

Short Communication

Pioglitazone-Induced Reductions in Atherosclerosis Occur via Smooth Muscle Cell-Specific Interaction With PPAR γ

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Rationale: Peroxisome proliferator-activated receptor (PPAR) γ agonists attenuate atherosclerosis and abdominal aortic aneurysms (AAAs). PPAR γ , a nuclear receptor, is expressed on many cell types including smooth muscle cells (SMCs).

Objective: To determine whether a PPAR γ agonist reduces angiotensin II (Ang II)-induced atherosclerosis and AAAs via interaction with SMC-specific PPAR γ .

Methods and Results: Low-density lipoprotein receptor (LDLR) $^{-/-}$ mice with SMC-specific PPAR γ deficiency were developed using PPAR γ floxed (PPAR $\gamma^{fl/fl}$) and SM22 Cre $^{+}$ mice. PPAR $\gamma^{fl/fl}$ littermates were generated that did not express Cre (Cre $^{0/0}$) or were hemizygous for Cre (Cre $^{+/0}$). To assess the contribution of SMC-specific PPAR γ in ligand-mediated attenuation of Ang II-induced atherosclerosis and AAAs, both male and female Cre $^{0/0}$ and Cre $^{+/0}$ mice were fed a fat-enriched diet with or without the PPAR γ agonist pioglitazone (Pio) (20 mg/kg per day) for 5 weeks. After 1 week of feeding modified diets, mice were infused with Ang II (1000 ng/kg per minute) for 4 weeks. SMC-specific PPAR γ deficiency or Pio administration had no effect on plasma cholesterol concentrations. Pio administration attenuated Ang II-increased systolic blood pressure equivalently in both Cre $^{0/0}$ and Cre $^{+/0}$ groups. SMC-specific PPAR γ deficiency increased atherosclerosis in male mice. Pio administration reduced atherosclerosis in only the Cre $^{0/0}$ mice, but not in mice with SMC-specific PPAR γ deficiency. SMC-specific PPAR γ deficiency or Pio administration had no effect on Ang II-induced AAA development. Pio also did not attenuate Ang II-induced monocyte chemoattractant protein-1 production in PPAR γ -deficient SMCs.

Conclusions: Pio attenuates Ang II-induced atherosclerosis via the interaction with SMC-specific PPAR γ , but has no effect on the development of AAAs. (*Circ Res.* 2010;107:953-958.)

Key Words: Ang II ■ atherosclerosis ■ smooth muscle cell ■ PPAR γ ■ Pioglitazone

Thiazolidinediones (TZDs), including rosiglitazone and pioglitazone (Pio), are used widely to improve insulin sensitivity in patients with type 2 diabetes. Experimentally, TZDs reduce atherosclerosis in both low-density lipoprotein receptor (LDLR) $^{-/-}$ and apolipoprotein (Apo)E $^{-/-}$ mice.^{1,2} Recent studies have demonstrated that TZDs also reduce Ang II-induced abdominal aortic aneurysm (AAA) development in ApoE $^{-/-}$ mice.^{3,4} The molecular target for TZD is PPAR γ , a nuclear receptor that is highly expressed in all cell types involved in vascular pathologies, including macrophages, endothelial cells, and smooth muscle cells (SMCs).⁵ Currently, it has not been defined whether the beneficial effects of TZDs are attributable to PPAR γ agonism in a specific cell type. Furthermore, it has been

suggested that TZDs may exert some of their biological effects through PPAR γ -independent mechanisms, although this has not been defined in vascular pathologies.⁶

TZDs have been demonstrated to regulate important SMC functions, including proliferation and migration.⁷ SMC-specific genetic manipulations have resulted in changes in both atherosclerosis^{8,9} and AAAs in mice.⁹ Furthermore, SMC-specific gene deletion of PPAR γ results in changes in blood pressure and injury-induced vascular hyperplasia.^{10,11} However, no studies have currently determined whether the benefits of TZDs on vascular pathologies are mediated via a SMC-specific PPAR γ -dependent mechanism.

