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Dominant negative PPARγ promotes atherosclerosis, vascular dysfunction, and hypertension through distinct effects in endothelium and vascular muscle

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Pelham CJ, Keen HL, Lentz SR, Sigmund CD. Dominant negative PPARγ promotes atherosclerosis, vascular dysfunction, and hypertension through distinct effects in endothelium and vascular muscle. Am J Physiol Regul Integr Comp Physiol 304: R690–R701, 2013. First published February 27, 2013; doi:10.1152/ajpregu.00607.2012.—Agonists of the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) have potent insulin-sensitizing effects and inhibit atherosclerosis progression in patients with Type II diabetes. Conversely, missense mutations in the ligand-binding domain of PPARγ that render the transcription factor dominant negative (DN) cause early-onset hypertension and Type II diabetes. We tested the hypothesis that DN PPARγ-mediated interference of endogenous wild-type PPARγ in the endothelium and vascular smooth muscle exacerbates atherosclerosis in apolipoprotein E-deficient (ApoE−/−) mice. Endothelium-specific expression of DN PPARγ on the ApoE−/− background unmasked significant impairment of endothelium-dependent relaxation in aortic rings, increased systolic blood pressure, altered expression of atherogenic markers (e.g., Cds3, Mcp1, Catalase), and enhanced diet-induced atherosclerotic lesion formation in aorta. Smooth muscle-specific expression of DN PPARγ, which induces aortic dysfunction and increased systolic blood pressure at baseline, also resulted in enhanced diet-induced atherosclerotic lesion formation in aorta on the ApoE−/− background that was associated with altered expression of a shared, yet distinct, set of atherogenic markers (e.g., Cds3, Mcp1, Osteopontin, Vcam1). In particular, induction of Osteopontin expression by smooth muscle-specific DN PPARγ correlated with increased plaque calcification. These data demonstrate that inhibition of PPARγ function specifically in the vascular endothelium or smooth muscle may contribute to cardiovascular disease. PPARγ; atherosclerosis; endothelium; smooth muscle

RISK FACTORS for cardiovascular diseases encompass many components of metabolic syndrome, including hypertension, obesity, diabetes, and atherosclerosis. The ligand-activated nuclear hormone receptor PPARγ plays critical roles in each of these. Treatment of Type II diabetes patients with thiazolidinedione (TZD) drugs, high-affinity agonists of PPARγ, has been shown to improve insulin sensitivity, reduce blood pressure, and decrease carotid intima-media thickness and coronary atheroma volume (30, 33). Likewise, TZD treatment inhibits atherosclerotic lesion formation in aorta and aortic root of apolipoprotein E (ApoE)-deficient and low-density lipoprotein receptor (LDLR)-deficient mouse models of hypercholesterolemia (12, 24). The observation that TZDs exert some cardiovascular protective effects is consistent with genetic evidence indicating that subjects who carry dominant negative (DN) mutations develop early-onset Type II diabetes and hypertension (2). Despite the antiatherosclerotic effects of PPARγ activation, TZD treatment is also associated with adverse effects including increased incidence of systemic edema, making them contraindicated for patients with heart failure (18).

Because PPARγ is expressed in monocytes/macrophages, T lymphocytes, vascular endothelial cells, and smooth muscle cells, all of which comprise atherosclerotic lesions, it remains unclear which cell type is the target of the beneficial actions of TZD. It also remains unclear to what extent the anti-atherosclerotic effects of TZD treatment are due to changes in systemic metabolism (e.g., glycemic control) as opposed to direct activation of PPARγ on cells encompassing the lesion. Previous studies have made use of the Cre-loxP system to generate mice lacking PPARγ specifically in cells encompassing atherosclerotic lesions, including endothelial and smooth muscle cells. Disruption of endothelial PPARγ in LDLR-deficient mice resulted in upregulation of pro-inflammatory genes in aorta, hypertension, and increased atherosclerosis (38). Smooth muscle-specific PPARγ-deficient LDLR−/− mice treated with ANG II displayed increased atherosclerotic lesion area in aorta (41). While the TZD pioglitazone significantly attenuated the increase in atherosclerosis induced by ANG II in control LDLR−/− mice, deletion of PPARγ in smooth muscle abrogated this effect. This suggests that the beneficial anti-atherosclerotic effects of TZD may be mediated by its actions on vascular smooth muscle.

One of the limitations of the PPARγ knockout approach is the induction (or derepression) of actively repressed target genes, resulting from abrogation of PPAR-dependent active repression. In the absence of ligand, PPAR/retinoid X receptor heterodimers on chromatin bind with corepressors to silence transcription of PPARγ target genes. Ligand activation stimulates replacement of corepressors with coactivators allowing transcription (25). Thus knockout of PPARγ could potentially mimic TZD-mediated activation of some genes. Indeed, knockout of PPARγ in smooth muscle induced expression of the
PPARγ target genes encoding β2 adrenergic receptor and cathepsin S (10, 17). To overcome the potential limitations of PPARγ deficiency in this study, we employed rare but naturally occurring DN mutations in the ligand-binding domain of PPARγ (i.e., V290M and P467L) that in heterozygous patients cause early-onset hypertension, partial lipodystrophy, and Type II diabetes (2). Both mutations cause impaired basal and agonist-induced transcriptional activity and repression of many PPARγ target genes (2, 21, 25). Knock-in mice homozygous for an equivalent DN mutation (P465L) die in utero (44), whereas heterozygous mice are hypertensive and exhibit cerebrovascular dysfunction and remodeling (4, 44). In contrast to the effects of PPARγ agonists, aortic smooth muscle cells isolated from these mice exhibited increased proliferation and migration (31). Atherosclerotic lesion area at the aortic root was not significantly altered in ApoE-deficient mice carrying the P465L PPARγ mutation in all cells though effects on systemic metabolism (i.e., reduced plasma triglycerides) are confounding factors (36).

