Interference with PPARγ in Endothelium Accelerates Angiotensin II-induced Endothelial Dysfunction

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Abstract

The ligand activated nuclear receptor peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) in the endothelium regulates vascular function and blood pressure (BP). We previously reported that transgenic mice (E-V290M) with selectively targeted endothelial-specific expression of dominant negative PPAR\(\gamma\) exhibited endothelial dysfunction when treated with a high-fat diet, and exhibited an augmented pressor response to angiotensin II (Ang II). We hypothesize that interference with endothelial PPAR\(\gamma\) would exacerbate Ang II-induced endothelial dysfunction.

Endothelial function was examined in E-V290M mice infused with a subpressor dose of Ang II (120 ng/kg/min) or saline for 2 weeks. Ang II infusion significantly impaired the responses to the endothelium-dependent agonist acetylcholine both in basilar and carotid arteries from E-V290M but not NT mice. This impairment was not due to increased BP, which was not significantly different in Ang II-infused E-V290M compared with NT mice. Superoxide levels, and expression of the pro-oxidant Nox2 gene was elevated, whereas expression of the anti-oxidant genes Catalase and SOD3 decreased in carotid arteries from Ang II-infused E-V290M mice. Increased p65 and decreased I\(\kappa\)-B\(\alpha\) suggesting increased NF-\(\kappa\)B activity was also observed in aorta from Ang II-infused E-V290M mice. The responses to acetylcholine were significantly improved both in basilar and carotid arteries after treatment with Tempol (1 mmol/L), a scavenger of superoxide. These findings provide evidence that interference with endothelial PPAR\(\gamma\) accelerates Ang II-mediated endothelial dysfunction both in cerebral and conduit arteries through an oxidative stress-dependent mechanism, suggesting a role for endothelial PPAR\(\gamma\) in protecting against Ang II-induced endothelial dysfunction.

Keywords: PPAR\(\gamma\), angiotensin II, oxidative stress, endothelium, and endothelial dysfunction
Abbreviations:

Ang II: angiotensin II  
ACh: acetylcholine  
BP: blood pressure  
DN: dominant negative  
ET-1: endothelin 1  
$E_{\text{max}}$: maximal relaxation effect  
EC: endothelial cell  
eNOS: endothelial nitric oxide synthase  
HFD: high-fat diet  
NO: nitric oxide  
NT: non-transgenic  
PPAR$\gamma$: peroxisome proliferator-activated receptor $\gamma$  
ROS: reactive oxygen species  
SNP: sodium nitroprusside  
5-HT: serotonin  
TZD: thiazolidinedione  
VSMC: vascular smooth muscle cell
Introduction

Peroxisome proliferator-activated receptor \( \gamma \) (PPAR\( \gamma \)) is a ligand-inducible transcription factor highly expressed in adipocytes (12). By forming a heterodimer with the retinoid X receptor at the regulatory region (i.e., PPAR response elements) of target genes, PPAR\( \gamma \) regulates expression of genes involved in adipogenesis, lipid metabolism, and insulin sensitivity (1). Although the identity of endogenous PPAR\( \gamma \) ligands remains unclear, fatty acids and their metabolites have been shown to bind and activate PPAR\( \gamma \) (29, 30). Thiazolidinediones (TZDs) are synthetic activators of PPAR\( \gamma \) (29, 45) that were frequently used clinically to treat type 2 diabetes. A substantial number of clinical as well as animal studies have reported protective effects of TZDs in the vasculature, including lowering of blood pressure (BP), inhibition of oxidative stress, and slowing of the atherosclerotic process (2, 23, 32, 56, 58, 65). Unfortunately however, adverse effects including weight gain and fluid retention have also been reported in some patient subpopulations. Although concerns were previously raised regarding their clinical safety (i.e., acute myocardial infarction) (33), the Food and Drug Administration has lifted the restrictions on one member of this class of drugs. Despite this reversal, TZDs have become less appealing as initial therapy or for persistent hyperglycemia control for type 2 diabetic patients.

