Endothelial PPAR-γ provides vascular protection from IL-1β-induced oxidative stress

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Mukohda M, Stump M, Ketsawatsomkron P, Hu C, Quelle FW, Sigmund CD. Endothelial PPAR-γ provides vascular protection from IL-1β-induced oxidative stress. Am J Physiol Heart Circ Physiol 310: H39–H48, 2016. First published November 13, 2015; doi:10.1152/ajpheart.00490.2015.—Loss of peroxisome proliferator-activated receptor (PPAR)-γ function in the vascular endothelium enhances atherosclerosis and NF-κB target gene expression in high-fat diet-fed apolipoprotein E-deficient mice. The mechanisms by which endothelial PPAR-γ regulates inflammatory responses and protects against atherosclerosis remain unclear. To assess functional interactions between PPAR-γ and inflammation, we used a model of IL-1β-induced aortic dysfunction in transgenic mice with endothelium-specific overexpression of either wild-type (E-WT) or dominant negative PPAR-γ (E-V290M). IL-1β dose dependently decreased IkB-α, increased phospho-p65, and increased luciferase activity in the aorta of NF-κB-LUC transgenic mice. IL-1β also dose dependently reduced endothelial-dependent relaxation by ACh. The loss of ACh responsiveness was partially improved by pretreatment of the vessels with the PPAR-γ agonist rosiglitazone or overexpression of PPAR-γ (E-WT). Conversely, IL-1β-induced endothelial dysfunction was worsened in the aorta from E-V290M mice. Although IL-1β increased the expression of NF-κB target genes, NF-κB p65 inhibitor did not alleviate endothelial dysfunction induced by IL-1β. Tempol, a SOD mimetic, partially restored ACh responsiveness in the IL-1β-treated aorta. Notably, tempol modestly improved protection in the E-WT aorta but had an increased protective effect in the E-V290M aorta compared with the aorta from nontransgenic mice, suggesting that PPAR-γ-mediated protection involves antioxidant effects. IL-1β increased ROS and decreased the phospho-endothelial nitric oxide synthase (Ser1177)-to-endothelial nitric oxide synthase ratio in the nontransgenic aorta. These effects were completely abolished in the aorta with endothelial overexpression of WT PPAR-γ but were worsened in the aorta with E-V290M even in the absence of IL-1β. We conclude that PPAR-γ protects against IL-1β-mediated endothelial dysfunction through a reduction of oxidative stress responses but not by blunting IL-1β-mediated NF-κB activity.

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is an anti-inflammatory, and we show here that treatment of the aorta with IL-1β activates NF-κB and NF-κB-dependent transcription. We show that the protective actions of PPAR-γ activation, induced by either rosiglitazone or overexpression of PPAR-γ in the endothelium, occurs independently of NF-κB activity, by reducing oxidative stress and preserving the phospho-endothelial nitric oxide synthase-to-endothelial nitric oxide synthase ratio.

THIAZOLIDINEDIONES (TZDs), a class of synthetic ligands of peroxisome proliferator-activated receptor (PPAR-γ), were previously considered highly effective oral medications for type 2 diabetes due to their robust insulin-sensitizing activities (23). PPAR-γ is a ligand-activated transcription factor of the nuclear hormone receptor superfamily that is expressed in many tissues, including adipocyte, macrophage, vascular endothelial cell (EC), and smooth muscle cell (SMC) lineages (1, 21). Classically, PPAR-γ is known to induce adipocyte differentiation and to regulate lipid metabolism (43). In macrophages, PPAR-γ 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 the 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 end points in pioglitazone-treated populations, reported significantly lower blood pressure and reduced rates of all-cause mortality, myocardial infarction, and stroke (9). In contrast, patients with dominant negative (DN) mutations in PPAR-γ (V290M or P467L) (4) exhibit severe early onset hypertension and insulin resistance. Other mutations in human PPAR-γ (R165T or L339X) cause hypertension and lipodystrophies (3). Taken together, these observations indicate a significant requirement for functional PPAR-γ in the regulation of cardiovascular homeostasis.

