Disruption of Endothelial Peroxisome Proliferator-Activated Receptor γ Accelerates Diet-Induced Atherogenesis in LDL Receptor-Null Mice

Aijuan Qu, Yatrik M. Shah, Soumen K. Manna, Frank J. Gonzalez

Objective—Peroxisome proliferator-activated receptor γ (PPARγ) is widely expressed in vessel walls, and its activation by agonists showed beneficial effects in cardiovascular diseases. However, the role of endothelial cell (EC) PPARγ in atherogenesis is not fully understood.

Methods and Results—To assess the contribution of endothelial-specific PPARγ in atherosclerosis, EC-specific PPARγ disruption and LDL receptor (LDLR) double-knockout (PPARγEC/LDLR−/−) mice were developed. When challenged with a high-cholesterol diet for 4 weeks, PPARγEC/LDLR−/− mice exhibited severe atherosclerotic lesions compared to either their littermate controls or macrophage-specific PPARγ disruption and LDLR double knockout (PPARγΔM/M/LDLR−/−) mice. Metabolic analysis showed severe dyslipidemia and significant increase in systolic blood pressure in the PPARγEC/LDLR−/− mice. Histological analysis and real-time quantitative PCR suggested an exacerbated inflammation in PPARγEC/LDLR−/− mice, as revealed by the increases of proinflammatory gene expression and macrophage infiltration in vivo and in vitro. Furthermore, in vivo endothelial permeability was also increased by endothelial PPARγ disruption. Bone-marrow transplantation studies, which reconstituted hematopoietic PPARγ, demonstrated that the accelerated atherogenesis was due to endothelial PPARγ deficiency.

Conclusion—Endothelial PPARγ plays an important protective role in atherogenesis. (Arterioscler Thromb Vasc Biol. 2012;32:65-73.)

Key Words: atherosclerosis • endothelial function • hypertension • lipids • lipoproteins

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated nuclear transcription factor that has a central role in controlling adipocyte differentiation, lipid metabolism, and insulin sensitivity.1 Clinically, PPARγ agonists, thiazolidinedione (TZD) class of drugs are used to improve insulin resistance and treat type 2 diabetes. PPARγ is also highly expressed in endothelial cells (ECs), macrophages (Mφ), and smooth muscle cells, all of which are critical in atherosclerosis. Several studies have shown that TZD administration reduces atherosclerosis in both apolipoprotein E (apoE)−/− and low-density lipoprotein receptor (LDLR)−/− mice.2–4 Recently, conditional disruption of PPARγ in macrophages demonstrated an increase in atherosclerosis under conditions of mild and severe hypercholesterolemia.5 Moreover, disruption of PPARγ in smooth muscle cells augments angiotensin-II induced atherosclerosis in LDLR−/− mice6 and exacerbates vascular lesion formation.7

EC activation and dysfunction on exposure to atheroprone factors, such as oxidized lipids and proinflammatory stimuli, play a critical role in the initiation and progression of atherosclerosis.8,9 Increasing data have shown that PPARγ agonists inhibit inflammation in ECs and suppress the expression of vasoconstrictors endothelin-1 and angiotensin-II, but enhance the expression and activity of vasodilator nitric oxide in cultured ECs.10–14 Because PPARγ agonists demonstrate receptor-independent functions the direct role of PPARγ in ECs remains unknown. Moreover, in the PROACTIVE trial, the PPARγ agonist pioglitazone had beneficial effects on cardiovascular diseases,15 but a recent meta-analysis demonstrates that the PPARγ agonist rosiglitazone was associated with an increase in risk of myocardial infarction and a trend toward a higher risk of cardiovascular death in patients with type 2 diabetes.16 These clinical trials raise the importance of understanding the role of PPARγ in cell types critical in the progression of cardiovascular diseases.

