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NADPH Oxidase 4 Promotes Endothelial Angiogenesis Through Endothelial Nitric Oxide Synthase Activation

Siobhan M. Craige, PhD*; Kai Chen, MD, PhD*; Yongmei Pei, MS; Chunying Li, PhD; Xiaoyun Huang, MD; Christine Chen; Rei Shibata, MD, PhD; Kaori Sato, MD, PhD; Kenneth Walsh, PhD; John F. Keaney, Jr, MD

Background—Reactive oxygen species serve signaling functions in the vasculature, and hypoxia has been associated with increased reactive oxygen species production. NADPH oxidase 4 (Nox4) is a reactive oxygen species–producing enzyme that is highly expressed in the endothelium, yet its specific role is unknown. We sought to determine the role of Nox4 in the endothelial response to hypoxia.

Methods and Results—Hypoxia induced Nox4 expression both in vitro and in vivo and overexpression of Nox4 was sufficient to promote endothelial proliferation, migration, and tube formation. To determine the in vivo relevance of our observations, we generated transgenic mice with endothelial-specific Nox4 overexpression using the vascular endothelial cadherin promoter (VECad-Nox4 mice). In vivo, the VECad-Nox4 mice had accelerated recovery from hindlimb ischemia and enhanced aortic capillary sprouting. Because endothelial nitric oxide synthase (eNOS) is involved in endothelial angiogenic responses and eNOS is activated by reactive oxygen species, we probed the effect of Nox4 on eNOS. In cultured endothelial cells overexpressing Nox4, we observed a significant increase in eNOS protein expression and activity. To causally address the link between eNOS and Nox4, we crossed our transgenic Nox4 mice with eNOS−/− mice. Aortas from these mice did not demonstrate enhanced aortic sprouting, and VECad-Nox4 mice on the eNOS−/− background did not demonstrate enhanced recovery from hindlimb ischemia.

Conclusions—Collectively, we demonstrate that augmented endothelial Nox4 expression promotes angiogenesis and recovery from hypoxia in an eNOS-dependent manner. (Circulation. 2011;124:731-740.)

Key Words: angiogenesis ■ endothelium ■ eNOS ■ reactive oxygen species ■ NADPH oxidase 4

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From the Division of Cardiovascular Medicine, Department of Medicine, University of Massachusetts Medical School, Worcester (S.M.C., K.C., Y.P., C.L., X.H., C.C., J.F.K.), and Boston University School of Medicine, Whitaker Cardiovascular Institute, Boston, MA (K.S., K.W.).

*Drs Craige and Chen contributed equally to this work.

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Correspondence to Siobhan M. Craige, PhD, University of Massachusetts Medical School, Department of Medicine/Division of Cardiovascular Medicine, 381 Plantation St, Biotech 5, Suite 200, Worcester, MA 01605. E-mail Siobhan.Craige@umassmed.edu

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response to environmental cues. In general, these enzymes are governed by the requirement for accessory proteins that facilitate full enzyme activity via the transfer of NADPH-derived electrons to molecular oxygen, initially resulting in superoxide formation. Among the Nox family, the Nox4 isoform is unique in that it requires little regulation from accessory proteins and primarily releases H2O2 into the cytosol. Multiple lines of evidence suggest that Nox4 is involved in the adaptation to hypoxia as it is upregulated in tissue ischemia, and Nox4 inhibition prevents endothelial-derived tumor formation. The vascular endothelium contains abundant Nox4, and this oxidase mediates endothelial responses to transforming growth factor-β and epidermal growth factor, which include migration and proliferation. Despite these data linking Nox4 to hypoxic responses, its specific role is not yet known, and therefore we sought to identify the implications of endothelial Nox4 in the adaptation to hypoxia.