To elucidate a role of SMC-specific PPAR γ expression on TZD-induced reductions in atherosclerosis and AAAs,

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Non-standard Abbreviations and Acronyms	
AAA	abdominal aortic aneurysm
Ang II	angiotensin II
ApoE	apolipoprotein E
IFN	interferon
LDLR	low-density lipoprotein receptor
MCP	monocyte chemoattractant protein
Pio	pioglitazone
PPAR	peroxisome proliferator-activated receptor
SBP	systolic blood pressure
SMC	smooth muscle cell
TZD	thiazolidinedione

we bred female LDLR^{-/-} mice harboring PPAR γ floxed genes to similarly genetically manipulated males that were hemizygous for Cre regulated by the SM22 promoter. This breeding strategy generated littermate controls that were either wild-type or SMC-specific deficient in PPAR γ . Using these mice, we determined the contribution of PPAR γ expression in SMCs to the effects of Pio on Ang II-induced atherosclerosis and AAAs.¹² The results demonstrate that SMC-PPAR γ deficiency resulted in increased Ang II-induced atherosclerosis. Furthermore, these data demonstrate that PPAR γ expression in SMCs is a major contributor to Pio-induced reduction in atherosclerosis. Contrary to previous studies, we did not discern an effect of Pio on Ang II-induced AAAs.

Methods

An expanded Methods section is available in the Online Data Supplement at <http://circres.ahajournals.org>.

Results

Generation of LDLR^{-/-} Mice With SMC-Specific PPAR γ Deficiency

To verify the genotype of mice, aortas were dissected free, adventitia and endothelium were removed, and DNA was isolated from SMC-containing media. PCR analyses were performed on DNA isolated from the arch, thorax, suprarenal, and infrarenal aortic regions to determine the uniformity of Cre-based exon excision. These analyses demonstrated the presence of nonfunctional alleles (240-bp amplicon) throughout aortas of Cre-expressing mice. In contrast, aortas from nontransgenic littermates generated 215-bp amplicons derived from intact floxed genes (Figure 1A).

RT-PCR analyses showed complete deletion of PPAR γ mRNA in SMC aortic medias of Cre⁺⁰ mice (Figure 1B), indicating that functional PPAR γ transcripts were ablated. Western blot analyses demonstrated that PPAR γ protein was ablated in aortic SMCs from Cre⁺⁰ mice, while not influencing abundance in liver, kidney, and adipose tissue (Figure 1C and Online Figure 2).

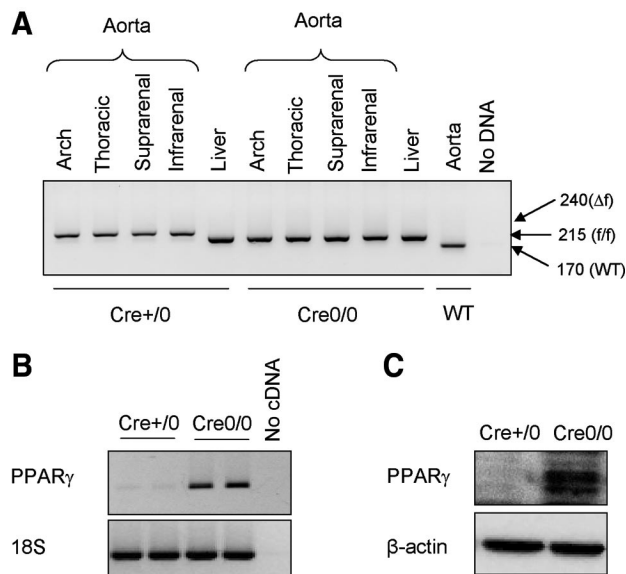


Figure 1. Generation of SMC-specific PPAR γ -deficient mice. SMC-specific PPAR γ -deficient mice were generated as described in the Methods. **A**, PCR analyses of DNA demonstrated complete deletion of PPAR γ in SMC-containing medial layer from all aortic regions. A 215-bp amplicon was generated from Cre^{0/0} mice, whereas a 240-bp amplicon was obtained from Cre⁺⁰ mice. **B**, RT-PCR analyses showed deletion of PPAR γ mRNA in aortic medias. **C**, Western blot analyses showed deletion of PPAR γ protein in Cre⁺⁰ SMCs.