Accumulating evidence indicates that PPAR\( \gamma \) is a critical regulator of BP and vascular function (6, 7, 22, 38, 39, 48, 57). The significance of PPAR\( \gamma \) on glycemic and BP regulation was highlighted by the observation that subjects carrying dominant negative (DN) mutants in PPAR\( \gamma \) (i.e., P467L or V290M), which block the action of endogenous ligands and partially of TZDs, cause severe insulin resistance and early onset hypertension (4). PPAR\( \gamma \) is expressed in vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) in the vasculature (44, 49, 51). We have shown that expression of DN mutations in PPAR\( \gamma \) specifically in VSMC causes...
hypertension, vascular dysfunction, and vascular remodeling under baseline conditions by altering expression of multiple PPARγ target genes (34, 38, 57). In endothelium, PPARγ is reported to exert protective effects by its anti-inflammatory and anti-oxidant actions as well as by maintenance of the balance of vasodilators and constrictors (9, 13, 18, 36, 50). Deletion of PPARγ specifically in ECs results in hypertension and endothelial dysfunction on both normal and high-fat diet (HFD) (37, 39, 55).

Since complete loss of PPARγ is lethal (3), our laboratory developed a model to emulate what occurs in the endothelium of human subjects carrying naturally occurring DN mutations in PPARγ. We generated transgenic mice (E-V290M mice), expressing the DN V290M mutation in PPARγ selectively in the endothelium using an endothelial-specific promoter (7). These mice are normotensive and exhibit normal endothelial function under baseline conditions. However, in response to a HFD, E-V290M mice exhibit profound endothelial dysfunction in the cerebral circulation caused by an oxidative stress-dependent mechanism (7). Thus, we hypothesize that PPARγ in the endothelium (particularly in the cerebral circulation) serves a protective mechanism which protects the endothelium in the face of physiologic stressors, such as a HFD. This hypothesis predicts that a pathological phenotype would emerge in response to other physiologic stressors when the function of PPARγ in the endothelium is impaired.

Angiotensin II (Ang II) increases production of reactive oxygen species (ROS) (43, 63) and plays a crucial role in the progression of vascular diseases, such as hypertension and atherosclerosis (52, 60). Increased ROS is associated with endothelial dysfunction, and NADPH oxidase is known to be a major source of ROS in endothelium (31). Previous evidence has shown that activation of PPARγ by TZDs can attenuate increases in BP and ameliorate vascular damage induced by Ang II (5, 22, 59). Moreover, deletion (39) or interference with
PPARγ (7) in the endothelium can enhance the pressor response to Ang II. Therefore, in the present study, we tested the hypothesis that interference with PPARγ in the endothelium would exacerbate Ang II-induced endothelial dysfunction via a ROS dependent mechanism even when a dose of Ang II which does not raise arterial pressure is used.
Materials and Methods

Experimental animals. E-V290M transgenic mice expressing a DN mutation in human PPARγ (V290M) under the control of the endothelial-specific vascular cadherin promoter were described previously and were maintained on a C57BL/6J background (7). Non-transgenic (NT) littermates were utilized as controls. Compared with C57BL/6 female mice, male mice are more sensitive to chronic infusion of Ang II, displaying higher BP (24). Ang II increased cardiac NADPH oxidase activity in males, but not females (24). To be consistent with previous reports where HFD-treated male E-V290M mice exhibited vascular dysfunction (7), male mice aged 3-6 months were utilized for the current studies. All mice were housed under 12:12 light/dark cycle at controlled temperature (23ºC), and had free access to water and standard mouse chow (7013, Teklad Premier Laboratory Diets). Care of the mice used in this study met the standard guidelines for the care and use of experimental animals by the National Institutes of Health (NIH). All procedures used were approved by the University of Iowa Institutional Animal Care and Use Committee.

Administration of Ang II. Ang II was infused through an osmotic minipump (Alzet, model 1002) as previously described (7). Following anesthesia with isoflurane, a minipump was placed subcutaneously in the midscapular region to administer vehicle (isotonic saline) or Ang II (subpressor or pressor dose at a rate of 120 or 500 ng/kg/min for 14 days, respectively). This dose of Ang II is comparable to that used in other studies which investigate vascular effects of Ang II treatment (41, 52). Separate cohorts of mice were utilized for vascular function and arterial pressure measurements.

