Endothelium-Dependent Coronary Vasodilatation Requires NADPH Oxidase–Derived Reactive Oxygen Species

Jun Feng, Scott M. Damrauer, Monica Lee, Frank W. Sellke, Christiane Ferran, Md. Ruhul Abid

**Objective**—To determine the functional significance of physiological reactive oxygen species (ROS) levels in endothelium-dependent nitric oxide (NO)-mediated coronary vasodilatation.

**Methods and Results**—Endothelium-derived NO is important in regulating coronary vascular tone. Excess ROS have been shown to reduce NO bioavailability, resulting in endothelial dysfunction and coronary diseases. NADPH oxidase is a major source of ROS in endothelial cells (ECs). By using lucigenin-based superoxide production and dichlorofluorescein diacetate (DCFH-Da) fluorescence-activated cell sorter assays, we found that mouse heart ECs from NADPH oxidase–knockdown (p47(phox)−/−) animals have reduced NADPH oxidase activity (>40%) and ROS levels (>30%) compared with wild-type mouse heart ECs. Surprisingly, a reduction in ROS did not improve coronary vasomotion; rather, endothelium-dependent vascular endothelial growth factor–mediated coronary vasodilatation was reduced by greater than 50% in p47(phox)−/− animals. Western blots and L-citrulline assays showed a significant reduction in Akt/protein kinase B (PKB) and endothelial NO synthase phosphorylation and NO synthesis, respectively, in p47(phox)−/− coronary vessels and mouse heart ECs. Adenoviral expression of constitutively active endothelial NO synthase restored vascular endothelial growth factor–mediated coronary vasodilatation in p47(phox)−/− animals.

**Conclusion**—Endothelium-dependent vascular endothelial growth factor regulation of coronary vascular tone may require NADPH oxidase–derived ROS to activate phosphatidylinositol 3-kinase–Akt–endothelial NO synthase axis. *(Arterioscler Thromb Vasc Biol. 2010;30:1703-1710.)*

**Key Words:** coronary artery disease ■ endothelial function ■ nitric oxide ■ reactive oxygen species ■ signal transduction ■ NADPH oxidase

Reactive oxygen species (ROS) include molecules that have unpaired electrons, such as superoxide (O2•−), hydroxyl anion (HO•), and nitric oxide (NO•); or that have the oxidizing ability but do not possess free electrons, such as hydrogen peroxide (H2O2), hypochlorous acid, and peroxynitrite. ROS have long been implicated in the pathogenesis of cardiovascular diseases, including hypertension, atherosclerosis, diabetic vasculopathy, and heart failure.1–3 However, recent findings4–13 suggest that ROS play critical roles in signal transduction in vascular cells, including endothelial cells (ECs). Researchers14–17 have identified NADPH oxidase as a major source of superoxide in ECs, thus 1 of the important determinants of the oxidation-reduction (redox) state of the endothelium. The NADPH oxidase-enzyme complex consists of 2 membrane-bound components (gp91(phox) [also known as NADPH oxidase (Nox)-2] and p22(phox)) and several cytosolic regulatory subunits, including p47(phox), p67(phox), and the small GTPase Rac (Rac1 or Rac2). On enzyme activation, the cytoplasmic subunits translocate to the cell membrane and the resulting complex transfers electrons from NADPH to molecular oxygen to form O2•−. More recently, NADPH oxidase–derived ROS have also been implicated in EC proliferation, migration, and angiogenesis.14,15,17

ROS-mediated vascular dysfunction is, in part, caused by reduction in NO• bioavailability as the result of redox (O2•−)–catalyzed formation of peroxynitrite.18–22 NO plays important roles in several vascular functions, including regulation of vasomotor tone and maintenance of vascular health.23,24 Endothelial NO synthase (eNOS) can be stimulated to produce NO by hemodynamic forces, hormones, cytokines, and growth factors, including vascular endothelial growth factor (VEGF). Once NO diffuses from the endothelium into the adjacent vascular smooth muscle cell layer, NO mediates vasorelaxation and regulates the balance between vascular smooth muscle cell proliferation and apoptosis; this later function governs important aspects of vessel caliber and

Received on: February 23, 2010; final version accepted on: May 27, 2010.

