Endothelial PPARγ Provides Vascular Protection from IL-1β-Induced Oxidative Stress

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Running title: Endothelial PPARγ Reduces IL-1β-induced Oxidative Stress

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Abstract

Loss of peroxisome proliferator-activated receptor gamma (PPARγ) function in vascular endothelium enhances atherosclerosis and nuclear factor-kappa-B (NF-κB) target gene expression in high fat diet fed apolipoprotein-E-deficient mice. The mechanisms by which endothelial PPARγ regulates inflammatory responses and protects against atherosclerosis remain unclear. To assess functional interactions between PPARγ and inflammation, we employed a model of interleukin-1β (IL-1β)-induced aortic dysfunction in transgenic mice with endothelial-specific overexpression of either wildtype (E-WT) or dominant negative PPARγ (E-V290M). IL-1β dose-dependently decreased IkBα, increased phospho-p65, and increased luciferase activity in aorta of NF-κB-Luc transgenic mice. IL-1β also dose-dependently reduced endothelial-dependent relaxation by acetylcholine (ACh). The loss of ACh responsiveness was partially improved by pretreating vessels with the PPARγ agonist, rosiglitazone, or by endothelial overexpression of wildtype PPARγ. Conversely, IL-1β–induced endothelial dysfunction was worsened in aorta from E-V290M mice. Although IL-1β increased expression of NF-κB target genes, an NF-κB p65 inhibitor did not alleviate endothelial dysfunction induced by IL-1β. Tempol, a superoxide dismutase mimetic partially restored ACh responsiveness in IL-1β-treated aorta. Notably, Tempol only modestly improved protection in E-WT but had an increased protective effect in E-V290M compared to aorta from non-transgenic mice (NT), suggesting that PPARγ-mediated protection involves antioxidant effects. IL-1β increased ROS and decreased phospho-eNOS (ser1177)/eNOS ratio in NT aorta. These
effects were completely abolished in E-WT aorta, but were worsened in E-V290M aorta even in the absence of IL-1β. We conclude that PPARγ protects against IL-1β-mediated endothelial dysfunction through a reduction of oxidative stress responses, but not by blunting IL-1β-mediated NF-κB activity.

New and Noteworthy

PPARγ is antiinflammatory and we show herein that treatment of aorta with IL-1β activates NF-κB and NF-κB-dependent transcription. We show that the protective actions of PPARγ activation, induced either by rosiglitazone or over-expression of PPARγ in the endothelium occurs independently of NF-κB activity, by reducing oxidative stress and preserving phospho-eNOS/eNOS ratio.

Keywords: PPARγ, vascular dysfunction, endothelium, inflammation
Introduction

Thiazolidinediones (TZDs), a class of synthetic ligands of peroxisome proliferator-activated receptor gamma (PPARγ) were previously considered highly effective oral medications for type 2 diabetes due to their robust insulin-sensitizing activities (23). PPARγ is a ligand-activated transcription factor of the nuclear hormone receptor superfamily that is expressed in many tissues including adipocyte, macrophage, vascular endothelial (EC) and smooth muscle cell (SMC) lineages (1, 21). Classically, PPARγ is known to induce adipocyte differentiation and to regulate lipid metabolism (43). In macrophages, PPARγ exerts strong anti-inflammatory effects (45).

Accumulating evidence from human and animal studies has shown that TZDs also attenuate vascular diseases including atherosclerosis. TZD treatment inhibits the formation of atherosclerotic lesions in aorta and aortic root of apolipoprotein E (ApoE)-deficient and low density lipoprotein receptor-deficient mouse models of hypercholesterolemia (10, 26). The PROactive trial, the largest clinical trial to measure cardiovascular (CV) endpoints in pioglitazone-treated populations, reported significantly lower BP and reduced rates of all-cause mortality, myocardial infarction, and stroke (9). In contrast, patients with dominant-negative (DN) mutations in PPARγ (V290M or P467L) (4) exhibit severe early onset hypertension and insulin resistance. Other mutations in human PPARγ (R165T or L339X) cause hypertension and lipodystrophies (3). Together, these observations indicate a significant requirement for functional PPARγ in the regulation of cardiovascular homeostasis.
To explore the mechanistic importance of PPARγ in the vasculature, we had generated several mouse models expressing DN PPARγ (human PPARγ P467L or V290M) specifically in SMCs or EC. Our data clearly support the concept that PPARγ plays a critical role in both SMC and EC but that it has a distinct mechanism of action in each cell type. Interference with PPARγ in SMC caused a significant increase in arterial blood pressure and severe vascular dysfunction at baseline (15). In aorta, loss of PPARγ function in SMC decreased RhoBTB1 expression and Cullin-3 activity which increased RhoA/Rho kinase activity (36). Loss of PPARγ in the mesenteric circulation decreased expression of regulator of G-protein signaling (RGS)-5 mRNA which enhanced myogenic tone and Ang-II-mediated contraction (20). In contrast, EC-specific interference with PPARγ (E-V290M) led to cerebral vascular dysfunction in response to high-fat diet (HFD) (5) through decreased NO bioavailability caused by an increase in superoxide. Loss of PPARγ function in EC caused an up-regulation of NADPH oxidase subunits and a decrease in catalase and superoxide dismutase (SOD) expression (5, 21). Atherosclerosis was exacerbated and inflammatory markers were significantly increased in aorta when both mouse models where crossed with ApoE-deficient mice and treated with a high cholesterol diet (35). The precise mechanism by which loss of PPARγ function exacerbates inflammatory signals and augments atherosclerosis remains unclear.