To explore the mechanistic importance of PPAR-γ in the vasculature, we generated several mouse models expressing DN PPAR-γ (human PPAR-γ P467L or V290M) specifically in SMCs or ECs. Our data clearly support the concept that PPAR-γ plays a critical role in both SMCs and ECs but that it has a distinct mechanism of action in each cell type. Interference with PPAR-γ in SMCs caused a significant increase in arterial blood pressure and severe vascular dysfunction at baseline (15). In the aorta, loss of PPAR-γ function in SMCs decreased RhoBTB1 expression and cullin-3 activity, which increased RhoA/Rho kinase activity (36). Loss of PPAR-γ in the mesenteric circulation decreased the expression of regulator of G protein signaling-5 mRNA, which enhanced myogenic tone and ANG II-mediated contraction (20). In contrast, EC-specific interference with PPAR-γ (E-V290M) led to cerebral vascular dysfunction in response to a high-fat diet (5) through decreased nitric oxide (NO) bioavailability caused by an increase in superoxide. Loss of PPAR-γ function in ECs caused...
an upregulation of NADPH oxidase (NOX) subunits and a
decrease in catalase and SOD expression (5, 21). Atheroscle-
rosis was exacerbated and inflammatory markers were signif-
ically increased in the aorta when both mouse models were
crossed with ApoE-deficient mice and treated with a high-
cholesterol diet (35). The precise mechanism by which loss of
PPAR-γ function exacerbates inflammatory signals and aug-
ments atherosclerosis remains unclear.

Inflammation is a risk factor for cardiovascular diseases, and
NF-κB is known as a central regulator of inflammation. NF-κB
can be detected in the cytoplasm of many cells in association
with IkB factors, which inhibit their DNA-binding activity
(31). Cellular activation by cytokines (33) or virus (6) can
induce the phosphorylation of IkB, leading to its dissociation
and release of active NF-κB transcription factors. Activation of
NF-κB increases proinflammatory mediators such as ICAM-1,
VCAM, and monocyte chemoattractant protein (MCP)-1 in
vascular cells (14, 37). NF-κB activity is also augmented in
ECs, SMCs, monocytes/macrophages, and T lymphocytes
in atherosclerotic plaques (7). PPAR-γ has been reported to
regulate NF-κB activity in macrophages by a transrepres-
sion mechanism involving an interaction between PPAR-γ
and NF-κB that does not require binding of the PPAR-γ/
retinoid X receptor heterodimer to a PPAR response element
(34). Moreover, PPAR-γ has recently been reported to act as
an E3 ubiquitin ligase that regulates the stability of the p65
subunit of NF-κB (18). Taken together, we hypothesized that
vascular PPAR-γ protects against inflammation by decreasing NF-κB activity. In the present study, we assessed
the interactions between PPAR-γ and NF-κB in a model of
IL-1β-induced vascular dysfunction. IL-1β is a NF-κB
activator in ECs (37) and contributes to the development of
atherosclerosis (29). This study was facilitated using trans-
genome mouse models that specifically expressed either wild-
type (WT) PPAR-γ overexpressed in the endothelium (E-
WT) or E-V290M in ECs, which caused a significant de-
crease in PPAR-γ target gene expression in aortic ECs (5).
Our data show that PPAR-γ in ECs 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 PPAR-γ-dependent regulation of ROS.

**MATERIALS AND METHODS**

**Animals.** The mice used in this study included male C57BL/6J
(12–20 wk of age), male and female NF-κB-LUC mice that expressed
luciferase under the control of the NF-κB responsive promoter (a gift
from Dr. Timothy Blackwell, Vanderbilt University) (40), and male
and female transgenic mice (3–7 mo of age) carrying E-WT or the
E-V290M form of human PPAR-γ under the control of the endothe-
lum-specific vascular cadherin promoter as previously described (5).
Age-matched nontransgenic (NT) littermates were used as controls.
Care of these mice met standards set forth by the National Institutes
of Health guidelines for the care and use of experimental animals. All
procedures were approved by Animal Care and Use Committee of
the University of Iowa.