In the present study, EC-specific PPARγ deficient mice were generated using the Cre/loxP system regulated by the Tie2 promoter on the LDLR−/− background. The data demonstrate that PPARγ deficiency in ECs significantly accelerates the initiation of atherosclerosis in LDLR−/− mice challenged with an atherogenic diet, demonstrating a protective role for endothelial PPARγ against the initiation and development of atherosclerosis.
Methods and Materials

Animals and Diets
PPARγF/F, PPARγΔC/H9253 and PPARγΔM/H11002 mice were previously described. LDLR−/− mice on a C57BL/6 background were purchased from Jackson Laboratories Inc (Bar Harbor, Me). PPARγEC and PPARγMP mice were crossed with LDLR−/− mice to obtain PPARγΔC/LDLR−/− and PPARγΔM/LDLR−/− mice and the littermate PPARγF/F/LDLR−/− were used as controls for all studies. All mice were maintained in microisolator cages with free access to rodent chow and water. Atherosclerosis was induced by feeding male mice an atherogenic rodent diet containing 1.25% cholesterol and 0.5% cholic acid (TD 02028, Harlan Teklad, Madison, WI) from 6 weeks old for the indicated times. All animal studies were carried out in accordance with Institute of Laboratory Animal Resources guidelines and approved by the National Cancer Institute Animal Care and Use Committee.

Serum Lipids and Lipoprotein Profiles
Mice were fasted overnight before blood sample collection. Total serum cholesterol and triglycerides were measured using kits from Wako (Wako Chemicals USA, Richmond, VA) according to the manufacturer’s instructions. Lipoprotein profile was assessed by fast performance liquid chromatography using a Superox 6 column (Pharmacia, Piscataway, NJ) on a high-performance liquid chromatography system model 600 (Waters, Milford, MA).

Blood Pressure Measurement
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Bone Marrow Transplantation
Six-week-old male PPARγF/F/LDLR−/− and PPARγΔC/LDLR−/− mice were lethally irradiated with 9.5 Gy from a cesium gamma source 6 hours before transplantation. The 137Cs source was in a Mark I Model 68 small animal irradiator (J. L. Shepherd & Associates, San Fernando, CA) operating at 2.57 Gy/min. This unit has a single 137Cs source and provides uniform doses to small animals centered on the revolving turntable revolving at a constant rate of 4.75 revolutions per minute within the chamber. Bone marrow was collected from femurs of donor PPARγF/F/LDLR−/− or PPARγΔC/LDLR−/− mice by flushing with sterile medium (RPNI 1640, 5 U/mL heparin, 50 U/mL penicillin, and 50 μg/mL streptomycin). Each recipient mouse was injected with 5×10⁶ bone marrow cells through the tail vein. Four weeks after BMT, peripheral blood was collected for PCR analysis of bone marrow reconstitution. For atherosclerosis study, the mice were fed with high-cholesterol diet for another 4 weeks after confirming they were fully chimeric.

Analysis of Atherosclerotic Lesions
After 4 weeks of high-cholesterol diet, anesthetized mice were perfused with 10 mL of PBS via left ventricle, followed by 10 mL of 10% buffered formalin phosphate. After removal of the adventitia, the aorta was opened longitudinally, pinned flat onto a black-wax plate and stained with oil red O (Sigma) as previously described. Plaques were analyzed under the Zeiss stemi 200-C microscope connected to an Olympus BX41 digital camera, and the lesions were quantified with Image J analysis software. To analyze atherosclerosis in the outflow tract and valve area of the heart, the top half of the heart was embedded in freezing media and stored at −80°C until sectioning. Serial 10-μm-thick cryosections were cut and stained with hematoxylin and eosin as well as oil red O for quantifications of the lesion areas with the Image J analysis software. The aortic lesion size of each animal was obtained by the average in 6 sections from the same mice.

Isolation and Culture of Primary ECs
Primary ECs were isolated from the lungs of male PPARγF/F, LDLR−/− or PPARγΔC/LDLR−/− mice. The mice were killed with carbon dioxide and the lungs were harvested and placed in DMEM. The tissues were trimmed, cut into small pieces, and incubated at 37°C with collagenase I (Invitrogen) for 45 minutes. The suspension was triturated with 30 cc syringe for 12 times, filtered through sterile 70-μm disposable cell strainer (BD Biosciences, San Jose, CA), and centrifuged at 400 g for 8 minutes. The cell pellet was resuspended in Dulbecco’s PBS and incubated with dynabeads (Invitrogen) coated with antinouse CD31 antibody (BD Biosciences) on a rotator for 10 minutes at room temperature. The cell-bound beads were washed 5 times with DMEM and cultured in Medium 200 (Invitrogen) with the addition of low-serum growth supplement (Invitrogen). Cells were grown in a collagen type I-coated plate and used at passage 2 for the experiments. The purification of ECs were >90% as confirmed by CD31 immunofluorescence staining.