Methods

Cell Culture

Cultured human and bovine endothelial cells (ECs) were obtained from Lonza (Basel, Switzerland) and used between passages 3 and 8. Human cells were cultured in endothelial growth medium-2 containing 2% fetal bovine serum, and bovine cells were cultured in endothelial growth medium with 5% fetal bovine serum with the included bullet kit supplements (Lonza). Mouse lung ECs were harvested via 2 consecutive selections with the use of intercellular adhesion molecule-2 and cultured on 0.2% gelatin-coated dishes in endothelial growth medium-2 containing 10% fetal bovine serum. The cells were used for experiments between passages 2 and 6.

Adenoviral Constructs

The adenoviral vector expressing Nox4 was a gift from Dr Barry Goldstein (Thomas Jefferson University). The adenoviral vector expressing Nox4 RNA interference (Nox4i) was constructed as described in Chen et al. Cells were typically infected at a multiplicity of infection of 10 to 50 with a control adenovirus at the multiplicity of infection of 1. The resultant fragment was inserted into the Nox4 adenoviral constructs (2×10⁹ plaque-forming units) were injected into 5 sites of the thigh adductor muscle.

Polymerase Chain Reaction

Total RNA was extracted with the use of Qiagen RNeasy Mini Kits for cells and with the use of TRizol (Invitrogen) for tissue samples according to the manufacturer’s protocol. Total RNA was reverse-transcribed to cDNA with the use of Qiagen Omniscript RT Kit at 37°C for 60 minutes. The primers used for polymerase chain reaction were as follows: Nox4 forward: tgcctgctgacatcat; Nox4 reverse: gagggcattcaccagat; GAPDH forward: acgtcctgtggct; GAPDH reverse: ggctaccacatccaaggaa; 18S forward: gctggaattaccgcggct; 18S reverse: gctggaattaccgcggct. The real-time polymerase chain reaction primers were as follows: human Nox4 forward: cagagcttccagcagag; human Nox4 reverse: ggtcggcagctccagagt; GAPDH forward: acagggcagctggatgagg; GAPDH reverse: aggcctgctccaatgcagctg; and Hprt1 reverse: ggcctctggcaatcaaacg. The real-time polymerase chain reaction reactions were run on a Bio-RAD 97Q iCycler.

Determination of Reactive Oxygen Species

As an index of ROS generation, we used the Amplex Ultra Red reagent 10-acetyl-3,7-dihydroxyphenoxazone (Molecular Probes; A36006), which reacts with hydrogen peroxide (1:1 stoichiometry) in the presence of horseradish peroxidase to form resorufin. ECs were cultured to confluence in 12-well plates and incubated with Krebs’ HEPES buffer (mmol/L: NaCl 118, HEPES 22, KCl 4.6, MgSO4 2.1, NaH2PO4 0.15, KH2PO4 0.41, NaHCO3 5, glucose 5.6, CaCl2 1.5) for 30 minutes. The Amplex Ultra Red and horseradish peroxidase were then added, and fluorescence (excitation 544 nm; emission 590 nm) was determined as a function of time (2 hours) in 96-well black plates (Corning) at 37°C in a fluorescent plate reader (Spectramax, Molecular Devices).

Endothelial Tube Formation and Migration

We utilized the spontaneous organization of ECs into capillary-like tubules when grown in vitro on Matrigel. Cells were cultured alone or with adenoviral constructs for 24 hours. Cells were then trypsinized and plated on Matrigel and monitored over time (6 to 12 hours) for tube formation as described. At least 5 random fields of vision were then analyzed for tube length with the use of NIH Image J software for quantification. For migration, we utilized the scratch assay. Bovine aortic ECs (Lonza) were infected with LacZ, Nox4, or Nox4i adenovirus for 48 hours. Cells were then treated with 50 μg/mL mitomycin C to inhibit proliferation and scratched with a 200-μL pipet. Migration was quantified as the extent of gap closure with NIH Image J software.

