**Vascular Medicine**

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

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**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

Reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide (H₂O₂) have been linked to the onset and progression of cardiovascular disease, yet antioxidant strategies to reduce ROS levels have failed to limit cardiovascular events. Thus, ROS levels alone are not a determinant of pathology, suggesting that the role of ROS in disease processes is complex. One reason that ROS may have unpredictable consequences in the vasculature is that they also serve as second messengers in multiple cellular processes such as proliferation, differentiation, and apoptosis. In addition, ROS are important for cytoskeletal rearrangement and cell migration in response to angiogenesis-inducing growth factors, including vascular endothelial growth factor (VEGF) and angiopoietin-1. Thus, there is evidence that ROS are involved in vascular physiology.

**Clinical Perspective on p 740**

Tissue ischemia and the resultant hypoxia initiate a tightly regulated response that includes both cellular adaptation and efforts to improve oxygen delivery, such as angiogenesis. Evidence now indicates that the response to hypoxia is dependent, in part, on ROS production. For example, the upregulation of VEGF and VEGF receptors is linked to a concomitant increase in ROS production during hypoxia. Ligand engagement of the VEGF receptor results in ROS production, and exogenous application of ROS promotes endothelial responses that mimic angiogenesis such as increased cell proliferation, migration, and tube formation. The specificity of hypoxia-inducible factor-1α, the master regulator of hypoxia-induced genes, is derived from protein stabilization due to an ROS flux during hypoxia, which is absent in anoxia. Thus, ROS are clearly involved in signaling responses important for angiogenesis. However, the specific ROS sources that mediate these physiological events are not completely defined.

Many members of the NADPH oxidase (Nox) enzyme family are expressed in the vasculature and produce ROS in

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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 $H_2O_2$ 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-$\beta$ 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 same multiplicity of infection. For in vivo administration of adenovirus, 3 days before surgery, either LacZ or Nox4 adenoviral virus, 3 days before surgery, either LacZ or Nox4 adenoviral constructs (2×10⁷ 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: tacctccgaggatcacagaa; Nox4 reverse: ggaagctgggtccacagcaga. The resultant construct was confirmed by sequencing. A linearized DNA construct 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/Bl6 background (University of Massachusetts Transgenic Core Facility). The primers used for genotyping are as follows: 5’-ctctcggacggacgatac-3’ and 5’-gtatgacgcgaccggttc-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 (14-μm cryosection) were incubated with Roche probe overnight at 50°C and blocked with maleic acid buffer with Tween 20/2% blocking reagent (Roche)/20% sheep serum at room temperature for 2 to 3 hours; 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 μL/mL) overnight at 37°C, and images were obtained with a 40×1.30 objective (Nikon) on an inverted fluorescence (excitation 544 nm; emission 590 nm) microscope (TE-2000; Nikon) with a camera (Cool-SPAP 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 peri-vascular fat and adventitia with the use of a dissecting microscope. 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 (Fluor-888- or Fluor-594-tagged goat anti-rabbit or donkey anti-sheep IgG; Invitrogen) were diluted 1:200.
Fluorescence images were obtained with the use of a 20×1.30 objective on the inverted aforementioned microscope system.

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.27 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.28

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-mL vessel myograph (Danish Myo Technology) containing physiological salt solution (mmol/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 for 1 hour, followed by 2 consecutive contractions with physiological salt solution containing 60 mmol/L potassium and 1 μmol/L phenylephrine, then with physiological salt solution containing 60 mmol/L potassium alone. Rings were then washed, allowed to return to basal tension, and subjected to concentration-response curves to phenylephrine.

cGMP Measurement

To measure cGMP in vitro, we cultured human aortic ECs with and without adenoviral constructs and rat aortic smooth muscle cells to confluence in 6-well plates. Cells were then washed twice with Locke’s buffer (mmol/L: NaCl 154, KCl 5.6, CaCl2 2. MgCl2 1, HEPES 10; pH 7.4) and allowed to equilibrate in Locke’s buffer containing 200 μmol/L 3-isobutyl-1-methylxanthine (IBMX), 100 U/mL superoxide dismutase, and 200 μmol/L L-arginine for 30 minutes. The calcium ionophore (A23187; 10 μmol/L; Sigma) was then added to the stimulated wells of the human endothelial aortic ECs for 30 minutes. The endothelial bathing medium was transferred immediately to the rat aortic smooth muscle cells for 3 minutes. The reaction was stopped with the immediate removal of buffer and addition of ice-cold 5% trichloracetic acid. cGMP was measured with the use of commercial enzyme-linked immunosorbent assay kits 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 Nω-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 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.
Results

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 IB to ID in the online-only Data Supplement) under conditions that do not affect Nox2 expression (Figure IB 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 \(\mu\)mol/L) or polyethylene glycol–catalase (200 U/mL) and assessed for tube formation at 6 hours, \(P<0.05\) vs Nox4 by one-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.