SMC-Specific Deficiency of PPAR γ Augmented Ang II-Induced Atherosclerosis Without Affecting AAAs

SMC-specific PPAR γ deficiency in LDL receptor^{-/-} mice resulted in significant ($P < 0.05$) increases in Ang II-induced atherosclerotic lesion areas in male mice, but had no effect in females (Figure 2, A and B). SMC-specific deletion of PPAR γ had no effect on body weight, plasma total cholesterol concentrations (Online Table I), or lipoprotein-cholesterol distributions (data not shown). Ang II infusion significantly increased systolic blood pressure (SBP) in male mice of both groups (Online Table I). SMC-specific PPAR γ deficiency had no effect on Ang II-induced AAA formation (Figure 2C) or aortic rupture (Cre^{0/0}, 25%; versus Cre⁺⁰, 28%) in either sex.

Pio Attenuated Ang II-Induced Atherosclerosis Only in the Presence of PPAR γ in SMCs

In Ang II-infused mice fed a fat-enriched diet, PPAR γ mRNA abundance was not significantly increased in peritoneal macrophages (Online Figure III). Pio administration to these mice induced PPAR γ mRNA abundance and activity in selected cell types and tissues, including macrophages, liver, kidney, and adipose. These inductions did not differ between Cre^{0/0} and Cre⁺⁰ mice (Figure 3A and 3B; Online Figure IV). Increased PPAR γ activity was demonstrable by increased mRNA abundance of PPAR γ target genes: AP2 and CD36 in macrophages (Online Figure V) and selected tissues (Online Figure VI).

Pio administration profoundly reduced atherosclerosis only in Cre^{0/0} mice, but not in mice with SMC-specific