We previously showed that transgenic mice that express DN PPARγ under control of the endothelium-specific vascular endothelial cadherin (Ve-cad) promoter (E-V290M or E-P467L mice) exhibited normal endothelial function in the basilar artery and aorta and normal blood pressure (5). High-fat diet feeding unmasked impaired endothelium-dependent vasodilation in basilar artery and aorta from E-V290M and E-P467L mice through a mechanism involving oxidative stress. Thus, consistent with its role as a lipid sensor, endothelial PPARγ protects the vessel against the harmful effects of a high-fat diet. In contrast, transgenic mice that express DN PPARγ under control of the smooth muscle-specific smooth muscle myosin heavy chain (SMMHC) promoter (S-V290M or S-P467L mice) displayed a significant increase in systolic blood pressure as well as enhanced agonist-mediated contraction in aorta and enhanced myogenic tone in resistance mesenteric arteries through mechanisms involving RhoA/Rho-kinase and protein kinase C, respectively (16, 35). In the current study, we crossed the E-V290M and S-P467L transgenic mouse models to ApoE-deficient mice to test the hypothesis that endothelial- or smooth muscle cell-specific interference with PPARγ exacerbates atherosclerosis.

MATERIALS AND METHODS

Transgenic mouse models. Generation of transgenic mice (S-P467L and E-V290M) and confirmation of tissue specificity of transgene expression was described previously (5, 16). Both the P467L and V290M mutations in PPARγ destabilize helix 12 and act in a DN manner (2). We have reported that both the P467L and V290M mutants act similarly when placed under either promoter and therefore function interchangeably (5, 16). ApoE−/− mice were obtained from The Jackson Laboratory. S-P467L, E-V290M, and E-P467L mice were maintained by backcross breeding to C57BL/6J for at least 10 generations. S-P467L and E-V290M transgenic mice were crossed for two successive generations to ApoE−/− mice. Genotyping on the human PPARγ transgene and ApoE alleles was performed by PCR of tail DNA using the following primers: S-P467L transgene: 5′-TATCTTCTAATCTGGTTGGTGTTG-3′ and 5′-GACGAGGATTTAGTTGCTGTTCA-3′; E-V290M transgene: 5′-CAGCTCTACAAAGGAAACAATAACAG-3′ and 5′-CTCCATAGGTGAATTTCCAGAAG-3′; and ApoE gene: 5′-GCCTAGCGCGGAGAGGCGG-3′ and 5′-TGTGTCGCGTGGACCTGCAGC-3′. Care of the mice used in the experiments met the standards set forth by the National Institutes of Health (NIH) guidelines for the care and use of experimental animals. All procedures were approved by the University of Iowa Institutional Animal Care and Use Committee.

Mouse diets. One cohort of mice was fed standard mouse chow diet (7013, Teklad Premier Laboratory Diets) and water ad libitum and euthanized at 24 wk of age to examine spontaneous atherosclerosis. To induce extensive plaque formation, a separate cohort of mice was fed an atherogenic “Western diet” (TD.88137, Harlan Teklad; high-fat, 21%; high-cholesterol, 0.2%) and water ad libitum starting at 8 wk until 24 wk of age. All experiments were carried out using similar numbers of male and female age-matched and sex-matched mice.

Aortic lesion analysis. Mice (24 wk of age) were given a lethal dose of pentobarbital (50 mg/mouse ip) and were perfused with phosphate-buffered saline (PBS) through a cannula inserted into the left ventricle, with perfusate draining from the severed right atrium (15). The aorta was dissected extending from the aortic arch to the iliac bifurcation, placed in fixative overnight (4% paraformaldehyde in PBS), and carefully dissected free of adventitial fat. Then the aorta was cut longitudinally and pinned to a black wax surface to expose the intimal surface. Oil Red O was used to stain lipids, and an image was recorded (15). Lesion area was quantified in the aorta by en face measurement as percentage of surface area using NIH Image J. Plaque composition was analyzed by histology of the aortic root. As above, the base of the heart from perfused animals was fixed and embedded in paraffin. Serial sections (8 μm) spanning from the origin of the aortic valve leaflets to the ascending aortic arch were mounted on slides. Aortic root sections were stained for elastic fibers by the Verhoeff-Van Gieson (VVG) method or stained for calcification by Alizarin Red. Total intimal lesion area (mm²) per cross section was quantified by drawing boundary lines using NIH Image J and calculated as the mean of 4 VVG-stained sections (80 μm apart), spanning 320 μm of the aortic root (47). Necrotic area was quantified by measuring the clear acellular/anuclear areas within lesions from VVG-stained sections (43). Calcification was evaluated by quantifying the Alizarin Red-positive areas within lesions from Alizarin Red-stained sections.

Metabolic analyses. Mice (24 wk of age) were fasted (overnight), and at the time of euthanasia, body mass and organ masses were recorded and plasma samples were collected and stored at −80°C. Blood glucose levels were determined using an Accu-Check meter (Roche). Plasma total triglyceride levels were measured enzymatically using Free Glycerol Reagent (Sigma) and Triglyceride Reagent (Sigma). Plasma total cholesterol was also measured enzymatically using Infinity Cholesterol Reagent (Thermo Electron) and cholesterol reference standards (Verichem).

