Inflammation

Hypertension-Causing Mutation in Peroxisome Proliferator–Activated Receptor γ Impairs Nuclear Export of Nuclear Factor-κB p65 in Vascular Smooth Muscle

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Abstract—Selective expression of dominant negative (DN) peroxisome proliferator–activated receptor γ (PPARγ) in vascular smooth muscle cells (SMC) results in hypertension, atherosclerosis, and increased nuclear factor-xB (NF-xB) target gene expression. Mesenteric SMC were cultured from mice designed to conditionally express wild-type (WT) or DN-PPARγ in response to Cre recombinase to determine how SMC PPARγ regulates expression of NF-xB target inflammatory genes. SMC-specific overexpression of WT-PPARγ or agonist-induced activation of endogenous PPARγ blunted tumor necrosis factor α (TNF-α)–induced NF-xB target gene expression and activity of an NF-xB–responsive promoter. TNF-α–induced gene expression responses were enhanced by DN-PPARγ in SMC. Although expression of NF-xB p65 was unchanged, nuclear export of p65 was accelerated by WT-PPARγ and prevented by DN-PPARγ in SMC. Leptomycin B, a nuclear export inhibitor, blocked p65 nuclear export and inhibited the anti-inflammatory action of PPARγ. Consistent with a role in facilitating p65 nuclear export, WT-PPARγ coimmunoprecipitated with p65, and WT-PPARγ was also exported from the nucleus after TNF-α treatment. Conversely, DN-PPARγ does not bind to p65 and was retained in the nucleus after TNF-α treatment. Transgenic mice expressing WT-PPARγ or DN-PPARγ specifically in SMC (S-WT or S-DN) were bred with mice expressing luciferase controlled by an NF-xB–responsive promoter to assess effects on NF-xB activity in whole tissue. TNF-α–induced NF-xB activity was decreased in aorta and carotid artery from S-WT but was increased in vessels from S-DN mice. We conclude that SMC PPARγ blunts expression of proinflammatory genes by inhibition of NF-xB activity through a mechanism promoting nuclear export of p65, which is abolished by DN mutation in PPARγ. (Hypertension. 2017;70:174-182. DOI: 10.1161/HYPERTENSIONAHA.117.09276.)

Key Words: aorta ■ atherosclerosis ■ hypertension ■ inflammation ■ mutation

Peroxisome proliferator–activated receptor γ (PPARγ) is a ubiquitously expressed ligand-activated transcription factor. PPARγ is well known to induce adipocyte differentiation and to regulate lipid metabolism, but other studies indicate roles and sites of PPARγ activity in other tissues, such as macrophages and brain.1-3 PPARγ plays a protective role in the vasculature, and PPARγ activators can protect against atherosclerosis and lower blood pressure.4,5 In contrast, patients carrying mutations in PPARγ exhibit severe early-onset hypertension and insulin resistance, and others exhibit hypertension and lipodystrophies.6,7 Taken together, experimental and clinical evidence points to a significant role for PPARγ in the regulation of cardiovascular homeostasis.

To explore the role of PPARγ in the vasculature, we generated mouse models expressing dominant negative (DN) mutations in PPARγ (either P467L or V290M) specifically in endothelium or vascular smooth muscle cells (SMC). Endothelial-specific interference with PPARγ led to cerebral vascular dysfunction in response to either high-fat diet or angiotensin II,8,9 whereas overexpression of PPARγ in endothelium had a protective effect on interleukin-1β–induced endothelial dysfunction.10 Transgenic mice expressing DN-PPARγ selectively in SMC (S-DN) exhibited systolic hypertension and severe vascular dysfunction through a RhoA/Rho kinase–dependent mechanism.11-14 When bred with ApoE-deficient mice and treated with a high cholesterol diet, both endothelial and SMC models exhibit exaggerated atherosclerosis associated with elevated expression of inflammatory markers in the vessel.15 Importantly, protection from atherosclerosis by a PPARγ agonist was dependent on PPARγ activity in SMC.16 However, the precise mechanism by which DN-PPARγ function exacerbates inflammatory signals and augments atherosclerosis remains unclear.