**Western blot analysis.** The frozen aorta (excluding perivascular fat)
was homogenized in lysis buffer containing 50 mmol/l Tris-Cl buffer,
0.1 mmol/l EDTA (pH 7.5), 1% (wt/vol) Na deoxycholic acid, 1%
(vol/vol) Nonidet P-40, and 0.1% (vol/vol) SDS with protease inhib-
itor (Roche) and phosphatase inhibitors (Roche). Supernatants were
collected after sonication for 10 s and centrifuged (20,000 g) for 10
min at 4°C. The protein concentration in the lysis buffer was deter-
mined by a Lowry assay (Bio-Rad). Equal amounts of proteins (15–35
μg) were separated by SDS-PAGE (8–12%) and transferred to a
nitrocellulose membrane (GE Healthcare). After being blocked 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, 1 h). Anti-phos-
pho-p65 (no. 3033, Cell Signaling), anti-p65 (no. 3034, Cell Signali-
ging), anti-IkB-α (no. 9242, Cell Signaling), endothelial NO synthase
(eNOS; no. 9572, Cell Signaling), and phospho-eNOS (no. 9571, Cell
Signaling) were used for these experiments. β-Actin was used as a
loading control (ab16039, Abcam).

**Bioluminescence imaging.** A luciferase assay was performed using
NF-κB-LUC mice. After treatment of the isolated aorta with IL-1β
(0–500 pg/ml) for 24 h, samples were washed using ice-cold Dulbec-
co’s PBS (dbPBS) and incubated with dbPBS including 1.5 mg/ml
α-luciferin (Gold Biotechnology) (42). Bioluminescence imaging was
performed on a Xenovis IVIS-200 System. Luminescence was quan-
titated where the peak of the luminescent signal occurred.

**Vascular function.** Aortic function was assessed using a wire
myograph preparation. The thoracic aorta was dissected free of
perivascular fat and cut into four segments. After three washes with
sterile PBS, aorta rings were placed in 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 h). Aortic rings were pretreated
with the indicated agents (rosiglitazone, NF-κB p65 inhibitor, tempol,
or vehicle) for 1 h at 37°C in an atmosphere of 95% air:5% CO2 and
then incubated in the absence (control) or presence of IL-1β (0.1–500
pg/ml, 24 h). Aortic rings were then equilibrated for 45 min under
a resting tension of 0.5 g, and vasoconstriction was recorded in response
to KCl (10–100 mM). Concentration-dependent response curves to ACh
(1 nM–30 μM) or sodium nitroprusside (SNP; 0.1 nM–30 μM) were
performed after an initial submaximal precontraction (40–60%)
with PGF2α (3–10 μM).

**Real-time RT-PCR.** Total RNA was extracted from the thoracic aorta
using RNasea spin columns (RNasea 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. Quantitative PCRs were performed in duplicate using Taq-
man 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, quantitative PCRs 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 were used
as follows: GAPDH, forward 5'-GCTACACGCTACACCAACA-3'
and reverse 5'-AAGGAAGGCTGGAAAAGGC-3'; murine MCP-1,
forward 5'-CCCCAATGAGTGCGCTGGAAG-3' and reverse 5'
-TCTGAGACATCTCTCTTG-3'; murine ICAM, forward 5'
-TTCACACTGAAGCAGCTC-3' and reverse 5'-GTCGC
GAGACCCCTCTTG-3'; and inducible NO synthase, forward 5'
-CACCTTGGAGTCCACCAGT-3' and reverse 5'
-GGAGGATCCACCAGT-3'. ΔΔCT values (where CT is threshold cycle)
were calculated using GAPDH or β-actin as a reference gene to deter-
mine relative mRNA expression levels.

**Fluorometric measurement of ROS.** ROS accumulation was as-
sessed using dihydroethidium (DHE; Invitrogen). Aortic rings were
pretreated with the indicated agents (apocynin or vehicle) for 1 h at
37°C and then incubated in the absence (control) or presence of IL-1β
(20–500 pg/ml, 24 h) before treatment with DHE. ImageJ software
was used for quantitative analysis.