Generation of Endothelial-Specific Nox4 Transgenic Mice

Full-length human Nox4 cDNA was subcloned into the pCR3.1 vector (Invitrogen) followed by restriction digestion with Kpn1 and Xhol. The resultant fragment was inserted into the NoI site between the mouse vascular endothelial cadherin (VE-Cad) promoter and the SV40 polyadenylation sequence of the pShmVE vector, and the resultant construct was confirmed by sequencing. A linearized DNA fragment containing the intact mouse VE-Cad promoter–human Nox4–SV40pA cassette was used for pronuclear injection to generate multiple founders with endothelial-specific expression of human Nox4 in the C57/B6 background (University of Massachusetts Transgenic Core Facility). The primers used for genotyping are as follows: 5'-ctagacctgagagagcctca-3' and 5'-gtcactcaagcacgttt-3'.

In Situ Hybridization

To detect human Nox4 expression specifically, a 750-bp probe was designed that contained 530 bp of the human Nox4 sequence and 220 bp of the VE-Cad vector. The fragment was cloned into pBlueScript SK (Stratagene), and riboprobes were synthesized following the digoxigenin labeling manufacturer’s protocol (Roche). Slides were treated with acetic anhydride before hybridization at 37°C overnight. The slides were then incubated with 40% SSC at 65°C for 1 hour; anti-digoxigenin alkaline phosphatase was then added to the slides (1:3000 dilution), followed by incubation overnight at 4°C. Slides were then stained with 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue (3.5 μM/mL) overnight at 37°C, and images were obtained with a 40×1.3 objective (Nikon) on an inverted microscope (TE-2000; Nikon) with a camera (Cool-SNAP HQ; Photometrics). Images were captured with the use of NIS Elements software (Nikon).

Immunofluorescence

Aortas were perfused with 4 mL of 0.9% sterile saline solution before excision and removal of perivascular fat and adventitia with the use of a dissecting microscope. The tissue was then formalin-fixed for 12 hours, and paraffin was embedded. Nox4 antibody (Novus) was diluted 1:50, and von Willebrand factor was diluted 1:100 (abcam). Secondary antibodies (Fluor488- or Fluor594-tagged goat anti-rabbit or donkey anti-sheep IgG; Invitrogen) were diluted 1:200.
Capillary Sprouting
The aortas were perfused and cleaned as described above, then 1-mm segments were placed in 300 μL cold Matrigel in a 48-well plate. After 30 minutes in a 37°C incubator, endothelial basal medium-2/10% fetal bovine serum was added, and the medium was changed every other day. Capillary sprouting was counted by phase-contrast microscopy with the use of at least 6 segments of aorta from 4 mice per group. Aortic ring sprouts were analyzed carefully on the basis of morphological differences in growth between the endothelial sprouts and fibroblast sprouts based on greater thickness and a uniform pattern of growth. Sprout counting was confirmed with CD31 staining (data not shown).

Hindlimb Ischemia and Laser Doppler Imaging
Animals were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) via intraperitoneal injection and placed on a heated water blanket to maintain body temperature. The left femoral artery was exposed via an inguinal incision (1.5 to 2 cm), and the femoral artery was ligated and removed from its origin to the proximal portion of the saphenous artery (along with the adjacent veins). For ENOS−/− animals, the femoral artery and vein were simply ligated (5.0 nylon suture) at the origin to prevent limb loss. Animals underwent periodic assessment of blood flow with laser Doppler imaging (a noninvasive technique that allows a user to monitor how the perfusion gradually recovers over time) as described.

Immunohistochemistry
Ischemic and nonischemic muscle was removed and immediately frozen in Tissue Tek OCT, and sections (7 μm/L) were subsequently stained with antibody to platelet EC adhesion molecule-1 (CD31; BD Pharmingen) and counterstained with hematoxylin and eosin. Cells positive for CD31 antigen were counted by phase-contrast microscopy with at least 4 different microscopic fields from each animal, and capillary density was expressed as the capillary number/muscle fiber (×40) or as normalized to nonischemic capillary density per field of vision (×20). Images were obtained with the use of the aforementioned inverted microscope system.