Monocyte-EC Adhesion Assay
Monocytes were isolated from C57/B6 mice as previously described and labeled with calcine-AM (invitrogen). Confluent ECs were treated with control or lipopolysaccharide for 16 hours and incubated with calcine-AM-labeled monocytes for 30 minutes. After washing with PBS monocytes bound to ECs were visualized on fluorescence microscopy. The number of bound monocytes was quantified by counting 5 microscopic fields per well in triplicates.

In Vivo Vascular Permeability Assay
Six-week-old male PPARγF/F or PPARγΔC mice were injected with Evans blue dye (30 mg/kg in 100 μL normal saline). Mustard oil diluted to 5% in corn oil or vehicle (corn oil) was applied to the dorsal and ventral surfaces of the ear and photographs were taken 15 minutes after dye injection. After mice were euthanized, ears were removed, dried, and weighed. Evans blue dye was extracted with 1 mL of formamide overnight at 55°C and measured spectrophotometrically at 620 nm. Values were expressed as μg of dye/mg of ear tissue.

Immunohistochemistry
Snap-frozen fixed aortic rings embedded in OCT were sectioned, fixed in 10% buffered formalin phosphate and processed for antibody staining according to standard protocols. The following antibodies were used: anti-CD31 (BD Bioscience, San Jose, CA), F4/80 (Serotec, Raleigh, NC), VCAM-1 and ICAM-1 (R&D System, Minneapolis, MN). Positive cells and total cells were quantified from the aortic arch (5 different sections) from 4 different mice of each genotyping using IMAGE J software.

RNA Analysis
Total RNA was extracted from aortas, liver, or primary ECs using Trizol reagent (Invitrogen, Carlsbad, CA) and further purified by RNeasy columns (QIAGEN, Valencia, CA). Quantitative real-time PCR (qPCR) were performed using cDNA generated from 1 μg total RNA with SuperScript II Reverse Transcriptase kit (Invitrogen). qPCR reactions were performed by use of SYBR green PCR master mix (Applied Biosystems, Foster City, CA) in an ABI Prism 7900HT sequence detection system (Applied Biosystems). Values were quantified by the comparative CT method and normalized to β-actin. Sequences for primers used to quantify mRNA are listed in Supplemental Table, available online at http://atvb.ahajournals.org.

Data Analysis
Results are expressed as means±SD. Statistical analysis was performed using the Student t test (2 groups) or one-way ANOVA with Bonferroni procedure for multiple comparison tests (≥3 groups)
with GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA). Value of \( P<0.05 \) was considered statistically significant.

**Results**

**Deletion of PPAR\( \gamma \) by the Tie2-Driven Cre Recombinase Accelerates the Initiation of Atherosclerosis in LDLR\( ^{-/-} \) Mice**

To investigate the contribution of EC PPAR\( \gamma \) in the development of atherosclerosis, PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) were generated by crossing PPAR\( \gamma ^{\text{EC}} \) with LDLR\( ^{-/-} \) mice. To verify tissue-specific PPAR\( \gamma \) deficiency, genomic DNA recombination and mRNA levels were assessed in aorta, spleen, macrophage, liver, brown adipose tissue, and white adipose tissue (Supplemental Figure IA). qPCR showed that Tie2-Cre directs an efficient PPAR\( \gamma \) disruption in ECs and bone marrow-derived hematopoietic cells, but not in liver, brown adipose tissue, or white adipose tissue from PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice (Supplemental Figure IB). These results are consistent with previous data, in which Southern blot analysis revealed recombination in spleen and ECs.\(^{18}\)

Comprehensive analysis of atherosclerotic lesions was performed. Atherosclerotic plaques were not observed in PPAR\( \gamma ^{\text{EC}/LDLR^{+/+}} \) on high-cholesterol diet for 4 weeks (Supplemental Figure IIA) or PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice on standard chow diet for 26 weeks (Supplemental Figure IIB). To investigate the role of EC PPAR\( \gamma \) in the initiation and early stage of atherosclerosis, PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice and littermate controls were challenged with high-cholesterol diet for 4 weeks. Atherosclerotic plaques were clearly visible by light microscopy in the aortic arch, innominate artery, left carotid artery, left subclavian artery, thoracic aorta, and abdominal aorta in PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice. However, no visual lesions were observed in the thoracic or abdominal aorta with any littermate controls. En face analysis of atheromatous plaques by oil red O staining showed a marked increase of plaques in the aorta from PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice when compared to control littermates (Figure 1A and 1B). Quantification of atherosclerotic lesions relative to aortic cross sections (Figure 2D, \( 0.079 \pm 0.003 \text{ mm}^2 \)) revealed a dramatic increase of lesions in the aortic arch (Figure 1C), thoracic aorta (Figure 1D) as well as abdominal aorta (Figure 1E) in PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice. Histological assessment of atherosclerotic lesions at the aortic sinus by hematoxylin and eosin staining confirmed the results of en face analysis, revealing an increase of plaque formation in PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice compared to their PPAR\( \gamma ^{\text{F/F}/LDLR^{-/-}} \) littermate controls (Figure 1F). Similarly, there was a significant increase of lipid-burden plaque area in PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice compared with their littermate controls as revealed by oil red O staining on cross-sectional aorta sinus (Figure 1G). These data demonstrate an accelerated atherogenesis in PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice, suggesting an important role for endothelial PPAR\( \gamma \) in protecting against atherogenesis.

