Dominant-Negative Loss of PPARγ Function Enhances Smooth Muscle Cell Proliferation, Migration, and Vascular Remodeling

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Objective—The peroxisome proliferator activated receptor-gamma (PPARγ) protein is a nuclear transcriptional activator with importance in diabetes management as the molecular target for the thiazolidinedione (TZD) family of drugs. Substantial evidence indicates that the TZD family of PPARγ agonists may retard the development of atherosclerosis. However, recent clinical data have suggested that at least one TZD may increase the risk of myocardial infarction and death from cardiovascular disease. In this study, we used a genetic approach to disrupt PPARγ signaling to probe the protein’s role in smooth muscle cell (SMC) responses that are important for atherosclerosis.

Methods and Results—SMC isolated from transgenic mice harboring the dominant-negative P465L mutation in PPARγ (PPARγL/+) exhibited greater proliferation and migration then did wild-type cells. Upregulation of ETS-1, but not ERK activation, correlated with enhanced proliferative and migratory responses PPARγL/SMCs. After arterial injury, PPARγL/+ mice had a ≈4.3-fold increase in the development of intimal hyperplasia.

Conclusion—These findings are consistent with a normal role for PPARγ in inhibiting SMC migration and proliferation in the context of restenosis or atherosclerosis.

Key Words: PPARγ ■ smooth muscle cell ■ restenosis

PPARγ, a dynamic nuclear transcriptional regulator, has well characterized roles in adipocyte differentiation, lipid metabolism, and insulin sensitivity. Clinically, PPARγ is the molecular target of the insulin sensitizing thiazolidinedione (TZD) class of drugs that includes rosiglitazone and pioglitazone. TZD and other PPARγ agonists effectively increase insulin sensitivity in type 2 diabetic patients. Many preclinical and clinical studies have suggested that PPARγ agonists protect against the development of atherosclerosis and reduce the development of intimal hyperplasia. In the PROACTIVE trial, the PPARγ agonist pioglitazone had beneficial effects on the combined secondary end points of myocardial infarction, stroke, and death. However, a recent meta-analysis suggested that the use of the PPARγ agonist rosiglitazone for the treatment of diabetes was associated with an increase in risk of myocardial infarction and a trend toward a higher risk of cardiovascular death. Endogenous ligands for PPARγ include unsaturated and oxidized fatty acids, eicosanoids, prostaglandins, and possibly lysophospholipids. The putative PPARγ agonist lysophosphatidic acid (LPA) accelerates neointimal formation after vessel injury in rodents in a PPARγ-dependent manner, suggesting that activation of PPARγ may promote the development of intimal hyperplasia. However, at present, the role that PPARγ plays in regulating vascular responses that underlie atherosclerosis and restenosis remain incompletely understood.

Proliferation and migration of vascular smooth muscle cells (SMCs) are key events in the development of intimal hyperplasia that occurs in the context of atherosclerosis and restenosis. PPARγ is present in vascular SMCs, and the atheroprotective effects of PPARγ ligands have been proposed to relate in part to beneficial effects on SMC biology. Indeed, studies have shown that TZDs inhibit vascular SMC proliferation and migration and apoptosis. It is not clear whether this is a direct effect of TZDs on PPARγ or an “off-target” effect of the drugs, as most studies have inferred a role for PPARγ in atherosclerosis on the basis of results with pharmacological interventions. Homozygous deficiency of PPARγ is embryonic lethal. Atherosclerosis and the development of intimal hyperplasia in a vascular SMC-specific PPARγ knock-out mouse have not been reported. However, there is genetic evidence to support a role for PPARγ in SMC biology. For example, a generalized PPARγ knock-out model was created by breeding floxed PPARγ mice to Mox2-Cre mice to inactivate PPARγ in the embryo but not in trophoblasts. The resulting mice have...
hypotension and impaired SMC contraction. PPARγ variants with dominant negative function have been documented in families of humans with insulin resistance, type 2 diabetes, and hypertension. Dominant-negative loss-of-function PPARγ mutations have also been introduced into mice to phenocopy abnormalities identified in humans. In humans, the substitution of a leucine for proline at amino acid 465 (P465L) is associated with severe insulin resistance and significantly reduced PPARγ activity.23 The homologous mutation in mice, P467L, causes hypertension, abnormal fat distribution but not insulin resistance,24 and cerebral vascular dysfunction.25 Thus, mice with the PPARγ-P465L mutation (PPARγL/+ mice) may be a useful model to examine the role of PPARγ signaling in SMC function.