Isometric Measurements of Endothelial Function
Thoracic aortic rings (2 mm in length) were mounted on 200-μm/L pins in a 6-μL vessel myograph (Danish Myo Technology) containing physiological salt solution (mM/L; NaCl 130, KCl 4.7, KHPO4 1.18, MgSO4 1.17, CaCl2 1.6, NaHCO3 14.9, dextrose 5.5, CaNa2/EDTA 0.03). Vessels were stretched to 1-g basal tension at 37°C and aerated with 95% O2/5% CO2. Vessels were equilibrated in physiological salt solution containing 200 μmol/L L-arginine and incubated for 20 minutes. Aortas were then snap-frozen in liquid nitrogen and immediately placed in ice-cold 0.1 mol/L HCl and homogenized. Samples were diluted 1:3, and cGMP was measured with a commercial enzyme-linked immunosorbent assay kit according to the manufacturer’s instructions (Cayman Chemicals, Ann Arbor, MI). Results are expressed as normalized to the adenoviral untreated control of 3 separate experiments run in triplicate.

To measure tissue cGMP, aortas were removed and cleared of connective tissue as described above. Aortic rings 2 mm in width were cut and placed into individual wells of a 96-well plate with 100 μL of Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 500 μmol/L IBMX plus 200 μmol/L L-arginine. Aortic rings were allowed to equilibrate for 30 minutes, and then fresh medium was added with or without 500 μmol/L N ω-nitro-L-arginine methyl ester and incubated for 20 minutes. Aortas were then snap-frozen in liquid nitrogen and immediately placed in ice-cold 0.1 mol/L HCl and homogenized. Samples were diluted 1:3, and cGMP was measured with a commercial enzyme-linked immunosorbent assay kit according to the manufacturer’s instructions (Cayman Chemicals, Ann Arbor, MI). At least 3 segments in each condition from 4 mice per group were measured.

Statistical Analysis
All data are presented as mean±SEM. Images shown are representative of ≥3 independent experiments. Comparisons among 2 treatment groups were performed with a Student’s t test, and comparisons among ≥3 groups involved 1-way ANOVA with a post hoc Dunnett comparison. Dose-response curves were compared with 2-way ANOVA with or without repeated measures as appropriate. Statistical significance was accepted if the null hypothesis was rejected with P<0.05.
Hypoxia Upregulates Nox4

To examine whether Nox4 regulation is a part of the cellular hypoxic response, cultured ECs were subjected to hypoxia (1% oxygen), and we observed a marked increase in Nox4 mRNA and protein expression coincident with hypoxia-inducible factor-1α upregulation (Figure 1A and 1B). We did not see increased expression of other Nox isoforms (Figure 1A in the online-only Data Supplement). We then probed the in vivo relevance of these observations in wild-type C57/B16 mice with hindlimb ischemia and found increased Nox4 expression in ischemic gastrocnemius (5-fold) and thigh adductor muscles (2-fold) compared with nonischemic muscles (Figure 1C; $P<0.05$; n=4). Thus, hypoxia is associated with Nox4 upregulation in vitro and in vivo.

Nox4 Stimulates Angiogenesis

Previously, we have shown that Nox4 overexpression potentiates endothelial proliferation.24 To determine whether Nox4 also drives EC behaviors important for angiogenesis, we overexpressed or knocked down Nox4 using Nox4 and Nox4i adenovirus in ECs (Figure 1B to ID in the online-only Data Supplement) under conditions that do not affect Nox2 expression (Figure 1B in the online-only Data Supplement) and examined EC tube formation and migration. Under basal conditions, increasing Nox4 expression alone increased endothelial tube formation (Figure 2A) and migration (Figure 2B). Conversely, with knockdown of Nox4, we observed blunted tube formation (Figure 2C) and migration (Figure 2D). To examine the role of ROS in Nox4-mediated tube formation, we overexpressed Nox4 in ECs and found that the antioxidants taxifolin (250 μmol/L) or polyethylene glycol–catalase (200 U/mL) decreased Nox4-induced tube formation (Figure 2E). Thus, Nox4 promotes angiogenesis in a ROS-dependent manner.

