The Cytosolic NADPH Oxidase Subunit NoxO1 Promotes an Endothelial Stalk Cell Phenotype

Ralf P. Brandes, Sabine Harenkamp, Christoph Schürmann, Ivana Josipovic, Beliza Rashid, Flavia Rezende, Oliver Löwe, Franziska Moll, Jeremy Epah, Jeanette Eresch, Arnab Nayak, Irakli Kopaliani, Cornelia Penski, Michel Mittelbronn, Norbert Weissmann, Katrin Schröder

Objective—Reactive oxygen species generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases contribute to angiogenesis and vascular repair. NADPH oxidase organizer 1 (NoxO1) is a cytosolic protein facilitating assembly of constitutively active NADPH oxidases. We speculate that NoxO1 also contributes to basal reactive oxygen species formation in the vascular system and thus modulates angiogenesis.

Approach and Results—A NoxO1 knockout mouse was generated, and angiogenesis was studied in cultured cells and in vivo. Angiogenesis of the developing retina and after femoral artery ligation was increased in NoxO1−/− when compared with wild-type animals. Spheroid outgrowth assays revealed greater angiogenic capacity of NoxO1−/− lung endothelial cells (LECs) and a more tip-cell–like phenotype than wild-type LECs. Usually signaling by the Notch pathway switches endothelial cells from a tip into a stalk cell phenotype. NoxO1−/− LECs exhibited attenuated Notch signaling as a consequence of an attenuated release of the Notch intracellular domain on ligand stimulation. This release is mediated by proteolytic cleavage involving the α-secretase ADAM17. For maximal activity, ADAM17 has to be oxidized, and overexpression of NoxO1 promoted this mode of activation. Moreover, the activity of ADAM17 was reduced in NoxO1−/− LECs when compared with wild-type LECs.

Conclusions—NoxO1 stimulates α-secretase activity probably through reactive oxygen species–mediated oxidation. Deletion of NoxO1 attenuates Notch signaling and thereby promotes a tip-cell phenotype that results in increased angiogenesis. (Arterioscler Thromb Vasc Biol. 2016;36:1558-1565. DOI: 10.1161/ATVBHA.116.307132.)

Key Words: ADAM17 protein ■ angiogenesis effect ■ dibenzazepine ■ Notch ■ NoxO1 protein, mouse ■ reactive oxygen species

Angiogenesis, ie, the formation of new blood vessels by endothelial sprouting into a nonvascularized tissue, is a complex process. It involves specific endothelial migration and proliferation patterns and complex environment cues, such as hypoxia or gradients in growth factors like vascular endothelial growth factor (VEGF) that direct the cells. Another factor influencing angiogenesis and endothelial cell function are reactive oxygen species (ROS). ROS can both enhance and impair angiogenesis depending on their source, the amount of ROS, and the specific species formed. ROS can also alter the activity of matrix metalloproteases, such as MMP2 and MMP9. These molecules are essentially involved in matrix remodeling and therefore play an important role in angiogenesis.

The best studied source of controlled cellular ROS formation in the vasculature is the family of Nox NADPH oxidases. It compromises 7 members; of those, Nox5, DUOX1, and DUOX2 are Ca2+-dependent, and Nox4 is constitutively active. In contrast, Nox1, Nox2, and Nox3 have to be activated in a process depending on cytosolic subunits. In the case of Nox2, those cytosolic subunits are Rac1 or Rac2 and p67phox tethered to Nox2 by p47phox. The assembly process is initiated by serine phosphorylation of the adapter protein p47phox that subsequently unfolds and only then interacts with p67phox and Nox2.6

For each of the classic cytosolic proteins, homologues have been cloned: NADPH oxidase organizer 1 (NoxO1) is homologous to p47phox, and NoxA1 (NADPH oxidase activator) is an isoform of p67phox. Being a p47phox homologue, NoxO1 facilitates the assembly of the complex between the NADPH oxidase activator and the catalytic Nox subunit, in this case, mainly Nox1. Different from p47phox, however, NoxO1 lacks the autoinhibitory region, and the complex of Nox1, NoxO1, and NoxA1 is therefore constitutively active.