Studies of vascular function. Detailed methods used to measure vascular responses have been described previously (7, 27, 28). Briefly, mice were euthanized with pentobarbital (50 mg/mouse IP) followed by removal of brain and carotid arteries. Basilar arteries were isolated.
and cannulated onto glass micropipettes filled with oxygenated Krebs buffer (in mmol/L: 118.3 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂ and 11 glucose) in an organ chamber. Arteries were transferred to a pressurized myograph system (DMT), equilibrated for 30 min at 60 mmHg under no-flow conditions, and viability was first examined by exposure to KCl (100 mmol/L). Relaxation was recorded for acetylcholine (ACh, 0.001-100 µmol/L), sodium nitroprusside (SNP, 0.001-100 µmol/L) after initial pre-constriction by ~30% with U46619, a thromboxane A2 mimetic. The level of pre-constriction was similar in NT and E-V290M mice. At the end of the experiment, arteries were exposed to Ca²⁺-free Krebs buffer containing 2 mmol/L EGTA and 10 µmol/L SNP to obtain maximum passive diameter. In a separate set of experiments, contraction was recorded for Ang II (0.01-3 nmol/L). To investigate the effect of a superoxide scavenger on ACh-induced relaxation, the basilar arteries were pre-incubated with Tempol (1 mmol/L) for 30 min.

For studies of carotid arteries, the left and right carotid arteries were quickly removed and placed in oxygenated Krebs buffer, the loose tissue of the adventitia layer was removed, and the vessels were cut into two rings (3–4 mm in length). Vascular rings were suspended in an organ bath containing 20 ml Krebs buffer (maintained at 37°C and 95% O₂/5% CO₂) and connected to force transducers to measure isometric tension. Resting tension was increased stepwise to reach the final tension of 0.25 g, and the rings were allowed to equilibrate for 45 min. Relaxation was recorded for ACh (0.001-100 µmol/L) or SNP (0.001-100 µmol/L) after initial submaximal pre-contraction (~50-60% of max) using U46619. Contraction was recorded for KCl (10-100 mmol/L), 5-HT (0.01-30 µmol/L), and endothelin 1 (ET-1, 0.1-100 nmol/L). In some experiments, carotid arteries were pre-incubated with Tempol (1 mmol/L) for 30 min. Data were collected with PowerLab/8SP and analysed with Chart 5 software (AD Instruments). The maximal relaxation effect (Eₘₐₓ) of ACh and SNP was assessed from cumulative concentration-effect curves in basilar or carotid arteries. Curve fitting by non-linear regression for the
calculation of $E_{\text{max}}$ was performed with SigmaPlot.

**BP and Heart Rate Measurements.** BP and heart rate in conscious mice were measured by radiotelemetry (TA11PA-C10, Data Sciences International) by implanting a catheter into the left carotid artery as previously described (7). After one week of recovery, baseline BP was continuously recorded (sampling every 5 min for 10-second intervals) for 7 days. Then, the same cohort of mice underwent Ang II (120 ng/kg/min) infusion as described above, and BP was continuously measured during the following two weeks. Data were collected and stored using Dataquest ART. Data from each mouse was averaged hourly, and corresponding times across 7 days were averaged for each mouse to create a single composite 24-hour tracing.

**Detection of ROS by dihydroethidium (DHE) fluorescence.** The relative amount of ROS in carotid arteries was determined using DHE (Sigma-Aldrich Biochemical) as described (16). Carotid arteries were embedded in OCT compound and kept at -80°C until sectioning. Frozen sections (20 µm thick) of the carotid arteries were cut using a cryostat and incubated for 15 minutes at room temperature in PBS (1X) containing 8 µmol/L DHE. Images were visualized using confocal microscopy (Zeiss LSM710) at an excitation/emission of 488/568 nm, and analysed using ImageJ software. We have shown previously that the DHE signal is marked reduced by polyethylene glycol-SOD and in mice that overexpress SOD1 (15, 20, 21).

**Real-Time PCR (qPCR).** The carotid arteries of mice were dissected and snap-frozen in liquid nitrogen. RNA was extracted in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was prepared using a RNA mini kit (Invitrogen) following the manufacturer’s protocol. RNA concentrations were examined using a NanoDrop spectrophotometer, with an OD260/OD280 ratio of greater than 1.9 (indicating very high quality RNA). cDNA was generated from total RNA by RT-PCR using SuperScript III (Invitrogen) and qPCR was conducted by Taqman Gene
Expression Assays (Applied Biosystems). The assay numbers for TaqMan were as follows:

Mm00476361_m1 (mouse NF-κB1), Mm01287743_m1 (mouse Nox2), Mm00479246_m1 (mouse Nox4), Mm01344233_g1 (mouse SOD1), Mm01313000_m1 (mouse SOD2), Mm00448831_s1 (mouse SOD3), Mm00437992_m1 (mouse Catalase), and Mm00435217_m1 [mouse endothelial nitric oxide (NO) synthase (eNOS)]. All data were normalized by β-actin and quantified by the 2^ΔΔCT method (47).