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. Human 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 \(\mu\)mol/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.
that exhibited increased Nox4 mRNA (Figure 4B) and protein (Figure 4C). We then assayed ROS production in mouse lung ECs from 2 lines of transgenic animals versus control mice and observed a 20% to 30% increase in hydrogen peroxide production as assessed by Amplex Ultra Red reagent (Figure 4D). In aortic sections from wild-type and VE-Cad-Nox4 mice, we observed increased Nox4 mRNA (Figure 4E) and protein (Figure 4F) expression. We then removed the endothelium from the aorta (deendothelialization), which is a small percentage of the total aortic tissue, and compared this with lung (highly endothelialized) tissue from the same mice and demonstrated clear mRNA overexpression in the transgenic lung tissue, which was not seen in the deendothelialized aorta (Figure II A in the online-only Data Supplement). We also assessed lung tissue expression in both young (3 months) and old mice (1 year) and found sustained increased expression at both the mRNA and protein levels (Figure II B and II C in the online-only Data Supplement).

**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, and there is evidence that ROS may regulate eNOS activity. 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).

**Figure 3. NADPH oxidase 4 (Nox4) improves recovery from hindlimb ischemia in C57Bl/6 mice.** C57Bl/6 mice underwent hindlimb ischemia as in Methods. Three days before surgery, LacZ or Nox4 adenovirus (2 \times 10^6 plaque-forming units) was injected into 5 sites of the thigh adductor muscle. Mice were monitored for return of blood flow with laser Doppler imaging (A), and recovery was quantified as percent blood flow return in ischemic vs control leg (B; **P<0.05 vs LacZ by 2-way repeated-measures ANOVA; n=6 per group). C, At day 28, mice were euthanized, and gastrocnemius muscle was stained for CD31. D, Composite data of CD31-positive capillaries/muscle fiber is shown.

**Figure 4. Endothelial-specific NADPH oxidase 4 (Nox4) overexpression.** A, Construct for creation of endothelial-specific human Nox4 overexpression. VE-Cad indicates vascular endothelial cadherin; sv40pA, SV40 polyadenylation sequence. Mouse lung endothelial cells were harvested from wild-type (WT) and Nox4 transgenic (TG) mice and human Nox4 (hNox4) mRNA (B), and protein expression (C) was confirmed by real-time polymerase chain reaction and immunoblot, respectively. D, Mouse lung endothelial cells from 2 distinct transgenic founder lines were assessed for extracellular H2O2 production with the use of Amplex Red (**P<0.005; n=3). E, Segments of wild-type and Nox4 transgenic aortas were isolated, fixed, and assessed for Nox4 gene expression with the use of in situ hybridization (×40). E indicates the endothelium. F, Aortic segments from wild-type and transgenic mice were fixed and paraffin embedded and co-stained with von Willebrand factor (VWF), an endothelial marker, and Nox4 for immunofluorescence (×20).
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 probe the mechanisms of eNOS regulation by Nox4, we introduced catalase into our Nox4 overexpression system and observed an attenuation of both expression and phosphorylation of eNOS (Figure 6C), indicating that Nox4-derived ROS were responsible for eNOS activation. Under hypoxic conditions, adenoviral knockdown of Nox4 led to a decrease of both total and Ser-1177 phosphorylated eNOS (Figure 6D).

Consistent with the observation that Nox4 enhances eNOS activity, aorta from VE-Cad-Nox4 transgenic mice demonstrated increased basal NO production manifest as enhanced cGMP production (Figure 6E). This increase in basal eNOS activity appeared functional because intact aortic rings from VE-Cad-Nox4 mice exhibited less phenylephrine-induced contraction than vessels from littermate controls (Figure 6F). To further examine the role of basal NO bioactivity, we conducted functional aortic assays on VE-Cad-Nox4 transgenic mice bred onto the eNOS-null (eNOS−/−) background and found no effect of Nox4 in the absence of eNOS (Figure 6G).