Inflammation is a risk factor for cardiovascular diseases and nuclear factor-kappa B (NF-κB) is known as a central regulator of inflammation. NF-κB can be detected in the cytoplasm of many
cells in association with Iκ-B factors, which inhibit their DNA binding activity (31). Cellular activation
by cytokines (33) or virus (6) can induce the phosphorylation of Iκ-B, leading to its dissociation and
release of active NF-κB transcription factors. Activation of NF-κB increases pro-inflammatory
mediators such as ICAM1, VCAM and MCP1 in vascular cells (14, 37). NF-κB activity is also
augmented in EC, SMC, monocytes/macrophages, and T lymphocytes in atherosclerotic plaques (7).
PPARγ has been reported to regulate NF-κB activity in macrophages by a transrepression
mechanism involving interaction between PPARγ and NF-κB which does not require binding of the
PPARγ/RXR heterodimer to a PPAR response element (PPRE) (34). Moreover, PPARγ was
recently reported to act as an E3 ubiquitin ligase which regulates the stability of the p65 subunit of
NF-κB (18). Taken together, we hypothesized that vascular PPARγ protects against inflammation
by decreasing NF-κB activity. In this study, we assessed the interactions between PPARγ and
NF-κB in a model of IL-1β-induced vascular dysfunction. IL-1β is an NF-κB activator in EC (37) and
contributes to the development of atherosclerosis (29). This study was facilitated by using
transgenic mouse models specifically expressing either wildtype PPARγ (E-WT) or DN-V290M
PPARγ (E-V290M) in EC, which caused a significant decrease in PPARγ target gene expression in
aortic EC (5). Our data show that PPARγ in EC protects against IL-1β-induced endothelial
dysfunction. However, we conclude that the mechanism mediating this protection does not depend
on interference with NF-κB activity, but rather functions through EC-PPARγ-dependent regulation of
ROS.
Materials and Methods

Animals: The mice used in this study included male C57BL/6J (12-20 week of age), male and female NF-κB-LUC mice expressing luciferase under the control of NF-κB responsive promoter (the gift of Dr. Timothy Blackwell, Vanderbilt University)(40), and male and female transgenic mice (3-7 months of age) carrying wide-type (E-WT) or dominant negative (E-V290M) form of human PPARγ under the control of the endothelium-specific vascular cadherin (Ve-cad) promoter as described previously (5). Age-matched non-transgenic (NT) littermates were used as controls. Care of these mice 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 Animal Care and Use Committee.

Western Blotting: Frozen aorta (excluding perivascular fat) was homogenized 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). Supernatants were collected after sonication for 10 second and centrifuged (20,000 g) for 10 min at 4°C. Protein concentration in lysis buffer was determined by Lowry assay (Biorad). Equal amounts of proteins (15-35 µg) were separated by SDS-PAGE (8-12 %) and transferred to a nitrocellulose membrane (GE healthcare). After blocking with 5% skim milk, membranes were incubated with primary antibodies at 4°C overnight and then visualized using horseradish peroxidase-conjugated
secondary antibodies (1:10,000 dilution, 1h). Anti-phospho-p65 (#3033, Cell Signaling), anti-p65 (#3034, Cell Signaling), anti-Iκ-Bα (#9242, Cell Signaling), endothelial NOS (eNOS) (#9572, Cell Signaling), and phospho-eNOS (#9571, Cell Signaling) were used for these studies. β-actin was utilized as a loading control (ab16039, Abcam).

**Bioluminescence imaging:** Luciferase assay was performed using NF-κB-LUC mice. After treatment of isolated aorta with IL-1β (0-500 pg/ml) for 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) (42). Bioluminescence imaging was performed on a Xenogen IVIS-200 System. Luminescence was quantitated where peak of the luminescent signal occurred.

**Vascular function:** Aortic function was assessed using a wire myograph preparation. Thoracic aorta was dissected free of perivascular fat and cut into 4 segments. After three washes with sterile phosphate buffered saline, aorta rings were placed in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 1% penicillin–streptomycin in the absence (control) or presence of IL-1β (0.1-500 pg/ml, 30 min-24 hr). Aortic rings were pre-treated with indicated agents (rosiglitazone, NF-κB p65 inhibitor, tempol, or vehicle) for 1 hr at 37°C in an atmosphere of 95% air and 5% CO2 and then incubated in the absence (control) or presence of IL-1β (0.1-500 pg/ml, 24 hr). Aortic rings were then equilibrated for 45 min under a resting tension of 0.5 grams, and
vasoconstriction was recorded in response to KCl (10-100 mM). Concentration-dependent response curves to acetylcholine (ACh, 1 nM–30 μM) or sodium nitroprusside (SNP, 0.1 nM-30 μM) were performed following initial submaximal pre-contraction (40-60 %) with prostaglandin-F2α (PGF2α, 3–10 μM).