Western Blotting. Thoracic aortas were cleaned of perivascular adipose tissue in Krebs buffer described above and snap-frozen in liquid nitrogen. Frozen aortas were crushed and solubilized 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 Complete protease inhibitors and PhosSTOP phosphatase inhibitors (Roche). Homogenates were centrifuged at 4°C, and supernatant total protein was quantified by Lowry assay (Biorad), using bovine serum albumin as a standard. Equal amounts of proteins (25 µg) were separated by SDS-PAGE 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, 1 h). Anti-p50 (Santa Cruz: sc-114), anti-p65 (#3034, Cell Signaling), anti-IκBα (#9242, Cell Signaling), and anti-phospho-p65 (#3033, Cell Signaling) were used in this study. β-actin was utilized as a loading control (ab16039, Abcam). Blots were quantified using ImageJ software and were internally normalized to individual blots.

Drugs and Reagents. Ang II, ACh, SNP, KCl, 5-HT, and Tempol were obtained from Sigma-Aldrich Biochemical; and Y-27632 from Calbiochem; all of these reagents were dissolved in saline. ET-1 was from Peninsula Laboratories Inc. and dissolved in water. U46619 (Cayman
Chemical, Ann Arbor, MI) was dissolved in ethanol with subsequent dilutions made in saline.

**Statistical analysis.** All data are expressed as mean ± SEM. Data were analyzed with 1- or 2-way ANOVA (repeated measures when appropriate) using Bonferroni post hoc tests. Student's t test was used where required. *P*<0.05 was considered statistically significant. Data were analyzed using SigmaStat (Systat Software).
Results

Ang II impairs endothelium-dependent dilation in basilar arteries from E-V290M transgenic mice.

Preliminary studies were first conducted to determine if there is a dose of Ang II that specifically impair vascular function in E-V290M but not in NT mice. According to previous studies, infusion of Ang II at a dose of 500 ng/kg/min for 2 weeks caused an elevation in mean BP of about 45 mmHg in control littermates, but not in Nox1 knockout mice (52). Moreover, our preliminary data showed that this dose significantly impaired endothelium-dependent ACh-induced vasodilation in basilar arteries from both E-V290M and NT mice (data not shown). Since we would not be able to mechanistically distinguish between endothelial dysfunction caused by PPARγ interference in the endothelium or the hypertension caused by a pressor dose of Ang II, a lower dose of Ang II (120 ng/kg/min) was utilized. This dose did not raise mean arterial pressure after one or two weeks of infusion in NT nor E-V290M mice (Figure 1A). Heart rate was modestly elevated in E-V290M mice but Ang II significantly decreased heart rate in both groups equally (Figure 1B).

At baseline, ACh caused equal concentration-dependent dilation of the basilar artery from both NT and E-V290M (Figure 2A). At the subpressor dose, Ang II markedly impaired vasodilation to ACh only in E-V290M mice (Figure 2A). Endothelial-independent dilation to SNP (a NO donor) in basilar arteries was not altered in Ang II-infused E-V290M mice compared with other groups (Figure 2B). Constriction of the basilar artery to KCl (100 mmol/L, Figure 2C), Ang II (Figure 2D), and 5-HT (data not shown) was not altered in Ang II-infused E-V290M mice compared with other groups. Structural analysis of the basilar artery revealed that there were no differences in the lumen diameter, external diameter, or wall thickness among the different groups with or without Ang II infusion (Figure 2E).

Ang II impairs carotid artery responses to NO in E-V290M transgenic mice. Carotid artery
dysfunction and cerebrovascular diseases are major causes of stroke and dementia (35, 40). Therefore, we next examined carotid artery function ex vivo. Concentration-dependent relaxation to ACh, as well as maximal relaxation to ACh (ACh $E_{\text{max}}$), was significantly impaired in Ang II-infused E-V290M mice compared with other groups (Figure 3A). There was a rightward shift in the dose-dependent relaxation to SNP in carotid arteries from Ang II-infused E-V290M mice (Figure 3B, left panel). However, Ang II-infusion did not alter the maximal relaxation response to SNP (SNP $E_{\text{max}}$) in E-V290M mice compared with other groups (Figure 3B, right panel). Contraction to KCl and ET-1 was not altered in Ang II-infused E-V290M mice compared with other groups (Figure 3C and D). In contrast, contraction to 5-HT was significantly increased in Ang II-infused mice, and this was augmented in E-V290M mice (Figure 3E).