We then probed the in vivo consequences of acute Nox4 overexpression utilizing a hindlimb ischemia–induced angiogenesis model.28 We observed enhanced blood flow recovery in mice receiving local adenoviral Nox4 injection compared with LacZ injection (Figure 3A and 3B). Blood flow recovery

\[ \text{Figure 2. NADPH oxidase 4 (Nox4) expression promotes reactive oxygen species–dependent endothelial migration and tube formation. Human aortic endothelial cells were treated with LacZ or Nox4 adenovirus as above for 24 hours, then trypsinized and plated on Matrigel for 6 hours, and tube formation was imaged and quantified with the use of NIH Image J software to assess tube length (A). B, Bovine aortic endothelial cells were treated with Lac Z (control [Ctl]) or Nox4 adenovirus for 24 hours. Confluent cells were then scratched with a 200-μL pipet, and cell migration was imaged after 8 hours. Gap closure was quantified at 8 hours with the use of NIH image J software. C, Human aortic endothelial cells were treated with scrambled (control) or Nox4 short interfering RNA (Nox4i) adenovirus for 48 hours and then plated on Matrigel for 6 hours before imaging for tube formation as above. D, Bovine aortic endothelial cells were treated for 48 hours with Nox4i as above and then scratched as in B and imaged after 24 hours. E, Human aortic endothelial cells were treated with Nox4 adenovirus as in A and then plated on Matrigel with or without taxifolin (250 μmol/L) or polyethylene glycol–catalase (200 U/mL) and assessed for tube formation at 6 hours. \( ^*P<0.05 \) vs Nox4 by 1-way ANOVA with a post hoc Dunnett test. For all other comparisons, \( ^*P<0.05 \) vs control by Student t test with n=3 for each group. }\]
Endothelial Nox4 Is Sufficient to Promote Angiogenesis

We probed capillary sprouting from intact aorta as a model of early angiogenesis. Aorta from VE-Cad-Nox4 mice demonstrated enhanced ex vivo capillary sprouting compared with wild-type littermates (Figure 5A). To evaluate the physiological relevance of this observation, we utilized the hindlimb ischemia model and found that blood flow recovery after femoral artery excision was significantly hastened in VE-Cad-Nox4 mice compared with littermate controls (Figure 5B). Consistent with increased angiogenesis, there was increased capillary density in gastrocnemius muscle harvested from VE-Cad-Nox4 compared with wild-type animals (Figure 5C and 5D).

Nox4 Increases Endothelial Nitric Oxide Synthase Activity and Expression

Endothelial nitric oxide synthase (eNOS) is a key regulator of angiogenesis,29 and there is evidence that ROS may regulate
eNOS expression and activity. Therefore, we probed eNOS regulation by Nox4. In cultured ECs, Nox4 overexpression produced increased total eNOS expression and activity (Figure 6A). To further assess Nox4 activation of eNOS, we measured cGMP accumulation in a coculture system using human aortic ECs and rat aortic smooth muscle cells. Under basal conditions, we saw a 2-fold increase in cGMP production, which was increased dramatically on stimulation with the calcium ionophore A23187 (Figure 6B).

To further prove the mechanisms of eNOS regulation by Nox4, we introduced catalase into our Nox4 overexpression system and observed an attenuation of both expression and catalase activity appeared functional because intact aortic rings from VE-Cad-Nox4 mice exhibited less phenylephrine-induced contraction with the use of bright-field microscopy (Figure 6F). Capillary sprouts were then counted in 6 separate aortic sections