Real-time RT-PCR: Total RNA was extracted from thoracic aorta using RNeasy spin columns (RNeasy Mini Kit, QIAGEN). cDNA was synthesized from 500-800 ng of total RNA by RT-PCR using Superscript III (Invitrogen), RNaseOUT (Invitrogen), and oligo(dT) primers. Q-PCR 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, the Applied Biosystems StepOnePlus System was used (4352932-0905028 for GAPDH). In some experiments, Q-PCR reactions were performed using Fast SYBR Green Master Mix (Applied Biosystems), target gene primers, and 10 ng of cDNA in a total volume of 10 µl. The primers are: GAPDH: Forward: 5′-gctacagcttcaccaccaca-3′ and Reverse: 5′-aaggaaggctggaaaagagc-3′; MCP1: Forward: 5′-cccaatgagtagggaga-3′ and Reverse: 5′-tctggacccattccttcttg-3′; mLCAM: Forward: 5′-ttcacactgaatgcca-3′ and Reverse: 5′-gctgctgagacccctcttg-3′; INOS: Forward: 5′-caccttggagtctcctagc-3′ and Reverse: 5′-cgccctgagttccctagc-3′. ∆∆CT were calculated using GAPDH or β-actin as a reference gene to determine relative mRNA expression levels.
Fluorometric measurement of ROS: ROS accumulation was assessed using dihydroethidium (DHE, Invitrogen). Aortic rings were pre-treated with indicated agents (Apocynin or vehicle) for 1 hr at 37°C and then incubated in the absence (control) or presence of IL-1β (20-500 pg/ml, 24 hr) before treatment with DHE. Image J software was used for the quantitative analysis.

Chemicals: IL-1β was purchased from R&D Systems (Minneapolis, MN). ACh, SNP, KCl and tempol were from Sigma (St. Louis, MO). PGF2α were acquired from Pfizer (New York, NY). We used an NF-κB p65 inhibitor (NBP2-29321) from Novus Biologicals (Littleton, CO). Rosiglitazone was from Cayman (Ann Arbor, MI) and it was dissolved in DMSO according to manufacturer’s instruction.

Statistical analysis: Experiments were performed in similar numbers between male and female mice. We observed that there was no difference between the sex of mice, therefore, data were pooled from both. Results are expressed as mean±SEM. Statistical evaluation of the data was performed using GraphPad Prism. Where appropriate, a paired or unpaired Student’s t-test was used to compare between two groups. In other studies, ANOVA followed by Tukey’s test for comparisons was performed. Differences were considered significant when $P$ value was less than 0.05.
Results

IL-1β induces NF-κB activity and vascular dysfunction

To examine whether IL-1β activates NF-κB, isolated aortas from C57BL/6J mice were treated with IL-1β (0.1-100 pg/ml) in vitro and NF-κB activity was examined. IL-1β, starting at 5 pg/mL, increased p65 phosphorylation without affecting total expression of p65, and reduced the levels of Iκ-Bα (Figure 1A). This indicates that IL-1β activated NF-κB signaling in the whole aorta. IL-1β treatment of aorta isolated from NF-κB-LUC mice consistently caused a dose-dependent increase in NF-κB transcriptional activity (Figure 1B and C).

Aorta isolated from C57BL/6J mice was incubated with IL-1β in vitro to determine if IL-1β can induce endothelial dysfunction. IL-1β dose-dependently impaired vasodilation to ACh (Figure 2). IL-1β treatment caused a much smaller decrease in endothelium-independent vasodilation induced by SNP, suggesting possible additional impairment of smooth muscle function (Figure 2). KCl and PGF2α-induced contraction were not altered by IL-1β at any concentrations (1-500 pg/ml, n=5-6, data not shown). These data show that IL-1β treatment of the aorta ex vivo causes endothelial dysfunction.