From the Department of Surgery (J.F. and F.W.S.), Beth Israel Deaconess Medical Center, Boston, Mass; Alpert School of Medicine of Brown University (J.F. and F.W.S.), Providence, RI; Department of Surgery (S.M.D.), Massachusetts General Hospital, Boston, Mass; Department of Surgery (S.M.D. and C.F.), Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Mass; and The Center for Vascular Biology Research, Department of Medicine (M.L. and M.R.A), Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Mass.

Drs Feng and Damrauer contributed equally to this study.

Correspondence to Ruhul Abid, MD, PhD, The Center for Vascular Biology Research, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, RN-232, 330 Brookline Ave, Boston, MA 02215. E-mail rabid@bidmc.harvard.edu

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*Arterioscler Thromb Vasc Biol* is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.110.209726

1703
remodeling. NO-induced vasodilatation occurs via activation of soluble guanylyl cyclase, thereby elevating cGMP levels. Indeed, mice deficient in eNOS demonstrated impaired endothelium-dependent relaxation in conduit arteries. NO also exerts its effects on the cardiovascular system by S-nitrosylation of protein thiols in a cGMP-independent manner. As a major source of endothelial superoxide, increases in NADPH oxidase activity contribute to the impairment of endothelium-dependent vasodilatation by reducing NO levels. The resultant endothelial dysfunction is believed to be involved in the pathophysiology of vascular diseases. Thus, the balance between ROS and NO is critical for optimal endothelial and vascular function.

VEGF plays important roles in vascular protection, hemo-
stasis, microvascular permeability, wound repair, and angiogenesis of ischemic tissue; and also regulates vasomo-
stasis, microvascular permeability, wound repair, and these vessels.

Methods

Mouse Heart EC Isolation and Culture

Mouse heart ECs (MHECs) were isolated from the heart specimens of 3-week-old animals, as previously described. For each experiment, primary cultures of both genotypes were started simulta-
neously from 2 animal hearts each.

Also, NADPH oxidase activity and intracellular levels of ROS were determined in MHEC, as previously described. Western blot analyses were performed using Western blotting methods described in the supplemental data. Details of the experiments can be found in the supplemental materials (available online at http://atvb.ahajournals.org).

Ex Vivo Coronary Microvessel Relaxation Studies

After cardiac harvest from 6- to 8-week-old mice, coronary arterioles (diameter, 80 to 120 μm; length, 2 mm) from wild-type (WT) C57BL/6 (n = 6) and p47phox−/− (n = 8) mice were dissected from the surrounding tissue. Microvessel studies were performed using in vitro organ bath videomicroscopy, as previously described. Where isolated, isolated coronary vessels were pretreated with membrane-
permeant ROS scavengers (4-hydroxy-2,2,6,6-tetramethylpiperidine-
1-oxyl [TEMPOL], 1 μmol/L) and manganese (Mn) (II)tetrakis (4-benzoic acid)porphyrin chloride (MnTTPA, 1 μmol/L) (EMD Bioscience, Darmstadt, Germany). More details can be found in the supplemental data.

eNOS Activity Assay

Mouse heart specimens were harvested and homogenized in 20 mL of ice-cold homogenization buffer (25-mmol/L Tris-Cl, 1-mmol/L EDTA, and 1-mmol/L EGTA, pH 7.4) per gram of tissue. eNOS activity was determined using the NOS assay kit (CalBiochem, San Diego, Calif) that measures conversion of L-[3H]arginine to L-[3H]citrulline, according to the manufacturer’s directions. Detailed methods can be found in the supplemental data.

Western Blot Analyses

Mouse whole hearts and coronary vessels with surrounding cardiac tissues were harvested separately for total protein content. Western blots were performed as previously described. Coronary vessels from 2 hearts were pooled for each sample. Antiphosphorylated (473) Akt, antiphosphorylated (1179) eNOS, antiphosphorylated ERK1/2, anti-Akt, and anti-eNOS antibodies were obtained from Cell Signaling (Beverly, Mass). MHECs were grown on gelatin-coated plates to 80% or 90% confluence, serum starved for 24 hours, and then treated with VEGF, 100 ng/mL, for the times indicated. Cell lysates were prepared for Western blot analysis, as previously described.