Nox4 Stimulation of Angiogenesis Requires Endothelial Nitric Oxide Synthase

To test the hypothesis that eNOS activation is required for Nox4 stimulation of angiogenesis in vivo, we utilized the VE-Cad-Nox4 transgenic mice on the eNOS−/− background. In aorta from mice lacking eNOS, there was no difference in capillary sprouting as a function of the transgene (Figure 7A). Moreover, endothelial Nox4 overexpression no longer accelerated the recovery from hindlimb ischemia in mice lacking eNOS (Figure 7B and 7C). Collectively, these data demonstrate that Nox4 stimulation of angiogenesis requires eNOS (Figure 7D).

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 increased Nox4 expression promoted 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).
cancer cells limits angiogenesis.\textsuperscript{32} 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. \textbf{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; $n=3$. \textbf{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 $\mu$mol/L). *$P<0.05$ by Student t test; $n=3$. \textbf{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\textsuperscript{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 H$_2$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 NO are known to limit NO consumption, it is surprising that one particular ROS-producing enzyme promotes an NO-dependent process. However, evidence in the literature helps to reconcile these seemingly contradictory results because one particular ROS, H$_2$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 H$_2$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 H$_2$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 H$_2$O$_2$, and H$_2$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 H$_2$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.
NADPH Oxidase 4 Promotes Endothelial Angiogenesis Through Endothelial Nitric Oxide Synthase Activation

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SUPPLEMENTAL MATERIAL

NADPH Oxidase 4 Promotes Endothelial Angiogenesis Through eNOS Activation

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Supplementary Figure 1
**A**

Comparison of gene expression between wild type (WT) and transgenic (TG) mice in Aorta (endothelium) and Lung tissues.

**B**

Expression of Nox4 and 18S in Lung and Heart tissues at 3 months and 1 year.

**C**

Western blot analysis showing expression levels of Nox4 and Actin in WT and TG mice at 3 months and 1 year.

*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.
카인과 아벨: 혈관내피세포에 대해 Nox2와 Nox4는 상반된 영향을 미친다

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Summary

배경
Reactive oxygen species(ROS)는 혈관의 신호전달 기전의 중요한 부분으로 혈관 허혈상태에서 ROS의 생성이 증가되는 것으로 보고되었다. Nox4의 발현은 혈관내피세포에서 매우 높으므로 허혈상태에서의 Nox4의 역할을 검증하였다.

방법 및 결과
저산소 상태에서 Nox4의 발현이 증가하였으며, in vitro, in vivo Nox4의 과발현은 혈관내피세포의 증식, 이동 및 혈관생성을 유발하는 데 충분하였다. 본 연구진은 Nox4 과발현 마우스를 제작하였으며, 이를 이용한 결과 VECad-Nox4 마우스는 hindlimb ischemia에서의 회복이 빠르고, aortic capillary sprouting이 효과적이었다. Nitric oxide synthase(eNOS)는 허혈에서의 혈관생성 효과에 기여하므로 Nox4가 eNOS의 발현에 미치는 영향을 연구한 결과, Nox4 과발현 상태에서 eNOS 발현이 증가함을 관찰하였다. Nox4 과발현 마우스를 eNOS 결핍 마우스와 교배하면 이들 동물에서는 aortic sprouting이 관찰되지 않으며, hindlimb ischemia의 개선이 빠르지 않았다.

결론
Nox4의 발현 증가는 신혈관생성이 촉진되는 현상과 함께 허혈상태를 개선시킬 수 있음이 증명되었다.
NADPH(nicotinamide adenine dinucleotide phosphate) oxidase(Nox)는 세포내 ROS를 생성하는 중요한 시스템이다. 거의 모든 세포에서 발현하며, 그 기능을 수행하는 것으로 추정되는 이 시스템은 안정적인 생리적 상태에서 세포의 정상적인 활동을 유지하는 데 필수적인 세포신호를 생성하지만, 과도한 자극 또는 산화/염증 자극을 받을 때의 반응은 병적인 결과를 초래할 수도 있다. 이러한 Nox의 양면적인 성격은 혈관내피세포에서의 발현에 있어 서도 동일한 현상을 보인다.