In the present study, we examined the response of primary cultures of SMCs obtained from aortas of mice harboring the PPARγL/+ mutation or sibling controls. We report increases in proliferation and migration that translated into enhanced neointimal hyperplasia following vascular injury in vivo.

Methods

Animals

The generation and genotyping of 129/SvEv mice containing the P465L mutation in PPARγ (PPARγL+/−) has been previously described.26,27 To minimize background-specific effects, the PPARγL/+ mice on the 129/SvEv background were crossed with female C57BL/6J mice from Jackson Laboratory (Bar Harbor, Maine) to generate F1 mice with the same complement of genes varying only in the presence (PPARγL+/+) or absence (PPARγL−/−) of the P465L mutation. Carotid ligation was performed in 8-week-old wild-type PPARγL+/− and PPARγL/− sibling matched mice and analyzed as previously described.26,27 Carotid arteries were stained with combined Mason Trichrome and elastin stain. Serial sections taken at 0.4 mm intervals from the ligation for histomorphometric analysis.

Vascular Smooth Muscle Cells

Aortas from sibling-paired mice aged 4 and 6 weeks old were digested in collagenase (175 U/mL) and their proliferative properties examined. PPARγ activity was attenuated by the introduction of a dominant-negative P465L mutation in PPARγ (PPARγL/−) and PPARγL/− sibling matched mice and analyzed as previously described.26,27 Carotid arteries were stained with combined Mason Trichrome and elastin stain. Serial sections taken at 0.4 mm intervals from the ligation for histomorphometric analysis.

Statistical Analysis

In vitro experiments were performed a minimum of 3 times with cells prepared from at least 3 different cohorts of animals. All results were expressed as mean±SEM. All data were analyzed using nonpaired t test or ANOVA, where indicated, using Sigma-STAT software, version 3.5 (Systat Software Inc). A probability value of less than 0.05 was considered significant.

Results

PPARγL/+ Vascular Smooth Muscle Cells Exhibit Enhanced Proliferation and Migration

A key element in the development of neointima is the proliferation and migration of SMCs from the vessel media to intima. Studies using synthetic PPARγ agonists such as TZDs have identified a potential role for PPARγ as a regulator of SMC proliferation and migration. To probe the normal role of PPARγ in SMC responses, we used a genetic strategy in which PPARγ activity was attenuated by the introduction of a dominant-negative PPARγ mutation in mice. SMCs were isolated from aortas of wild-type mice or heterozygous knock-in mice carrying the dominant negative P465L mutation in PPARγ (PPARγL/−) and their proliferative properties examined. PPARγL/− SMCs displayed a modest 1.6-fold increase in proliferation in response to 10% FBS as compared to wild-type PPARγL/+ cells at 72 hours (P=0.003; supplemental Figure I). The enhanced proliferation was not observed in vehicle-treated PPARγL/+ cells or in the presence of PDGF (supplemental material available online at http://atvb.ahajournals.org).

PPARγL/+ SMCs also displayed enhanced wound closure in a scratch assay (Figure 1A). Twelve hours after a single
Figure 1. Enhanced wound closure in PPARγ+/+ aortic SMCs. SMCs were grown to confluence, serum starved, and then a 400-μm scratch was made through the lawn of confluent cells. After 12 hours, the region containing the scratch was imaged. (A) The number of migrated cells was measured from digital images (B). Significantly more PPARγ+/+ SMCs migrated into the wound (108±4 PPARγ+/+ cells versus 73±3 wild-type PPARγ+/+ cells, P=0.002, Figure 1B), and they migrated a greater distance (132±7 μm for PPARγ+/+ cells compared 101±7 μm for PPARγ+/+ cells; P=0.035; Figure 1C). PDGF (20 ng/mL) increased the number of PPARγ+/+ cells and the distance they migrated and nearly rescued the defect in the PPARγ+/+ cells. In the presence of PDGF, 130±4 PPARγ+/+ cells and 110±2 PPARγ+/+ cells migrated further into the wound than did PPARγ+/+ cells in the absence (P=0.035) but not the presence of PDGF (P=0.49). Values are presented as mean±SE.