Adenoviruses and Expression of Constitutively Active S1179D eNOS in Coronary Vessels

Replication-deficient adenoviruses encoding the cDNAs of β-galactosidase (adenovirus-β-galactosidase gene [Ad-lacZ]), green fluorescent protein (GFP) (Ad-GFP), and S1179D eNOS (Ad-S1179D eNOS-GFP) were previously described. Mice were anesthetized, exsanguinated, and perfused with saline via infusion into the apex of the left ventricle. Recombin

Statistical Analyses

Data are given as the mean±SEM, where appropriate. P<0.05 between experimental groups was considered a significant difference. Nonlinear regression modeling using the extra sum-of-squares F test to compare slopes (Prism 5, Graph Pad Software) was used to analyze microvessel reactivity data.

Results

p47phox−/− MHECs Have Reduced NADPH Oxidase Activity and ROS Levels

First, we wanted to determine the levels of NADPH oxidase activity and total ROS in ECs of genetically modified p47phox subunit-knockout (p47phox−/−) animals. By using a low concentration-based lucigenin assay and dichlorfluor-

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VEGF-Induced Vasodilatation Is Inhibited in Coronary Vessels of p47phox−/− Mice

Endothelium-dependent vasodilatation is dependent on NO bioavailability. Because MHECs from p47phox−/− mice have significantly reduced NADPH oxidase activity and ROS levels, we wanted to examine endothelium-dependent coronary vasodilatation in these animals. Surprisingly, VEGF-induced coronary vasodilatation was reduced by 50.0±6.4% in p47phox−/− mice compared with WT mice (Figure 2A). This apparently counter-intuitive finding led us to examine whether NO production in endothelium and NO response in vascular smooth muscle cells are intact in p47phox−/− mice. Thus, coronary arterioles from WT and p47phox−/− mice were subject to relaxation responses to ADP, a nucleotide that releases NO from the endothelium in a PI3K-Akt–independent manner; and to sodium nitroprusside (SNP), an NO donor that acts directly on vascular smooth muscle. ADP− (Figure 2B) and SNP-induced (Figure 2C) vasodilatation of the coronary arterioles from p47phox−/− mice was comparable to that of WT animals, suggesting that the ability of the endothelium to produce NO in response to non-VEGF stimuli and that the response of smooth muscle cells to NO in these vessels were unaffected. Furthermore, the endothelium dependence of VEGF- and ADP-mediated, but not SNP-mediated, vasodilatation was confirmed by endothelial denudation in WT coronary vessels, as described in the supplemental methods. Taken together, these findings suggest that VEGF-stimulated endothelium-dependent vasodilatation is specifically affected in the coronary blood vessels in p47phox−/− mice.

PI3K-Akt-eNOS Signaling and NO Synthesis Are Reduced in Coronary Vessels of p47phox−/− Mice

NO synthesis in the endothelium is known to be induced by PI3K-Akt–mediated activation of eNOS. Recently, it was demonstrated that VEGF-mediated activation of PI3K-
Akt is dependent on NADPH oxidase–derived ROS in human ECs in vitro. Combined with our current findings, this led us to examine whether the observed defect in coronary vessel relaxation in p47phox−/− mice resulted from reduced basal activation of redox-sensitive PI3K-Akt-eNOS. To test this, we measured activation levels of PI3K-Akt-eNOS signaling and NO levels in p47phox−/− coronary vessels using Western blots and citrulline assays, respectively. Figure 3A demonstrates that phosphorylation levels of Akt and eNOS in p47phox−/− coronary vessels are reduced by 61.0±4.8% and 34.5±6.3%, respectively, compared with that of WT mice. NO synthesis in coronary vessels of p47phox−/− mice was also reduced by 30.8±4.2% compared with that of WT mice (Figure 3B). These findings suggest that genetic deletion of the NADPH oxidase subunit, p47phox, results in reduced activation of PI3K-Akt-eNOS signaling and NO synthesis in the coronary vasculature. However, there was no difference in the expression levels of VEGF receptors 1 and 2 (supplemental Figure IA) or phosphorylation levels of ERK1/2 (Figure 3A) in coronary vessels of WT and p47phox−/− mice, suggesting that NADPH oxidase activity selectively modulates the PI3K-Akt-eNOS pathway.