Nevertheless, phosphorylation events can modulate this activity by ≥50%.7,8
Not much has been reported on these cytosolic homologues in the vascular system, and data in knockout mice are totally lacking. In fact, to our knowledge, a NoxA1 knockout mouse has not yet been generated. NoxA1 is expressed in smooth muscle cells and substitutes for p67phox. For NoxO1, a role of streptozotocin diabetes mellitus–induced eNOS uncoupling has been suggested on the basis of in vivo siRNA experiments. Moreover, NoxO1 is induced by oscillatory flow and contributes to superoxide anion (O$_2^-$) formation that limits NO availability. In the inner ear, NoxO1 may interact with Nox3, which is involved in the proper formation of otoconia. Mice expressing a dysfunctional mutant of NoxO1 indeed present with a similar phenotype as Nox3$^{-/-}$ mice: both have major balance problems and a head-tilt phenotype. In the vasculature, NoxO1 is primarily thought to activate Nox1, and thus most of its functions should be analogous to those of Nox1. Nox1 is a mediator of angiotensin II–induced hypertension and endothelial dysfunction. Genetic deletion of Nox1 promotes angiogenesis in xenograft tumor models and after femoral artery ligation. Nox1 thus has opposing functions to Nox4, which rather promotes angiogenesis. The basis of this difference is probably the fact that Nox4 produces H$_2$O$_2$, whereas Nox1 primarily generates O$_2^-$, which scavenges NO and results in peroxynitrite formation.

Based on these considerations, we speculate that NoxO1 limits angiogenesis and studied this and its underlying mechanisms in a newly generated NoxO1 knockout mouse and in cultured endothelial cells.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

**NoxO1 Is Expressed in the Vascular System**

To determine the contribution of NoxO1 to vascular function, a global knockout mouse was generated by deletion of exons 2 to 9 through homologous recombination in C57/BL6 stem cells according to the strategy outlined in Figure 1A by Artemis. Integration of the neomycin cassette and germ-line transmission was confirmed by Southern blot, and global knockout mice were generated by crossing with Cre-deleters. NoxO1$^{-/-}$ mice were viable and showed the expected head-tilt phenotype as consequence of the absence of otoliths (data not shown). qRT-PCR from the carotid artery of wild-type (WT) animals demonstrated NoxO1 mRNA expression in the endothelium and in the smooth muscle layer, whereas NoxO1 mRNA from the region exon 2 to 9 was not detected in vessels of knockout mice (Figure I in the online-only Data Supplement). To determine whether mRNA expression in WT animals was sufficient to result in protein formation, a NoxO1 antibody was raised in rabbits that yielded only a low background signal after affinity purification (Figure 1B; Figure IB in the online-only Data Supplement). In the skeletal muscle of WT animals, the antibody gave basically no staining of the skeletal fibers; the capillary compartment, comprising endothelium and pericytes, in contrast, stained NoxO1 positive. Importantly, this staining was not observed in tissue from NoxO1$^{-/-}$ mice (Figure 1B).

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NoxO1 Contributes to Endothelial Superoxide Formation

To determine a potential contribution of NoxO1 to basal ROS formation, endothelial cells (LEC) from WT and NoxO1 knockout mice were isolated from the lung. The luminol/horse radish peroxidase signal of intact cells, which largely reflects O$_2^-$ generation, was identical between the 2 lines, whereas lucigenin chemiluminescence of LECs demonstrated an $\approx$50% lower O$_2^-$ generation of the NoxO1−/− cells. To determine whether this difference in O$_2^-$ levels is functionally relevant, the Click-iT Lipid Peroxidation Imaging Kit (ThermoFisher) was used. This assay indeed revealed a slightly, yet significantly, lower rate of lipid peroxidation in LECs from NoxO1−/− mice when compared with WT LEC (Figure 1C). Collectively, the findings suggest that NoxO1 is expressed in the murine vascular endothelium and contributes to basal O$_2^-$ formation.