**Chemicals.** IL-1β was purchased from R&D Systems (Minneapo-
lis, MN). ACh, SNP, KCl, and tempol were from Sigma (St. Louis,
MO). PGF2α were acquired from Pfizer (New York, NY). We used the
NF-κB p65 inhibitor NBP2-29321 from Novus Biologicals (Littleton,
Rosiglitazone was from Cayman (Ann Arbor, MI) and dissolved in DMSO according to the 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 means ± SE. 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 experiments, ANOVA followed by Tukey’s test for comparisons was performed. Differences were considered significant when P values were <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 IκB-α (Fig. 1A). This indicates that IL-1β activated NF-κB signaling in the whole aorta. IL-1β treatment of aortas

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**ENDOTHELIAL PPAR-γ REDUCES IL-1β-INDUCED OXIDATIVE STRESS**

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**Fig. 1.** Effect of IL-1β on NF-κB activity in the aorta. A: Western blot detecting phosphorylated and total p65, total IκB-α, and β-actin in IL-1β (0.1–100 pg/ml, 2 h)-treated aortas from C57BL/6J mice. B: Effect of IL-1β on NF-κB-LUC reporter mice and nontransgenic (NT) littermate controls (n = 6). All data are means ± SE. #P < 0.05 and *P < 0.01 vs. no treatment.

**Fig. 2.** Effect of IL-1β on vascular relaxation. Isometric tension experiments 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) doses of IL-1β (1–500 pg/ml) for 24 h. Concentration-dependent relaxation to ACh (1 nM–30 μM) or sodium nitroprusside (SNP; 0.1 nM–30 μM) was recorded after precontraction with PGF2α. All data are means ± SE; n = 5–6. #P < 0.05 and *P < 0.01 vs. control.
isolated from NF-κB-LUC mice consistently caused a dose-dependent increase in NF-κB transcripational activity (Fig. 1, B and C).

Aortas isolated from C57BL/6J mice were incubated with IL-1β in vitro to determine if IL-1β can induce endothelial dysfunction. IL-1β dose dependently impaired vasodilation to ACh (Fig. 2). IL-1β treatment caused a much smaller decrease in endothelium-independent vasodilation induced by SNP, suggesting possible additional impairment of smooth muscle function (Fig. 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.

**PPAR-γ in ECs protects against IL-1β-induced vascular dysfunction.** Next, we investigated whether PPAR-γ activation can protect against IL-1β-induced EC dysfunction. Rosiglitazone, a potent PPAR-γ agonist, did not affect relaxation to ACh or SNP in vehicle-treated aortas from C57BL/6J mice (Fig. 3A). However, pretreatment with rosiglitazone before IL-1β modestly but significantly improved ACh-induced vasodilation. To eliminate the possibility of an off-target effect of rosiglitazone, we used a transgenic mouse model that expressed WT PPAR-γ specifically in ECs. At baseline, there was no difference in ACh- or SNP-induced vasodilation between E-WT and NT littermate controls (Fig. 3, A and B). KCl-induced contraction was similar between genotypes (NT + vehicle: 0.84 ± 0.08 g, E-WT + vehicle: 0.95 ± 0.11 g; NT + IL-1β: 0.82 ± 0.09 g, and E-WT + IL-1β: 0.81 ± 0.06 g, n = 6–8, P > 0.05). Consistent with the results from the rosiglitazone experiment, overexpression of WT PPAR-γ in the endothelium significantly improved ACh-induced relaxation in IL-1β (500 pg/ml)-treated aortas (Fig. 3B). A higher dose of IL-1β was used in the E-WT experiments because overexpression of WT PPAR-γ provides protection from IL-1β-induced endothelial dysfunction. There was no further

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**Fig. 3.** Vasomotor function with peroxisome proliferator-activated receptor (PPAR)-γ activation. A: ACh (1 nM–30 µM) or SNP (0.1 nM–30 µM) responses in aortas from NT mice. Aortic rings were treated with or without rosiglitazone (Rosi; 1 µM, 25 hr) or control treatment (n = 6–8). B: concentration-dependent relaxation to ACh or SNP in aortas from NT or transgenic mice overexpressing wild-type (WT) PPAR-γ specifically in the endothelium (E-WT). Aortic rings were treated with or without IL-1β (500 pg/ml) for 24 h (n = 6). C: ACh or SNP response in aortas from E-WT mice. Aortic rings were treated with or without Rosi (1 µM) for 1 hr before IL-1β (500 pg/ml, 24 h) or control treatments (n = 6). All data are means ± SE. *P < 0.01, IL-1β vs. Rosi + IL-1β (A) or NT IL-1β vs. E-WT IL-1β (B).