**VEGF-Induced Activation of PI3K-Akt-eNOS, But Not ERK1/2, Is Inhibited in p47phox−/− MHECs**

Next, we wanted to examine whether VEGF-induced activation of PI3K-Akt-eNOS signaling required NADPH oxidase activity in murine endothelium. We performed Western blots for the phosphorylation of Akt, eNOS, and ERK1/2 using cell lysates from WT and p47phox−/− MHECs. VEGF-induced phosphorylation of Akt and eNOS, but not ERK1/2, was inhibited in MHECs from p47phox−/− mice, suggesting that selective activation of PI3K-Akt-eNOS by VEGF depends on NADPH oxidase activity in murine ECs (supplemental Figure IB). However, there was no difference in the expression levels of VEGF receptors 1 and 2 between WT and p47phox−/− MHECs (supplemental Figure IA). These results suggest that the VEGF-PI3K-Akt-eNOS (but not ERK1/2) axis in MHECs is dependent on NADPH oxidase activity.

**Expression of Activated eNOS Compensates for p47phox−/− Effects on Coronary Vasodilatation**

To examine whether reduced activation of PI3K-Akt-eNOS is responsible for the inhibition of VEGF-induced vasodilatation, we introduced replication-deficient control adenovirus (Ad-lacZ or Ad-GFP) or adenovirus expressing constitutively active eNOS (Ad-S1179D eNOS-GFP) into the coronary vessels of p47phox−/− mice (Figure 4), as described in the supplemental methods. Gene transfer to the endothelium was further confirmed by β-galactosidase staining (data not shown) and immunofluorescence staining for GFP and anti-CD31 (Figure 4A). Overexpression of eNOS in the endothelium of the Ad-S1179D eNOS-transduced coronary vessels was confirmed by Western blots (Figure 4B) and immunohistochemistry (Figure 4C). Ad-S1179D eNOS–transduced p47phox−/− coronary vessels demonstrated a 1.84±0.22-fold increase in eNOS activity compared with Ad-GFP–transduced vessels, as measured by the citrulline assay (Figure 4D).

Finally, we examined whether expression of activated eNOS in the endothelium of intact coronary blood vessels could compensate for the reduced vasodilatory activity in NADPH oxidase knockdown mice. Figure 5A demonstrates that expression of activated eNOS (recombinant adenovirus [rAd].S1179D NOS3) restored VEGF response in the coronary vessels of p47phox−/− mice to the WT levels. Together, these findings
suggest that activation of PI3K-Akt-eNOS signaling is inhibited in p47phox
coronary vessels, which results in defective vasodilatation in response to VEGF.

Reduction in ROS Inhibits VEGF-Induced Coronary Vasodilatation

Next, we wanted to test whether ROS have a direct role on VEGF-induced coronary vasodilatation. To that end, we treated the coronary arteries of WT mice with 2 different cell-permeant ROS scavengers, TEMPO (broad-spectrum free radical scavenger) and MnTBAP (superoxide scavenger/dismutase that does not scavenge NO), and measured VEGF-induced vasodilatation. Both TEMPO and MnTBAP significantly inhibited VEGF-induced coronary vasodilatation (Figure 6), but not SNP-mediated vasodilatation (data not shown), providing direct evidence that decreasing ROS impairs endothelium-dependent coronary vasodilatation. Although TEMPO decreases ROS levels, MnTBAP specifically reduces superoxide levels by converting \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) and, thus, increases overall \( \text{H}_2\text{O}_2 \) levels. The inhibitory effects of both TEMPO and MnTBAP suggest a greater role for superoxide compared with \( \text{H}_2\text{O}_2 \) in VEGF-induced coronary vasodilatation. However, our data cannot exclude the requirement for proper subcellular localization of \( \text{H}_2\text{O}_2 \) in VEGF-induced activation of signaling pathways and coronary vasodilatation. Future studies will address these important questions.

