Disruption of endothelial peroxisome proliferator-activated receptor-γ reduces vascular nitric oxide production

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Kleinhenz JM, Kleinhenz DJ, You S, Ritzenthaler JD, Hansen JM, Archer DR, Sutliff RL, Hart CM. Disruption of endothelial peroxisome proliferator-activated receptor-γ reduces vascular nitric oxide production. Am J Physiol Heart Circ Physiol 297: H1647–H1654, 2009. First published August 7, 2009; doi:10.1152/ajpheart.00148.2009.—Vascular endothelial cells express the ligand-activated transcription factor, peroxisome proliferator-activated receptor-γ (PPARγ), which participates in the regulation of metabolism, cell proliferation, and inflammation. PPARγ ligands attenuate, whereas the loss of function mutations in PPARγ stimulate, endothelial dysfunction, suggesting that PPARγ may regulate vascular endothelial nitric oxide production. To explore the role of endothelial PPARγ in the regulation of vascular nitric oxide production in vivo, mice expressing Cre recombinase driven by an endothelial-specific promoter were crossed with mice carrying a floxed PPARγ gene to produce endothelial PPARγ null mice (ePPARγ−/−). When compared with littermate controls, ePPARγ−/− animals were hypertensive at baseline and demonstrated comparable increases in systolic blood pressure in response to angiotensin II infusion. When compared with those of control animals, aortic ring relaxation responses to acetylcholine were impaired, whereas relaxation responses to sodium nitroprusside were unaffected in ePPARγ−/− mice. Similarly, intact aortic segments from ePPARγ−/− mice released less nitric oxide than those from controls, whereas endothelial nitric oxide synthase expression was similar in control and ePPARγ−/− aortas. Reduced nitric oxide production in ePPARγ−/− aortas was associated with an increase in the parameters of oxidative stress in the blood and the activation of nuclear factor-κB in aortic homogenates. These findings demonstrate that endothelial PPARγ regulates vascular nitric oxide production and that the disruption of endothelial PPARγ contributes to endothelial dysfunction in vivo.

endothelial nitric oxide synthase

THE LIGAND-ACTIVATED transcription factor, peroxisome proliferator-activated receptor-γ (PPARγ), is a member of the nuclear hormone receptor superfamily. PPARγ is expressed in numerous cells and tissues including endothelial (34) and smooth muscle cells (30, 35) comprising the vascular wall. PPARγ is activated by a diverse spectrum of ligands including fatty acids and their derivatives and by synthetic ligands from the thiazolidinedione (TZD) class of medications such as rosiglitazone and pioglitazone. These drugs are widely employed in the treatment of type 2 diabetes where they enhance insulin sensitivity through the modulation of lipid metabolism in fat, liver, and skeletal muscle (19). In addition to insulin sensitization, TZD-mediated activation of PPARγ modulates inflammation as well as cell signaling and proliferation (54), suggesting that the activation of PPARγ in vascular endothelial and smooth muscle cells can modulate vascular function independent of insulin sensitization (17).

Consistent with this concept, TZDs have been reported to exert beneficial effects in experimental animal models of non-diabetic vascular disease (8, 10, 17, 31) and to reduce surrogate markers of vascular dysfunction in several clinical studies. TZD therapy in animal and human subjects improved endothelial function; reduced surrogate markers of vascular disease; improved flow-mediated, endothelium-dependent vasodilation; and reduced carotid intimal thickening and neointimal formation after coronary stent placement (1, 11, 13, 29, 39, 41, 52, 55). The vascular protective effects of TZDs have been reported in nondiabetic subjects with documented coronary disease (36, 49), cerebrovascular disease (38), or risk factors for atherosclerosis (7). In diabetic subjects, rosiglitazone may worsen (22, 44, 48), whereas pioglitazone reduced (15, 18, 33, 57), cardiovascular end points. Additional clinical trials will be required to further clarify the impact of TZD therapy on cardiovascular outcomes. Further clarification of the role of PPARγ in the regulation of vascular function will advance efforts to optimize the therapeutic potential of PPARγ activation in vascular disease.