Ang II-infusion causes oxidative stress and inflammation in E-V290M transgenic mice. We previously reported that cerebral vascular dysfunction in HFD treated E-V290M mice was due to oxidative stress (7). To determine whether a similar mechanism is operant in Ang II-infused E-V290M mice, we first measured relative superoxide levels in carotid arteries by DHE fluorescence. ROS levels in carotid arteries were significantly increased in Ang II-infused E-V290M mice compared with other groups (Figure 4). To determine if oxidative stress plays a role in endothelial dysfunction in E-V290M mice after chronic Ang II treatment, we examined the response to ACh in basilar arteries before and after treatment with Tempol (1 mmol/L), a SOD mimetic. As described above, the response to ACh was impaired in the basilar artery from Ang II-infused E-V290M mice (Figure 5A). Tempol significantly improved the dose-dependent vasodilation to ACh in Ang II-infused E-V290M mice, and increased the ACh $E_{\text{max}}$ (Figure 5A-B). A similar result was observed in carotid arteries from Ang II-infused E-V290M mice (Figure 5C-D).

We next measured the level of expression of genes related to inflammation and oxidative stress
in carotid artery using qPCR. This included a pro-inflammatory marker p50 (NF-κB1), catalytic subunits of NADPH oxidases (Nox2 and Nox4), antioxidants (SOD1, SOD2, SOD3, and Catalase), as well as eNOS. Significant increases in expression of p50 (Figure 6A), Nox2 (Figure 6B) and Nox4 (Figure 6C) were observed in untreated E-V290M compared with NT mice. Thus, even in the absence of Ang II and the absence of endothelial dysfunction, expression of proinflammatory and pro-oxidant genes were increased in carotid artery from E-V290M. Expression of both p50 and Nox2 were further increased in Ang II-infused E-V290M mice compared with other three groups. There was no significant difference in expression of SOD1, SOD2 and eNOS in any group. There was a significant decrease in SOD3 and Catalase expression in Ang II-infused E-V290M compared with saline treated mice (Figure 6D-H).

Given the increase in p50 mRNA expression, we measured the level of NF-κB signaling proteins as an index of inflammatory signalling related to the NF-κB pathway in aorta harvested from the same mice used for gene expression analysis. As shown in Figure 7A, the levels of p65, phospho-p65, and p50 protein were increased whereas the level of Iκ-Bα was decreased in aorta from Ang II-infused E-V290M mice. Quantification of a total of 6 independent samples revealed greater heterogeneity among samples and confirmed the trends shown in Figure 7A, but only the increase in p65 reached statistical significance.
Discussion

The importance of PPARγ in mediating adipogenesis, lipid metabolism and glucose homeostasis is widely accepted, but its role in endothelium to regulate vascular tone remains incomplete. In this study, we utilized transgenic mice with EC-specific expression of DN PPARγ (V290M) to investigate the effect of endothelial PPARγ on vascular function in an Ang II-infused model. We conclude that specific interference with PPARγ in ECs enhanced Ang II-induced endothelial dysfunction both in cerebral and conduit arteries through an oxidative stress-dependent mechanism, independent of changes in BP. Moreover, selective impairment in endothelial PPARγ causes a predisposition to increased inflammatory signaling in response to Ang II. In concert with our previous studies (7), these results reveal a role for endothelial PPARγ in protection from endothelial dysfunction caused by proinflammatory and prooxidant stressors such as HFD and Ang II.