**Deficiency of PPAR\( \gamma \) in EC Accelerates the Initiation of Atherosclerosis in \textit{Ldlr}^{-/-} \) mice**

Recent studies demonstrate that Tie2 is expressed in 20% of human blood monocytes.\(^{26}\) To test whether a PPAR\( \gamma \)-dependent mechanism in macrophages accounts for the accelerated atherogenesis in the PPAR\( \gamma ^{\text{AF}C/LDLR^{-/-}} \) mice, mice with a conditional disruption of PPAR\( \gamma \) in macrophages, were bred to the LDLR\( ^{-/-} \) background (PPAR\( \gamma ^{\text{AMe}/LDLR^{-/-}} \)). Interestingly, as revealed by en face staining with aorta (Figure 2A), PPAR\( \gamma ^{\text{AMe}/LDLR^{-/-}} \) mice on 4 weeks of high-cholesterol diet did not develop an increase in atherosclerotic lesions compared to PPAR\( \gamma ^{\text{F/F}/LDLR^{-/-}} \) littermate control mice. Although a previous study showed that macrophage-specific PPAR\( \gamma \) deficiency could increase atherosclerosis in LDLR\( ^{-/-} \) mice challenged with 16 weeks of butterfat diet,\(^{5}\) our data suggested that disruption of PPAR\( \gamma \) in macrophage might not be essential for the initiation of atherogenesis.

PPAR\( \gamma \) is disrupted in bone-marrow-derived hematopoietic cells by the Tie2-driven Cre recombinase.\(^{18,19}\) To further elucidate whether the large increase in atherogenesis in PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice was due to decreased PPAR\( \gamma \) in hematopoietic cells, bone marrow transplantation was undertaken. Bone marrow from PPAR\( \gamma ^{\text{F/F}/LDLR^{-/-}} \) or PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) was transplanted into 6-week-old, lethally irradiated PPAR\( \gamma ^{\text{F/F}/LDLR^{-/-}} \) or PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \), respectively. Four weeks after transplantation, peripheral blood was collected confirming full reconstitution of donor hematopoietic cells in the recipient mice. (Figure 2B). Mice were fed high-cholesterol diet for 4 weeks following bone marrow transplantation. PPAR\( \gamma ^{\text{F/F}/LDLR^{-/-}} \) mice reconstituted with PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) bone marrow exhibited 3.4\%\( \pm \)0.26% of lesion areas in aortic arch (Figure 2C, left panel) and 0.008\%\( \pm \)0.003 mm\(^2\) of lesion in cross sections (Figure 2D, left panel). However, PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice transplanted with PPAR\( \gamma ^{\text{F/F}/LDLR^{-/-}} \) bone marrow maintained larger lesion areas both in aortic arch (Figure 2C, 17.0\%\( \pm \)0.90%) and in cross sections (Figure 2D, 0.079\%\( \pm \)0.004 mm\(^2\)). These data suggest that loss of PPAR\( \gamma \) in bone-marrow-derived hematopoietic cells could not account for the enhanced aortic atherosclerosis in PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice. These data indicate that EC dysfunction in host mice lacking PPAR\( \gamma \) is likely a major cause of lesion initiation in PPAR\( \gamma ^{\text{EC}/LDLR^{-/-}} \) mice.