NoxO1 Limits the Angiogenic Function of Murine Lung Endothelial Cells

To characterize the functional importance of NoxO1 in the vascular system, we focused on angiogenesis in cultured cells and in vivo. Proliferation of LEC from WT and NoxO1−/− cells in response to serum was similar (Figure 2A), and there was also no difference in the behavior of NoxO1−/− and WT LEC in the matrigel tube formation assay (Figure II in the online-only Data Supplement). As the latter assay, however, only reflects aspects of cell–cell interaction in a disintegrating network but not a true angiogenic response, endothelial sheet migration in the scratch wound assay was studied. Basal and VEGF-stimulated migration of NoxO1−/− LECs was higher than that of WT LECs (Figure 2B). Moreover, in the spheroid outgrowth assay, NoxO1−/− LECs produced more sprouts and longer tubes in response to VEGF than WT LECs (Figure 2C). Thus, NoxO1-deficient LECs have a greater angiogenic function than WT LECs. This effect probably is a consequence of a reduced formation of O$_2^-$ as treatment of human umbilical vein endothelial cells with the redox cycler menadione prevented sprouting (Figure III in the online-only Data Supplement).

NoxO1 Deficiency Accelerates Angiogenesis

Given that NoxO1 affects migration and spheroid outgrowth, it could be inferred that also retina angiogenesis in vivo is limited by this NADPH oxidase subunit. Indeed, retina vascularization was quicker in NoxO1−/− pups than in WT pups (Figure 3A). Interestingly, retinas of NoxO1−/− pups also exhibited a higher density of the vascular plexus with a higher number of branches than their WT littermates (Figure 3B). This combination may suggest a more tip-cell–like behavior of NoxO1−/− than that of WT cells.

To determine whether NoxO1 also limits pathophysiolog-ical angiogenesis in mice, the femoral artery ligation model was performed. Laser Doppler flow imaging revealed that restoration of flow was indeed faster in NoxO1−/− mice when compared with their WT littermates (Figure 3C). This was most likely a consequence of a greater degree of angiogenesis: NoxO1−/− mice had a slightly higher endothelial cell:muscle fiber ratio at baseline (Figure 3D), and this difference was much more pronounced after femoral artery ligation. NoxO1 mRNA expression was lower in the operated than in the control leg of WT animals (Figure 3E). The angiogenic phenotype was paralleled by corresponding differences in VEGF mRNA expression: VEGF mRNA expression in muscles of the control leg of NoxO1−/− mice was higher than that of WT littermates 2 weeks after the operation. In WT animals, VEGF mRNA expression in muscles of the operated leg was still significantly higher than from the control leg. In NoxO1−/− animals, in contrast, no difference in VEGF mRNA expression was detected at this late time point (Figure 3F).

Interestingly, NoxO1 also had an impact on mononuclear angiogenic cells, which contribute to revascularization. Two weeks after femoral artery ligation, the number of bone marrow resident mononuclear cells was reduced in NoxO1−/− mice, whereas the number of angiogenic myeloid cells and circulating mononuclear cells was increased (Figure IV in the online-only Data Supplement). Such a difference could be a reflection of a difference in the release of chemokines and mobilizing factors from the ischemic leg. Thus, NoxO1 deficiency results in increased angiogenesis, and this could be a consequence of enhanced VEGF expression and mobilization of angiogenic cells from the bone marrow.

NoxO1 Deficiency Results in Attenuated Notch Signaling

In order to determine the mechanistic basis of the increased angiogenesis after NoxO1 deletion, experiments in cultured
LECs were performed. Given that NoxO1 deficiency increased migration of endothelial cells in culture and in vivo without affecting proliferation, we hypothesized that NoxO1 deficiency may promote an endothelial tip-cell phenotype. Indeed, VEGF receptor 2 mRNA expression was elevated in NoxO1−/− cells. This effect was a consequence of the deletion of NoxO1 as overexpression of NoxO1 in NoxO1−/− LECs rescued the phenotype (Figure 4A). Other markers of tip cells, such as EGF receptor or MMP2 and 9, had increased levels of mRNA and activity (Figure V A–VC in the online-only Data Supplement). Importantly, the expression of angiopoietin 1, which in general is important for developmental angiogenesis, was unchanged, as was the expression of angiopoietin 2, which is required for angiogenic sprouting and vascular regression (Figure VD in the online-only Data Supplement).