scratch injury, significantly more PPARγL/+ SMCs migrated into the wound (108±4 PPARγL/+ cells versus 73±3 wild-type PPARγ+/+ cells, P=0.002, Figure 1B), and they migrated a greater distance (132±7 μm for PPARγL/+ cells compared 101±7 μm for PPARγ+/+ cells; P=0.035; Figure 1C). PDGF (20 ng/mL) increased the number of PPARγ+/+ cells and the distance they migrated and nearly rescued the defect in the PPARγL/+ cells. In the presence of PDGF, 130±4 PPARγL/+ cells and 110±2 PPARγ+/+ cells migrated further into the wound than did PPARγ+/+ cells in the absence (P=0.035) but not the presence of PDGF (P=0.49). Values are presented as mean±SE.

Figure 2. Enhanced migration in PPARγ+/+ SMC. SMCs were stained with Diff-Quik on the undersurface of a membrane with a 5-μm pore after migration to media containing vehicle, 10% FBS, 20 ng/mL PDGF, or 1 μmol/L LPA. Representative images of migrated PPARγ+/+ SMCs (A) and PPARγL/+ SMCs (B). The area occupied by migrated cells is presented as mean±SE (C). Combined results from 3 experiments are shown. *P<0.05 vs vehicle treatment by ANOVA. Values are presented as mean±SE.

(P=0.011) migrated a distance of 158±3 μm and 153±6 μm (P=0.49), respectively (Figure 1C).

The behavior of wild-type PPARγ+/+ (Figure 2A) and mutant PPARγL/+ (Figure 2B) cells was also measured in a chemotactic assay in which the cells migrated toward a gradient of FBS, PDGF, or lysophosphatidic acid (LPA), which, in addition to its well-described actions at cell surface receptors, has been proposed to also serve as a PPARγ agonist.28 In wild-type PPARγ+/+ cells, migration increased 5.2-fold in the presence of FBS and 10-fold with LPA, whereas migration increased ~20-fold with the combination of FBS and LPA (Figure 2C). In comparison to wild-type cells, the migration of PPARγL/+ cells was 6.1-fold higher toward FBS (P=0.002, by ANOVA), 4-fold higher to PDGF (P=0.004 by ANOVA), and 2.6-fold higher to LPA (P=0.002 by ANOVA).

Mitogen-activated protein kinase pathways involving ERK contribute to SMC proliferative and migratory responses. We previously reported that SMC migration to FBS and LPA requires ERK activity.27 In keeping with these observations, the MEK1 inhibitor PD 98059 reduced chemotactic migration of wild-type PPARγ+/+ and PPARγL/+ SMCs by 62% and 58%, respectively (supplemental material). In addition, the TZDs rosiglitazone and pioglitazone inhibited wild-type PPARγ+/+ cell migration by ~50% and 40%. Interestingly, rosiglitazone but not pioglitazone partially blocked PPARγL/+ cell migration by ~40%.