Aortic ring wire myograph preparation. Vessel function was examined using a wire myograph preparation of thoracic or distal abdominal aorta segments (16) from mice (24 wk of age) on standard diet. The aorta was removed and placed in oxygenated Krebs physiological buffer, dissected free of perivascular fat, and cut into two abdominal aortic rings and two thoracic aortic rings 4–5 mm in length each. Aortic rings were suspended in organ baths containing Krebs physiological buffer (maintained at 37°C and 95% O2–5% CO2) and connected to a force transducer via steel hooks to measure isometric tension. Resting vessel tension was set to 0.5 g during a 45-min equilibration period. Contraction was recorded in response to endothelin-1 (0.1 nM–0.1 μM), serotonin (0.01–30 μM), ANG II (0.1 nM–0.1 μM), prostaglandin-F2α (0.1–30 μM), and KCl (10–100 mM). Relaxation was recorded in response to acetylcholine (0.01–30 μM) and sodium nitroprusside (1 nM–10 μM) after initial submaximal precontraction (40%–50% of max) with prostaglandin-F2α (3–10 μM).

Blood pressure analysis. Systolic blood pressure and heart rate were recorded in conscious restrained mice (20–22 wk of age) using the tail-cuff method (Visitech Systems BP-2000) (26). Thirty cycles of measurements were made for a period of 10 days. The first 5 days constituted the training period, and the mean of the final 5 days of
baseline recordings was calculated for the final determination of systolic blood pressure and heart rate.

**Real-time RT-PCR.** Tissue samples of the entire length of the aorta (including arch, thoracic, and abdominal regions) were removed from the mice, placed in PBS, dissected free of perivascular fat, and snap frozen in liquid nitrogen before storage at −80°C. Total RNA was extracted from entire aorta using RNeasy spin columns (RNeasy Mini Kit, QIAGEN). cDNA was synthesized from 400 ng of total RNA by RT-PCR using Superscript III (Invitrogen), RNaseOUT (Invitrogen), and oligo(dT) primers. After initial RNA denaturation at 65°C for 5 min, reverse transcription was performed for 60 min at 37°C followed by heat inactivation of the enzyme at 70°C for 15 min. cDNA samples were stored at −20°C until further use. Quantitative real-time PCR (qPCR) reactions were performed in duplicate using Taqman Fast Advanced Master Mix (Applied Biosystems), TaqMan Gene Expression Assays (Applied Biosystems) and 10 ng of cDNA in a total volume of 10 µL. For Taqman assays, after denaturation at 95°C for 20 s, 40 cycles at 95°C for 1 s, and 60°C for 20 s were carried out using the Applied Biosystems StepOnePlus System. Also, qPCR reactions were performed in duplicate using Fast SYBR Green Master Mix (Applied Biosystems), target gene primers, and 10 ng of cDNA in a total volume of 10 µL. For SYBR green assays were the following: GAPDH: 5′-CCTGCCCTCCGTGTTCTCTA-3′ (forward) and 5′-CCTGCTTCACCACCTTCTTGAT-3′ (reverse); ICAM1: 5′-CATAAGGA-3′ (forward) and 5′-CAGGGAGGTTGTG-3′ (reverse); and GAPDH: 5′-GACTTGGA-3′ (forward) and 5′-TAGAGAATGGATGAACA-3′ (reverse), as reported previously (7, 23, 24).

**Drugs and reagents.** ACh, SNP, KCI, and 5-HT were obtained from Sigma, PGF2α, was from Pfizer, and these reagents were dissolved in physiological saline. ET-1 was obtained from Peninsula Laboratories and dissolved in water.

**Gene expression signatures.** To determine whether the gene expression profiles in our E-PPAR aortic endothelial cells (accession GSE11870) and S-PPAR aorta (accession GSE37196) are similar to that observed in other disease models, we used the Microarray Rank Query (MarQ, available at http://marq.cnb.csic.es/) (45). The gene signatures from our data consisted of the highest ranked differentially expressed genes (100 genes from both up- and downregulated sets). For the endothelium DN (E-DN) group, the genes were ordered by fold change relative to nontransgenic samples. For the smooth muscle DN (S-DN) group, the gene list was ordered by aggregating data from two separate microarray experiments as previously described (8). Functional enrichment analysis (DAVID at NIH) using the top 100 up- and downregulated genes was performed for dataset GDS1300 (similar to S-DN aorta) and GDS1239 (similar to E-DN aortic endothelial cells). Genes were ordered by fold change as calculated by MarQ.

**Statistical analysis.** Experiments were performed on similar numbers of male and female littermate mice. The sex of the mice had no effect when comparing differences between transgenic mice to control littermates; therefore, data from male and female mice were grouped together. Data were analyzed using SigmaStat (Systat Software), and all data are expressed as means ± SE. Data were analyzed with Student’s unpaired t-test or Mann Whitney rank sum test where required. Aortic function data were analyzed with two-way repeated measures analysis of variance (ANOVA) using a Tukey post hoc test or t-test where appropriate. P values less than or equal to 0.05 (P ≤ 0.05) were considered statistically significant.

**RESULTS**

To investigate the functions of vascular cell-specific PPARγ in atherosclerosis progression, we used transgenic mice expressing DN PPARγ mutants (V290M or P467L) targeted to vascular endothelium (E-V290M mice) or smooth muscle (S-P467L mice) bred onto the ApoE-deficient background. Herein

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**A**

![Graph A](https://example.com/graphA.png)

**B**

![Graph B](https://example.com/graphB.png)

Fig. 1. Quantification of atherosclerosis in aorta. A: atherosclerotic lesion area was quantified by en face method in Oil red O-stained aortas from endothelium dominant negative (E-DN) ApoE mice and their control NT-ApoE mice, and from smooth muscle (S-DN) ApoE mice and their control NT-ApoE mice. Mice (24 wk old) were maintained on standard (Stan) chow diet or Western (West) diet (TD.88137) starting at 8 wk of age (n = 8–14 for E-DN groups; n = 10–14 for S-DN groups). B: includes representative Oil red O-stained aortas. *P < 0.05 Western vs. standard diet. #P < 0.05 E-DN or S-DN vs. NT. All data are means ± SE.