On such a basis, we focused on the Notch signaling system, which mediates tip-stalk cell specification. The protein abundance of the tip-cell marker and Notch ligand Delta-like 4 (DLL4) was higher in NoxO1−/− cells than in WT cells (Figure 4B). Accordingly, expression of the Notch target gene, Hey, was lower in LEC of NoxO1−/− mice than in WT LEC (Figure 4C). Importantly, overexpression of NoxO1 in NoxO1−/− LEC increased Hey expression over the expression level of WT cells, suggesting that NoxO1 promotes Notch signaling (Figure 4C).

On stimulation with its agonist DLL4, the Notch receptor is cleaved by an α- and γ-secretase, and the free Notch intracellular domain (NICD), the cleaved part of the receptor, mediates further downstream signaling. Interestingly, when WT LECs were stimulated with DLL4, the abundance of...
of NICD increased, whereas this was not the case in NoxO1−/− LECs (Figure 4D). As Notch1 mRNA expression was rather higher than lower in NoxO1−/− cells (data not shown), we speculate that potentially impairment of Notch cleavage is responsible for the attenuated Notch signaling in NoxO1−/− LECs.

NoxO1 Knockout Promotes an Endothelial Tip-Cell Phenotype

To dissect the presentation of Notch ligand from Notch signaling in this system, we generated mixed spheroids of LECs from WT and NoxO1−/− mice stained with cell tracker green or red, respectively. Subsequently, the number of WT and NoxO1−/− cells at the tip position was counted (Figure 4E). Under native conditions, NoxO1−/− contributed >80% of the tip cells. Importantly, in the presence of the γ-secretase inhibitor dibenzazepine, the number of WT tip cells increased, whereas the number of NoxO1−/− tip cells remained constant, so that the distribution was similar between the 2 genotypes (Figure 4F). To validate this effect, we utilized cells from the naturally occurring NoxO1 mutant mouse.14 Because of the genetic background of this strain, these animals were not suited for in vivo experiments, as the WT controls had a poor angiogenic response, which is also illustrated by the failure of the WT cells to sprout from spheroids under native conditions (Figure 4G). Dibenzazepine treatment, however, increased sprouting of WT cells. Similar as in the C57/BL6-strain, sprouting of NoxO1-mutant cells was much better, and dibenzazepine failed to affect this process (Figure 4G). Given that NoxO1 knockout and mutant cells were unresponsive to dibenzazepine, it seems that the tip-cell specification observed after loss of NoxO1 is a consequence of a failure to cleave the Notch receptor and thus to release NICD with no effect on the γ-secretase itself.

NoxO1 Maintains α-Secretase (ADAM10/17) Activity

To identify the possible mechanisms leading to attenuated Notch receptor cleavage, we focused on the sheddases. mRNA expression of the γ-secretase coactivators presenelin 1 and 2, as well as mRNA and protein of the α-secretase ADAM17/TACE, was similar between WT and NoxO1−/− LECs (Figure 5A and 5B). Given that ADAM17/TACE also cleaves pro-TNFα to the active cytokine, we measured this active TNFα in the murine plasma after femoral artery ligation. Importantly, NoxO1−/− mice had significantly lower TNFα plasma level than WT animals (Figure 5C), pointing to a potential defect in ADAM17/TACE activity after deletion of NoxO1. Myers et al22 found that ROS facilitate activation of ADAM17/TACE. In pilot experiments in our hands, H₂O₂ increased, whereas unspecific inhibition of all flavor enzymes, including NADPH oxidases by diphenylene iodonium (10 μmol/L), decreased ADAM17/TACE oxidation (Figure VI in the online-only Data Supplement). Given the lower O₂ level in LECs of NoxO1−/− mice, the impact of NoxO1 overexpression on ADAM17/TACE oxidation was analyzed in human umbilical endothelial cells. NoxO1 overexpression had no effect on ADAM17/TACE expression but increased not only the formation of O₂ but also the activity of ADAM17/TACE (Figure VII in the online-only Data Supplement). Importantly, NoxO1 overexpressed tripled ADAM17 oxidation as observed by redox-BIAM labeling followed by ADAM17/TACE IP and streptavidin detection (Figure 5D). The remaining fraction of the NoxO1...
overexpressing cells after immunoprecipitation contained less ADAM17/TACE when compared with GFP overexpressing cells suggesting a functional important amount of oxidation of the protein. These data demonstrate that NoxO1 overexpression promotes ADAM17/TACE oxidation.