While PPARγ stimulation appears to be vasoprotective, the disruption of PPARγ within the vascular wall may promote vascular dysfunction. Dominant negative mutations in human PPARγ are associated with hypertension and insulin resistance (3), conditions commonly associated with endothelial dysfunction. Emerging studies in genetically engineered mice have provided additional evidence for an important regulatory role of PPARγ in the vasculature. Although a global deletion of PPARγ results in embryonic lethality (2), PPARγ knockout mice that are rescued by preserving PPARγ expression in the trophoblast demonstrate a complex phenotype characterized by severe lipodystrophy, insulin resistance, and hypotension (16). In heterozygous mice with global dominant negative PPARγ expression, the cerebral vessels demonstrated endothelial dysfunction and an enhanced vascular remodeling that could be attenuated with superoxide inhibitors (4). Similarly, targeting dominant negative PPARγ expression to smooth muscle cells impaired nitric oxide (NO)-mediated vasodilation and caused systolic hypertension (21), whereas targeted interference with endothelial PPARγ led to enhanced endothelial dysfunction in response to high-fat diets (5). Similarly, endothelial PPARγ null (ePPARγ−/−) mice, created using the Cre-loxP system (42), were sensitized to the hypertensive effects of a high-fat diet, and treatment with rosiglitazone, while lowering blood pressure in control mice, did not lower blood pressure in ePPARγ−/− mice (43). These studies suggest that PPARγ...
plays a critical role in the regulation of normal vascular function and tone although the mechanisms for these effects remain to be defined.

NO, a critical endothelial-derived mediator, reduces vessel tone, platelet activation and aggregation, smooth muscle cell proliferation, and leukocyte adherence. Under pathophysiologic conditions, the biological activity of NO is reduced. Previous in vitro studies demonstrated that the activation of PPARγ in vascular endothelial cells increased endothelial NO release (6, 9) by increasing the activity, but not the expression, of endothelial NO synthase (eNOS) through PPARγ-dependent mechanisms involving posttranslational eNOS regulation (46).

The current study extends previous reports to further clarify the role of endothelial PPARγ in normal vascular function in vivo. Our findings indicate that a disruption of endothelial PPARγ reduces NO production in the vascular wall and causes oxidative stress and vascular dysfunction. These findings emphasize the importance of endothelial PPARγ in the regulation of normal vascular function and suggest that this receptor may serve as a target for novel pharmacological approaches to improving endothelial dysfunction.

MATERIALS AND METHODS

Generation of ePPARγfl/fl mice. All studies were performed according to protocols reviewed and approved by the Atlanta Veterans Affairs Medical Center Animal Care and Use Committee. Mice from the C57Bl/6 strain with the loxP-flanked (floxed) PPARγ allele were obtained from the Jackson Laboratory (Bar Harbor, ME). The floxed PPARγ allele was limited to the vascular endothelium, the aortas of male and female animals were fixed in 0.2% glutaraldehyde and stained with ethidium bromide, and visualized on a UV light box.

Blood pressure monitoring and angiotensin II treatment. Data Sciences International (St. Paul, MN) PA-C10 blood pressure probes were used for telemetric blood pressure monitoring as previously reported (28) (see online data supplement for experimental details). Blood pressures were monitored for 10 s each minute for two 24-h periods, and these results were analyzed by two-way ANOVA with repeated measures or averaged over that duration to obtain baseline hemodynamic data. In selected ePPARγ−/− and control animals, osmotic pumps (Alzet, Cupertino, CA) were implanted for subcutaneous infusion of angiotensin II (ANG II, 0.7 mg·kg⁻¹·day⁻¹). Sigma), an intervention previously shown to generate hypertension and endothelial dysfunction in the mouse (40). Hemodynamic data were then collected 4, 6, 8, and 10 days following the onset of ANG II infusion. Isometric forces in aortic rings were measured as described previously (51). Aortic rings were threaded onto two triangular stainless steel wires and then mounted on hooks attached to a Harvard Apparatus (Holliston, MA) differential capacitor force transducer. The resting tension of each aortic ring was set to 40 mN and maintained throughout the experiment. Relaxation responses to graded concentrations of the endothelium-dependent vasodilator, acetylcholine, and to the endothelium-independent vasodilator, sodium nitroprusside, were determined in aortic rings contracted with l-phenylephrine. In selected studies, the relaxation responses of rings from littermate control and ePPARγ−/− mice were examined following an ex vivo treatment with rosiglitazone (10 μM for 1 h) or Tempol (1 mM for 30 min) in the muscle bath. Data were obtained using MP100W hardware and analyzed using AcqKnowledge software (Biopac, Goleta, CA).