Figure 5. Endothelial NADPH oxidase 4 (Nox4) overexpression promotes angiogenesis. Aortas were removed from wild-type (WT) and vascular endothelial cadherin Nox4 transgenic (TG) littermates and cultured in Matrigel for 7 days (A, top). Capillary sprouts were then counted in 6 separate aortic sections with the use of bright-field microscopy (A, bottom) (*P<0.05 by Student t test; n=3 per group). B, Wild-type and vascular endothelial cadherin Nox4 transgenic (Nox4 TG) mice were subjected to hindlimb ischemia (HLI) as in Methods, and blood flow recovery was monitored with the use of laser Doppler imaging over 28 days (*P<0.05 vs wild-type by 2-way ANOVA; n=9 to 10 per group). C, At 28 days, gastrocnemius muscle was harvested from the control and ischemic legs of wild-type and vascular endothelial cadherin Nox4 transgenic animals and stained for CD31 as an index of capillary density (×20 objective). D, Composite data from C per ×20 field (*P<0.05 by Student t test; n=3 per group).

Discussion

Our data demonstrate that hypoxia is associated with upregulation of Nox4 and that endothelial Nox4 is sufficient to promote angiogenesis in an eNOS-dependent manner. In cultured ECs and in vivo, hypoxia was associated with robust upregulation of Nox4, suggesting that Nox4 upregulation may be an adaptive response to tissue ischemia and hypoxia. The endothelium responds to ischemia by promoting angiogenesis, and in the present study we demonstrate that endothelial Nox4 upregulation was sufficient to promote many salient features of the angiogenic process. Specifically, endothelial proliferation, migration, and tube formation were promoted by Nox4 in a ROS-dependent manner. Importantly, these findings were physiologically relevant because Nox4 overexpression in vivo led to enhanced angiogenesis in response to hypoxia. The vascular EC appeared central in mediating this effect because endothelial-specific Nox4 overexpression promoted angiogenesis both in vitro and in vivo. Finally, we were able to demonstrate that the mechanism whereby Nox4 promotes angiogenesis was eNOS dependent, mediated by H2O2. Thus, our data implicate Nox4 as an important adaptive response to ischemia that coordinates the endothelial contribution to new vessel formation.

Data in the literature have demonstrated that Nox4 is upregulated in hypoxic settings such as ischemia and tumor angiogenesis. Ischemic brain injury is associated with an upregulation of Nox4 expression that corresponds temporally to the initiation of angiogenesis. These data, coupled with the fact that Nox4 expression is increased in newly formed capillaries, suggest that Nox4 is linked to angiogenesis. Moreover, hypoxia-induced tumor angiogenesis is associated with Nox4 upregulation and Nox4 suppression in ovarian
Our data demonstrate a causal role for endothelial Nox4 in promoting angiogenesis because Nox4 overexpression is sufficient to promote key endothelial phenotypes (eg, proliferation, migration, tube formation), and Nox4 knockdown hinders these processes that are important in new vessel formation. These findings are of physiological relevance because we found that mice with endothelial-specific Nox4 overexpression exhibited significant promotion of blood flow recovery in response to hindlimb ischemia.