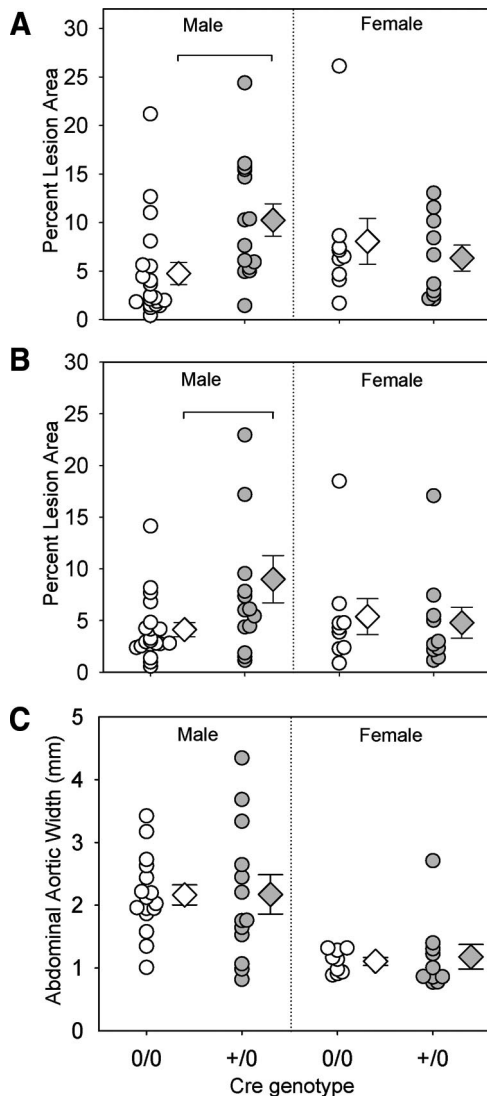


Figure 2. SMC-PPAR γ deficiency augmented Ang II-induced atherosclerosis but had no effect on AAAs in male LDLR $^{-/-}$ mice. Atherosclerotic lesion area was measured on aortic arch (A) and thoracic (B) intimal surfaces (n=9 to 20). C, Measurements of maximal external width of abdominal aortas (n=9 to 16). Open circles (Cre $^{0/0}$) and gray circles (Cre $^{+0}$) represent individual mice, diamonds represent means, and bars are SEMs. Statistical analyses were performed using Mann-Whitney rank sum analyses. Horizontal bars represent significance of $P < 0.05$.

PPAR γ deficiency (Figure 3C and 3D). In contrast, Pio administration significantly attenuated Ang II–increased SBP equivalently in both Cre $^{0/0}$ and Cre $^{+0}$ groups (Online Table II). Pio administration had no effect on body weight, plasma total cholesterol concentrations (Online Table II), or lipoprotein cholesterol distributions (data not shown). AAA formation (Figure 3E) or aortic rupture (Cre $^{0/0}$, 11%; versus Cre $^{+0}$, 20%) was not different between groups.

Immunostaining of atherosclerotic lesions with α -actin demonstrated uniform reactivity throughout the medial intralaminar spaces of all groups, but minimal SMC immunostaining was detected in atherosclerotic lesions from any group. Although PPAR γ deficiency increased

lesion size, immunostaining for macrophages was dominant in atherosclerosis from both Cre $^{0/0}$ or Cre $^{+0}$ mice.

Ang II Augmented Monocyte Chemoattractant Protein-1 Production in PPAR γ -Deficient SMCs

To define potential mechanisms of Pio reducing atherosclerosis, plasma monocyte chemoattractant protein (MCP)-1 concentrations were measured. No significant difference was observed among groups demonstrating no systemic effect on MCP-1 (Online Figure VII).

Aortic SMCs cultured from either Cre $^{0/0}$ or Cre $^{+0}$ mice were incubated with Pio (20 μ mol/L) for 24 hours, and with or without Ang II (1 μ mol/L) for a further 18 hours. Ang II significantly increased MCP-1 concentrations from Cre $^{+0}$ SMCs but had no significant effect on Cre $^{0/0}$ SMCs (Figure 4). Coincubation with Pio had no effect on Ang II–induced MCP-1 production in Cre $^{+0}$ SMCs.

Consistent with SMCs harvested from Cre $^{0/0}$ and Cre $^{+0}$ mice, Ang II increased MCP-1 concentrations in media of SMCs cultured from mice expressing a dominant-negative mutation of PPAR γ P465L (PPAR γ^{L+})¹³ but not in cells isolated from nontransgenic littermates. To determine whether PPAR γ has a dominant effect on MCP-1 secretion, SMCs were incubated with interferon (IFN) γ . In contrast to Ang II, IFN γ (300 U/mL) significantly increased MCP-1 concentrations in media of SMCs from both strains (Figure 4B). Pio had no effect on IFN γ –induced MCP-1 (Figure 4C).

To confirm that the effects of Pio on MCP-1 were attributable to interactions with PPAR γ , Cre $^{0/0}$ and Cre $^{+0}$ SMCs were incubated with Pio and Ang II as described above. The absence of PPAR γ in SMCs significantly lowered AP2 mRNA abundance but failed to affect CD36 (Online Figure VIII). Ang II incubation significantly reduced mRNA abundance of both these target genes in Cre $^{+0}$ SMCs. Coincubation of Ang II and Pio significantly attenuated the reduced mRNA abundance of target genes in SMCs from Cre $^{0/0}$ but not Cre $^{+0}$ mice.