**REFERENCE**

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we will use the acronyms “E” for endothelium, “S” for smooth muscle, “DN” for dominant negative PPARγ, and “NT” for nontransgenic. The experimental groups are the following: 1) E-DN.ApoE^{-/-} and E-DN.ApoE^{-/-} mice along with their NT.ApoE^{-/-} and NT.ApoE^{-/-} littermate controls; and 2) S-DN.ApoE^{-/-} and S-DN.ApoE^{-/-} mice along with their respective NT.ApoE^{-/-} and NT.ApoE^{-/-} littermate controls.

En face lesion area, determined by Oil Red O-positive staining for lipids, was examined in the entire length of the aorta from the arch to the iliac bifurcation. There were no significant differences in en face aorta lesion area comparing E-DN.ApoE^{-/-} mice or S-DN.ApoE^{-/-} mice with their respective controls (NT.ApoE^{-/-}) mice at 24 wk of age on a standard chow diet (Fig. 1). On the contrary, en face lesion area in the entire aorta was significantly increased in Western diet-fed E-DN.ApoE^{-/-} mice and S-DN.ApoE^{-/-} mice compared with their matched NT.ApoE^{-/-} control mice (Fig. 1). Transgenic and NT mice on the heterozygous ApoE^{-/-} background failed to develop any gross lesions on standard chow diet or Western diet (data not shown). The data indicate that interference with PPARγ-dependent pathways in vascular endothelium or smooth muscle enhances lesion area in diet-induced atherosclerosis.

To determine features of plaque composition and stability, histological analyses were performed on samples of the aortic root. Cross-sectional lesion area and necrotic area were quantified from VVG-stained sections. Lesion area was significantly increased by Western diet treatment, and there was a trend toward greater lesion area in the aortic root from S-DN.ApoE^{-/-} and E-DN.ApoE^{-/-} mice compared with their NT.ApoE^{-/-} control mice in response to Western diet (Fig. 2). Necrotic and Alizarin Red-positive area was significantly greater in aortic root from S-DN.ApoE^{-/-} mice suggesting increased medial calcification.

Since the development of atherosclerosis is accelerated by insulin resistance, hyperglycemia, and dyslipidemia, we next assessed if interference with endothelial or smooth muscle PPARγ had an effect on systemic metabolism (Table 1).

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**Table 1**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Lesion Area (mm²)</th>
<th>Necrotic Area (mm²)</th>
<th>Alizarin Red Area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet</strong></td>
<td><strong>Lesion Area</strong></td>
<td><strong>Necrotic Area</strong></td>
<td><strong>Alizarin Red Area</strong></td>
</tr>
<tr>
<td>Stan</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>West</td>
<td>0.4</td>
<td>0.1</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Diet</strong></td>
<td><strong>Lesion Area</strong></td>
<td><strong>Necrotic Area</strong></td>
<td><strong>Alizarin Red Area</strong></td>
</tr>
<tr>
<td>Stan</td>
<td>0.8</td>
<td>0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>West</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
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</table>

*P < 0.05 Western vs. Standard diet; #P < 0.05 E-DN or S-DN vs. NT. All data are means ± SE. The scale bar represents 1 mm.

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**Fig. 2.** Plaque necrosis and calcification at the aortic root. A: paraffin sections of aortic root were stained for elastic fibers by the Verhoeff-Van Gieson method (top) or stained for calcification by Alizarin Red (bottom). Representative images of aortic root from E-DN.ApoE^{-/-} mice, S-DN.ApoE^{-/-} mice and their respective control NT.ApoE^{-/-} mice maintained on Western diet. B: Quantification of lesion area, necrotic area, or Alizarin Red-positive area. Mice were maintained on standard chow diet or Western diet (TD.88137) starting at 8 wk of age (n = 8 for all groups). *P < 0.05 Western vs. Standard diet; #P < 0.05 E-DN or S-DN vs. NT. All data are means ± SE. The scale bar represents 1 mm.
Although body weight and fasting plasma glucose increased in Western diet fed mice, there was no effect of PPARγ genotype. Total plasma cholesterol and triglycerides increased in ApoE-deficient mice, and these measures further increased in ApoE-deficient mice fed a Western diet. However, there was no effect of PPARγ genotype. There were no changes in heart mass or heart-to-body mass ratio. These data indicate that the increase in diet-induced atherosclerotic lesion formation caused by endothelium-specific DN PPARγ or smooth muscle-specific DN PPARγ is independent of systemic changes in cholesterol, triglyceride, or glucose levels.

Endothelial dysfunction is a hallmark associated with the development of hypertension and atherosclerosis (6, 14). Furthermore, an enhanced contractile response to endothelin-1 (ET-1) is observed in aortic rings from young ApoE−/− mice before lesion development, suggesting it may contribute to disease progression (28). To evaluate a possible imbalance in relaxation or contraction, we implemented a wire myograph preparation of aortic rings ex vivo. ACh was used to assess endothelium-dependent relaxation, whereas sodium nitroprusside (SNP) was used to assess direct nitric oxide-dependent relaxation of the smooth muscle. The effect of ApoE-deficiency and PPARγ interference was measured in mice fed a standard diet.