Figure 6. NADPH oxidase 4 (Nox4) promotes endothelial nitric oxide synthase (eNOS) activity. A, Human aortic endothelial cells were infected with LacZ or Nox4 adenovirus for 24 hours; cells were lysed and assessed for phosphorylated (Ser-1177; p-eNOS) and total eNOS as well as actin by immunoblotting. *P<0.05 vs control (Ctl) by Student t test; n=3. B, Human aortic endothelial cells and rat aortic smooth muscle cells were cocultured and assessed for cGMP accumulation as described in Methods. The left panel represents basal activity, and the right panel demonstrates the cells after stimulation with the calcium ionophore A23187 (10 μmol/L). *P<0.05 by Student t test; n=3. C, Human aortic endothelial cells were infected with Nox4 alone or Nox4 with catalase (+Ad-Cat) for 48 hours and assessed for total and phosphorylated (Ser-1177) eNOS, catalase, and actin by immunoblotting. D, Human aortic endothelial cells were cultured for 18 hours in hypoxic conditions with and without short interfering RNA (Nox4i) adenovirus and probed for total and phosphorylated (Ser-1177) eNOS, Nox4, and actin by immunoblotting. E, Aortas were harvested from wild-type (WT) and vascular endothelial cadherin Nox4 transgenic (TG) mice and assessed for cGMP accumulation (P<0.05 vs wild-type by Student t test; n=4). LNAME indicates N^G-nitro-L-arginine methyl ester. F, Aortas were harvested from wild-type and vascular endothelial cadherin Nox4 transgenic mice and assessed for contraction in response to phenylephrine (PE) (P<0.05 vs wild-type by 2-way repeated-measures ANOVA; n=7 per group). G, Aortas were harvested from eNOS−/− and vascular endothelial cadherin Nox4 transgenic eNOS−/− mice and assessed for contraction in response to phenylephrine (n=5 per group).
In multiple ischemia/reperfusion studies, excessive ROS production is known to cause tissue damage. However, in this study we observed a beneficial response with overexpression of a ROS-producing enzyme after hypoxia. The manner in which Nox4 promotes tissue repair rather than injury is not yet clear but is likely related to the unique characteristics of the enzyme. For example, Nox4 releases \( \text{H}_2\text{O}_2 \), and this species is a 2-electron oxidant well suited for cell signaling owing to its preferred target (thiols) and relatively longer half-life compared with other ROS such as superoxide. Moreover, Nox4 seems to generate ROS constitutively rather than producing a high-level burst as in the neutrophil enzyme Nox2. It is therefore plausible that Nox4-derived ROS are not cytotoxic and, as a consequence, are involved in mediating reparative signaling.

Our data identify eNOS as a downstream component of Nox4 signaling. eNOS and its production of bioactive NO contribute to angiogenesis through enhancing EC proliferation and migration. As a consequence, mice lacking eNOS exhibit a significant impairment in ischemia-induced angiogenesis. Given that pathological increases in vascular ROS are known to limit NO bioactivity, it is surprising that a ROS-producing enzyme promotes an NO-dependent process. However, evidence in the literature helps to reconcile these seemingly contradictory results because one particular ROS, \( \text{H}_2\text{O}_2 \), is known to both activate eNOS and upregulate its transcription. Consistent with these published data, we observed increased eNOS expression and activity in ECs overexpressing Nox4, which was reduced in the presence of the \( \text{H}_2\text{O}_2 \) scavenger catalase. In hypoxia, we found Nox4 upregulation that coincided with eNOS phosphorylation, whereas Nox4 RNAi limited the phosphorylation of eNOS. Collectively, these data imply a physiological role for Nox4 in mediating the endothelial response to hypoxia.

The data presented here also generally agree with a recent report demonstrating that Nox5, a calcium-dependent Nox isoform, enhances eNOS catalytic activity in both cultured endothelium and mouse aorta. Despite this increase in eNOS catalytic activity, Zhang and colleagues observed reduced NO bioactivity suggesting extracellular consumption of NO. One distinguishing feature of the present study is that Nox4 is thought to produce \( \text{H}_2\text{O}_2 \), a ROS species not associated with NO consumption. Thus, our observations of enhanced NO bioactivity with Nox4 may reflect the nature of the Nox product. Collectively, these 2 studies indicate that the phenotypic implications of ROS are contextual and depend on the Nox isoform involved.

Nox4 is known to produce \( \text{H}_2\text{O}_2 \) and \( \text{H}_2\text{O}_2 \) is known to activate Akt and AMPK, 2 kinases important in eNOS regulation. Thus, our data are consistent with the idea that Nox4-derived \( \text{H}_2\text{O}_2 \) is responsible for promoting angiogenesis. With regard to the mechanism, we and others have demonstrated that Nox4 targeting to the endoplasmic reticulum facilitates protein tyrosine phosphatase 1B (PTP1B) inhibition. In mice lacking PTP1B, Akt activation is prolonged. Because Nox4-mediated PTP1B inhibition enhances endothelial proliferation and PTP1B inhibition stimulates VEGF-induced endothelial proliferation and angiogenesis, it is plausible that PTP1B inhibition is a mechanism for Nox4-mediated eNOS activation.