TZDs have been variably proposed to alter ERK activity in SMCs, with some studies suggesting a role for TZDs in...
regulating cytosolic ERK\(^{29}\) and other studies showing an effect downstream of cytosolic ERK.\(^{30,31}\) Therefore, we examined the effects of the PPAR\(\gamma\) P465L mutation on ERK activation in SMCs. In wild-type PPAR\(\gamma^{+/+}\) cells, FBS elicited an increase in ERK activity at 10 minutes that persisted for 60 minutes (Figure 3A). PDGF also increased ERK activity rapidly and resulted in a slight but sustained elevation in ERK activity at 6 hours (Figure 3B). LPA stimulated a rapid increase in ERK phosphorylation that was maximal at 5 to 10 minutes (Figure 3C) and a second phase

Figure 3. Normal ERK activation in PPAR\(\gamma^{+/+}\) SMCs. Time course of activation of ERK, as measured by phosphoERK/total ERK ratios, in cells after exposure to 10% FBS (A), 20 ng/mL PDGF (B), or 1 \(\mu\)mol/L LPA (C). Results are presented as mean±SE and representative of 3 experiments with independent cultures of SMCs of each genotype.

Figure 4. Alterations in ETS-1 in PPAR\(\gamma^{+/+}\) SMC may account for enhanced migration. A, Time course of effects of 20 ng/mL PDGF on ETS-1 levels. Results are presented as mean±SE and are representative of 3 experiments. B, Migration of PPAR\(\gamma^{+/+}\) was performed 72 hours after transfection with scrambled control siRNA or siRNA to ETS-1. Images are representative of results obtained in 3 experiments. Results are graphed as mean±SD *P<0.05.
of late ERK activation. Biphasic ERK activation also occurs in thrombin stimulated SMCs, where the second phase of ERK activation is triggered by HB-EGF expression. No substantial differences were observed in ERK activation in mutant PPARγ/H9253L/H11001 cells in response to FBS (Figure 3A), PDGF (Figure 3B), or LPA (Figure 3C). Based on these results, the enhanced migration observed in the presence of the PPARγ dominant negative mutation is unlikely to be the result of increased ERK activity. Others have reported that PPARγ agonists prevent SMC migration by acting downstream of ERK to block expression of ETS-1. ETS-1 also regulates the ability of PPARγ agonists to inhibit telomerase activity in SMCs. We therefore examined the effects of the dominant-negative PPARγP465L mutation on ETS-1. After stimulation with FBS or PDGF, PPARγL/H11001 cells upregulated ETS-1 to a significantly greater extent at earlier (≈1 hour; P=0.028) and later (≈12 hours; P=0.041) time points than did PPARγ+/+ cells (Figure 4A). Moreover, downregulation of ETS1 using RNA-dependent gene silencing (siRNA) blunted the enhanced PPARγL/+ cell migration (Figure 4B).

PPARγL/+ Mice Show Increased Neointimal Formation During Vascular Remodeling

To determine whether the enhanced SMC proliferation and migration observed in PPARγL/+ SMCs in culture also occurs in vivo, we examined the response to arterial injury in PPARγ+/+ and PPARγL/+ mice using a well-characterized mouse model in which robust development of intimal hyperplasia occurs. At 4 weeks after carotid injury, intimal hyperplasia develops along the length of the vessel (Figure 5A and 5B). In response to injury, PPARγL/+ mice develop more extensive neointima with a statistically greater intima/media ratio (Figure 5A and 5B; n=8 PPARγ+/+, n=9 PPARγL/+). At 10 mm from the ligation, the PPARγL/+ vessels display a 4.3-fold increase in neointima formation compared to the wild-type PPARγL/+ vessel (P=0.005 by ANOVA). The difference in intima:media ratio persisted until 24 mm from the ligation (Figure 5B). No difference in lumen (P=0.317; Figure 5C) or medial (P=0.447; Figure 5D) areas were observed between mice of different genotypes. Consistent with the observations in isolated cells, ETS-1 expression was higher in PPARγL/+ vessels after injury (Figure 6).