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**Fig. 4.** Vasomotor function with PPAR-γ interference. Concentration-dependent relaxation to ACh (1 nM–30 µM) or SNP (0.1 nM–30 µM) in aortas from NT or transgenic mice expressing dominant negative PPAR-γ specifically in the endothelium (E-V290M) is shown. Aortic rings were treated with IL-1β (20 pg/ml) or control for 24 h (n = 6). All data are means ± SE. *P < 0.01, NT + IL-1β vs. E-V290M + IL-1β.
improvement in vasodilation by rosiglitazone treatment in aortas from E-WT mice (Fig. 3C).

Conversely, overexpression of a DN mutant form of PPAR-γ specifically in the endothelium exacerbated endothelial dysfunction caused by low-dose IL-1β (20 pg/ml; Fig. 4). Low-dose IL-1β was used in experiments using aortas from E-V290M mice because interference with PPAR-γ caused increased susceptibility to IL-1β-induced endothelial dysfunction. Indeed, even very low doses of IL-1β (5 pg/ml) caused a modest impairment in ACh-induced vasodilation in aortas from E-V290M compared with NT mice (maximum relaxation by ACh: 68.21 ± 4.11% in NT mice and 53.30 ± 4.01% in E-V290M mice, n = 6, P < 0.05). In contrast, high-dose IL-1β (500 pg/ml) caused an almost complete ablation of ACh-induced vasodilation in NT aortas, and this maximal level of dysfunction could not be worsened in aortas from E-V290M mice (maximum relaxation by ACh: -5.17 ± 9.96% in NT mice and 3.90 ± 6.25% in E-V290M mice, n = 3). There were no differences in SNP-induced relaxation (Fig. 4) and KCl-induced contraction (data not shown) between NT and E-V290M mice. These data support the hypothesis that endothelial PPAR-γ 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 the aorta (Fig. 1). To determine if IL-1β-induced endothelial dysfunction requires activation of NF-κB, we used a NF-κB p65 inhibitory peptide at a concentration that blunted IL-1β (20 pg/ml)-induced luciferase activity in aortas from NF-κB-LUC mice (Fig. 5A). However, the NF-κB inhibitor did not affect endothelial dysfunction induced by any dose of IL-1β in aortas from NT mice (Fig. 5, B and C). The inhibitor modestly improved endothelium-independent relaxation in aortas treated with low-dose IL-1β (Fig. 5B), suggesting that NF-κB activity may contribute to IL-1β-induced dysfunction in SMCs. Importantly, IL-1β-induced impairment of vasodilation to ACh was
also not altered by the NF-κB inhibitor in aortas from E-WT mice (n = 6, data not shown), suggesting that the protective actions of endothelial PPAR-γ are not dependent on altering NF-κB activity. Consistent with this, endothelium-specific overexpression of WT PPAR-γ in E-WT mice did not blunt the induction of the NF-κB target genes iNOS, MCP-1, or ICAM-1 in IL-1β-treated aortas (Fig. 6). Similarly, there was no effect on IL-1β-induced iNOS protein levels in aortas from E-WT mice (NT, vehicle: 1.00 ± 0.90, NT + IL-1β: 4.02 ± 0.67, E-WT + vehicle: 1.08 ± 0.69, and 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 aortas from NT mice (Fig. 7A) but had a blunted effect on aortas from E-WT mice (Fig. 7B) when

Fig. 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 h before high (500 pg/ml; A and B) or low (20 pg/ml; C and D) doses of IL-1β or control treatments for 24 h. Isometric tension experiments were performed with ACh (1 nM–30 μM) or SNP (0.1 nM–30 μM). All data are means ± SE; n = 5–6. *P < 0.01, IL-1β vs. tempol + IL-1β.