Aortic rings from E-DN.ApoE−/− mice exhibited impaired ACh-mediated relaxation compared with NT.ApoE−/−, E-DN.ApoE−/−, and NT.ApoE−/− control mice (Fig. 3A). SNP-mediated relaxation was normal in E-DN.ApoE−/− mice (Fig. 3B). There were no alterations in contraction to KCl, 5-HT, ET-1, or ANG II in E-DN.ApoE−/− mice (Fig. 4A). These data suggest that the combination of endothelial PPARγ interference and ApoE deficiency causes endothelial dysfunction.

Consistent with our previous findings (16, 35), aortic rings from S-DN.ApoE−/− mice displayed impaired relaxation to ACh (Fig. 3C) and SNP (Fig. 3D) in addition to increased agonist-induced contractile responses to 5-HT, ET-1, ANG II, which were maintained in S-DN.ApoE−/− mice (Fig. 4B). Results from concurrent experiments using thoracic aortic rings (data not shown) were the same as those from abdominal aortic rings (Figs. 3 and 4), although the contractile responses to ET-1 and ANG II were much lower in thoracic segments, consistent with previous reports (39).

We next tested if the impairments in vascular function were associated with any functional changes in hemodynamics (Fig. 5). E-DN.ApoE−/− mice on standard diet had normal baseline systolic blood pressure (Fig. 5A). In contrast, E-DN.ApoE−/− mice on both standard and Western diets exhibited a significant increase in systolic blood pressure, suggesting that hypercholesterolemia and hyperlipidemia contributes to hypertension in the presence of endothelial PPARγ interference. The presence of smooth muscle PPARγ interference led to a significant increase in systolic blood pressure in mice irrespective of ApoE genotype and diet (Fig. 5B).

It is not clear if vascular endothelial cells, smooth muscle cells, and macrophages share the same PPARγ-dependent pathways that regulate inflammatory signaling and lipid metabolism. PPARγ binds to PPAR response elements within regulatory regions of target genes to activate transcription. PPARγ can also mediate transcription of pro-inflammatory genes by antagonizing other transcription factors, including activator protein-1 (AP-1), signal transducers and activators of transcription (STAT), and nuclear factor (NF)-κB (3). We next performed gene expression analysis using entire aorta from mice in each of the experimental groups on standard diet to characterize the transcriptional changes induced by vascular endothelium-specific or smooth muscle-specific expression of endogenous PPARγ. The aortic tissue samples contained the endothelial, smooth muscle, and adventitial layers but were dissected free of the perivascular adipose. Aortas collected from the ApoE−/− experimental groups were devoid of any lesions, whereas aortas collected from the ApoE−/− groups contained a minimal amount of lipid-rich atherosclerotic lesion areas (as depicted in Fig. 1). ApoE−/− experimental groups on standard diet were analyzed because they displayed similar values in terms of aortic lesion area.

We first determined the mRNA expression levels of endogenous mouse PPARγ (mPPARγ) and of the human DN PPARγ (hPPARγ) transgenes using PCR primers that distinguish between the two (Fig. 6). ApoE deficiency did not alter the expression of endogenous mPPARγ. Expression of the human PPARγ transgene was detected in the aorta from E-DN.

### Table 1. Metabolic profile

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Body Mass, g</th>
<th>Heart Mass, g</th>
<th>Glucose, mg/dl</th>
<th>Total CHO, mg/dl</th>
<th>Triglycerides, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT.ApoE+/+</td>
<td>Standard</td>
<td>27.3 ± 1.5</td>
<td>0.116 ± 0.004</td>
<td>106.1 ± 6.3</td>
<td>85.9 ± 5.1</td>
<td>70.5 ± 2.8</td>
</tr>
<tr>
<td>E-DN.ApoE−/−</td>
<td>Standard</td>
<td>28.5 ± 1.3</td>
<td>0.120 ± 0.005</td>
<td>106.6 ± 5.1</td>
<td>86.2 ± 6.9</td>
<td>68.2 ± 5.4</td>
</tr>
<tr>
<td>NT.ApoE+/+</td>
<td>Standard</td>
<td>26.9 ± 1.3</td>
<td>0.125 ± 0.005</td>
<td>111.9 ± 5.0</td>
<td>391.5 ± 21*</td>
<td>84.6 ± 4.5*</td>
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<tr>
<td>E-DN.ApoE−/−</td>
<td>Standard</td>
<td>25.6 ± 1.2</td>
<td>0.124 ± 0.006</td>
<td>108.5 ± 4.6</td>
<td>379.2 ± 15*</td>
<td>85.5 ± 2.9*</td>
</tr>
<tr>
<td>NT.ApoE−/−</td>
<td>Western</td>
<td>30.9 ± 1.6†</td>
<td>0.141 ± 0.007</td>
<td>129.4 ± 4.7†</td>
<td>739.9 ± 26†</td>
<td>111.9 ± 9.0†</td>
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<tr>
<td>E-DN.ApoE−/−</td>
<td>Western</td>
<td>30.3 ± 1.8†</td>
<td>0.137 ± 0.006</td>
<td>125.8 ± 2.4†</td>
<td>70.6 ± 32†</td>
<td>118.5 ± 4.4†</td>
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<tr>
<td>NT.ApoE−/−</td>
<td>Standard</td>
<td>27.9 ± 1.4</td>
<td>0.120 ± 0.004</td>
<td>107.0 ± 5.8</td>
<td>84.5 ± 5.7</td>
<td>73.4 ± 2.4</td>
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<td>S-DN.ApoE+/+</td>
<td>Standard</td>
<td>27.0 ± 1.4</td>
<td>0.117 ± 0.004</td>
<td>100.3 ± 4.3</td>
<td>82.3 ± 4.6</td>
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<tr>
<td>NT.ApoE−/−</td>
<td>Standard</td>
<td>26.4 ± 1.4</td>
<td>0.129 ± 0.006</td>
<td>113.0 ± 4.4</td>
<td>380.7 ± 18*</td>
<td>86.8 ± 4.9*</td>
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<td>S-DN.ApoE−/−</td>
<td>Standard</td>
<td>26.6 ± 1.1</td>
<td>0.130 ± 0.008</td>
<td>107.5 ± 5.9</td>
<td>368.4 ± 16*</td>
<td>85.2 ± 3.6*</td>
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<tr>
<td>NT.ApoE−/−</td>
<td>Western</td>
<td>31.4 ± 1.7†</td>
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<td>132.1 ± 6.1†</td>
<td>770.7 ± 25†</td>
<td>110.0 ± 4.8†</td>
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<tr>
<td>S-DN.ApoE−/−</td>
<td>Western</td>
<td>31.4 ± 1.8†</td>
<td>0.142 ± 0.006</td>
<td>120.7 ± 5.2†</td>
<td>747.2 ± 30†</td>
<td>115.3 ± 4.9†</td>
</tr>
</tbody>
</table>