**Deficiency of PPAR\( \gamma \) in EC Increases Serum Cholesterol and Triglycerides Following High-cholesterol Diet**

Multiple metabolic parameters were assessed following high-cholesterol diet administration. No changes in body weights were observed between PPAR\( \gamma ^{\text{AF}C/LDLR^{-/-}} \) and littermate control mice on standard chow or high-cholesterol diet (Figure 3A). At 1 week of high cholesterol feeding, both genotypes developed dyslipidemia (Figure 3B). PPAR\( \gamma ^{\text{AF}C/LDLR^{-/-}} \) mice manifested more marked accumulation of serum cholesterol (Figure 3C, 2880\%\( \pm \)572 versus \( 1420\%\pm 139 \text{ mg/dL} \)) and TG (Figure 3D, 1170\%\( \pm \)209 versus 732\%\pm 171 \text{ mg/dL}) than PPAR\( \gamma ^{\text{F/F}/LDLR^{-/-}} \) controls. Cholesterol concentrations were also measured in lipoprotein fractions separated by fast protein liquid chromatography. PPAR\( \gamma ^{\text{AF}C/LDLR^{-/-}} \) mice exhibited more VLDL and LDL than PPAR\( \gamma ^{\text{F/F}/LDLR^{-/-}} \) mice (Figure 3E). Previous studies indicated that PPAR\( \gamma \) in the endothelium regulates the metabolic response to high-fat diet in mice.\(^{18,19}\) In primary ECs

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isolated from PPARγEC/LDLR−/− mice, a series of known PPARγ target genes involved in cholesterol and triglyceride metabolism were analyzed. No changes were found in mRNA expression of ABCA1 and ABCG1, which are known as cholesterol reverse transporters. However, CD36, a long-chain fatty acid receptor that facilitates fatty acid uptake, was significantly decreased in ECs from PPARγEC/LDLR−/− mice. Fatty acid binding protein 4 was also repressed in the PPARγ-deficient ECs (Figure 3F).

**Figure 1.** Deficiency of peroxisome proliferator-activated receptor γ (PPARγ) in endothelial cells (ECs) accelerates atherogenesis in LDL receptor (LDLR)−/− mice. PPARγF/F/LDLR−/− and PPARγEC/LDLR−/− mice consumed a high-cholesterol diet for 4 weeks and underwent analysis of lesion size in the aorta and aortic root. A, Representative images of entire aortas stained with oil red O for lipid deposition. B–E, Quantification of lesion area for entire aorta (B), aortic arch (C), thoracic aorta (D), and abdominal aorta (E), respectively. All the quantification was expressed as % of corresponding area (n=8). F, Representative examples of cross-sections from the aortic root stained with hematoxylin and eosin H&E. Black arrows indicate atherosclerotic lesions. G, Representative examples of cross-sections from the aortic root stained with oil red O to show the lipid deposition.

EC PPARγ Disruption Is Associated With Increased Blood Pressure After High-Cholesterol Diet Feeding

Because hypertension is a major risk factor for atherosclerosis, systolic BP was analyzed using tail-cuff plethysmography. Without high-cholesterol diet challenge, there was no significant difference between PPARγEC/LDLR−/− (124±6.2 mm Hg) and PPARγF/F/LDLR−/− (120±3.4 mm Hg). High-cholesterol diet feeding increased systolic BP in both
groups, however, PPAR\textsubscript{γ}\textsuperscript{AEc/LDLR}\textsuperscript{-/-} mice showed a higher mean systolic BP compared with their littermate controls (Table). This is consistent with previous data showing that PPAR\textsubscript{γ} deficiency in EC does not influence basal BP but augments high-fat diet-induced hypertension.\textsuperscript{18} Measurement of AngII type 1 receptor (AT\textsubscript{1}R) and eNOS mRNAs in the aortas from mice on standard chow diet or high-cholesterol diet revealed no statistical difference in levels between PPAR\textsubscript{γ}\textsuperscript{F/F}/LDLR\textsuperscript{-/-} and PPAR\textsubscript{γ}\textsuperscript{AEc/LDLR}\textsuperscript{-/-} mice on standard chow diet. When challenged with a high-cholesterol diet, an increase of AT1R mRNA was detected in PPAR\textsubscript{γ}\textsuperscript{AEc/LDLR}\textsuperscript{-/-} mice transplanted with PPAR\textsubscript{γ}\textsuperscript{AEc/LDLR}\textsuperscript{-/-} bone marrow. DNA was extracted from 100 μL of peripheral blood 4 weeks after bone marrow transplantation. The PCR product for the Ppar\textsubscript{γ} allele is 285 bp and for the Ppar\textsubscript{γ}AEc allele is 400 bp.