Role of PPARγ in the vasculature. Results from clinical and animal studies using TZD treatment revealed that in addition to improved glycemic control, TZDs have non-metabolic actions which include lowering of BP (23, 32, 58, 59, 65). Given that PPARγ is the molecular target of TZDs, this highlighted a possible functional role for PPARγ in the vasculature. Indeed, PPARγ is expressed both in ECs and VSMCs. Mice with tissue-specific PPARγ-deficiency (i.e., tissue-specific knockouts) or PPARγ-interference (using PPARγ mutants which interfere with endogenous PPARγ) provided strong evidence that PPARγ directly participates in BP regulation and prevents vascular dysfunction through actions in ECs and VSMCs (6-8, 17, 34, 37, 39, 55, 57). Selective expression of DN PPARγ (P467L) in vascular muscle results in hypertension and vascular dysfunction both in resistance (cerebral arteries) and conduit vessels (aorta) via a Rho kinase-dependent pathway (17, 34, 57). In contrast, expression of DN PPARγ (V290M) selectively in the endothelium caused vascular endothelial dysfunction in the basilar artery.
through an oxidative stress-dependent mechanism when combined with a HFD (7). Both of the
DN PPARγ mutants mentioned above are functionally equivalent and naturally occurring, and
cause early-onset hypertension and insulin resistance in humans (4). Moreover, we have
previously shown that the V290M and P467L mutants in PPARγ are equally effective in inhibiting
PPARγ in both smooth muscle and endothelium (7, 34). In the current study, we focused on the
effect of PPARγ in endothelium, and extended the above findings to demonstrate that DN
interference with endothelial PPARγ can accelerate and amplify Ang II-induced endothelial
dysfunction.

**DN PPARγ in ECs exacerbates Ang II-mediated endothelial dysfunction.** Ang II is recognized as
a key cause of endothelial dysfunction in many cardiovascular diseases. Activation of PPARγ by
TZD attenuated hypertension and improved endothelial dysfunction in both an Ang II-infused
model (22) and in mice overexpressing human renin and human angiotensinogen transgenes
(59). TZD treatment reduced Ang II-induced elevation of BP, and attenuated Ang II-mediated
endothelial dysfunction in mesenteric arteries (22), consistent with the finding that PPARγ
activation down-regulates Ang II type 1 receptor in VSMCs (62). In contrast, genetic deficiency
(39) or interference with EC PPARγ (7) enhanced the pressor response to Ang II. Our data has
extended these findings to show that interference with EC PPARγ impairs endothelial function in
the cerebral circulation (basilar artery) in response to Ang II-induced oxidative stress, without
altering structure of the basilar artery. To further investigate whether conduit arteries exhibit the
same response in this Ang II-infused model, we examined carotid artery function. We
considered this to be translationally important because carotid artery disease (i.e.,
atherosclerosis) is a major cause of stroke and dementia (35, 40). A more modest endothelial
dysfunction was observed in carotid artery from two-week Ang II-infused E-V290M mice. It is
worth noting that SNP-induced relaxation was modestly impaired in carotid artery, but not in
basilar artery, from Ang II-infused E-V290M mice. This suggests the possibility of some smooth
muscle dysfunction in carotid artery perhaps as a result of oxidative stress or inflammation
induced by a loss of endothelial PPARγ in concert with Ang II treatment. The NO/cGMP/cGMP-
dependent protein kinase pathway plays an important role in VSMC relaxation (53). Future
studies could be designed to assess if this pathway is selectively impaired.

Importantly, we found that Ang II infusion increased 5-HT induced contraction in carotid arteries,
which was further enhanced by endothelial DN PPARγ. The 5-HT response in the vasculature is
a sensitive indicator of altered endothelial function (42), which is the result of a balance between
its relaxation actions on ECs and its contractile effects on VSMCs (64). Thus, these findings
suggest that interference with EC PPARγ can worsen endothelial dysfunction after chronic Ang II
treatment. Although the cause of enhanced 5-HT response in Ang II-infused E-V290M mice is
unclear, one might hypothesize a possible role for RhoA/Rho-kinase signaling. Taken together,
while altered endothelial function is observed in both a conduit and resistance vessel from Ang
II-infused E-V290M mice, the degree of dysfunction observed is more profound in cerebral
circulation.

Hypertension is associated with endothelial dysfunction (19) and an enhanced pressor response
to high dose Ang II was found in E-V290M mice (7). In the present study, a relatively low dose
of Ang II was utilized which did not affect BP either in control or E-V290M mice. Thus,
interference with EC PPARγ can enhance Ang II-induced endothelial dysfunction independent of
changes in BP. These findings highlight a critical role for EC PPARγ in protecting against
endothelial dysfunction produced by an established mediator of vascular dysfunction (i.e., Ang
II). Taken together with our previous study showing that E-V290M mice exhibit endothelial
dysfunction after HFD (7) suggests the endothelial PPARγ may provide protection from a variety
of physiologic stressors (or cardiovascular risk factors).