PPARγ in EC protects against IL-1β-induced vascular dysfunction

Next, we investigated whether PPARγ activation can protect against IL-1β-induced EC dysfunction.
Rosiglitazone, a potent PPAR\(\gamma\) agonist, did not affect the relaxation to ACh or SNP in vehicle treated aorta from C57BL/6J mice (Figure 3A). However, pre-treatment with rosiglitazone prior to IL-1\(\beta\) modestly but significantly improved ACh-induced vasodilation. To eliminate the possibility of an off-target effect of rosiglitazone, we employed a transgenic mouse model (E-WT) expressing wildtype PPAR\(\gamma\) specifically in EC. At baseline, there was no difference in ACh- or SNP-induced vasodilation between E-WT and non-transgenic (NT) littermate control (Figure 3A and B). KCl-induced contraction was similar between genotypes (n=6-8, NT+vehicle: 0.84±0.08 g; E-WT+vehicle: 0.95±0.11 g; NT+IL-1\(\beta\): 0.82±0.09 g; E-WT+IL-1\(\beta\): 0.81±0.06 g, p>0.05). Consistent with the results from the rosiglitazone experiment, overexpressing wildtype PPAR\(\gamma\) in endothelium significantly improved ACh-induced relaxation in IL-1\(\beta\) (500 pg/mL) treated aorta (Figure 3B). A higher dose of IL-1\(\beta\) was employed in the E-WT studies because over-expression of wildtype PPAR\(\gamma\) provides protection from IL-1\(\beta\)-induced endothelial dysfunction. There was no further improvement in vasodilation by rosiglitazone treatment of aorta from E-WT mice (Figure 3C).

Conversely, overexpressing a dominant negative mutant form of PPAR\(\gamma\) specifically in the endothelium exacerbated endothelial dysfunction caused by low-dose IL-1\(\beta\) (20 pg/mL, Figure 4). Low dose IL-1\(\beta\) was employed in studies using aorta from E-V290M mice because interference with PPAR\(\gamma\) caused increased susceptibility to IL-1\(\beta\)-induced endothelial dysfunction. Indeed, even very low doses of IL-1\(\beta\) (5 pg/ml) caused a modest impairment in ACh-induced vasodilation in aorta from
E-V290M compared to NT mice (n=6, maximum relaxation by ACh; NT: 68.21±4.41%; E-V290M: 53.30±4.01%, p<0.05). By contrast, high-dose IL-1β (500 pg/ml) caused an almost complete ablation of ACh-induced vasodilation in NT aorta, and this maximal level of dysfunction could not be worsened in aorta from E-V290M mice (n=3, maximum relaxation by ACh; NT, -5.17±9.96%; E-V290M, 3.90±6.25%). There was no difference in SNP-induced relaxation (Figure 4) and KCl-induced contraction (data not shown) between NT and E-V290M. These data support the hypothesis that endothelial PPARγ functions to protect against IL-1β-induced endothelial dysfunction in the aorta.

NF-κB activity does not affect EC dysfunction by IL-1β

We showed that IL-1β induces NF-κB activity in aorta (Figure 1). In order to determine if IL-1β induced endothelial dysfunction requires activation of NF-κB, we utilized an NF-κB p65 inhibitory peptide at a concentration which blunted IL-1β (20 pg/ml)-induced luciferase activity in aorta from NF-κB-LUC mice (Figure 5A). However, the NF-κB inhibitor did not affect endothelial dysfunction induced by IL-1β at any dose in aorta from NT mice (Figure 5B and C). The inhibitor modestly improved endothelial-independent relaxation in aorta treated with low-dose IL-1β (Figure 5B), suggesting that NF-κB activity may contribute to IL-1β-induced dysfunction in SMC. Importantly, IL-1β-induced impairment of vasodilation to ACh was also not altered by the NF-κB inhibitor in aorta from E-WT mice (n=6, data not shown), suggesting that the protective actions of endothelial PPARγ
are not dependent on altering NF-κB activity. Consistent with this, endothelial-specific over-expression of WT PPARγ in E-WT mice did not blunt the induction of the NF-κB target genes iNOS, MCP1, or ICAM1 in IL-1β-treated aorta (Figure 6). Similarly, there was no effect on IL-1β-induced iNOS protein levels in aorta from E-WT mice (NT+vehicle: 1.00±0.90; NT+IL-1β: 4.02±0.67; E-WT+vehicle: 1.08±0.69; E-WT+IL-1β: 3.57±0.33, n=3).

IL-1β induces vascular dysfunction via increasing ROS

Oxidative stress has been implicated in endothelial dysfunction and cardiovascular diseases. We next used a ROS scavenger to test if increased ROS plays a role in IL-1β-mediated endothelial dysfunction. Tempol significantly improved ACh-mediated dilation of IL-1β-treated aorta from NT mice (Figure 7A), but had a blunted effect on aorta from E-WT mice (Figure 7B) when both were treated with the same dose of IL-1β (500 ng/ml). Although the maximal relaxation in response to tempol was similar in both groups (maximum relaxation by ACh; NT tempol+IL-1β: 47.98±4.57%; E-WT tempol+IL-1β: 51.59±5.55%), the effect size of tempol was greater on NT aorta compared to E-WT (difference in maximum relaxation by ACh between tempol+IL-1β and IL-1β alone, NT: 54.39±4.03%; E-WT, 19.86±7.75%, p<0.05). Analysis of area under the curve also showed that the effect of tempol was greater on NT (IL-1β: 1083±24 vs tempol+IL-1β: 810±75) aorta compared to E-WT (IL-1β: 904±34 vs tempol+IL-1β: 806±29). This suggests that there is at least some redundancy in the protection mediated by tempol and endothelial PPARγ. Consistent with this,
tempol also improved IL-1β-induced impairment in E-V290M aorta (Figure 7D), but with larger effect compared to NT (Figure 7C) when tested at the same low dose of IL-1β (20 pg/ml, difference in maximum relaxation by ACh between tempol+IL-1β and IL-1β alone, NT: 12.79±5.43%; E-V290M, 25.65±12.42%). Similarly, analysis of area under the curve showed that tempol caused a greater improvement in the IL-1β-induced impairment in E-V290M aorta (IL-1β: 999±42 vs. tempol+IL-1β: 812±33) compared to NT (IL-1β: 835±35 vs. tempol+IL-1β: 765±38). Tempol had no effect on the response to SNP in any groups (Figure 7).