To determine whether NoxO1 also affects the activity of the α-secretase, cleavage of a fluorescence-tagged substrate was measured in LECs (Figure 5E). The absence of NoxO1 resulted in an ≈25% reduction of ADAM17/TACE activity (Figure 5E). To determine whether this response is specific for NoxO1, LECs from p47phox−/− and NoxO1-p47phox double knockout mice were also included in the assay. Whereas activity in the double knockout mice was similar to that of NoxO1 LECs, deletion of p47phox failed to reduce ADAM17/TACE activity. Thus, a basal ROS formation facilitated by NoxO1-dependent activation of a Nox enzyme results in ADAM17/TACE oxidation and thus activation of this sheddase. Lack of NoxO1 results in attenuated ADAM17/TACE oxidation and less activation and thus attenuated Notch signaling. The consequence is a more tip-cell–like endothelial phenotype and enhanced angiogenesis.

**Discussion**

This study provides evidence that the cytosolic NADPH oxidase subunit NoxO1 limits both developmental and reparative angiogenesis. As a potential mechanism, we identified that NoxO1 is required to maintain α-secretase activity that is required for Notch signaling. Impaired Notch activity promotes endothelial migration and angiogenesis.

In the course of angiogenesis, tip cells pave a way for the following endothelial cells in the direction of a chemoattractant cue like a gradient of VEGF.23 Tip cells produce factors, such as the Notch ligand DLL4, that promote the specification of the following cells into stalk cells.24 Binding of DLL4 to the extracellular domain of the Notch receptor triggers a series of proteolytic cleavages of Notch. The first cleavage step is mediated by an α-secretase, a member of the disintegrin and metalloproteases (ADAM) family within the juxtamembrane region,25 followed by a cleavage by a proteolytical active γ-secretase within the transmembrane domain.26 As a consequence, the NICD is released and translocates to the nucleus where it activates the transcription factor CSL (CBF1/RBP-Jk/Suppressor of Hairless/LAG-1) by displacing a corepressor.27

In NoxO1-deficient cells, DLL4 expression was increased, whereas NICD release on DLL4 stimulation was reduced, indicating a defect in Notch receptor function. Accordingly, expression of Notch target genes, such as the basic helix-loop-helix proteins Hairy/Enhancer of Split (Hes)–related protein Hey, was reduced. Proteins encoded by the Hes and Hey genes are, in turn, transcriptional repressors of both their own expression and further downstream genes.24 Among others, activation of Notch results in the downregulation of VEGFR2,23 which then may act as an off switch for sprouting angiogenesis. Here, we found an enhanced expression of

![Figure 5. NoxO1 enhances α-secretase ADAM17/TACE activity. A, mRNA expression of the genes indicated, and (B) representative images and statistics (below the blot) of Western blots for ADAM17 of wild-type (WT) and NoxO1−/− lung endothelial cells (LECs). C, TNFα plasma levels of WT and NoxO1−/− mice (KO) 14 d after femoral artery ligation. D, Representative images and statistics of a redox immunoprecipitation of ADAM17 in human umbilical vein endothelial cells overexpressing GFP as a control or NoxO1. Reduced thiols were saturated with NEM, and oxidized thiols were labeled with biotinylated iodoacetamide followed by streptavidin immunoprecipitation. Numbers indicate abundance of ADAM17 in the initial loading (preimmunoprecipitation [pre-IP]), the IP, and the remaining fraction (post-IP) relative to GFP and β-actin, if applicable. E, ADAM10/17 activity as measured by conversion of an artificial fluorogenic substrate by WT, NoxO1−/−, p47phox−/−, and NoxO1 and p47phox double KO LECs within 16 h. n=4 to 9; *P<0.05 WT vs KO.](http://atvb.ahajournals.org/)

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VEGFR2 indicating a defect in Notch signaling that promotes a tip-cell–like phenotype.