Discussion

The goal of the present study was to determine the role of endogenous NADPH oxidase–derived ROS (ie, physiological levels of ROS in coronary vascular function). Specifically, we wanted to examine whether reduction in endogenous NADPH oxidase activity would improve coronary vascular function (eg, vasodilatation, by increasing NO bioavailability). To that end, we have used an NADPH oxidase–knockdown mouse model (p47phox−/−) that has significantly reduced ROS levels in the coronary endothelium. The results presented herein using intact coronary blood vessels and isolated MHECs suggest that endothelium-dependent coronary vasodilatation operates through NADPH oxidase–mediated activation of PI3K-Akt-eNOS signaling and NO synthesis in ECs. To our knowledge, these data also provide the first
evidence that reduction in endogenous NADPH oxidase activity can result in coronary vascular dysfunction by decreasing redox-mediated activation of eNOS. Our data demonstrate that genetic knockdown of NADPH oxidase subunit, p47phox, results in significant decrease in NADPH oxidase activity and ROS levels in coronary blood vessels. We also show that coronary vessels of these animals have reduced PI3K-Akt-eNOS activity, NO production, and VEGF-induced vasodilation. Interestingly, coronary vasodilation by ADP, a PI3K-Akt–independent activator of eNOS,58 was unaffected in p47phox−/− mice, providing additional support for the specificity of the defective VEGF activation of the PI3K-Akt-eNOS axis in this animal. Furthermore, our results demonstrate that VEGF-induced activation of PI3K-Akt-eNOS, but not ERK1/2, signaling is impaired in NADPH oxidase–knockdown MHECs. These findings are in accordance with a previous report46 that VEGF-mediated activation of PI3K-Akt-forkhead signaling, but not ERK1/2, is dependent on NADPH oxidase activity in human coronary artery ECs in vitro. Taken together, we hypothesize that NADPH oxidase–derived ROS modulate activation of PI3K-Akt-eNOS in endothelium and, thus, regulate VEGF-induced coronary vasodilation. Our data demonstrating that expression of activated eNOS compensates for the functional loss of upstream PI3K-Akt-eNOS activity in p47phox−/− coronary vessels support this hypothesis. Future studies aimed at modulating coronary vasodilation using loss-of-function or gain-of-function mutants of eNOS-activating signaling intermediates (eg, Akt, PI3K, or cellular sarcoma [c-Src]), including upstream serine and threonine kinases (eg, protein kinase Cβ, AMP-activated protein kinase, and protein kinase A), should further our understanding about their individual contributions to ROS-mediated eNOS activity and, thus, the regulation of coronary vasomotor tone.

Figure 5. A, Activated eNOS expression compensates for the NADPH oxidase knockdown effects on coronary vessels. VEGF-induced endothelium-dependent dilation of coronary arterioles from p47phox−/− mice transduced with Ad-GFP (n = 4) or Ad-S1179D nos3-GFP (n = 4), P < 0.001. B, Proposed model for NADPH oxidase–dependent activation of eNOS and coronary vasodilation. Reduction in coronary vasodilatation in NADPH oxidase–knockdown mice appears to be because of defective activation of the redox-sensitive VEGF signaling pathway (shown in the colored box), PI3K-Akt-eNOS, in the coronary endothelium. ERK1/2 activation is independent of the redox level in the endothelium.

Figure 6. ROS scavengers inhibit VEGF-induced vasodilation in coronary vessels of WT mice. Isolated coronary vessels were treated with membrane-permeant ROS scavenger TEMPOL, 1 μmol/L, and MnTBAP, 1 μmol/L, for 15 minutes. Endothelium-dependent dilation of coronary arterioles from WT (n = 4) mice in response to VEGF was assayed as described in “Ex Vivo Coronary Microvessel Relaxation Studies” under Methods. section. For statistical significance, nonlinear regression modeling was used to compare slopes (Prism 5, Graph Pad Software) of the microvessel reactivity data.

ROS have been previously shown to increase NO release in blood vessels and in EC culture in vitro.20,60,62 A recent study by Zhang and coworkers63 reported that superoxide production by overexpression of nox5 increased eNOS activity in aorta and cultured ECs. However, nox5-induced activation of eNOS was mediated through enhanced association of eNOS with heat shock protein-90 without altering eNOS phosphorylation.63 Another study64 reported that Rac1 regulated vasomotor response and angiogenesis by transcriptional upregulation of eNOS. To our knowledge, the current study is the first to demonstrate that endogenous NADPH oxidase–derived ROS play an important role in endothelium-dependent vasodilation in intact coronary blood vessels. Our data also provide evidence for a novel mechanism by which NADPH oxidase–derived ROS regulate PI3K-Akt activation and, thus, regulate downstream phosphorylation and posttranslational activation of eNOS in coronary endothelium. In addition, ROS scavengers inhibited endothelium-dependent coronary vasodilation in WT animals, providing further support for a positive role for ROS in coronary vasomotion.
These results are in contrast with a long-held notion that reduction of ROS will improve global vascular functions by increasing bioavailability of NO. Rather, our data support a critical positive role for endogenous NADPH oxidase-derived ROS in NO production in intact coronary vessels.