Discussion

Studies in animal models have shown that PPARγ agonists inhibit atherosclerotic development. However, the precise role for PPARγ signaling in vascular SMCs remains unclear. In this report, we used a strain of mice harboring a dominant-negative acting PPARγ allele that suppresses endogenous
PPARγ function to probe the normal role of PPARγ signaling in SMC phenotypic modulation that is important for atherosclerosis and restenosis. SMCs with the PPARγ P465L mutation display dramatically enhanced migration and a modest increase in proliferation in vitro assays. These in vitro observations appear relevant in vivo because mice with the PPARγ P465L mutation exhibit exaggerated development of intimal hyperplasia after arterial injury. Upregulation of ETS-1 correlates with the enhanced migration in PPARγ L/−/− SMCs, and transient reduction of ETS-1 inhibited PPARγ L/−/− SMC migration. ETS-1 levels were also higher in PPARγ L/−/− arteries after injury. Our findings are consistent with previous demonstrations that ETS-1 mediates the effects of PPARγ agonists on migration and telomerase activity. Interestingly, although ERK activation contributes to SMC migration, we did not observe substantial differences in ERK activation in WT and PPARγ L/−/− SMCs. Others have also suggested that the antimigratory effects of TZDs occur downstream of ERK.

Our results are consistent with a normal role for PPARγ in attenuating injury-induced SMC proliferation, migration, and vascular remodeling. Our findings are in agreement with previous studies documenting inhibition of neointimal formation by TZD agonists in animal models and a slowing of progression of carotid intima-media thickness by TZDs in individuals with type 2 diabetes. The beneficial effects of TZDs in nondiabetic preclinical models and our results in nondiabetic mice suggest that the reduction in intimal hyperplasia associated with PPARγ activity is not simply a consequence of normalizing blood glucose and other metabolic abnormalities of diabetes. Because the PPARγ P465L mutation may affect signaling through other PPAR isoforms, we cannot exclude a role of PPARα or PPARδ in regulating SMC responses. However, our results, when considered in the context of the beneficial effects of TZDs, are most consistent with a central role of PPARγ in attenuating SMC proliferative and migratory responses in the context of vascular injury. Although our results suggest a direct role for PPAR signaling in SMCs, effects in other vascular cells, such as monocytes and endothelial cells, may also indirectly influence SMC function after arterial injury. Together with published experiments using pharmacological approaches, our observations in a genetic model of altered PPARγ activity support a role for endogenous PPARγ in modulating important pathophysiological processes underlying restenosis and atherosclerosis.

Surprisingly, rosiglitazone but not pioglitazone was able to suppress migration and proliferation in cells from mice with the PPARγ P465L mutation. The lack of effect of pioglitazone was specific, in that under identical conditions, both pioglitazone and rosiglitazone inhibited wild-type SMC migration and proliferation. These findings would be consistent with rosiglitazone eliciting “off-target” effects that are not mediated by PPARγ. In this context it is interesting to note that clinical trials have tended to show a beneficial vascular effect of pioglitazone but not of rosiglitazone. It is possible, however, that the PPARγ L/−/− cells have low levels of PPARγ activity that for unknown reasons are selectively enhanced by rosiglitazone but not pioglitazone.

Our findings are also relevant to the role of the lysolipid mediator LPA in regulating SMC responses. In addition to acting on G protein–coupled receptors, LPA has been proposed as an endogenous agonist of PPARγ. In contrast to the inhibitory effects on the development of intimal hyperplasia reported with the TZD class of agonists, infusion of LPA into rodent carotid arteries has been associated with the formation of neointimal hyperplasia in a PPARγ-dependent manner. We observed enhanced LPA-migration in SMCs with the dominant-negative loss-of-function PPARγ L/−/− mutation, which appears to be nonselective with regards to agonists in that the PPARγ L/−/− SMC migration to PDGF was also enhanced. Our results suggest that LPA, FBS, and PDGF–promoted chemotactic migration of SMCs is normally inhibited by PPARγ-dependent pathways. Whether PPARγ mediates other effects of LPA on vascular SMC function or regulates the effects of endogenous LPA is not known.

In conclusion, our findings are consistent with a normal role for PPARγ in attenuating SMC migratory and proliferative properties which translate into beneficial vascular effects in terms of limiting the development of neointima. If future studies substantiate the initial reports of an association of at least one TZD with higher rates of adverse cardiovascular events, our results might suggest that the adverse cardiovascular profile for TZDs are attributable either to “off-target” effects or are the result of proatherothrombotic effects mediated by PPARγ agonism in other blood and vascular cells.

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**Disclosures**

None.

**References**


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