Nuclear factor-xB (NF-xB) is recognized as a central regulator of inflammation, a risk factor for cardiovascular
inactive NF-κB is retained in the cytoplasm through association with its inhibitory factor Iκ-B, whereas phosphorylation of Iκ-B promotes dissociation of NF-κB and its import into the nucleus as an active transcription factor. In vascular cells, NF-κB activation increases proinflammatory mediators, such as VCAM-1 (vascular cell adhesion molecule 1), MCP-1 (monocyte chemotactic protein 1), and matrix metalloproteinase. PPARγ has been reported to regulate NF-κB activity in macrophages by a transrepression mechanism involving the interaction between PPARγ and NF-κB, which does not require binding of the PPARγ/RXR (retinoid X receptor) heterodimer to DNA. PPARγ was also reported to act as an E3 ubiquitin ligase regulating the ubiquitination and degradation of the p65 subunit of NF-κB. Here, we tested the hypothesis that PPARγ controls the expression of inflammatory genes in SMC by regulating the activity of NF-κB p65. We provide evidence supporting the concept that PPARγ directly inhibits p65 in SMC, not through ubiquitination or altered expression, but by facilitating nuclear to cytoplasm transport of p65. This mechanism is impaired in SMC expressing the P467L DN mutant in PPARγ which does not bind p65.

Methods

Details of the experiments using cell culture, Western blotting and immunoprecipitation, real-time PCR, NF-κB promoter activity, immunostaining, bioluminescence imaging, and chemicals are described in the expanded Methods in the online-only Data Supplement.

Results

Analysis of SMC from Transgenic Mice Inducibly Expressing WT-PPARγ or DN-PPARγ

We previously reported the generation of transgenic mice carrying conditionally activatable transgenes designed to express either WT-PPARγ or DN-PPARγ, each also expressing tdTomato (Figure 1A). First, we cultured mesenteric SMC from these mice and activated the transgene by infecting the cells with an adenovirus encoding Cre recombinase (AdCre). Transgene expression remained silent in SMC infected with control adenovirus (AdGFP [adenovirus expressing green fluorescence protein]) but was induced in AdCre-infected SMC (Figure 1B through 1D). As evidence of PPARγ activation, expression of FABP4 (fatty acid binding protein 4), a known PPARγ target, was induced in SMC derived from WT-PPARγ (Figure 1E). Consistent with transcriptional impairment of DN-PPARγ, there was no induction of FABP4 expression in mesenteric SMC derived from DN-PPARγ mice (Figure 1F).

WT-PPARγ Antagonizes NF-κB–Mediated Inflammatory Pathways in SMC

To examine whether PPARγ can inhibit expression of NF-κB target genes in SMC, expression of the proinflammatory markers VCAM-1, MCP-1, and matrix metalloproteinase 9 was evaluated in AdCre- or AdGFP-treated SMC cultured from WT-PPARγ and DN-PPARγ mice after stimulation by TNF-α, interleukin-1β, or lipopolysaccharide. Expression of all 3 genes were robustly induced by TNF-α in AdGFP-treated mesenteric SMC from both WT-PPARγ and DN-PPARγ mice (Figure 2A through 2C). Similarly, expression of all 3 genes were robustly induced by interleukin-1β or lipopolysaccharide in AdGFP-treated mesenteric SMC from WT-PPARγ mice (Figure S1 in the online-only Data Supplement). AdCre-mediated activation of WT-PPARγ blunted the induction of VCAM-1, MCP-1, and matrix metalloproteinase 9 by all 3 cytokines. Similarly, WT-PPARγ prevented the TNF-α–mediated increase in activity of an NF-κB–responsive promoter in SMC (Figure 2D). By contrast, expression of DN-PPARγ augmented the TNF-α–induced expression of NF-κB target genes (Figure 2A through 2C) and TNF-α–induced activity of an NF-κB–responsive promoter (Figure 2D). There was no significant change in the level of mRNA expression of TNF-α receptor 1a or 1b in cultured SMC from transgenic mice inducibly expressing WT-PPARγ or DN-PPARγ compared with control cells (data not shown).

We next used pioglitazone, a PPARγ agonist, and GW9662, a PPARγ antagonist, to assess the activity of endogenous PPARγ in SMC. Consistent with effects of overexpression, pioglitazone decreased TNF-α–induced gene expression in SMC, an effect that was blocked by GW9662 (Figure 2E). Pioglitazone also induced the expression of the canonical PPARγ target gene FABP4, an effect blocked by GW9662 (Figure 2F). These data suggest that PPARγ activity in SMC attenuates NF-κB–dependent gene expression.