In order to determine if IL-1β can induce ROS accumulation in aorta, DHE staining was performed in aorta from NT mice. ROS was increased by IL-1β (Figure 8A), and its accumulation was significantly blunted in the presence of the NADPH oxidase inhibitor, apocynin. This suggests that this effect depends on NADPH oxidase activity. We next investigated whether PPARγ in endothelium decreases IL-1β-induced ROS generation. Consistent with the vascular function data, ROS accumulation in response to IL-1β was reduced in E-WT compared to NT aorta (Figure 8B). In contrast, DN PPARγ in EC significantly increased ROS in aorta even without IL-1β treatment (Figure 9). Taken together, these data suggest that IL-1β causes oxidative stress, and the oxidative stress component of IL-1β-induced endothelial dysfunction is blunted by PPARγ.

NADPH oxidase is one of the major sources of ROS in vascular tissues (24) and antioxidant
enzymes such as catalase and SOD are known as a critical determinant of redox status in the vasculature (8). IL-1β decreased expression of catalase and NOX4 and increased expression of SOD2, without changing expression of NOX1, NOX2, SOD1 and SOD3 mRNAs (n=3, data not shown). Although IL-1β-induced ROS accumulation was dependent on NADPH oxidase activity (Figure 8A), there was no difference in the IL-1β-induced changes in oxidant or antioxidant gene expression between NT and E-WT mice. These data suggest that the reduced ROS accumulation mediated by PPARγ activity is not dependent on changes in expression of these genes.

Overexpressing endothelial PPARγ preserved activity of eNOS

In aorta, ACh-induced relaxation mainly depends on eNOS activity, which is partly determined by the prevalence of phospho-serine 1177 within the total eNOS population (11). In aorta from NT mice, IL-1β (500 pg/ml) caused an increase in eNOS expression, but with a concomitant decrease in the phospho-eNOS/eNOS ratio, consistent with a marked EC dysfunction (Figure 10). Interestingly, the ratio of phospho-eNOS/eNOS was preserved in IL-1β-treated aorta from E-WT mice. Conversely, loss of PPARγ function in the endothelium (E-V290M) resulted in a sharp reduction in the ratio of phospho-eNOS/eNOS even in the absence of IL-1β. These data suggest that endothelial PPARγ preserves eNOS activity, which is reduced in aorta of NT mice in response to IL-1β.
Discussion

TZDs are pharmacological activators of PPAR\(\gamma\) used to treat patients with type-2 diabetes. As reported in the PROactive clinical trial, TZDs not only exhibited unparalleled glycemic control but also decreased macrovascular events and lowered blood pressure and cardiovascular risk (9). In contrast, we previously showed that interference with PPAR\(\gamma\) function in SMC or EC exacerbated aortic atherosclerosis with increased NF-κB target gene expression in ApoE-deficient mice (35). Because IL-1\(\beta\) is an NF-κB activator and contributes to the development of atherosclerosis (29), the present study examined the effect of EC-PPAR\(\gamma\) on vascular dysfunction induced by IL-1\(\beta\) using transgenic mice over-expressing WT- or DN-PPAR\(\gamma\) in EC. Treatment with IL-1\(\beta\) impaired vascular function which was improved by increased endothelial PPAR\(\gamma\) activity. Our results suggest that the IL-1\(\beta\) induced EC dysfunction was dependent on ROS formation but not on NF-κB activity, and that PPAR\(\gamma\)-mediated protection involves suppressing effects of IL-1\(\beta\)-induced ROS.