Discussion

In the present study, we demonstrate that SMC-specific PPAR γ deficiency augments Ang II–induced atherosclerosis in male LDLR $^{-/-}$ mice. Interestingly, Pio administration attenuates Ang II–induced atherosclerosis only in wild-type mice, but not in SMC-specific PPAR γ -deficient mice, which characterizes SMC-specific PPAR γ as the key molecular target for the ligand-mediated attenuation of atherosclerosis.

SMC-specific PPAR γ deficiency augmented Ang II–induced atherosclerosis only in male mice. This is in agreement with the study by Li et al, in which the attenuation of atherosclerosis by a PPAR γ ligand was observed only in male LDLR $^{-/-}$ mice.¹ The basis for these sex differences have not been defined.

Pio administration activates PPAR γ in both Cre $^{0/0}$ and Cre $^{+0}$ genotypes, which was evidenced by increased PPAR γ expression observed in peritoneal macrophages

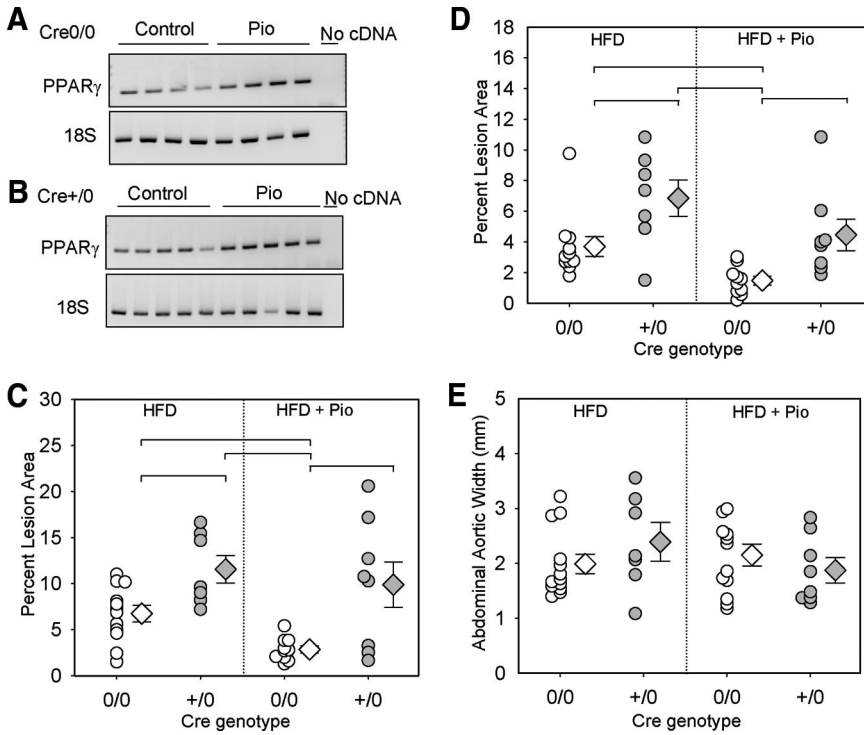


Figure 3. Pio attenuated Ang II-induced atherosclerosis via SMC-PPAR γ . Peritoneal macrophages were harvested from Cre^{0/0} (**A**) and Cre⁺⁰ (**B**) mice fed with or without Pio. Total RNA was extracted and analyzed by RT-PCR using 18S as an internal control. Atherosclerotic lesion areas were measured in aortic arch (**C**) and thoracic (**D**) intimal surfaces (n=7 to 12). **E**, Measurements of maximal external width of abdominal aortas (n=7 to 13). **Open circles** (Cre^{0/0}) and **gray circles** (Cre⁺⁰) represent individual mice, and **diamonds** represent means and bars are SEMs. **Horizontal bars** represent significance of $P < 0.05$ by 2-way ANOVA followed by Holm-Sidak post hoc tests.