Endothelial Nox4 is likely not the only ROS-sensitive component of angiogenesis. In fact, previous observations have demonstrated that Nox stimulation of angiogenesis may involve other tissues such as the bone marrow and other ROS-producing sources such as Nox2. Although in our system we cannot rule out a role for the bone marrow–derived cells, our work in cultured cells coupled with observations of capillary sprouting in intact vessels indicates that the bone marrow is not necessary for our observations. With regard to Nox2, we find that manipulation of Nox4 expression does not affect Nox2 expression (Figure 2A), and therefore we conclude that the effect seen here is due specifically to Nox4 manipulation in the endothelium.
Collectively, the data presented here demonstrate that augmented endothelial Nox4 expression promotes angiogenesis and recovery from hypoxia through enhanced eNOS activation. Important questions remain such as the mechanism(s) whereby Nox4 is upregulated and the specific molecular targets between Nox4 and eNOS. Nevertheless, our findings support an adaptive role for Nox4 in response to tissue injury.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Reactive oxygen species (ROS) are involved in promoting many vascular disease states such as hypertension, diabetes mellitus, and atherosclerosis and consequently have been the subject of considerable investigation. Identification of ROS sources has led to the realization that “pathological” ROS are often produced by enzymes normally present in the vascular wall. These observations prompt the question: What is the physiological role of ROS producing enzymes in the vasculature? To address this, we investigated the role of NADPH oxidase 4 (Nox4), a ROS-producing enzyme located in multiple vascular cells. We found that tissue hypoxia resulted in increased Nox4 expression, suggesting a role for this enzyme in the response to injury. Indeed, we observed that increased levels of Nox4 proved important for endothelial cell migration and proliferation, 2 features needed for endothelial-mediated angiogenesis. In addition, mice with excess endothelial Nox4 demonstrated accelerated blood flow recovery from limb ischemia, consistent with enhanced angiogenesis. This effect was due to the ability of Nox4 to activate endothelial nitric oxide synthase, another enzyme known to be involved in angiogenesis and injury responses. Our study lends insight into the important roles of ROS in cardiovascular cell biology and suggests that Nox4 may be a potential therapeutic target for manipulating angiogenesis and tissue repair.
SUPPLEMENTAL MATERIAL

NADPH Oxidase 4 Promotes Endothelial Angiogenesis Through eNOS Activation

Siobhan M. Craig PhD; Kai Chen MD PhD; Yongmei Pei MS; Chunying Li PhD; Xiaoyun Huang MD; Christine Chen; Rei Shibata MD PhD; Kaori Sato MD PhD; Kenneth Walsh, PhD and John F. Keaney, Jr. MD
AORTA (-endothelium) LUNG

Nox4

18S

WT    TG    WT    TG

LUNG

A

Supplementary Figure 2

B

3 months 1 year

Lung Heart Lung Heart

Nox4

18S

C

3 MONTHS 1 YEAR

WT    TG    WT    TG

Nox4

Actin

Supplementary Figure 2
**Supplementary Figure 1: Manipulation of Nox4 expression.** Endothelial cells were cultured for 18h in hypoxic conditions and mRNA was harvested to examine Nox expression. Endothelial cells were cultured for 48h with Ad-Nox4 or Ad-Nox4i and Nox4 and Nox2 expression levels were assessed. Endothelial cultures were routinely tested for increased/decreased Nox4 expression (C) and H2O2 production (D) to ensure successful gene modulation. *p<0.05 vs. Ctl by one-way ANOVA with a post hoc Dunnet’s test (N = 3)

**Supplementary Figure 2: Tissue Nox4 expression.** (A)Aorta with the endothelium removed and lung tissue lysates were examined for Nox4 expression (mRNA). (B) Lung and heart tissue was isolated from WT and TG mice and assessed for Nox4 mRNA expression (C) Lung tissue from WT and TG mice was lysed and immunoblotted for Nox4 expression.