PPARγ Does Not Decrease Expression of p65 in SMC and Human Embryonic Kidney Cells

Previous reports have found that PPARγ can act as an E3 ubiquitin ligase which targets p65 for ubiquitination. To determine whether this mechanism was operant in our model, we assessed the effect of WT-PPARγ overexpression on p65 protein levels during TNF-α–induced NF-κB signaling. TNF-α–induced activation of NF-κB in SMC was evidenced by degradation of Iκ-Bα (Figure 3A). There was no alteration in the level of p65...
protein or mRNA by WT-PPARγ (Figure 3A through 3C). To assess whether PPARγ affects p65 turnover, we used human embryonic kidney 293T (HEK293) cells transfected with p65. Overexpression of WT-PPARγ in HEK293 cells failed to elicit a change in p65 protein expression levels under baseline conditions or alter p65 stability in cells treated with cycloheximide (Figure 3D). Similarly, there was no change in phospho-p65 or total p65 in the presence or absence of TNF-α in response to overexpression of either WT-PPARγ or DN-PPARγ (Figure 3E). Addition of resiglitazone, another PPARγ agonist, had no effect on phospho-p65 or total p65 levels. Combined treatment with resiglitazone and TNF-α had no effect on phospho-65 but modestly blunted total p65 irrespective of the presence of PPARγ. Contrary to a previous report, we were not able to detect any increase in p65 ubiquitination under any of these conditions (Figure S2).21 Our results suggest that degradation of p65 was not responsible for the PPARγ-mediated inhibition of expression of NF-κB target genes.

WT-PPARγ Accelerates Nuclear Export of p65 in SMC

To explore the molecular mechanism mediating the effects of PPARγ on NF-κB signaling, we determined the subcellular localization of p65 protein. Treatment with TNF-α induced rapid nuclear import of p65 in control (untransfected) primary SMC and in primary SMC induced to express WT-PPARγ (Figure 4A). In control cells, nuclear export of p65 was evident by 2 hours, and by 3 hours, there was little evidence of nuclear p65. Interestingly, nuclear export of p65 was accelerated in WT-PPARγ compared with control cells. In marked contrast, nuclear p65 was preserved 3 hours after TNF-α treatment in cultured SMC induced to express DN-PPARγ, a time point at which most nuclear p65 had been exported in control cells. The difference in nuclear export of p65 is evident when the data were quantified (Figure 4B). Pretreatment with leptomycin B, a nuclear export inhibitor, decreased the export of p65 and preserved significant levels of nuclear p65 3 hours after TNF-α treatment in control and WT-PPARγ SMC (Figure 4C). Notably, leptomycin B also increased expression of the NF-κB target gene MCP-1 in WT-PPARγ and control SMC treated with TNF-α (Figure 4D), indicating that nuclear export restricts NF-κB–mediated gene expression in SMC. It is interesting that the effect of leptomycin phenocopies the effect of DN-PPARγ, suggesting that accelerated nuclear export of p65 may contribute to the inhibitory effect of PPARγ on expression of proinflammatory genes, and thus, this may contribute to the anti-inflammatory effects of PPARγ.

WT-PPARγ Binds p65 and Promotes Cytoplasmic Export in TNF-α–Treated SMC

That PPARγ facilitated nuclear export of p65 suggested there might be a direct interaction between the proteins. Thus, we next determined whether PPARγ binds to p65 using HEK293 cells transfected with p65 and WT-PPARγ or DN-PPARγ. Coimmunoprecipitation showed that WT-PPARγ but not DN-PPARγ was associated with p65 (Figure 5A). Consistent with this association, treatment of SMC with TNF-α induced nuclear export of WT-PPARγ but not DN-PPARγ (Figure 5B). This export was blocked by either leptomycin B or by a p65-specific (pSer529, pSer536) inhibitor peptide. These data support the hypothesis that WT-PPARγ accelerates nuclear export of p65 by directly binding to p65.

WT-PPARγ Inhibits NF-κB Promoter Activity in Vessels

To examine the anti-inflammatory role of SMC PPARγ in whole vessels, we injected TNF-α intraperitoneally in our previously reported mouse models expressing DN-PPARγ (S-DN)
selectively in SMC. Injection with TNF-α (66.7 µg kg⁻¹ d⁻¹) for 3 consecutive days equivalently increased total leukocytes (CD45⁺) and monocytes/macrophages (CD45⁺/F4/80⁺) in aorta from non-transgenic and S-DN (Figure S3). Total T lymphocytes (CD3⁺), T helper (CD3⁺CD4⁺), and cytotoxic T cells (CD3⁺CD8⁺) were not altered in either group. These data indicate that the degree of leukocyte infiltration in aorta induced by systemic inflammatory activity is not altered by interference with SMC PPARγ.

To assess direct effects on inflammation-stimulated NF-κB activity in SMC of intact vessels, we bred transgenic mice expressing WT-PPARγ (S-WT) or DN-PPARγ (S-DN) specifically in SMC with mice expressing luciferase under control of a NF-κB–responsive promoter. TNF-α–induced NF-κB activity was decreased in aorta and particularly in carotid artery from S-WT×NF-κB-LUC mice compared with mice expressing only NF-κB-LUC.
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(Figure 6A). In contrast, S-DN exhibited enhanced TNF-α–induced NF-κB activity (Figure 6B).