All data are means ± SE. E, endothelium; S, smooth muscle; NT, normal; DN, dominant negative; CHO, cholesterol. Body mass, heart mass, fasting blood glucose, fasting plasma cholesterol, and fasting plasma triglycerides were measured in mice (24 wk old) maintained on standard chow diet or Western diet (TD.88137) starting at 8 wk of age (n = 8–13 for E-DN groups; n = 8–16 for S-DN groups). *P < 0.05 ApoE−/− standard vs. ApoE+/+ standard; †P < 0.05 ApoE−/− Western vs. ApoE+/+ standard. There were no significant changes between E-DN or S-DN vs. NT.
ApoE+/−, E-DN.ApoE+/−, S-DN.ApoE+/−, and S-DN.ApoE−/− mice but not in aorta from any of the NT mice. Transgene expression level was higher when comparing smooth muscle-specific DN PPARγ to endothelium-specific DN PPARγ, which is probably a result of the smooth muscle cells making up a large population of the aortic tissue sample. It is notable that ApoE deficiency caused a significant down-regulation of transgene expression in the aorta from S-DN.

Fig. 3. Vasomotor function-relaxation. Isometric tension studies were performed using abdominal aortic rings from E-DN.ApoE+/−, E-DN.ApoE−/− mice (A and B), S-DN.ApoE+/− mice, S-DN.ApoE−/− mice (C and D), and their respective NT control mice on standard diet. Dose-dependent relaxation was recorded following precontraction with prostaglandin F2α (PGF2α) in response to acetylcholine (Ach) (A and C) or sodium nitroprusside (SNP) (B and D) (n = 4–5 for E-DN groups; n = 4–7 for S-DN groups). *P < 0.05 ApoE−/− vs. ApoE+/−; #P < 0.05 E-DN or S-DN vs. NT. All data are means ± SE.

Fig. 4. Vasomotor function-contraction. Isometric tension studies were performed using abdominal aortic rings from E-DN.ApoE+/− and E-DN.ApoE−/− mice (A) and S-DN.ApoE+/− and S-DN.ApoE−/− (B) mice and their respective NT control mice on standard diet. Contraction was measured in response to KCl, serotonin (5-HT), endothelin 1 (ET-1), or angiotensin II (ANG II). (n = 4–5 for E-DN groups; n = 4–7 for S-DN groups). #P < 0.05 E-DN or S-DN vs. NT. There were no significant differences between ApoE−/− vs. ApoE+/−. All data are means ± SE.
mPPARγ binding cassette protein A1 (ABCA1) mediates cholesterol efflux and is upregulated by PPARγ activation in macrophages (11). While Abca1 expression was higher in aorta from ApoE−/− mice compared with ApoE+/− mice, it was not significantly altered by the presence of DN PPARγ in endothelium or smooth muscle. The Acaa1b gene encoding acetyl-CoA acyltransferase-1B was identified as a potential target of PPARγ through genome-wide microarray analyses (21). Its expression was upregulated in the aorta of wild-type mice treated with rosiglitazone and was downregulated in aorta from heterozygous knock-in mice carrying DN PPARγ (21). Acaal1b gene expression was selectively downregulated in aorta from S-DN.ApoE+/− and S-DN.ApoE−/− mice. Lipoprotein lipase (LPL) is a target gene of PPARγ in adipocytes that plays an important role in the hydrolysis of triglycerides in lipoproteins (40). Lpl expression in aortic tissue was constant among each of the experimental groups.

Osteopontin (OPN; also known as secreted phosphoprotein 1) and matrix metalloproteinases (MMPs) have key roles in determining the composition of the extracellular matrix, destruction of which can lead to an unstable plaque phenotype and plaque rupture. Opn expression was induced in aorta in all

ApoE−/− compared with S-DN.ApoE+/− mice. Smooth muscle-specific expression of DN PPARγ is controlled by the SMMHC promoter, and downregulation of smooth muscle-specific marker genes, including SMMHC and SM22α, is characteristic of phenotypic switching that occurs in smooth muscle cells from atherosclerotic lesions (46).