Fig. 8. Effect of IL-1β on ROS generation. Dihydroethidium (DHE) staining was used to detect ROS generation in the aorta. A: aortas from NT mice were pretreated with or without apocynin (0.3 mM) for 1 h before IL-1β (500 pg/ml, 24 h) or control treatments (n = 6). B: aortas from NT or E-WT were treated with IL-1β (500 pg/ml, 24 h) alone (n = 7). Samples labeled 1 and 2 refer to two independent replicates showing the range of DHE staining in the experiments. All data are means ± SE. *P < 0.05, IL-1β vs. no treatment or apocynin + IL-1β.
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: 47.98 ± 4.57% for NT tempol + IL-1β and 51.59 ± 5.55% for E-WT tempol + IL-1β), the effect size of tempol was greater on NT aortas compared with E-WT aortas (difference in maximum relaxation by ACh between tempol + IL-1β and IL-1β alone: 54.39 ± 4.03% in NT aortas and 19.86 ± 7.75% in E-WT aortas, *P* < 0.05). Analysis of the area under the curve also showed that the effect of tempol was greater on NT aortas (IL-1β: 1,083 ± 24 vs. tempol + IL-1β: 810 ± 75) compared with E-WT aortas (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 PPAR-γ. Consistent with this, tempol also improved IL-1β-induced impairment in E-V290M aortas (Fig. 7D) but with a larger effect compared with NT aortas (Fig. 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: 12.79 ± 5.43% in NT aortas and 25.65 ± 12.42% in E-V290M aortas). Similarly, analysis of the area under the curve showed that tempol caused a greater improvement in the IL-1β-induced impairment in E-V290M aortas (IL-1β: 999 ± 42 vs. tempol + IL-1β: 812 ± 33) compared with NT aortas (IL-1β: 835 ± 35 vs. tempol + IL-1β: 765 ± 38). Tempol had no effect on the response to SNP in any group (Fig. 7).

To determine if IL-1β can induce ROS accumulation in the aorta, DHE staining was performed in aortas from NT mice. ROS was increased by IL-1β (Fig. 8A), and its accumulation was significantly blunted in the presence of the NOX inhibitor apocynin. This suggests that this effect depends on NOX activity. We next investigated whether PPAR-γ in the 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 with NT aortas (Fig. 8B). In contrast, DN PPAR-γ in ECs significantly increased ROS in aortas even without IL-1β treatment (Fig. 9). Taken together, these data suggest that IL-1β causes oxidative stress and that the oxidative stress component of IL-1β-induced endothelial dysfunction is blunted by PPAR-γ.

NOX is one of the major sources of ROS in vascular tissues (24), and antioxidant enzymes such as catalase and SOD are known as critical determinants of the 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 mRNA (n = 3, data not shown). Although IL-1β-induced ROS accumulation was dependent on NOX activity (Fig. 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 PPAR-γ activity is not dependent on changes in the expression of these genes.

**Overexpression of endothelial PPAR-γ preserved activity of eNOS.** In the aorta, ACh-induced relaxation mainly depends on eNOS activity, which is partly determined by the prevalence of phospho-Ser1177 within the total eNOS population (11). In aortas from NT mice, IL-1β (500 pg/ml) caused an increase in eNOS expression but with a concomitant decrease in the phospho-eNOS-to-eNOS ratio, consistent with marked EC dysfunction (Fig. 10). Interestingly, the ratio of phospho-eNOS to eNOS was preserved in IL-1β-treated aortas from E-WT mice. Conversely, loss of PPAR-γ function in the endothelium (E-V290M) resulted in a sharp reduction in the ratio of phospho-eNOS to eNOS even in the absence of IL-1β. These data suggest that endothelial PPAR-γ preserves eNOS activity, which is reduced in aortas of NT mice in response to IL-1β.

**DISCUSSION**

TZDs are pharmacological activators of PPAR-γ 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 have previously shown that interference with PPAR-γ function in SMCs or ECs exacerbated aortic atherosclerosis with increased NF-κB target gene expression in ApoE-deficient mice (35). Because IL-1β is an NF-κB activator and contributes to the development of atherosclerosis (29), the present study...
examined the effect of EC PPAR-γ on vascular dysfunction induced by IL-1β using transgenic mice with overexpression of WT or DN PPAR-γ in ECs. Treatment with IL-1β impaired vascular function, which was improved by increased endothelial PPAR-γ activity. Our results suggest that IL-1β-induced EC dysfunction was dependent on ROS formation but not on NF-κB activity and that PPAR-γ-mediated protection involves suppressing effects of IL-1β-induced ROS.