and other tissues. Previous in vitro studies demonstrated that TZDs inhibited SMC proliferation and induced apoptosis through PPAR γ -dependent mechanisms.¹⁴ In the present study, Pio administration attenuates Ang II-in-

duced atherosclerosis only in Cre^{0/0} mice, but not in mice with SMC-specific PPAR γ deficiency. Considering that SMC proliferation constitutes an important cellular mechanism for atherosclerosis initiation,¹⁵ our findings not only

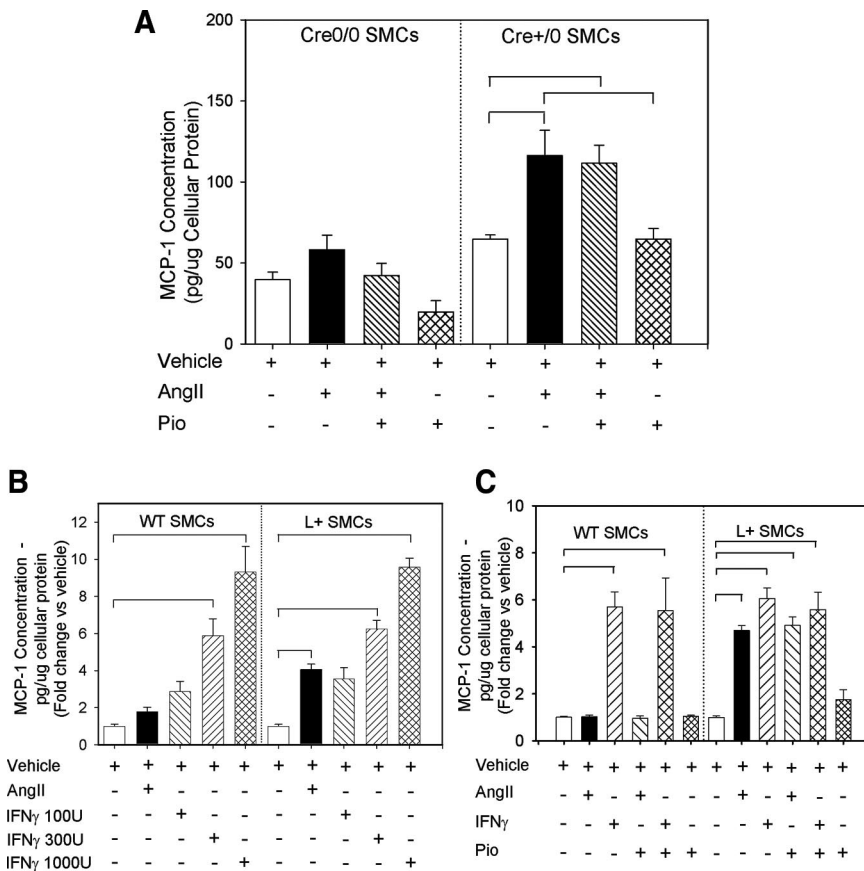


Figure 4. Ang II augmented MCP-1 production in PPAR γ -deficient SMCs. **A through C**, MCP-1 protein accumulated in cell culture media was measured by ELISA. Values are represented as means \pm SEMs. **Horizontal bars** represent significance of $P < 0.05$ by 1-way ANOVA followed by Holm-Sidak post hoc tests. WT indicates wild type.

demonstrated SMC-specific PPAR γ as an endogenous inhibitor of atherosclerosis but also established that TZDs exert antiatherosclerotic effects through this pathway.

Pio administration significantly suppresses Ang II–induced SBP in both genotypes. This result indicates that Pio-mediated SBP-lowering effect is independent of SMC-specific PPAR γ . In support of this observation, a recently published study using both SM22-Cre⁺ and Tie2-Cre⁺ PPAR γ flox mice showed that TZD-mediated the SBP-lowering effects via PPAR γ expressed in endothelium.¹⁶ Because endothelial PPAR γ is intact, Pio administration attenuates Ang II–induced SBP in both Cre^{0/0} and Cre⁺⁰ groups in our study.