**Discussion**

Thiazolidinediones, such as rosiglitazone and pioglitazone, are potent activators of PPARγ and were previously used to improve glycemic control in type 2 diabetes mellitus. The PROActive clinical trial (Prospective Pioglitazone Clinical Trial in Macrovascular Events) reported that pioglitazone decreased macrovascular events and lowered blood pressure and cardiovascular risk.4 In contrast, patients with PPARγ mutations exhibit hypertension.6 We previously showed that loss of PPARγ function in SMC exaggerated aortic atherosclerosis with increased NF-κB target gene expression in ApoE-deficient mice fed with western diet.15 Because NF-κB–induced proinflammatory signals drive initiation, progression, and development of atherosclerotic lesions,23 this study determined the effect of SMC-PPARγ on NF-κB activity using transgenic mice inductively overexpressing WT-PPARγ or DN-PPARγ in SMC. The main findings from our study are as follows: (1) cytokine-induced NF-κB activity in cultured SMC, as measured by expression of NF-κB target genes and the activity of an NF-κB–responsive promoter, was blunted by overexpression of WT-PPARγ but was increased by DN-PPARγ, (2) SMC-PPARγ protects against cytokine-induced NF-κB activity by facilitating nuclear export of p65 subunit, (3) PPARγ facilitates nuclear export of p65 by direct interaction, which is blocked by the DN P467L mutation in PPARγ, and (4) WT-PPARγ blunts, whereas DN-PPARγ augments TNF-α–induced activity of an NF-κB responsive promoter in aorta and carotid artery.

NF-κB is well known as a central regulator of inflammation, and several lines of evidence suggest that PPARγ protects against inflammation by interfering with NF-κB activity. Hou et al21 reported that PPARγ has a RING (really interesting new gene) domain similar to E3 ubiquitin ligases, can directly bind to the p65 subunit of NF-κB, and induce ubiquitination and degradation of p65. Although we can confirm that PPARγ and p65 can directly interact, we did not find evidence supporting ubiquitination of p65 by PPARγ. First, steady-state levels of p65 protein were not altered by overexpression of WT-PPARγ...

**Figure 3.** Nuclear factor-κB (NF-κB) p65 expression in peroxisome proliferator-activated receptor γ (PPARγ)-expressing smooth muscle cells (SMC). A, Western blot detecting the indicated proteins (representative of 6 experiments). B, Quantification of Western blots such as the representative shown in A (n=6). C, Quantitative real-time PCR detecting mouse p65 mRNA (n=6) in mesenteric artery SMC infected with either AdGFP (adenovirus expressing green florescence protein) or adenovirus encoding Cre recombinase (AdCre) from transgenic mice with inducible expression of WT-PPARγ. Data were normalized to the tumor necrosis factor α (TNF-α)–treated control. All data are mean±SEM. **D–E,** Western blots detecting the indicated proteins in human embryonic kidney 293T (HEK293T) cells transfected with p65 and WT-PPARγ or DN-PPARγ before treatment with cycloheximide (30 mg/mL, 0–12 h), TNF-α (50 ng/mL, 30 min) or rosiglitazone (1 µmol/L, 1 h) as indicated. p-p65 refers to the phosphorylated form of p65. Size markers transferred from the blots are shown. DN indicates dominant negative; and WT, wild type.
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or by rosiglitazone. Second, PPARγ did not alter the rate of p65 degradation in cells treated with the protein synthesis inhibitor cycloheximide. Third, PPARγ did not induce ubiquitination of p65. This suggests that an alternative mechanism accounts for PPARγ-mediated interference with NF-κB activity, at least in vascular SMC.

Even though the abundance of total cellular p65 was not altered by PPARγ, important changes in the temporal localization of p65 in SMC were observed. There was no difference between groups in the import of p65 into the nucleus at 30 minutes after TNF-α treatment. However, 1 to 2 hours after treatment, the amount of p65 remaining in the nucleus was significantly lower in WT-PPARγ overexpressing SMC compared with control cells. This finding is consistent with previous reports in Caco-2 cells and HUVECs (human umbilical vein endothelial cells).26,27 Interleukin-1β–induced nuclear p65 protein was also decreased by pioglitazone in vascular SMC from hypertensive rats.28 The functional importance of PPARγ to accelerate nuclear export of p65 is further illustrated by our finding that WT-PPARγ-mediated reduction in NF-κB target gene expression can be impaired by pharmacological inhibition of nuclear export. Moreover, that nuclear export of PPARγ was occurring with the same time course and could be blocked with an NF-κB inhibitor lends support for a PPARγ-dependent mechanism.