**Increased Inflammation and Macrophage Infiltration by PPAR\textsubscript{γ} Disruption In Vitro and In Vivo**

A significant increase in endothelial permeability was observed after disruption of PPAR\textsubscript{γ} in EC. Because no direct transcriptionally mediators were identified, inflammation was assessed. Proinflammatory mediators were shown to be direct repressive targets of PPAR\textsubscript{γ}\textsuperscript{27,28} and increased inflammation is a major mechanism leading to endothelial barrier dysfunction.\textsuperscript{28,29} To understand the mechanism by which ECs are activated, primary ECs were used and the expression patterns of proinflammatory genes were analyzed. PPAR\textsubscript{γ}-deficient ECs exhibited higher expression of inflammatory chemokines and adherent molecules, such as MCP-1, ICAM-1, and VCAM-1, which are important for monocyte recruitment and interaction with endothelium. Interestingly, iNOS, a key mediator critical for inflammation and barrier permeability,
was dramatically increased after EC-PPARγ disruption. Moreover, the expression of the above genes was further extended in response to lipopolysaccharide treatment for 16 hours (Figure 5A). Next, EC-monocyte adhesion assays were performed to determine the recruitment of monocytes to activated ECs. PPARγ disruption markedly increased the primary monocyte adhesion to ECs under basal level, and preadministration of lipopolysaccharide augmented the recruitment and adhesion of monocytes to ECs (Figure 5B). These data demonstrated an activated phenotype of ECs by PPARγ loss–of–function in ECs in vitro. Activation of ECs was further assessed by analysis of mRNAs encoding inflammatory modulators in the vessel wall of PPARγEC/LDLR−/− mice after high-cholesterol diet treatment. Consistent with in vitro data, several inflammatory molecules, MCP-1, iNOS, and TNFα, were significantly upregulated in the PPARγEC/LDLR−/− mice compared to PPARγFF/LDLR−/− mice after high-cholesterol diet treatment. VCAM-1, which is expressed after the activation of ECs and mediates the adhesion of leukocytes, was also significantly increased in PPARγEC/LDLR−/− aorta (Figure 5C). In addition, the expression of VCAM-1 and macrophage marker F4/80 was examined in aortic roots by immunofluorescent staining. PPARγEC/LDLR−/− mice demonstrated a greater area positive for VCAM-1 and F4/80 (Figure 5D),

**Table. Mean Systolic Blood Pressure for PPARγFF/LDLR−/− or PPARγEC/LDLR−/− Mice on High-Cholesterol Diet**

<table>
<thead>
<tr>
<th>Group</th>
<th>0 wk (mm Hg)</th>
<th>1 wk (mm Hg)</th>
<th>4 wk (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγFF/LDLR−/−</td>
<td>120 ± 3.4</td>
<td>128 ± 3.2**</td>
<td>134 ± 2.5**</td>
</tr>
<tr>
<td>PPARγEC/LDLR−/−</td>
<td>124 ± 6.2</td>
<td>139 ± 5.6†</td>
<td>149 ± 6.0††</td>
</tr>
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0 wk indicates standard chow diet; 1 wk, 1 wk treatment with high cholesterol diet; 4 wk, 4 wk of treatment with high cholesterol diet; PPAR, peroxisome proliferator-activated receptor; LDLR, LDL receptor.

Mean values were calculated by averaging the last 2-day values for each animal.

Values shown were means ± SEM. N = 6 per group. *P < 0.05 and **P < 0.01 vs PPARγFF/LDLR−/− (0 wk). †P < 0.05 vs PPARγFF/LDLR−/− (1 wk) and ††P < 0.01 vs PPARγFF/LDLR−/− (4 wk).
Atherosclerosis is a complex disease due to the formation of atherosclerotic lesions consisting of accumulated modified lipids, VSMCs, ECs, leukocytes, and foam cells. PPARγ is highly expressed in both the normal vasculature, including ECs, VSMCs, and macrophages, and in atherosclerotic plaques. Several studies were performed to investigate the role of PPARγ in the development of atherosclerosis. In macrophage, loss of PPARγ leads to reduced cholesterol efflux and decreased expression of lipoprotein lipase, liver X receptor, and ABCG1. Transplantation of PPARγ-deficient macrophage into LDLR−/− mice resulted in significant increase of atherosclerotic lesions. Others showed that PPARγ deficiency in VSMC specifically could augment angiotension II-induced atherosclerosis without affecting abdominal aortic aneurysms. Although these studies demonstrated a beneficial effect of PPARγ in more advanced stages of atherosclerotic lesions, little is known about the role of PPARγ in the initiation of atherosclerosis.