In summary, we demonstrate that endothelium-dependent coronary vasodilatation operates through NADPH oxidase-derived ROS-mediated activation of PI3K-Akt-eNOS signaling and NO synthesis in endothelium (Figure 5B). Together with previous findings using human coronary ECs, the present study suggests that redox sensitivity of PI3K-Akt-eNOS signaling may be evolutionarily conserved in mammalian coronary vessels. The redox dependence of eNOS in the coronary vasculature may have the following important functional implications: (1) it may help maintain critical balance between superoxide and NO levels in coronary vessels and, thus, ensure NO bioavailability during oxidative stress; (2) it may synchronize peroxynitrite levels with redox content of the vasculature; and (3) it may represent a coordinated feedback loop that enhances eNOS activity by ROS to counterbalance reduction in NO bioavailability. Studies are undergoing to address these important questions.

Acknowledgments
We thank William Sessa, PhD, Yale University School of Medicine, for the Ad-S1179D eNOS adenoviruses and for critically reading the manuscript and providing valuable suggestions.

Sources of Funding
This study was supported by grants SDG 0453284N and 10GRNT3640011 from the American Heart Association (Dr Abid); and also in part by grants ST32HL077734–14 (Dr Damrauer), HL–46716 and HL–09024 (Dr Selke), and HL080130 (Dr Ferran) from the National Institutes of Health.

Disclosures
None.

References


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Arterioscler Thromb Vasc Biol. 2010;30:1703-1710; originally published online August 11, 2010;
doi: 10.1161/ATVBAHA.110.209726
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/30/9/1703

Data Supplement (unedited) at:
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Methods:

Coronary Microvessel Relaxation Studies ex vivo

After cardiac harvest from 6-8 week-old mice, coronary arterioles (80-120 µm in diameter and 2 mm in length) from WT/C57 (n = 6) and p47phox-/- (n = 8) mice were dissected from the surrounding tissue with a X40 dissecting microscope (Olympus Optical, Japan). Microvessel studies were performed using in vitro organ bath videomicroscopy as described previously \textsuperscript{1-3}. Once the steady-state tone was reached, dose-dependent relaxation was measured in response to extraluminal application of the following vasodilators: nitroprusside (SNP; 1 nmol/L to 100 µmol/L or 10\textsuperscript{-9} to 10\textsuperscript{-4} M), adenosine 5'-diphosphate (ADP; 10\textsuperscript{-9} to 10\textsuperscript{-4} M) and VEGF (10\textsuperscript{-15} to 10\textsuperscript{-10} M). SNP, a nitric oxide (NO) donor, directly relaxes vascular smooth muscle via an endothelium-independent cGMP-mediated pathway. ADP, an endothelium-dependent vasodilator, relaxes vascular smooth muscle mainly via stimulation of endothelial NO synthase, which releases NO. VEGF relaxes vascular smooth muscle via an endothelium-dependent tyrosine receptor kinase (VEGFR-2)-PI3K-Akt-eNOS-mediated release of NO. The vessels were washed 3 times with KHB and allowed to equilibrate drug free for 30 minutes between interventions. Six vessels were used for each drug or intervention. Where indicated, isolated coronary vessels (n=4) were treated with membrane-permeant ROS scavenger 4-Hydroxy-2,2,6,6-tetramethylpieradine-1-oxyl (TEMPOL, 1 µM) and Mn(III)tetrakis (4-benzoic acid)porphyrin chloride (MnTBAP, 1 µM) (EMD Bioscience) for 15 min. Selected experiments were performed following 30 min of pretreatment with 10\textsuperscript{-4} M L-NAME (Sigma). Endothelial denudation was carried out by advancing a human hair into the lumen and gently abrading the luminal surface.
eNOS Activity Assay

eNOS activity was determined using a Nitric Oxide Synthase (NOS) Assay kit (CalBiochem, cat# 482700) that measures conversion of radioactive[^H]-L-arginine to[^H]-L-citrulline. This reaction involves a five-electron oxidation of a guanidino nitrogen of L-arginine to nitric oxide (NO), together with the stoichiometric production of L-citrulline. Mouse hearts and coronary vessels embedded in surrounding cardiac tissues were harvested en bloc and homogenized in 20 ml ice-cold 1X Homogenization Buffer containing 25 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, pH 7.4 per gram of tissue. After centrifugation for 5 in at 4°C, the freshly prepared supernatants were used for the assay as described in the manufacturer's protocol. A small aliquot from each sample was used to determine protein concentration.