NF-κB is a transcription factor known as a central regulator of inflammation, and several lines of evidence suggest that PPAR\(\gamma\) might exert anti-inflammatory effects by interfering with NF-κB activity in vascular cells. Indeed, expression of constitutively active PPAR\(\gamma\) in cultured ECs was reported to reduce NF-κB activity (46), although the mechanism of this effect was not determined. However, direct effects of PPAR\(\gamma\) on NF-κB have been documented in other cell types. In macrophages, inflammatory gene expression is inhibited through a “transrepression” mechanism in which PPAR\(\gamma\)
interacts with nuclear receptor corepressor (NCoR)-histone deacetylase-3 (HDAC3) complexes on NF-κB or AP1 (34). It has also been reported that PPARγ has a RING domain similar to E3 ubiquitin ligases, and can directly bind to the p65 subunit of NF-κB, causing its ubiquitination and degradation (18). However, we did not find evidence to support these mechanisms as mediators of PPARγ protection from IL-1β-induced EC dysfunction. Specifically, neither expression of p65 protein nor its activating phosphorylation (Ser536) was altered by overexpression of WT PPARγ in IL-1β-treated aorta (n=3, data not shown). We also did not detect any changes in NF-κB-mediated gene expression associated with PPARγ activity in aorta. Thus, we were not able to demonstrate that reported mechanisms of PPARγ-mediated interference with NF-κB are operative in vascular ECs. We also did not find evidence that NF-κB activity contributed to EC dysfunction induced by inflammatory cytokines, since this dysfunction was not reduced by an NF-κB p65 inhibitor. Similarly, suppression of vascular endothelial-specific NF-κB did not decrease blood pressure in a complex model of hypertension involving angiotensin-II infusion, high salt and treatment with L-NAME (16). Conversely, activation of NF-κB was reported to protect against endothelial apoptosis (25) through its target gene, A1 (41), or the immediate early response gene X-1 (39). These evidences indicate that NF-κB activity in vascular EC might not impair endothelial-dependent vasodilation directly, and that other pathways may be responsible for IL-1β-mediated vascular dysfunction.
The pathway mediating ACh-induced relaxation in aorta mainly depends on eNOS and its activity, which is regulated by protein-protein interactions and multisite phosphorylation (22). Activating (Ser1177) and inhibitory (Thr495) phosphorylations are the most thoroughly studied sites (11), and Ser1177 phosphorylation has been shown to be decreased in EC in models of atherosclerosis (30) and hypertension (2, 22). Moreover, cardiovascular risk factors including atherosclerosis (19), diabetes (17) and hypertension (44), are associated with an increase in eNOS expression rather than a decrease (27). Expression of eNOS is also increased in the presence of superoxide, through H$_2$O$_2$-mediated transcriptional and post-transcriptional mechanisms (13). Consistent with these reports, our present study showed that an inflammatory cytokine robustly decreased the phospho-eNOS$^{\text{Ser1177}}$/eNOS ratio. These findings are consistent with a marked endothelial dysfunction caused by an IL-1β-induced increased in ROS. Importantly, the non-inflammatory level of eNOS activity was preserved in aorta from E-WT mice.

We provided several lines of evidences to indicate that endothelial PPARγ diminished the effects of oxidative stress induced by IL-1β. First, a ROS scavenger, tempol significantly lessened the impairment of ACh relaxation induced by IL-1β in aorta from NT mice. Second, the magnitude of this tempol-mediated protection was markedly blunted in aorta from E-WT mice compared with aorta from NT mice. Third, endothelial PPARγ prevented the NAPDH oxidase-dependent increase in ROS induced by IL-1β. This suggests that over-expression of PPARγ in the endothelium may have contributed to the protection from oxidative stress-induced endothelial dysfunction. However,
this protection was not associated with altered expression of NADPH oxidase or anti-oxidant genes in IL-1β-treated aorta, suggesting that PPARγ reduces ROS production through some other mechanism. The ability of EC PPARγ to preserve a non-inflammatory profile of eNOS activity suggests possible protection against the uncoupling of eNOS activity associated with IL-1β-induced ROS. However, we also showed that ROS accumulation and decreased eNOS activity in aorta from E-V290M did not cause endothelial dysfunction in the absence of IL-1β. These data indicate that increased ROS and reduced eNOS activity by themselves don’t cause vascular dysfunction, but increase susceptibility to IL-1β-induced dysfunction. Moreover, that tempol nor expression of WT PPARγ did not fully correct endothelial dysfunction mediated by IL-1β suggests there may be an oxidant stress-independent component to the endothelial dysfunction caused by IL-1β.

In addition to causing vascular dysfunction, it is known that IL-1β contributes to the development of atherosclerosis, resulting from alteration of lipid metabolism and plaque development (29). Several rodent and human studies demonstrate that circulating IL-1β levels are also correlated with cardiovascular risk factors such as obesity, diabetes and hypertension. Notably, inflammatory cytokines, such as IL-1β and IL-6, are secreted from expanding adipose tissue and are elevated in obesity (47). Functionally, IL-1β is reported to contribute to the development of type-II diabetes mellitus and insulin resistance (38). An effect of inflammatory cytokines on vascular function is also suggested by a human study where serum IL-1β levels were correlated with essential hypertension,
although other possible risk factors of atherosclerosis could not be excluded (12). Concentrations of 500 pg/ml IL-1β are also commonly seen in a systemic inflammatory response syndrome (SIRS), which is associated with endothelial dysfunction in rat aorta (28). Serum levels of 30 pg/ml IL-1β have also been observed in young db/db mice compared to control mice, a 3-fold increase compared to control mice (32). Thus, the concentrations of IL-1β (5-500 pg/ml) used in the present study have pathobiological relevance. Given the protective effects of PPARγ observed in the present study against IL-1β-mediated EC dysfunction, future studies closely examining the interaction between IL-1β and PPARγ on cardiovascular disease are warranted.