The present study demonstrated that PPARγ in ECs is a critical player in protecting against the development of atherosclerosis, especially the initiation of atherogenesis using a loss-of-function strategy. LDLR−/− mice with EC-specific disruption of PPARγ develop atherosclerosis as early as 4 weeks on a high-cholesterol diet, suggesting an important vascular protective role of endothelial PPARγ against atherogenesis. Mechanistically, disruption of EC PPARγ leads to an increase of several proatherosclerotic pathways including exacerbated dyslipidemia, raised systolic BP, increased endothelial permeability, enhanced expression of proinflammatory cytokines, and increased monocyte/macrophage recruitment and infiltration into vessel walls.

ECs serve as an important barrier between vascular tissue and blood components as well as modulate the traffic of immune cells. Its activation on exposure to cardiovascular risk factors, such as hyperglycemia, hypercholesterolemia, hypertension, and smoking is the first step of atherogenesis. Disruption of PPARγ in ECs exacerbates dyslipidemia in LDLR−/− mice. Defective cholesterol and triglyceride metabolism in EC after PPARγ disruption might contribute to the large increases in serum lipid concentrations. A previous study showed PPARγ in ECs regulates metabolic responses to high-fat diet in mice, in which defective fatty acid uptake and triglyceride metabolism occurred only in the absence of EC PPARγ. The data in the present article further confirms the role of EC PPARγ in regulation of lipid homeostasis.

Hypertension is a major risk factor for atherosclerosis and cardiovascular disease. The current study showed that PPARγ disruption in EC raised systolic BP after a high-cholesterol diet challenge. In addition, AT1R mRNA was also significantly upregulated, suggesting an impaired vascular activity. Others revealed that PPARγ disruption in EC is associated with impaired vascular relaxation after a high-fat diet in which carotid artery dilation response to carbobach was measured after phenylephrine preconstriction. In addition, an increased contraction in aortic rings was demonstrated in PPARγ KO mice. These data support the current study of endothelial dysfunction and increased BP in PPARγ−/− mice.

Deficiency of EC PPARγ dramatically upregulated several proinflammatory molecules, such as vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), and TNFα in the vessel wall, all of which are critical for the recruitment, rolling, and trafficking of monocytes into subendothelium. This result is consistent with in vitro studies showing that PPARγ activation by its ligands inhibits proinflammatory cytokines and chemokines in ECs. Bioinformatics analysis of gene sets regulated by ligand-ac-

**Discussion**

Atherosclerosis is a complex disease due to the formation of atherosclerotic lesions consisting of accumulated modified lipids, VSMCs, ECs, leukocytes, and foam cells. PPARγ is highly expressed in both the normal vasculature, including ECs, VSMCs, and macrophages, and in atherosclerotic plaques. Several studies were performed to investigate the role of PPARγ in the development of atherosclerosis. In macrophage, loss of PPARγ leads to reduced cholesterol efflux and decreased expression of lipoprotein lipase, liver X receptor, and ABCG1. Transplantation of PPARγ-deficient macrophage into LDLR−/− mice resulted in significant increase of atherosclerotic lesions. Others showed that PPARγ deficiency in VSMC specifically could augment angiotension II-induced atherosclerosis without affecting abdominal aortic aneurysms. Although these studies demonstrated a beneficial effect of PPARγ in more advanced stages of atherosclerotic lesions, little is known about the role of PPARγ in the initiation of atherosclerosis.

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**Figure 4.** Increased permeability of endothelium in the absence of peroxisome proliferator-activated receptor γ (PPARγ). A, Photographs of ears after treatment with mustard oil (15 minutes) and vascular perfusion, showing relative amount of extravasated Evans blue tracer. B, Spectrophotometric measurement of amount of extravasated Evans blue in mouse ears 15 minutes after topical application of mustard oil. *P<0.05 compared with controls. C, Expression of genes related with adherens junctions and tight junctions in endothelial cells (ECs). NS indicates no statistic difference; LDLR indicates LDL receptor.
tivated and dominant-negative PPARγ in the mouse aorta is consistent with the present study. VCAM-1, ICAM-1, MCP-1, and TNFα mRNA expression were repressed by PPARγ ligand rosiglitazone and induced by PPARγ P465L dominant-negative mutation.40

Disruption of PPARγ in EC significantly raised vascular permeability as demonstrated by Evans Blue leakage assay. Although no transcriptional changes of barrier genes were observed, dyslipidemia and increased inflammation that are found in the result in impaired barrier function of ECs. Inflammation and iNOS can increase barrier permeability in EC.41 This is consistent with the present results showing increased proinflammatory cytokines, chemokines, and iNOS in PPARγΔEC mouse ECs that have elevated vascular permeability.