Measurement of aortic NO production using electron spin resonance spectroscopy. NO production was determined by incubating intact or demyled aortic segments with the NO spin trap, iron diethyldithiocarbamic acid [Fe(DETC)₂] and electron spin resonance (ESR) spectroscopy as reported (28). Detailed methods are provided in the online data supplement. The NO signal was derived from the amplitude of the peaks from the triplet ESR signal characteristic of the NO-Fe(DETC)₂ complex. ESR signals were normalized to the amount of aortic protein in each sample.

Electrophoretic mobility shift assay. Nuclear proteins were isolated from the aortas of littermate control and ePPARγ−/− mice and subjected to electrophoretic mobility shift assay analysis to examine the extent of NF-κB nuclear binding as previously reported (47) (see online data supplement for detailed methods).

Assays of oxidative stress in blood. Serum was assayed for derivatives of reactive oxygen metabolites (dROMs) by colorimetric assay, according to the manufacturer’s protocol (Diacron International, Grosseto, Italy). Plasma cysteine (Cys) and cystine (CySS) levels, the major plasma small molecular weight thiol couple, were measured as an index of oxidative stress with HPLC and fluorescence detection as previously reported (25). Plasma redox potential (E₀) was calculated using the Nernst equation, E₀ = Eₒ + RT ln [disulfide]/[thiol]², where Eₒ is the respective standard potential for the redox couple at pH 7.4, R is the gas constant, T is the absolute temperature, n is 2 (the number of electrons transferred), and F is Faraday’s constant. The standard potential for the Cys/CySS couple at pH 7.4 was −250 mV.

Statistical analysis. Statistical analyses of mean arterial pressure (MAP), contractility, and glucose tolerance experiments were performed using two-way ANOVA with repeated measures and Bonferroni’s posttest. A statistical analysis of NO levels by Fe(DETC)₂ was performed using one-way ANOVA with Newman-Keuls posttest. Statistics on all other experiments were performed using an unpaired
The ePPARγ−/− mice carry a Tie2-driven Cre gene identified by a 100-bp Cre PCR product (Fig. 1A) and two floxed PPARγ alleles identified by a 250-bp PPARγ PCR product (Fig. 1B). The specificity of Cre-recombinase expression in the vascular endothelium was supported by studies in Fig. 1C where the detection of the null PPARγ allele by PCR could be eliminated from aortas by a mechanical denudation of the endothelium. To further analyze Tie2-driven Cre recombinase expression, Tie2-Cre mice were bred with R26R mice, which express β-galactosidase activity only in tissues where Cre recombinase is expressed. A gross and microscopic examination of aortas from R26R+/−/Tie2-Cre−/− mice demonstrated no evidence of β-galactosidase activity (Fig. 1, D and F). In contrast, R26R+/−/Tie2-Cre+/− mice displayed intense β-galactosidase activity (blue staining) in the vascular endothelium.

**RESULTS**

- **A**
  - floxed PPARγ
  - Tie2Cre
  - ePPARγ−/
  - Control

- **B**
  - 100 bp

- **C**
  - 200 bp
  - Aorta 1
  - Aorta 2
  - Aorta 3
  - Aorta 4
  - Int
  - Den

- **D**
  - 300 bp

- **E**
  - 100 bp

- **F**

- **G**

All analyses were performed using GraphPad Prism v. 4.03 (GraphPad Software, San Diego, CA).

As previously reported, ePPARγ−/− mice were viable, fertile, and normal in size compared with littermate control animals (supplemental Table 1) (43). Although the average body weights of ePPARγ−/− mice were ~5% less than littermate controls, their fasting blood glucose levels and glucose tolerance tests were comparable with littermate controls (supplemental Table S1 and supplemental Fig. S2, respectively). A gross and microscopic analysis of the heart, liver, kidneys, lungs, and testicles revealed no differences between littermate control and ePPARγ−/− animals (supplemental Fig. S1, and data not shown). The organ weights were comparable between littermate control and ePPARγ−/− mice with the exception of the spleens that were significantly larger in ePPARγ−/− mice than in littermate controls (supplemental Table S1), consistent with depletion of endothelial rather than nonendothelial compartments.

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with a recent report demonstrating that Tie2-Cre-mediated PPARγ depletion caused splenic extramedullary hematopoiesis related to impaired osteoclast differentiation and reduced bone marrow medullary space (56). A hematological analysis of ePPARγ−/− mice also revealed a slight but significant reduction in hematocrit relative to littermate controls (supplemental Table S1).