EC dysfunction is a marker of cardiovascular disease and is closely associated with inflammation. Loss of PPARγ function in EC enhances atherosclerosis in ApoE-deficient mice fed HFD (35). This study shows that PPARγ also protects against inflammatory cytokine-mediated vascular dysfunction though reduction of ROS-mediated effects rather than direct antagonism of NF-κB activities. This contributes to an increasing understand for the potential roles of EC-PPARγ in the pathogenesis of vascular diseases.
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Disclosures:

None
Figure legends

**Figure 1. Effect of IL-1β on NF-κB activity in aorta.**

A: Western blot detecting phosphorylated and total p65, total Ik-Bα and β-actin in IL-1β (0.1-100 pg/ml, 2 hr)-treated aorta from C57BL/6J mice.  B-C: NF-κB activity determined by luciferase assay in IL-1β (5-500 pg/ml, 24 hr)-treated aorta isolated from NF-κB-LUC reporter mice (NF-κB-LUC) and non-transgenic (NT) littermate controls (n=6).  #, P<0.05; *, P<0.01 vs. no treatment. All data are mean±SEM.

**Figure 2. Effect of IL-1β on vascular relaxation.**

Isometric tension studies were performed using thoracic aortic rings from C57BL/6J mice treated ex-vivo with or without low (1-20 pg/ml, A) or high (50-500 pg/ml, B) dose of IL-1β (1-500 pg/ml) for 24 hr. Concentration-dependent relaxation to ACh (1 nM-3 μM) or SNP (0.1 nM-3 μM) was recorded following pre-contraction with prostaglandin F2α (PGF2α) (n=5-6).  #, P<0.05; *, P<0.01 vs. control. All data are mean±SEM.

**Figure 3. Vasomotor function with PPARγ activation.**

A: ACh (1 nM-3 μM) or SNP (0.1 nM-3 μM) response in aorta from NT mice. Aortic rings were treated with or without rosiglitazone (1 μM) for 1 hr prior to IL-1β (100 pg/ml, 24 hr) or control treatment (n=6-8).  B: Concentration-dependent relaxation to ACh or SNP in aorta from NT or
transgenic mice overexpressing WT-PPARγ specifically in endothelium (E-WT). Aortic rings were treated with or without IL-1β (500 pg/ml) for 24 hr (n=6). C: ACh or SNP response in aorta from E-WT mice. Aortic rings were treated with or without rosiglitazone (1 μM) for 1 hr prior to IL-1β (500 pg/ml, 24 hr) or control treatments (n=6). *, P<0.01 IL-1β vs. Rosi+IL-1β (A) or NT IL-1β vs E-WT IL-1β (B). All data are mean±SEM.

Figure 4. Vasomotor Function with PPARγ interference.

Concentration-dependent relaxation to ACh (1 nM-3 μM) or SNP (0.1 nM-3 μM) in aorta from NT or transgenic mice expressing dominant negative PPARγ specifically in endothelium (E-V290M). Aortic rings were treated with IL-1β (20 pg/ml) or control for 24 hr (n=6). *, P<0.01 NT+IL-1β vs. E-V290M+IL-1β. All data are mean±SEM.

Figure 5. Effect of NF-κB inhibition on vascular dysfunction induced by IL-1β.

Aortas were pretreated with vehicle (control) or the NF-κB p65 inhibitor (50 μM) for 1 hr prior to exposure to IL-1β or control treatments. A: NF-κB activity was determined by luciferase assay in aorta from NF-κB-LUC mice treated with IL-1β (20 pg/ml, 24 hr) in the presence or absence of the p65 inhibitor (n=3). B-C: Aorta from NT mice were treated with low (B: 20 pg/ml, 24 hr) or high (C: 500 pg/ml, 24 hr) dose of IL-1β in the presence or absence of the p65 inhibitor. Isometric tension studies were then performed with ACh (1 nM-3 μM) or SNP (0.1 nM-3 μM) (n=3-6). *, P<0.05 IL-1β
vs. p65 inhibitor+IL-1β. All data are mean±SEM.

**Figure 6. Analysis of NF-κB target genes**

Relative mRNA expression of mouse MCP1 (A), ICAM1 (B) and iNOS (C) were determined by quantitative real time RT-PCR in aorta from NT or E-WT mice treated ex vivo with or without IL-1β (500 pg/ml) for 24 hr (n=3). Data were normalized to NT which was set to 1.0. *, P<0.01 Control vs. IL-1β. Differences between IL-1β-treated NT and E-WT were not significant (NS). All data are mean±SEM.

**Figure 7. Effect of ROS scavenger on vascular dysfunction induced by IL-1β.**

Aortas from NT, E-WT and E-V290M mice were pretreated with or without tempol (1 mM) for 1 hr prior to high (500 pg/ml; A-B) or low (20 pg/ml; C-D) dose of IL-1β or control treatments for 24 hr. Isometric tension studies were performed with ACh (1 nM-3 μM) or SNP (0.1 nM-3 μM) (n=5-6). *, P<0.01 IL-1β vs. tempol+IL-1β. All data are mean±SEM.