The present study demonstrates PPARγ deficiency in EC leads to multiple endothelial dysfunctions in LDLR−/− mice exposed to high-cholesterol diet. Although further study is needed to explore which is the first step in this process, the current study provides first definitive evidence that endothelial PPARγ serves as a critical player for protecting against the initiation of atherosclerosis using EC-specific PPARγ disruption animal models and thus raises a potentially clinical therapeutic strategy to prevent atherogenesis.

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Figure 5. Lack of endothelial cell (EC) peroxisome proliferator-activated receptor γ (PPARγ) leads to increased inflammation in vessel wall. A, qPCR analysis for mRNA levels of proinflammatory genes in primary ECs from PPARγF/F/LDLR−/− or PPARγΔEC/LDLR−/− mice with or without 100 ng/mL lipopolysaccharide treatment. B, Endothelial-macrophage adhesion assay. Calcein AM-labeled primary macrophages bound to ECs were visualized on fluorescence microscopy. The number of bound macrophages was quantified by counting 5 microscopic fields per well in triplicates. C, qPCR analysis for mRNA levels of proinflammatory genes in the whole aortas from PPARγF/F/LDLR−/− or PPARγΔEC/LDLR−/− mice with 4-week high-cholesterol diet challenge. D, Representative examples of cross sections from the aortic sinus labeled for VCAM-1 and F4/80 (marker for macrophages), respectively. *P<0.05, **P<0.01, and ***P<0.001 vs the controls.

Disclosures

None.

References

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Supplemental Materials

Disruption of Endothelial Peroxisome Proliferator-activated Receptor γ Accelerates Diet-induced Atherogenesis in Low-density Lipoprotein Receptor-null Mice

Aijuan Qu, Yatrik M. Shah, Soumen K. Manna, and Frank J. Gonzalez
Supplemental Table 1 Primer List

<table>
<thead>
<tr>
<th>qPCR</th>
<th>Sequences</th>
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<tr>
<td>β-actin FWD</td>
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<td>β-actin REV</td>
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<td>5’-GACCGACACATTGGAAT-3’</td>
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Supplemental figure I. Disruption of PPARγ gene via Cre-loxP-mediated recombination.
A. PCR diagnostic for Tie2-Cre-mediated recombination of PPARγ allele in genomic DNA isolated from tissues of Pparγ<sup>F/F</sup>/Ldlr<sup>−/−</sup> or Pparγ<sup>EC</sup>/Ldlr<sup>−/−</sup> mice.
B. qPCR analysis of PPARγ mRNA expression in the tissues from Pparγ<sup>F/F</sup>/Ldlr<sup>−/−</sup> or Pparγ<sup>EC</sup>/Ldlr<sup>−/−</sup> mice. For qPCR analysis the expression was normalized to β-actin and each bar represents the mean value ± S.D. **P<0.01 compared to wild-type floxed littermates.
Supplemental figure II. No atherosclerotic lesions in $PPAR_\gamma^{EC}/Ldlr^{+/+}$ on high-cholesterol diet or $Ppar_\gamma^{EC}/Ldlr^{-/-}$ mice on standard chow diet.
A. Aortas from $Ppar_\gamma^{FF}/Ldlr^{+/+}$ or $Ppar_\gamma^{EC}/Ldlr^{+/+}$ mice fed with high cholesterol diet for 4 weeks. Of note, no lesions were observed in the aortic arch.
B. Aortas from $Ppar_\gamma^{FF}/Ldlr^{-/-}$ or $Ppar_\gamma^{EC}/Ldlr^{-/-}$ mice on standard chow diet for 26 weeks. No lesions were observed in the aortic arch.
Supplemental figure III. AT1R and eNOS mRNA levels in Pparγ^{FF}/Ldlr^-/- or Pparγ^{EC}/Ldlr^-/- mice.

Messenger RNA was isolated from Pparγ^{FF}/Ldlr^-/- or Pparγ^{EC}/Ldlr^-/- mice fed with standard chow diet or high cholesterol diet for 4 weeks, **p<0.01, n=5. AT1R and eNOS mRNAs were measured by qPCR.