To examine arterial pressure, telemetric blood pressure monitors were surgically implanted into 8-wk-old ePPARγ−/− and littermate control mice. In contrast to a previous study examining blood pressure in this model using tail-cuff measurements (43), ePPARγ−/− mice displayed higher baseline MAPs compared with those of littermate control animals. The MAPs of ePPARγ−/− mice were consistently higher than those of littermate controls whether examined throughout the diurnal cycle (Fig. 2A) or averaged over a 24-h period (Fig. 2B). After the baseline MAPs were obtained, selected animals were challenged with ANG II (0.7 mg·kg−1·day−1 subcutaneously via osmotic pump), a vasoconstrictive stimulus known to induce endothelial dysfunction. As expected, ANG II infusion caused progressive increases in MAP in both littermate control and ePPARγ−/− animals. The MAPs of ePPARγ−/− mice were consistently higher than those of littermate controls whether examined throughout the diurnal cycle (Fig. 2A) or averaged over a 24-h period (Fig. 2B). After the baseline MAPs were obtained, selected animals were challenged with ANG II (0.7 mg·kg−1·day−1 subcutaneously via osmotic pump), a vasoconstrictive stimulus known to induce endothelial dysfunction. As expected, ANG II infusion caused progressive increases in MAP in both littermate control and ePPARγ−/− mice. Consistent with the elevated baseline MAP in ePPARγ−/− mice, Fig. 2C illustrates that ePPARγ−/− mice also displayed higher MAP during ANG-II infusion compared with control mice (Fig. 2C) although this difference did not achieve a statistical significance.

To explore whether endothelial PPARγ regulates vasorelaxation responses, aortic rings from ePPARγ−/− and littermate control animals were mounted onto force transducers for the determination of endothelium-dependent and endothelium-independent vasorelaxation. The rings were first precontracted with l-phenylephrine to 80% maximal contraction. No difference was found in the sensitivity to l-phenylephrine-induced ring contraction between ePPARγ−/− and control animals (data not shown). As illustrated in Fig. 3A, when compared with those from littermate controls, aortic rings from ePPARγ−/− mice demonstrated an impaired endothelium-dependent, acetylcholine-stimulated vasorelaxation. Acetylcholine-mediated relaxation was fully inhibited in both control and ePPARγ−/− rings by a preincubation with the NO synthase inhibitor, NG-nitro-l-arginine methyl ester, confirming that these acetylcholine-induced relaxations were NO dependent (data not shown). In addition, an acute addition of the PPARγ ligand, rosiglitazone, to the muscle bath of aortic rings from control and ePPARγ−/− animals failed to alter the relaxation responses (supplemental Fig. S3), consistent with the absence of acute nonspecific or nongenomic effects of PPARγ activation on vascular regulation. In contrast, relaxation responses to the endothelium-independent vasodilator, sodium nitroprusside, were comparable in ePPARγ−/− and littermate control vessels (Fig. 3B). Impaired endothelium-dependent vasorelaxation in ePPARγ−/− mice suggested a reduced NO bioavailability in these vessels. To further examine these findings, ePPARγ−/− and control aortas were removed and incubated with the NO spin trap, Fe(DETC)2, to directly measure vascular NO release from these vessels (14, 28). In aortas from selected control animals, the luminal surface of the vessel was exposed and gently rubbed to remove the endothelium to provide a negative control. When compared with those from littermate controls, aortas from ePPARγ−/− mice released significantly less NO (Fig. 4), approaching levels observed in denuded control vessels. However, reductions in NO production observed in vessels from ePPARγ−/− mice

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**Fig. 2.** Blood pressure in control and ePPARγ−/− mice. Telemetric blood pressure monitors were surgically implanted into 8-wk-old ePPARγ−/− and littermate control mice. Hemodynamic data were collected for 2 days, and baseline mean arterial pressures (MAPs) were calculated. A: MAP over a 24-h period. Each point represents mean MAP ± SE (in mmHg) from 11 control and 8 ePPARγ−/− mice. When compared with that of controls, the MAP of ePPARγ−/− mice was significantly higher. B: each bar represents average mean MAP ± SE over 24 h (in mmHg) from 11 control and 8 ePPARγ−/− mice. *P < 0.05 vs. control. C: after baseline blood pressure was measured, an osmotic minipump containing angiotensin II (ANG II) was implanted into selected mice from each group, and blood pressures were recorded 4, 6, 8, and 10 days following the onset of ANG II infusion. Each point represents the mean MAP ± SE (in mmHg) from 3 control and 5 ePPARγ−/− mice during ANG II infusion.
were not associated with significant reductions in the level of eNOS in the vascular wall (supplemental Fig. S4).