**Effects of EC PPARγ interference on Ang II-infused model: role of oxidative stress.** Oxidative stress is associated with endothelial dysfunction (7, 39, 48). Accumulating evidence supports the hypothesis that activation of EC PPARγ exerts anti-inflammatory and anti-oxidative stress effects (13, 36, 50). Consistent with this, our data herein showed that interference with EC PPARγ elevated expression of NF-κB p50 mRNA, which was further increased by Ang II-infusion. Similarly, there was an increase in p65 protein and a trend toward an increase in phospho-p65 and p50, and a decrease in the NF-κB inhibitory protein Iκ-Bα. This pattern suggests loss of function of endothelial PPARγ predisposes to increased NF-κB-mediated signaling which worsens with Ang II. Importantly, we have previously shown that E-V290M mice exhibit increased susceptibility to HFD-induced endothelial dysfunction in an oxidative stress dependent manner (7). Ang II treatment causes oxidative stress and endothelial dysfunction in carotid arteries and cerebral circulation. This action is reversed by scavengers of ROS and augmented by genetic deletion of key antioxidants (10, 11, 14, 26). In our study, Tempol (a scavenger of ROS) reversed the Ang II-induced endothelial dysfunction observed in E-V290M mice both in cerebral circulation and carotid artery. Although we did not specifically define the source of superoxide in our study, expression of Nox2, an enzymatic subunit of NADPH oxidase and a key source of superoxide in cerebrovascular dysfunction (25), is increased in Ang II-infused E-V290M mice. This change in Nox2 is selective during Ang II as there was no Ang II-mediated increase in Nox4. Nox4 was recently shown to protect against Ang II-induced aortic endothelial dysfunction (61), and its increase in E-290M mice at baseline might represent a compensatory change. Increased Nox2 expression during Ang II infusion is interesting given that Nox2 has been associated with Ang II-induced endothelial dysfunction both in cerebral and conduit vessels (14, 54) and that Ang II can activate Nox2 in carotid arteries (46). Moreover,
expression of the genes encoding antioxidant enzymes SOD3 and Catalase were decreased by Ang II in E-V290M mice. Given these findings, we speculate that Nox2/ROS signalling is involved in the exacerbation of Ang II-induced endothelial dysfunction in cerebral and carotid arteries in our model. Overall, our findings provide additional insight into the mechanisms involved in endothelial dysfunction induced by interference with PPARγ in ECs, and highlight the importance of PPARγ in maintenance of endothelial function.

In conclusion, genetic interference with EC PPARγ exacerbates Ang II-induced endothelial dysfunction both in cerebral and conduit arteries through an oxidative stress-dependent mechanism. Our study supports the concept that EC PPARγ is a critical regulator of endothelial function particularly under conditions that induce oxidative stress and inflammation, and provides new insight into the importance of PPARγ in a model of Ang II-induced vascular disease. Because Ang II plays an important role in promoting cardiovascular diseases, therapeutic approaches targeting EC PPARγ or its downstream gene targets may be beneficial in preventing or slowing the progression of vascular diseases.
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Figure Legends

Figure 1. Effect of Ang II on Arterial BP.
(A) Twenty-four hour mean arterial pressure (MAP) and (B) heart rate were measured with radiotelemetry in E-V290M (n=6-8) and NT mice (n=6). Baseline measurements were taken for 7 days. Thereafter, Ang II (120 ng/kg/min) was continuously infused for two weeks. *P<0.05 E-V290M versus NT. #P<0.05 Ang II (week 2) versus baseline by genotype. All data are mean±SEM.

Figure 2. Vascular Function in Basilar Artery after Chronic Ang II Treatment.
(A) Cumulative concentration-response curves for acetylcholine (ACh) or maximum responses to ACh (n=7-9), and (B) cumulative concentration-response curves for nitroprusside (SNP) or maximum responses to SNP in basilar arteries (n=7-9) are shown. (C-D) Vasoconstriction in response to 100 mmol/L KCl (C, n=7-9) and Ang II (D, n=3) is shown. (E) Structural parameters of basilar arteries in Ca²⁺-free condition (n=7-9) are shown. *P<0.05 versus E-V290M Ang II. All data are mean±SEM.