Quantitative Real-Time PCR

Real time PCR was carried out as previously described[^4]. Briefly, total RNA was prepared using the RNeasy RNA extraction kit with DNase-I treatment following the manufacturer's protocol (Qiagen, Valencia, CA). Following primer sets were used for Flt-1: 5'-TGGCCAGAGGCATGGAGT-3 and 5'-TCGCA AATCTTCACCACATTG-3, and for Flk-1: 5'-CCTGCCTACCTCACCTGTTT-3 and 5'-CGGCTCTTTCGCTTACTGTT-3. The real-time PCR was carried out using ABI Prism 7700 Sequence Detection System. PCR reactions for each sample were performed in triplicate and the level of target gene expression was normalized against the 18S rRNA expression in each sample. The data presented are as mRNA copies per 10^6 18S rRNA.

Adenoviral Expression of Constitutively Active S1179D eNOS in Coronary Vessels

6-8 week old p47^{phox-/-} mice underwent coronary artery perfusion with either control recombinant adenovirus (Ad-lacZ; Ad-GFP) or Adv-S1179D eNOS[^5,^6]. After induction of
anesthesia the apex of left ventricle was cannulated and perfused with 10 mL of 0.9% saline at 4°C while the mouse was simultaneously exsanguinated via an incision in the abdominal aorta and inferior vena cava. The distal ascending aorta was then ligated and cannulated in a retrograde fashion to allow perfusion of the coronary arteries. The coronaries were perfused in situ with 5 x 10^9 pfu/ml recombinant adenovirus in 0.9% saline at a rate of 40 µL/min for 20 min before being excised and cultured with the ligature on in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin, and 25 µg/ml gentamicin for 16 hrs at 37°C, 5% CO₂. Four mice were used for Ad-lacZ adenovirus, and four mice were used for each of Ad-GFP and Ad-S1179D eNOS-GFP transduction. Three mice from each adenovirus transduced group were used to determine the expression of the transgene (lacZ, GFP and eNOS) using immunohistochemistry, immunofluorescence and Western blotting.

**Immunohistochemistry**

To confirm adenovirus (Ad-lacZ) transduced gene expression, snap-frozen fixed hearts embedded in OCT compound were sectioned and processed for X-gal staining (β-gal) using standard protocol . Paraformaldehyde-fixed semi-wet sections of Ad-S1179D eNOS-GFP-transduced hearts were used for visualizing GFP fluorescence. Serial frozen sections were acetone fixed and incubated overnight with a rat monoclonal anti-PECAM1/CD31 antibody (1:50 dilution) (Pharmpingen, San Diego, CA) at 4 °C to detect endothelium-specific GFP fluorescence. In order to detect adenovirus-mediated exogenous expression of S1179 eNOS in the coronary vessels, frozen sections were subject to immunohistochemistry using anti–eNOS antibody (ab50260; Abcam), biotinylated anti-IgG, and Standard Peroxidase Vecstatin ABC reagent from Vector Labs.
REFERENCES


Supplemental Figure I. A, There was no significant difference in VEGF receptor expression levels between WT and p47^{phox/-} mice. Quantitative real-time PCR (qPCR) for Flt-1 (VEGFR1) and Flk-1 (VEGFR2) was carried out using total RNA from MHEC (left four bars) and coronary arteries (right four bars) of WT and p47^{phox/-} mice as described in Methods. B, VEGF-induced Akt and eNOS activation, but not ERK1/2, was inhibited in MHEC from p47^{phox/-} mice. Western blots using extracts from WT and p47^{phox/-} MHEC treated with VEGF for the times indicated. The membrane was probed for phosphorylation of Akt S473 (p-Akt), then stripped and reprobed for phosphorylation of eNOS S1179 (p-eNOS) and ERK1/2 (p-ERK1/2). Anti-ERK1/2 antibody (ERK1/2) was used as loading control. The blots shown are representative of two independent experiments.