The mRNA expression levels of genes related to lipid and cholesterol metabolism was next examined (Fig. 7). CD68 (also known as macroisin in mice) is a scavenger receptor for oxidized low-density lipoprotein (LDL) with predominant expression in monocytes/macrophages. Expression of Cdl68 was significantly increased in aorta from the ApoE−/− mice, which contained macrophage-rich lesions compared with aorta from ApoE+/− mice, which were devoid of lesions. Cdl68 was not altered by PPARγ deficiency in either endothelium or smooth muscle. This may reflect the similar extent of aortic lesion area for each of the experimental ApoE−/− groups on standard diet. Another scavenger receptor, Cdl36, is a classic PPARγ target gene (32) that was also upregulated during atherosclerosis in NT.ApoE−/− mice. Endothelium- or smooth muscle-specific PPARγ interference significantly suppressed Cdl36 expression in aorta on either the ApoE−/− or ApoE+/− background consistent with the DN activity of the mutant PPARγ. ATP-
ApoE−/− mice and was further increased in S-DN.ApoE−/− mice (Fig. 8). The induction of Opn in aorta by smooth muscle-specific DN PPARγ is consistent with our previous data (16). Mmp9 expression was similar in each group. The antioxidant enzymes catalase (CAT) and Cu-Zn superoxide dismutase (SOD1) have potential roles in preventing oxidant modification of LDL. Expression of Cat or Sod1 was not affected by ApoE deficiency; however, Cat was selectively downregulated in aorta from mice carrying DN PPARγ in endothelium.

Finally, PPARγ-dependent regulation of adhesion molecules (e.g., VCAM-1, ICAM-1) and cytokines/chemokines (e.g., IL-1β, TNF-α, MCP-1) was examined in aortic tissue (Fig. 8). Vcam1 and Mcp1, unlike Icam1 and Tnfa, were upregulated in aorta from ApoE-deficient mice. Smooth muscle-specific interference with PPARγ caused a significant upregulation of Vcam1 in aorta whereas both smooth muscle- and endothelium-specific interference with PPARγ caused a significant upregulation of Mcp1. Mcp1 was also selectively upregulated in aorta from S-DN.ApoE−/− mice compared with their NT.ApoE−/− control mice. The signature of genes upregulated during atherosclerosis (e.g., Cd68, Cd36, Abca1, Opn, Vcam1, Mcp1) in the present study is consistent with results obtained from genome-wide microarray transcriptional profiling of aorta from ApoE−/− mice (42). Together, the data indicate that interference with PPARγ in endothelium or smooth muscle leads to altered transcriptional regulation of common (i.e., Cd36, Mcp1) and distinct gene sets (i.e., Cat, Opn, Vcam1) related to atherogenesis.

**DISCUSSION**

Administration of high-affinity PPARγ agonists cause a significant attenuation of atherosclerotic lesion formation in mouse models of hypercholesterolemia that is associated with altered transcriptional regulation of many genes encoding proatherogenic factors in aortic tissue (12, 23). However, whereas the role of PPARγ in atherosclerosis is well recognized, the cell-specific contributions of PPARγ in vascular endothelium and smooth muscle to initiation and progression of atherosclerosis remain less well defined. The conclusions from our study are that 1) interference with endogenous PPARγ in either endothelium or vascular muscle worsens atherosclerosis in ApoE-deficient mice, and 2) atherosclerosis was accompanied by a) increased lesion necrosis and calcification, particularly in S-DN.ApoE−/− mice; b) endothelial dysfunction; c) hypertension; and d) changes in the expression of genes controlling lipid and cholesterol metabolism, extracellular matrix, oxidative stress, and adhesion molecules. Expression of DN PPARγ in endothelium versus smooth muscle produced modest differential effects on vascular reactivity, blood pressure, and aortic gene expression. Particularly in vascular smooth muscle, the antiosteogenic role of PPARγ may be important in limiting the extent of plaque necrosis and calcification.

**Vascular function and blood pressure.** Our group previously reported that transgenic mice with endothelium-specific expression of DN PPARγ exhibit normal endothelial function in the basilar artery and aorta and normal blood pressure; however, prolonged high-fat diet feeding unmasked impairment of endothelium-dependent vasodilation in the basilar artery and aorta (5). Hyperlipidemia and hypercholesterolemia induced by crossing endothelium-specific DN PPARγ mice with ApoE−/− mice similarly unmasked endothelial dysfunction and hypertension even when on a standard chow diet. Consequently, either high-fat diet or dyslipidemia caused by ApoE deficiency have similar effects on vascular function and blood...
pressure when PPARγ function in endothelium is impaired. This suggests that endothelial PPARγ protects against the detrimental effects of a high-fat or high-cholesterol diet. Similarly, endothelial function was reported to be normal in aortic rings from ApoE−/− mice maintained on standard diet, whereas coupling ApoE deficiency with a Western diet causes impaired endothelium-dependent relaxation (6, 14). It is possible that sufficient PPARγ activity is retained in normal diet-fed ApoE-deficient mice, but that PPARγ becomes impaired in ApoE−/− mice fed a Western diet. Indeed, recent studies suggest that a high-fat diet can cause posttranslational modifications (phosphorylation and acetylation) of PPARγ in adipose tissue that result in impaired PPARγ activity (13, 37).

Smooth muscle-specific expression of DN PPARγ also has profound effects on vascular reactivity. We recently showed that aortic dysfunction induced by smooth muscle-specific DN PPARγ occurs through a mechanism involving increased RhoA and Rho-kinase activity in the smooth muscle (35). Importantly, treatment of LDLR−/− mice with the Rho-kinase inhibitor Y-27632 (for 9 wk) significantly inhibited lesion formation at the aortic root compared with saline-treated control LDLR−/− mice (29). Here we found that contractile responses to several agonists of GPCRs (e.g., ET-1, ANG II, 5-HT) were significantly enhanced in aortic rings from S-DN.ApoE+/− mice, and these changes persisted in S-DN.ApoE−/− mice. Thus it is possible that increased RhoA/Rho kinase activity may also play a mechanistic role in worsening atherosclerosis in the S-DN.ApoE−/− mice.