**Figure 8. Effect of IL-1β on ROS generation.**

Dihydroethidium (DHE) staining detecting ROS generation in aorta. A: Aortas from NT mice were pretreated with or without apocynin (0.3 mM) for 1 hr prior to IL-1β (500 pg/ml, 24 hr) or control treatments (n=6). B: Aorta from NT or E-WT were treated with IL-1β (500 pg/ml, 24 hr) alone (n=7).
Samples labeled 1 and 2 refer to two independent replicates showing the range of DHE staining in the experiments. *, P<0.05 IL-1β vs. no treatment or apocynin+IL-1β. All data are mean±SEM.

**Figure 9.** ROS generation with PPARγ interference.

ROS generation in aorta from NT or E-V290M was detected by DHE staining. Aortic rings were treated with IL-1β (20 pg/ml) or control for 24 hr (n=7). *, P<0.05 NT no treatment vs. NT+IL-1β or E-V290M+IL-1β. Samples labeled 1 and 2 refer to two independent replicates showing the range of DHE staining in the experiments. All data are mean±SEM.

**Figure 10.** Analysis of eNOS activity

A: Western blot detecting phosphorylated and total eNOS, and β-actin in IL-1β (500 pg/ml, 24 hr)-treated aorta from NT, E-WT or E-V290M. B: Total eNOS expression, its phosphorylated form, and phospho-eNOS/total eNOS ratio were determined from western blots of aorta treated with or without IL-1β (500 pg/ml, 24 hr). #, P<0.05; *, P<0.01; NS, not significant; IL-1β vs untreated control, or indicated comparisons. All data are mean±SEM.
References


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33


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34176-34181, 2002.

Fig. 1

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C

Fold increase

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* p < 0.05

# p < 0.01
Fig. 2

A
- ○ Control
- ♦ IL-1β (1 pg/ml, 24 hr)
- □ IL-1β (5 pg/ml, 24 hr)
- △ IL-1β (20 pg/ml, 24 hr)

Relaxation (%)

ACh (logM)

B
- ○ Control
- ✶ IL-1β (50 pg/ml, 24 hr)
- ◊ IL-1β (100 pg/ml, 24 hr)
- ▽ IL-1β (500 pg/ml, 24 hr)

Relaxation (%)

ACh (logM)

Fig. 2
Fig. 4

- ○ NT, control
- ● E-V290M, control
- △ NT, IL-1β (20 pg/ml, 24 hr)
- ▲ E-V290M, IL-1β (20 pg/ml, 24 hr)

Relaxation (%) vs. ACh (logM)

Relaxation (%) vs. SNP (logM)
Fig. 5

A

NT
NF-κB-LUC
p65 inhibitor - - +
IL-1β (pg/ml) 0 20 20

B

○ NT, control
○ NT, p65 inhibitor (50 μM, 25 hr)
△ NT, IL-1β (20 pg/ml, 24 hr)
△ NT, p65 inhibitor+IL-1β

Relaxation (%)

ACh (logM)

C

○ NT, control
○ NT, p65 inhibitor (50 μM, 25 hr)
▽ NT, IL-1β (500 pg/ml, 24 hr)
▽ NT, p65 inhibitor+IL-1β

Relaxation (%)

ACh (logM)

SNP (logM)

SNP (logM)
Fig. 6

(A) MCP1

(B) ICAM

(C) iNOS
Fig. 7

A
- NT, control
- NT, tempol (1 mM, 25 hr)
- NT, IL-1β (500 pg/ml, 24 hr)
- NT, tempol+IL-1β

B
- E-WT, control
- E-WT, tempol (1 mM, 25 hr)
- E-WT, IL-1β (500 pg/ml, 24 hr)
- E-WT, tempol+IL-1β

C
- NT, control
- NT, tempol (1 mM, 25 hr)
- NT, IL-1β (20 pg/ml, 24 hr)
- NT, tempol+IL-1β

D
- E-V290M, control
- E-V290M, tempol (1 mM, 25 hr)
- E-V290M, IL-1β (20 pg/ml, 24 hr)
- E-V290M, tempol+IL-1β

Graphs show relaxation as a function of Ach and SNP concentrations.
**Fig. 8**

**A**
- **NT**
  - Vehicle
  - IL-1β
  - Apocynin + IL-1β

**B**
- **E-WT**
  - Vehicle
  - IL-1β

**Fold increase normalized to NT**
- IL-1β (pg/ml): 0, 500, 500
- Apocynin: -, -+, +

**Graphs:**
- E-WT: 0, 500 (NT), 0, 500 (E-WT)
- NT: 0, 500
Fig. 9

Vehicle  IL-1β

1
NT
2
E-V290M
1
2

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Fold increase normalized to NT

- NS
- *
- **

Fig. 9
Fig. 10