Interestingly, nuclear export of p65 was severely impaired in SMC expressing DN-PPARγ. The DN mutation in PPARγ also affects its association with p65, which could coimmunoprecipitate with WT-PPARγ but not the P467L mutant form of PPARγ. The P467L mutation resides in the ligand binding domain of PPARγ. The P467L-PPARγ protein is transcriptionally defective and acts dominant negatively. Moreover, the mutation causes hypertension in subjects carrying the mutation and hypertension in mice expressing the mutant protein selectively in SMC.6,11 It was reported that WT-PPARγ can bind p65 in the absence of ligand, but that the ligand binding domain of PPARγ is nonetheless required.21,29 Thus, the loss of PPARγ-induced p65 nuclear export in SMC expressing the P467L mutation in PPARγ is consistent with this.

One potential limitation of our study is that the molecular details of how DN-PPARγ induces accumulation of nuclear p65 after TNF-α treatment remain unclear. Expression of DN-PPARγ prevents export of both p65 and DN-PPARγ. Yet, DN-PPARγ and p65 do not physically interact, at least in a stable way detected by coimmunoprecipitation. SMC endogenously express PPARγ and pioglitazone blunted induction of an

Figure 4. p65 subcellular localization. A, p65 immunostaining (green) in tumor necrosis factor α (TNF-α; 0–3 h)–treated cultured mesenteric artery smooth muscle cells (SMC) infected with adenovirus encoding Cre recombinase (AdCre) from transgenic mice with inducible expression of either WT-PPARγ or DN-PPARγ. B, The fraction of cells with nuclear p65 was determined in a blinded fashion (50–200 cells counted per condition, n=7). C, p65 immunostaining (green) in control and WT-PPARγ expressing mesenteric SMC treated with leptomycin B (10 nmol/L, 1 hour) before TNF-α treatment (5 ng/mL, 3 hours). D, Relative mRNA expression of mouse MCP-1 (monocyte chemotactic protein 1) was determined by quantitative real-time PCR in WT-PPARγ expressing cells treated with an inhibitor of nuclear export, leptomycin B (10 nmol/L, 1 h) before TNF-α (5 ng/mL, 6 h, n=6). Data were normalized to the TNF-α–treated control. All data are means±SEM. *P<0.05 vs TNF-α–treated control cells. #P<0.05 vs TNF-α–treated AdCre-infected cells. DN indicates dominant negative; PPARγ, peroxisome proliferator–activated receptor γ; and WT, wild type.
NF-κB target gene (VCAM-1) in response to TNF-α, suggesting that DN-PPARγ interferes with effects of PPARγ to block expression of inflammatory genes. Perhaps DN-PPARγ interferes with an association between endogenous PPARγ and p65. It is also possible that the effect of PPARγ on p65 involves a PPARγ target gene whose expression is altered by DN-PPARγ. Indeed, we reported that expression of the P465L (the mouse equivalent to human P467L) mutation in PPARγ results in the alteration of many genes in the aorta.³⁰,³¹ Future molecular studies closely examining the interaction between WT-PPARγ and p65 in the presence or absence of DN-PPARγ are warranted to fully define the mechanism of this anti-inflammatory activity.

It is well accepted that the proliferation and migration of VSMC, which is regulated in part by NF-κB, is critically

![Figure 5. Peroxisome proliferator–activated receptor γ (PPARγ) association with p65. A, Human embryonic kidney 293T (HEK293T) cells transfected with p65, WT-PPARγ, or DN-PPARγ were treated with a proteasome inhibitor, MG132 (5 μmol/L, 12 h). Proteins were immunoprecipitated with p65 antibody and immunoprecipitated proteins were Western blotted for the indicated protein. The top 2 blots represent immunoprecipitation with p65 and Western blot with the indicated antibody. The bottom 3 blots represent Western blots for the indicated protein from cell lysates. Size markers transferred from the blots are shown. B, PPARγ immunostaining (green) of WT-PPARγ or DN-PPARγ expressing SMC treated with tumor necrosis factor α (TNF-α) for the indicated times. Where indicated, cells were treated with an NF-κB inhibitor (50 μmol/L) or leptomycin B (5 nmol/L) for 1 h before TNF-α. DN indicates dominant negative; PPARγ, peroxisome proliferator–activated receptor γ; and WT, wild type.](image)