NoxO1 was initially reported as a cytosolic subunit of the Nox1 complex, but it is now known that NoxO1 is also involved in the activation of Nox3. Mutation of Nox3 results in a defect in matrix metalloproteinase (MMP) activity. An enhanced mobilization of endothelial precursors from bone marrow and increased transplantation of vascular niches facilitates the formation of new blood vessels.

In conclusion, with this work, we provide evidence that the constitutive formation of O2•− in the absence of NoxO1, angiogenesis is enhanced, indicating that NoxO1-mediated ROS formation limits angiogenesis. The latter is facilitated by the ability of NoxO1-dependent ROS formation to maintain α-secretase activity in endothelial cells, rather than to stall cells, which then promotes sprouting angiogenesis.

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Disclosures
None.

References
Nicotinamide adenine dinucleotide phosphate oxidase organizer 1 is expressed in vascular cells and facilitates basal superoxide anion formation.

Genetic deletion of nicotinamide adenine dinucleotide phosphate oxidase organizer 1 increases angiogenesis in vitro and in vivo.

Nicotinamide adenine dinucleotide phosphate oxidase organizer 1 is required for the oxidation of ADAM17/TNFα-converting enzyme.

This oxidation results in ADAM17/tumor necrosis factor (TNF)-α-converting enzyme activation and facilitates Notch signaling.
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ROS

Nox1/2

NoxA1

NoxO1

Rac

p22

in

out

proteolytical cleavage and release of NICD

DLL4

NTCH

ADAM17

y-Secretase

NICD

Trancription

i.e. Hes

Stalk cell phenotype of endothelial cells
Supplemental Figure I A: Expression of NoxO1 mRNA (exon 2-7) and eNOS mRNA in carotid arteries from WT and NoxO1-/- mice as determined by qRT-PCR. The endothelial fraction was analyzed separately from the remaining carotid artery tissue. n.d.: not detected. n=3.

Supplemental Figure I B: Validation of the rabbit anti-NoxO1 antibody. HEK cells were left untransfected (control) or were transfected with plasmids coding for murine NoxO1 (mNoxO1) or human NoxO1 (hNoxO1). Triton soluble (TL) and insoluble extras (TI) as well as cells directly lysed in Laemmli buffer (LB) were subjected to Western blot.

Supplemental Figure II: Representative microscopic images and statistical analysis of matrigel tube formation assays of WT and NoxO1-/- LECs stimulated with (VEGF) or without (CTL) VEGF (50ng, 8hours). n=7-9, *p<0.05 CTL vs. VEGF.
Supplemental figure III:
Effect of Menadione (30µmol/L) on spheroid sprouting of HUVECs in the presence of VEGF to induce sprouting and of catalase (100U/ml) to prevent positive angiogenic effects by H₂O₂. n=15; *p<0.05 treatment vs. control
Supplemental figure IV:
(A) Number of mononuclear cells in the bone marrow and (B) FACS analyses of CD45/Flk-1 positive circulating angiogenic myeloid cells and lineage negative/Sca-1/flk-1/c-kit positive angiogenic cells the peripheral blood of wildtype (WT) and NoxO1-/- mice 14 days after femoral artery ligation. n=7-9; *p<0.05 WT vs. NoxO1-/-
Supplemental figure V A: qRT-PCR for the mRNAs indicated in LECs of wildtype (WT) and NoxO1-/- (KO) mice. *p<0.05 WT vs. KO n>3.

Supplemental figure V B&C: Representative image and statistics of gelatinase assays for MMP2 and 9 expression (B) and activity (C) in the culture medium of wildtype (WT) and NoxO1-/- (KO) lung endothelial cells with or w/o 50ng/ml VEGF as indicated. *p<0.05 WT vs. KO n>3.
Supplemental figure VI:
Redox immunoprecipitation of ADAM17 in HUVECs treated with or without H$_2$O$_2$ and DPI. Reduced thiols were alkylated with NEM and oxidized thiols were labeled with biotinylated iodoacetamide after DTT-mediated reduction. Subsequently, streptavidin immunoprecipitation was carried out. Initial loading (pre-IP), the immunoprecipitation (IP).
Supplemental figure VII:
Relative $O_2^-$ production as measured by LO12 chemiluminescence and ADAM17/TACE activity as measured by conversion of an artificial substrate in not transfected HUVECs (Ctl) or those transfected with green fluorescent protein (GFP) or NoxO1. n=7; *p<0.05
The cytosolic NADPH oxidase subunit NoxO1 promotes an endothelial stalk cell phenotype.