혈관내피세포에서의 Nox

ROS의 생성은 apoptotic mitochondrial damage(cyt p450), SOD 등의 발현과 같이 세포손상 및 자극을 통하여도 생성된다. 그러나 Nox는 ROS만을 생성하는 특이적인 시스템이므로 정상적인 세포 기능을 평상시 담당하는 주된 역할을 한다. 현재까지 Nox 계열은 여러 가지의 isoform으로 발견되는데(Nox 1-5, Doux 1, 2) 혈관에 분포하는 Nox들은 다음과 같다.

• Nox 1: smooth muscle cell
• Nox 2: endothelial cell, adventitial fibroblast, macrophage
• Nox4: ubiquitous expression in adventitial fibroblast, smooth muscle cell, endothelial cell
• Nox5: endothelial cell, smooth muscle cell

혈관내피세포에는 Nox4의 발현도가 상대적으로 높다고 알려져 있다. Nox4는 일반적으로 발현하지만, platelet-derived growth factor(PDGF), tumor necrosis factor(TNF)-α, interleukin-1 등 혹은 shear stress에 의하여 발현도가 빠르게 상승할 수 있다. 또한, 세포 노화에 연관되어 있는 microRNA(miR)-146a에 의한 증가, 이와는 반대로 당뇨병 상태에서의 miR-25 발현 감소가 Nox4의 발현 억제를 유리시킨다는 보고도 있다.

혈관내피세포 기능과 Nox

Nox는 신혈관생성, 다양한 세포기전 등 그 역할이 매우 많지만, 혈관내피세포의 기능 이상을 매개할 수 있다. 과정은 복잡하나, Nox의 기능을 통하여 eNOS의 활성 감소가 이루어지고 NO의 결핍이 유발되기 때문에 혈관의 이완이 장애를 일으키는 것이 대표적인 현상 중 하나이다. 특히, 이러한 NO 결핍과 관련한 세포기전은 혈관내피세포의 adhesion molecule, chemoattractant(MCP-1 등)의 발현을 증가시키기 때문에, 죽상경화의 유발 증가로 나타난다. 이러한 기전은 주로 Nox2와 연관된 기전으로 연구가 이루어져 증명되었으며, Nox4 등의 기타 유형에 대한 증가는 미약하다. Nox4는 주로 혈관모델에서 연구가 이루어져 왔는데, Nox2와는 반대로 NO의 형성을 증가시키는 결과를 유도하여 혈압을 안정시키고, 아마도 죽상경화의 발생을 억제할 수 있을 것으로 믿어져 왔다.

Nox4와 혈관내피세포(신혈관생성)

본 논문에서는 Nox4의 발현증가로 인한 혈관내피세포의 증식 등으로 인해, 결과적으로 신혈관생성을 통한 혈혈상태의 개선을 촉진적으로 보여주었다. 주지하는 바와 같이, 혈관내피세포에서 Nox4의 발현이 상대적으로 높은 상태임에도 이의 발현을 더욱 증가시키면 더욱 효용이 높아지는 현상이 관찰된다. 논의 부분에서 언급되었듯이 Nox4의 발현 증가가 바로 Nox4의 기능 증가로 이어진다. Nox4 과발현 마우스에서 혈관 유발이전의 상태에서 이미 (마세)혈관의 밀도가 상승되어 있는 것은 아닐까 하는 점이 궁금하다. 한편으로는 주지하는 바와 같이 혈혈
자체가 혈관내피세포의 Nox4 발현도를 높이는 자극인데, 과발현 상태의 Nox4 발현도 및 기능 상승이 이러한 상태의 Nox4를 얼마나 상회하는지도 정확히 확인되어야 할 부분이다. Nox4의 특성이 밝혀지기 이전에는 단지 ROS의 양적인 부분에 의하여 세포의 기능이 정하여 지는 것으로 이해되고 있었다. Nox4의 발현이 증가하면 합수록 그 이득이 극대화되고, ROS의 과도한 생산에 의한 해악이 없는지에 대하여서는 Nox4에 의하여 H₂O₂가 생성되어 NO의 결핍이 유도되지 않는 특이한 현상으로 설명하고 있다.

Reference