![Figure 6. Nuclear factor-κB (NF-κB) activity in aorta and carotid artery. NF-κB activity was measured by luciferase assay in tumor necrosis factor α (TNF-α; 0–500 pg/mL, 16–24 h)–treated aorta and carotid arteries. Experimental mice are double transgenic mice carrying NF-κB-luciferase (LUC) reporter mice and either S-WT (A) or S-DN (B). Age- and sex-matched single NF-κB-LUC littermates from each breeding (n=8–9) were used as controls. Data were normalized to the samples from untreated single NF-κB-LUC mice. All data are mean±SEM. *P<0.05 vs untreated; #P<0.05, TNF-α (500 μg/mL) NF-κB-LUC vs S-WT×NF-κB-LUC or NF-κB-LUC vs S-DN×NF-κB-LUC. DN indicates dominant negative; PPARγ, peroxisome proliferator–activated receptor γ; and WT, wild type.](image)
involved in progression of atherosclerosis. It is also known that VSMC migration is increased in association with vascular remodeling when SMC PPARγ activity is impaired via DN P467L mutation. Activation of PPARγ is protective in atherosclerosis, and this protective action requires SM C PPARγ. Indeed, interference with PPARγ function by expression of DN-PPARγ in SMC enhances atherosclerosis and augments NF-κB target gene expression in aorta from ApoE-deficient mice fed a high-fat diet. Thus, it is tempting to speculate that macrophage recruitment and atherosclerotic development are inhibited by PPARγ-dependent antagonism of NF-κB activity in SMC. We did not find that expression of DN-PPARγ selectively in SMC enhanced TNF-α–induced macrophage infiltration in aorta, but this infiltration was likely a result of direct action of TNF-α on macrophages expressing only endogenous PPARγ. In this regard, it will be instructive to determine whether development of inflammatory lesions is affected by PPARγ activity in SMC when inflammation is initiated directly within the vasculature.

**Perspectives**

Activation of PPARγ is clinically important because it increases insulin sensitivity and improves glycemic control in type II diabetes mellitus. PPARγ activation also lowers blood pressure and protects against vascular diseases, such as atherosclerosis. However, adverse effects have also been reported. PPARγ activation, at least by thiazolidinediones, causes weight gain, water retention, bone fracture and has also been reported to increase the risk of heart failure. Newer, nonagonist activators of PPARγ have been reported to have similar potent antidiabetic action but without the adverse weight gain and water retention. PPARγ also has antioxidant and anti-inflammatory properties. Many mechanisms involving NF-κB have been proposed to explain the anti-inflammatory actions of PPARγ, including transrepression and p65 turnover. Herein, we examined a mechanism for the anti-inflammatory actions of PPARγ specifically in vascular SMC. Our results suggest that PPARγ protects against cytokine-induced activation of NF-κB–dependent inflammatory gene expression through a mechanism involving direct interaction and nuclear export of the p65 subunit. Thus, PPARγ does not seem to prevent the activation of NF-κB–dependent inflammatory gene expression but acts to terminate transcription by removal of the critical p65 subunit from the nucleus. The hypertension-causing P467L mutation in PPARγ resulted in accumulation of nuclear p65, presumably because of loss of p65 binding preventing export of a PPARγ:p65 complex, thus prolonging NF-κB–mediated inflammatory gene expression. These findings expand our understanding of SMC PPARγ activities and suggest potential beneficial actions of PPARγ independent of its well-characterized role as a regulator of transcription. Thus, although the global actions of PPARγ are complex, involving multiple cell types and molecular mechanisms, results from this study provide strong evidence that the direct actions of PPARγ in the vasculature are protective against cardiovascular disease.

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**Disclosures**

None.

**References**


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A Hypertension-causing Mutation in PPARγ Impairs Nuclear Export of NF-κB p65 in Vascular Smooth Muscle

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Supplemental Materials
1. Detailed Methods and References
2. Supplemental Figures S1-S3
Supplemental Methods

Cell culture: Mesenteric artery smooth muscle cells (SMCs) were isolated from transgenic mice carrying WT or DN PPARγ under the control of CAG promoter as previously described. Briefly, SMCs was isolated from first or secondary order mesenteric artery of male transgenic mice carrying human WT- or DN-PPARγ under the control of CAG promoter by the explant method. These cells were suspended with high-glucose DMEM supplemented with 10% FBS, 1% sodium pyruvate, 1% penicillin and streptomycin (100 units/ml) and plated to six-well plate at 37 °C in a 5% CO₂ incubator. After 3 days, nonadherent cells were removed by washing with PBS and the adherent cells were cultured until the cells reached 90% confluence, and then split for experiments. Cell passages (2-8) were used for experiments. SMCs were obtained from the transgenic mice containing a floxed STOP cassette upstream of PPARγ (either WT or DN) followed by the fluorescent protein, tdTomato. For induction of WT- or DN-PPARγ, these cells were infected with Cre adenovirus. AdGFP or AdmCherry adenovirus was used as controls. HEK293T cell were obtained from ATCC and maintained in high-glucose DMEM with 10% FBS and 1% penicillin and streptomycin at 37°C in a 5% CO₂ incubator. After reaching 70-80% confluency, cells were transfected using Lipofectamine LTX (Life Technologies) according to the manufacturer’s protocol. SMC and HEK293T cells were starved overnight before stimulations, and protein or RNA lysates were collected and stored at 80°C until further analysis.