Hypertension is a risk factor for cardiovascular diseases. Increased systolic blood pressure was detected in E-DN.ApoE−/− mice, S-DN.ApoE+/− mice, and S-DN.ApoE−/− mice maintained on standard diet. Western diet markedly increased aortic lesion formation in each experimental group of ApoE−/− mice but did not further increase blood pressure. Although models with elevated blood pressure often exhibit increased atherosclerosis, the relationship between increased blood pressure and the development of atherosclerosis is not a strict one particularly in mice (27). For example, infusion of ApoE−/− mice with ANG II for 4 wk caused hypertension, increased aortic lesion formation, and induced abdominal aortic aneurysms (9). Whereas coadministration of hydralazine significantly lowered blood pressure, it failed to reduce aortic lesion formation at the aortic root compared with saline-treated control LDLR−/− mice (29). Here we found that contractile responses to several agonists of GPCRs (e.g., ET-1, ANG II, 5-HT) were significantly enhanced in aortic rings from S-DN.ApoE+/− mice, and these changes persisted in S-DN.ApoE−/− mice. Thus it is possible that increased RhoA/Rho kinase activity may also play a mechanistic role in worsening atherosclerosis in the S-DN.ApoE−/− mice.

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area or the incidence of abdominal aortic aneurysms. Based on these and other findings, Cassis and Daugherty postulated that the stimulus underlying the increase in blood pressure (ANG II in the above example) may have more influence on atherosclerosis than the increase in blood pressure itself (27).

Transcriptional regulation of atherosclerosis marker genes. Endothelium-specific DN PPARγ decrease expression of the scavenger receptor Cd36 in aorta; downregulation of Cd36 expression and impaired fatty acid uptake in microvascular endothelial cells was reported in endothelium-specific PPARγ knockout mice (20). The observation that Cd36−/−ApoE−/− double knockout mice exhibit decreased aortic lesion formation (22) suggests that negative regulation of Cd36 would be protective. On the contrary, TZD treatment, which is atheroprotective upregulates Cd36 in aorta from ApoE−/− and LDLR−/− mice (12, 24). Thus it remains unclear if decreased Cd36 expression in endothelium-specific PPARγ deficiency or PPARγ interference plays a mechanistic role in atherosclerosis or is a consequence of being a PPARγ target gene.

Evidence suggests that PPARγ is an important regulator of oxidative metabolism in the vessel wall. PPARγ ligand treatment of cultured endothelial cells is generally antioxidant (19). Here we found that that expression of catalase was decreased in the aorta from E-DN.ApoE−/− mice. Transgenic overexpression of catalase in ApoE−/− mice inhibited atherosclerotic lesion formation and decreased the levels of F2-isoprostanes, a marker of lipid peroxidation (48). Consequently, decreased expression of catalase could lead to increased oxidant modification of lipoproteins, thus promoting foam cell formation. At the same time, expression of Mcp1 was increased in aorta from E-DN.ApoE−/− mice. MCP-1 is a pro-inflammatory chemo- kine expressed by each of the major cell types in the vessel wall (1). Transgenic overexpression of MCP-1 in leukocytes on the ApoE−/− background was sufficient to increase lesion area in the aorta and the aortic root (1). Thus whether increased expression of Mcp1 contributes to altered monocyte recruitment would need to be directly tested in future studies.

Similar to the endothelial model, smooth muscle-specific interference with PPARγ led to downregulation of Cd36 and upregulation of Mcp1. In addition, Vcam1 and Opm were upregulated in aorta from S-DN.ApoE−/− mice. Like Mcp1, increased expression of Vcam1 may contribute to increased leukocyte recruitment. Osteopontin is a pro-atherogenic extracellular matrix protein synthesized by endothelial cells, smooth muscle cells, and macrophages (7). ApoE−/−OPN−/− double knockout mice exhibit reduced ANG II-induced aortic lesion formation and reduced incidence and severity of ANG II-induced abdominal aortic aneurysms compared with control ApoE−/− mice (7). The induction of Opm is consistent with data showing that PPARγ activation negatively regulates Opm expression (34). Moreover, the induction of Osteopontin expression by smooth muscle-specific PPARγ interference led us to examine calcification of the aortic root. Interference with PPARγ in smooth muscle, but not endothelium, increased plaque necrosis and calcification in response to Western diet.

Perspectives and Significance

Activation of PPARγ is well known to have both antioxidant and anti-inflammatory properties through the induction of antioxidant and repression of pro-oxidant and pro-inflammatory gene expression. This made it logical to hypothesize that PPARγ interference or PPARγ deficiency would worsen a disease process characterized by both oxidative stress and inflammation. It is therefore quite interesting to note that the “gene signature,” that is, the top 100 upregulated and downregulated genes in S-DN aorta compared with the entirety of gene signatures in the Gene Expression Omnibus collection of microarray datasets, was similar to the gene signature in aorta from ApoE−/− mice on a normal diet (accession GDS1300, P < 0.0001). Moreover, functional enrichment analysis (DAVID) of the top upregulated genes in the GDS1300 dataset was statistically enriched in inflammatory response genes (P = 5.3 × 10⁻⁹). Cultured endothelial cells derived from the E-DN aorta exhibited a similar expression signature as that for lung samples from an LPS-induced model of acute lung injury and inflammation (accession GDS1239, P < 0.0001). Similarly, the upregulated gene signature in the GDS1239 dataset was statistically enriched in inflammatory response genes (P = 10⁻²¹). This suggests that the interference with PPARγ signaling in the endothelium and vascular smooth muscle may cause a predisposition to inflammation that gets exacerbated on the ApoE−/− genetic background. When S-DN.ApoE−/− or E-DN.ApoE−/− mice are subjected to a high cholesterol diet this may result in an additive or synergic effect to worsen atherosclerosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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