NF-κB is a transcription factor known as a central regulator of inflammation, and several lines of evidence suggest that PPAR-γ might exert anti-inflammatory effects by interfering with NF-κB activity in vascular cells. Indeed, expression of constitutively active PPAR-γ 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-γ on NF-κB have been documented in other cell types. In macrophages, inflammatory gene expression is inhibited through a “transrepression” mechanism in which PPAR-γ interacts with nuclear receptor corepressor-histone deacetylase-3 complexes on NF-κB or activator protein-1 (34). It has also been reported that PPAR-γ 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 PPAR-γ protection from IL-1β-induced EC dysfunction. Specifically, neither expression of p65 protein nor its activating phosphorylation (Ser536) was altered by overexpression of WT PPAR-γ in IL-1β-treated aortas (n = 3, data not shown). We also did not detect any changes in NF-κB-mediated gene expression associated with PPAR-γ activity in the aorta. Thus, we were not able to demonstrate that reported mechanisms of PPAR-γ-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 a NF-kB p65 inhibitor.

Similarly, suppression of vascular endothelium-specific NF-κB did not decrease blood pressure in a complex model of hypertension involving ANG II infusion, high salt, and treatment with N-nitro-l-arginine methyl ester (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 ECs might not impair endothelium-dependent vasodilation directly and that other pathways may be responsible for IL-1β-mediated vascular dysfunction.

The pathway mediating ACh-induced relaxation in the 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 ECs 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 H2O2-mediated transcriptional and posttranscriptional mechanisms (13). Consistent with these reports, our present study showed that an inflammatory cytokine robustly decreased the phospho-eNOS (Ser1177)-to-eNOS ratio. These findings are consistent with marked endothelial dysfunction caused by an IL-1β-induced increase in ROS. Importantly, the noninflammatory level of eNOS activity was preserved in aortas from E-WT mice.

We have provided several lines of evidences to indicate that endothelial PPAR-γ 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 aortas from NT mice. Second, the magnitude of this tempol-mediated protection was markedly blunted in aortas from E-WT mice compared with aortas from NT mice. Third, endothelial PPAR-γ prevented the NOX-dependent increase in ROS induced by IL-1β. This suggests that overexpression of PPAR-γ 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 NOX or antioxidant genes in IL-1β-treated aortas, suggesting that PPAR-γ reduces ROS production through some other mechanism. The ability of EC PPAR-γ to preserve a noninflammatory 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 aortas from E-V290M mice did not cause endothelial dysfunction in the absence of IL-1β. These data indicate that increased ROS and reduced eNOS activity by themselves do not cause vascular dysfunction but increase susceptibility to IL-1β-induced dysfunction. Moreover, the fact that neither tempol nor expression of WT PPAR-γ 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 alterations of lipid metabolism and plaque development (29). Several rodent and human studies have demonstrated 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 has been reported to contribute to the development of type II diabetes mellitus and insulin resistance (38). An effect of inflammatory cytokines on vascular function has also been suggested by a human study (12) where serum IL-1β levels were correlated with essential hypertension, although other possible risk factors of atherosclerosis could not be excluded. Concentrations of 500 pg/ml IL-1β are also commonly seen in a systemic inflammatory response syndrome, which is associated with endothelial dysfunction in the rat aorta (28). Serum levels of 30 pg/ml IL-1β have also been observed in young db/db mouse compared with control mice, a threefold increase compared with control mice (32). Thus, the concentrations of IL-1β (5–500 pg/ml) used in the present study have pathological relevance. Given the protective effects of PPAR-γ observed in the present study against IL-1β-mediated EC dysfunction, future studies closely examining the interaction between IL-1β and PPAR-γ on cardiovascular disease are warranted.

EC dysfunction is a marker of cardiovascular disease and is closely associated with inflammation. Loss of PPAR-γ function in ECs enhances atherosclerosis in ApoE-deficient mice fed a high-fat diet (35). This study shows that PPAR-γ also protects against inflammatory cytokine-mediated vascular dysfunction through a 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 PPAR-γ in the pathogenesis of vascular diseases.

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disclosures

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AUTHOR CONTRIBUTIONS


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