Western Blotting and Immunoprecipitation: Protein was extracted from primary cultures of SMCs or HEK cells and western blotting was performed as previously described. Briefly, protein was isolated in a lysis buffer containing 50 mmol/L Tris Cl buffer, 0.1 mmol/L EDTA (pH 7.5), 1% w/v NA deoxycholic acid, 1% v/v NP-40 and 0.1% v/v SDS, with protease inhibitor (Roche) and phosphatase inhibitors (Roche). Samples were centrifuged (12,000 g) for 10 min at 4°C and supernatants were collected. The protein concentration of lysates was determined by Lowry assay (Biorad). For immunoprecipitation, 1 mg of total protein lysate was incubated with agarose conjugated NF-κB p65 antibody (Santa Cruz, sc-109) for 2 h at 4°C. Beads were washed four times with lysis buffer, and immunoprecipitates were eluted for 10 min at 100°C in 50 µl of 2x sample buffer. Precipitated proteins or equal amounts of cell lysates (10-1000 µg) were separated by SDS-PAGE (8-12%) and transferred to a nitrocellulose membrane (GE Healthcare). After blocking with 5% non-fat milk, membranes were incubated with primary antibodies at 4°C overnight and then visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, at RT for 1 h). PPARγ (#2435, Cell Signaling), tdTomato (GTX127897, Gene Tex), GAPDH (sc-32233, Santa Cruz), Iκ-Bα (#9242, Cell Signaling), phospho-p65 (#3033, Cell Signaling) and p65 (#3034, Cell Signaling) were used for these studies. β-actin (ab16039, Abcam)
was utilized as a loading control.

**Real-time RT-PCR:** RNA was extracted from primary cultures of SMC or HEK cells and real-time RT-PCR (qPCR) was performed as previously described. Briefly, using oligo (dT) primers, RNaseOUT (Invitrogen), and Superscript III (Invitrogen), cDNA was synthesized from 500-1000 ng of total RNA extracted from the cells using RNeasy spin columns (RNeasy Mini Kit, QIAGEN). Each Q-PCR reaction was performed in duplicate. 10 ng of cDNA was subjected to TaqMan Gene Expression Assays using the Taqman Fast Advanced Master Mix (Applied Biosystems) and the targeted Taqman probes. The following Taqman probes were used on Applied Biosystems StepOnePlus System to evaluate gene expression level: GAPDH (4352932-0905028) and MMP9 (Mm0044299_m1). In some experiments, Q-PCR reactions were performed with 10 ng of cDNA and Fast SYBR Green Master Mix (Applied Biosystems) along with the targeted gene primers in a total volume of 10 µl. The primers are mMCP1: Forward: 5’-cccaatgagtaggctggaga-3’ and Reverse: 5’-cttgaccacctcttcttg-3’; mVCAM1: Forward: 5’-tcgagtcaccatgttcat-3’ and Reverse: 5’-catggtcagaacggacttgga-3’. ∆∆CT were calculated using GAPDH as a reference gene to determine relative mRNA expression levels.

**NF-κB Promoter activity:** Luciferase assays were performed using NF-κB-LUC adenovirus (gift from Dr. Steven Lentz and Dr. John F. Engelhardt, University of Iowa). Mouse mesenteric SMCs from transgenic mice carrying WT-or DN-PPARγ under the control of CAG promoter reached 50% confluence were infected with NF-κB-LUC adenovirus for 72 hrs, and then were treated with TNFα for overnight. NF-κB promoter activity was determined using a luciferase assay kit (Promega) and normalized to total cellular protein in the lysate.

**Immunostaining:** Cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes. Following this incubation, the cells were rinsed with PBS three times. p65 or PPARγ expression was determined using monoclonal anti-p65 (#6956 Cell Signaling) or polyclonal anti-PPARγ (#2435 Cell Signaling) diluted in 5% normal goat serum with 0.1% Triton X-100 to a final dilution of 1:250 and incubated at 4°C overnight. The cells were then rinsed in TBS-T (TBS with 0.3% Tween 20) three times for 10 min. The secondary antibody Alexa488 (Abcam) was diluted in 5% goat serum, 0.1% Triton X-100 in TBS to a final dilution of 1:1000 and incubated at room temperature for 1 h. All incubation and rinsing steps were performed under constant agitation. The cells were mounted with Vectashield (Vector Laboratories) and imaged for the presence of green fluorescence on a Zeiss LSM710 confocal microscope. Single-plane images were collected. When comparing detection of transgene expression between samples, we kept the microscope settings, including laser power, gain, and offset, constant throughout image collection. Final images were processed
using ImageJ software (version 1.48, National Institutes of Health; Java 1.6.0–20, 64-bit) to make adjustments to image size or linear parameters such as brightness and contrast. All adjustments were kept consistent across samples.