Figure 3. Vascular Function in Carotid Artery after Chronic Ang II Treatment.
(A) Cumulative concentration-response curves for acetylcholine (ACh) or maximum responses to ACh, and (B) cumulative concentration-response curves for nitroprusside (SNP) or maximum responses to SNP in carotid arteries are shown. (C-E) Contractile responses of carotid artery to KCl (C), endothelin-1 (D, ET-1) and serotonin (E, 5-HT) are shown. *P<0.05 versus E-V290M Ang II; †P<0.05 Ang II versus Saline; #P<0.05 E-V290M Ang II versus NT Ang II. N=5-6 each group. All data are mean±SEM.

Figure 4. Effect of Ang II on Relative Levels of Reactive Oxygen Species (ROS).
Representative pictures (A) and summarized data (B) show that ROS accumulation was
elevated in carotid arteries from Ang II-infused E-V290M mice. Scale bar indicates 100 µm.  

*P<0.05 versus E-V290M Ang II. N=6 each group. All data are mean±SEM.  

**Figure 5. Tempol Improves Endothelial Function.**  

(A-B) Cumulative concentration-response curves for acetylcholine (ACh) or maximum responses to ACh in basilar arteries from Ang II-infused E-V290M (n=6) and NT mice (n=6) ±Tempol (1 mmol/L, 30 min). (C-D) Cumulative concentration-response curves for ACh or maximum responses to ACh in carotid arteries from Ang II-infused E-V290M (n=7) mice ±Tempol (1 mmol/L, 30 min). For ease of comparison, the data from Ang II-infused NT mice shown in Figure 3A is reproduced here as the dashed line to show restoration of the Ach response to normal by Tempol. *P<0.05 versus E-V290M Ang II. All data are mean±SEM.  

**Figure 6. Gene Expression.**  
The level of expression of mRNAs from the indicated genes in NT and E-V290M ±Ang II is shown. *P<0.05 E-V290M versus NT; #P<0.05 E-V290M + Ang II versus E-V290M + Saline; †P<0.05 E-V290M Ang II versus both saline-treated groups; ‡P<0.05 E-V290M Ang II versus E-V290M Saline. N=6-9 each group. All data are mean±SEM.  

**Figure 7. Protein Expression Related to NF-κB Pathway.**  
The protein level of phosphorylated and total p65, p50, total IκBα and β-actin in aorta from NT and E-V290M ± Ang II is shown. The top 4 blots are derived from the same original gel, blotted and re-probed with p-p65, p65, p50, and β-actin antisera, respectively, whereas the bottom 2 blots are derived from the same gel, blotted and re-probed with IκBα and β-actin antisera. *P<0.05 versus other 3 groups. N=6 each group. All data are mean±SEM.
Figure 3

A. Relaxation (%) vs. ACh (-log mol/L) for NT Saline, E-V290M Saline, NT Ang II, and E-V290M Ang II.

B. Relaxation (%) vs. SNP (-log mol/L) for NT Saline, E-V290M Saline, NT Ang II, and E-V290M Ang II.

C. Contraction (g) vs. KCl (mM)

D. Contraction (g) vs. ET-1 (-log mol/L)

E. Contraction (g) vs. 5-HT (-log mol/L)
Figure 4

A

NT Saline  E-V290M Saline

NT Ang II  E-V290M Ang II

B

Relative DHE Fluorescence

Saline  Ang II
Figure 6

A

p50

Fold vs. NT-Saline

Saline

Ang II

*#

B

Nox2

Fold vs. NT-Saline

Saline

Ang II

*#

C

Nox4

Fold vs. NT-Saline

Saline

Ang II

*

D

SOD1

Fold vs. NT-Saline

Saline

Ang II

E

SOD2

Fold vs. NT-Saline

Saline

Ang II

F

SOD3

Fold vs. NT-Saline

Saline

Ang II

†

G

Catalase

Fold vs. NT-Saline

Saline

Ang II

‡

H

eNOS

Fold vs. NT-Saline

Saline

Ang II

NT

E-V290M
Figure 7

A

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B

- **p-p65/p65**
  - ![Image](image13.png)
  - ![Image](image14.png)
  - ![Image](image15.png)

C

- **p65**
  - ![Image](image16.png)
  - ![Image](image17.png)
  - ![Image](image18.png)

D

- **p50**
  - ![Image](image19.png)
  - ![Image](image20.png)
  - ![Image](image21.png)

E

- **Ik-Bα**
  - ![Image](image22.png)
  - ![Image](image23.png)
  - ![Image](image24.png)

- **NT**
  - ![Image](image25.png)

- **E-V290M**
  - ![Image](image26.png)

*Significant difference*