands had no effect on VSMC inflammatory responses, whereas we find that PPARγ ligands have antiproliferative and antiinflammatory activity in VSMCs.

The present results have important implications for diabetes-associated vascular disease. In type 2 diabetes, the development of both atherosclerosis and restenosis is substantially accelerated. We and others have suggested that TZD may retard arterogenesis and restenosis through their inhibitory effects on VSMCs and macrophages in the damaged vasculature. TZDs, therefore, may provide a dual benefit for type 2 diabetes by ameliorating insulin resistance and its metabolic sequelae, as well as directly protecting the vasculature from injury.

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References

Figure 9. Nuclear extracts from 3T3-L1 adipocytes block immunostaining of 14-day neointima by PPARγ antibody. Preabsorption of polyclonal antibody to human PPARγ with nuclear extracts from 3T3-L1 adipocytes substantially diminishes immunostaining in 14-day neointima (compare b vs c) (l indicates intima; M, media), whereas extract from undifferentiated 3T3-L1 cells lacked this effect (compare b vs d). Negative control (a) was stained with nonspecific rabbit IgG. Data are representative of 3 preabsorption experiments.

Negative Control

PPARγ

PPARγ + NE-diff.3T3

PPARγ + NE-non-diff.3T3

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