**Flow cytometry:** Mice were injected with TNFα (66.7 µg/kg/day) intraperitoneally for three consecutive days and were then sacrificed on the fourth day. Single cell suspensions were prepared from aortas as previously described. Briefly, the entire aorta with surrounding perivascular fat was minced with fine scissors and digested with 1 mg/mL collagenase A, 1 mg/ml collagenase B, and 100 µg/ml DNAase I in phenol-free RPMI 1640 medium with 5% FBS for 30 min at 37°C, with intermittent agitation. Fc receptors were blocked with anti-mouse CD16/CD32 (BD Biosciences, clone 2.4G2) for 20 min at 4°C prior to the staining of surface markers. The antibodies used were: Alexa Fluor 488 anti-mouse CD45, APC-Cy7 anti-mouse CD3, PE anti-mouse CD4, PE-Cy7 anti-mouse CD8, and APC anti-mouse F4/80. One million aortic cells were incubated in 100 µl of FACS buffer containing 1.5 µl of each antibody for 35 minutes. The cells were then washed twice with FACS buffer and immediately analyzed on an LSR II flow cytometer with DIVA software (BD Biosciences). Dead cells were eliminated from analysis using Hoechst 33528 (Sigma-Aldrich). For each experiment, we performed flow minus one (FMO) controls for each fluorophore to establish gates. Data analysis was performed using FlowJo 10.2 software (Tree Star, Inc.).

**Bioluminescence imaging:** Luciferase assay was performed using NF-κB-LUC mice as described previously. NF-κB-LUC mice were bred with S-WT or S-DN mice. After treatment of isolated aorta and carotid artery with TNFα (0-500 µM) for 16-24 hr, sample was washed using ice-cold Dulbecco’s phosphate-buffered saline (dPBS) and incubated with dPBS including 1.5 mg/ml D-luciferin (Gold Biotechnology). Bioluminescence imaging was performed on a Xenogen IVIS-200 System. Luminescence was quantitated where peak of the luminescent signal occurred.

**Chemicals:** Mouse TNFα (T7539) was from Sigma (St. Louis, MO). We used an NF-κB p65 inhibitor (NBP2-29321) from Novus Biologicals (Littleton, CO). Pioglitazone and GW9662 were from Cayman Chemical (Ann Arbor, MI) and were dissolved in DMSO according to the manufacturer’s instructions.
References


Figure S1. NF-κB Target Gene Expression and Promoter Activity

A-C) Relative mRNA expression of mouse vascular cell adhesion molecule 1 (VCAM-1) (A), monocyte chemotactic and activating factor 1 (MCP1) (B), and Matrix metallopeptidase 9 (MMP9) (C) were determined by quantitative real-time RT-PCR in interleukin 1β (IL-1β, 5 ng/ml)- or lipopolysaccharide (LPS, 1 µg/ml)-treated primary mesenteric SMC from transgenic mice with inducible expression of WT- or DN-PPARγ infected with AdGFP or AdCre. Data were normalized to the control value, which was set to 1.0. All data are means ± SEM. *p<0.05, vs. Control LPS or vs. Control IL-1β, #p<0.05, vs. Cre LPS or vs. Cre IL-1β.
Figure S2. Ubiquitination of p65
HEK293T cells were transfected with constructs expressing p65, WT-PPARγ, DN-PPARγ, and myc-tagged ubiquitin (C-myc-Ub), as indicated. Cells were treated with either MG132 (5 µM) or dimethyl sulfoxide for 12 hr as indicated. A) Proteins were immunoprecipitated with myc antibodies and Western blotted for the indicated protein. B) Cell lysates were Western blotted with the indicated antibodies. Size markers transferred from the blots are shown.
Figure S3. Inflammatory Cell Infiltration in Aorta
Quantification of flow cytometry for total leukocytes (CD45+ cells, A), monocytes/macrophages (CD45+F4/80+ cells, B), total T lymphocytes (CD45+CD3+ cells, C), T helper cells (CD3+CD4+ cells, D) and cytotoxic T cell (CD3+CD8+, E) in thoracic aortas of NT or S-DN mice infused with vehicle (circles) or TNFα (66.7 µg/kg/day, square) for 3 consecutive days. *p<0.05, vehicle vs. TNFα.