The absence of endothelial PPARγ also caused vascular oxidative stress. When compared with littermate controls, ePPARγ/−/− mice had significant reductions in plasma levels of Cys, whereas CySS levels were unchanged, resulting in an increased plasma redox potential (Fig. 5, A–C). This index of oxidative stress was supported by higher levels of dROMs in ePPARγ/−/− mice than in littermate controls (Fig. 5D). These markers of vascular oxidative stress were associated with evidence of an enhanced nuclear NF-κB binding activity in aortas from ePPARγ/−/− compared with control aortas (Fig. 6).

DISCUSSION

Our laboratory recently reported that PPARγ ligands stimulated the production of NO by endothelial cells in vitro through PPARγ-dependent mechanisms involving an increase in the activity, but not the expression, of eNOS (46). In addition, PPARγ ligands inhibited endothelial cell superoxide production and expression of selected subunits of the superoxide-generating enzyme, NADPH oxidase (23), a critical mediator of endothelial dysfunction (32). These findings suggested that endothelial PPARγ might regulate a program of gene expression in the vascular wall that coordinately controls the production of reactive oxygen and nitrogen species that participate in vascular function. The PPARγ ligand, rosiglitazone, also potently reduced vascular NADPH oxidase expression and superoxide production in vivo (24), providing additional evidence that PPARγ participates in the control of nitroso-redox balance in the vasculature.

The current study used a genetic approach to disrupt vascular endothelial PPARγ to further explore its role in the regulation of vascular function. Previously, ePPARγ/−/− mice were not hypertensive at baseline but were more susceptible to hypertension induced by high-fat diets. Furthermore, the disruption of endothelial PPARγ abrogated the ability of rosiglitazone therapy to lower blood pressure in animals fed high-fat diets (43), suggesting that the direct activation of endothelial PPARγ played a critical role in mediating the vascular effects of PPARγ ligands. The current study extends these findings to demonstrate that a disruption of endothelial PPARγ has significant effects on blood pressure, vascular relaxation, NO production, oxidative stress, and inflammation without altering insulin sensitivity. Our findings using telemetry to measure...
While our findings do not exclude the participation of additional mechanisms in the vascular dysfunction caused by endothelial PPARγ disruption, they emphasize the important relationship between endothelial PPARγ and vascular NO production.

Several limitations of these studies should be recognized. First, the Cre-lox approach employed to delete endothelial PPARγ in the current model could induce compensatory alterations in the expression of other vasoregulatory pathways that modulate the phenotype of this animal. Future studies employing strategies that permit inducible endothelial PPARγ disruption may permit a more accurate assessment of the vascular impact of endothelial PPARγ disruption in the absence of, or before, the development of such compensatory processes. Second, Tie2-Cre expression caused an efficient disruption of PPARγ in hematopoietic tissues in addition to vascular endothelial cells (56). Therefore, the contributions of PPARγ disruption in bone marrow-derived cells to the vascular derangements reported in the current study cannot be fully excluded. However, the similarity of vascular derangements in the current study with those in cerebral resistance vessels caused by targeted PPARγ interference using the vascular endothelial cadherin promoter to mediate endothelial specificity (5) suggests that PPARγ expressed in the vascular endothelium plays a critical role in the maintenance of normal vascular endothelial function.

In summary, this study demonstrates that PPARγ is required for normal vascular NO production. The targeted disruption of PPARγ was sufficient to cause mild hypertension, impair aortic endothelium-dependent vasodilation and NO production, and generate vascular oxidative stress and the activation of a proinflammatory transcription factor in the vascular wall. These findings suggest that the disruption of endothelial PPARγ may contribute to vascular dysregulation in clinical conditions associated with endothelial dysfunction. This study also provides additional evidence supporting the link between PPARγ and the production of NO in the vasculature, suggesting that selective PPARγ modulators may provide a novel therapeutic approach to restoring vascular function in conditions associated with reduced NO bioavailability and endothelial dysfunction.

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