SMC-specific PPAR γ deficiency or Pio administration did not influence aneurysm formation in LDLR^{-/-} mice, which is contrary to a recent publication in which Pio reduced suprarenal aortic expansion in Ang II–infused ApoE^{-/-} mice.⁴ The differences may be attributable to the lower dose used in the present study.⁴ Our dietary delivery was estimated to be \approx 20 mg/kg per day, whereas the drinking water delivery in the study by Golledge et al⁴ was estimated to be 50 mg/kg per day. In another study, rosiglitazone attenuated Ang II–induced AAA formation in ApoE^{-/-} mice, which was mainly associated with decreased expression of inflammatory mediators.³ The basis for the inconsistent effects of TZDs on Ang II–induced AAAs is unclear.

To further understand the mechanism by which Pio mediates its effect via SMC-PPAR γ on atherosclerosis, we examined the effect of Ang II on MCP-1 production in cultured Cre⁺ and PPAR γ ^{L+} SMCs. Interestingly, Ang II activates MCP-1 production only in Cre⁺⁰ and PPAR γ ^{L+} SMCs, but not in control SMCs, suggesting that endogenous SMC-PPAR γ regulates Ang II–induced MCP-1 production. In addition, Pio had no effect on Ang II–induced MCP-1 production in Cre⁺⁰ SMCs, which is consistent with this TZD requiring interaction with PPAR γ to reduce Ang II–induced atherosclerosis. The specificity of this pathway was demonstrated by the continued induction of MCP-1 secretion in PPAR γ ^{L+} cells during IFN γ incubation that signals via CD74 pathway in SMCs.¹⁷ This SMC-PPAR γ –dependent effect of Ang II is localized to SMCs, which is not reflected by plasma concentrations of MCP-1.

In summary, this study provides evidence that lack of PPAR γ in vascular SMCs results in significant increases in atherosclerosis associated with increased MCP-1 production. Furthermore, the study reveals that SMC-specific PPAR γ expression is a novel mediator of ligand-mediated attenuation of atherosclerosis.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Peroxisome proliferator-activated receptor (PPAR) γ , a nuclear receptor, is a target of therapeutic interventions to augment insulin sensitivity.
- PPAR γ expression in macrophages moderates the development of experimental atherosclerosis.
- Activation of PPAR γ by thiazolidinediones (TZDs) suppresses smooth muscle cell (SMC) proliferation.
- TZDs, PPAR γ agonists, attenuate atherosclerosis in male mice.

What New Information Does This Article Contribute?

- Pioglitazone-induced attenuation of atherosclerosis depends on PPAR γ in SMC.
- Selective deficiency of PPAR γ in SMC augments Ang II–aggravated atherosclerosis.

PPAR γ is a nuclear receptor that is highly expressed in many of cell types involved in vascular pathologies, including macro-

phages, endothelial cells, and SMCs. The TZDs (agonists of PPAR γ) have been shown to inhibit the development of atherosclerosis in male animals. Currently, it is unclear whether the beneficial effects of TZDs could be attributed to PPAR γ agonism in a specific cell type. In vitro, TZDs inhibit SMC proliferation and migration, the key events that promote intimal hyperplasia during atherogenesis; however, the contribution of SMC PPAR γ to the antiatherogenic effects of TZD has not been assessed. Because TZDs regulate SMC proliferation, which is a key step in the development of atherosclerosis, we hypothesized that SMC-specific PPAR γ is responsible for the beneficial effects of TZD on atherosclerosis. By generating SMC-specific PPAR γ -deficient mice, we show that SMC-specific PPAR γ plays a critical role in the development of Ang II–induced atherosclerosis. We demonstrate that PPAR γ expression in SMCs is required for the reduction in Ang II–induced atherosclerosis by pioglitazone. This is the first study to report that pioglitazone exerts its beneficial effect on atherosclerosis via a SMC-specific PPAR γ -dependent mechanism.