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Material and methods

Study Design and Animal Procedures

NoxO1-/- knockout mice were generated by Tarconic/Artemisin in C57/BL6 embryonic stem cells as outlined in Figure 1A. p47phox-/- were kindly provided by Ajay M. Shah, Kings college London, and NoxO1 mutant mice were obtained from Charles River Laboratories, Sulzfeld, Germany. NoxO1/p47phox double knockout mice were generated by crossbreeding the individual knockout strains. All mice were kept at the local facility. Experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the local government.

Hindlimb ischemia model

Neovascularization capacity was investigated in a murine model of hind limb ischemia using 6 to 8 week old mice. The deep femoral artery was ligated with an electric coagulator (ERBOTOM ICC50, ERBE) and subsequently the superficial femoral artery and vein as well as the epigastric arteries were completely excised after electrocoagulation. The overlying skin was closed with 3 surgical staples. Relative blood flow was determined by laser Doppler imaging (Laser Doppler Perfusion Imager System, Wilmington, Germany) at 7 and 14 days post-ligation. During the scan, mice were lying with their back on a heating pad with their legs
stretched and fixed. To determine capillary density, cross-sections of adductor and semimembranous muscles embedded in Tissue Tek (Sakura, Heppenheim, Germany) were stained. After fixation in phosphate buffer (100mmol/L, pH 7.3) containing 4% formalin the tissue was blocked with 3% BSA and permeabilized with 0.5% Triton X-100 followed by incubation with anti-CD31 (BD Pharmingen), anti-Smooth muscle alpha-actin (Sigma) and anti-laminin (Abcam, Cambridge, UK) and imaged by confocal microscopy.

**Retinal angiogenesis**

Eyes from postnatal day 2, 5 and 7 mice were fixed for 2 hours in 4% paraformaldehyde and subsequently permeabilized in 3% BSA and 0.5% Triton X-100. The vasculature of the retina was then stained with fluorescein isothiocyanate Griffonia (Bandeiraea) simplicifolia BS-I lectin (1:100) in 1% Triton X-100 (both Sigma-Aldrich) in 0.1 M PBS overnight, washed and mounted with mounting media (DakoCytomation). Images were taken with the aid of a digital microscope (Carl Zeiss). Image J software (National Institutes of Health) together with appropriate plugins and makros was used to analyze vessel regrowth and neo-angiogenesis.

**FACS Analyses of mononuclear cells**

Circulating mononuclear cells were prepared from blood obtained by cardiac puncture in heparinized syringes. The plasma was separated by centrifugation at 800g for 10min. The remaining blood fraction was diluted with 1 mL PBS containing 0.5% BSA and 2 mmol/L EDTA and was overlaid on top of 3 mL LSM 1077 lymphocyte separation medium (PAA Laboratories, Pasching, Austria) and subjected to density gradient centrifugation (800g, 10min). Mononuclear cells were counted, and lineage negative cells were enriched using a lineage cell depletion kit (Miltenyi, Bergisch- Gladbach) according to the manufacturer’s instruction. All antibodies were from Beckton-Dickinson and used in a dilution of 1:100 in the PBS buffer. After washing, FACS-analysis was performed with the aid of a FACS-Calibur (Beckton-Dickinson).

**Determination of TNFα plasma-level**

TNFα plasma levels were determined with the aid of a CBA-kit (R&D) following the manufacturer’s instructions.

**Cell culture**

Lung endothelial cells were isolated from freshly prepared murine lungs as described before. In brief, the tissue was minced and dispase digested at 37°C. After several washing
steps, LECs were separated magnetically using CD144 coated Dynabeads (MACS). LECs were used between passages 5-9. To analyze proliferation cells were seeded in a density of 1x10^4 per cm^2 trypsinized and subsequently counted by an automated cell counter (casy, Schärfe) after 1 day of culture.

**Cell Migration**

Scratch wound-healing assays were performed in 24-well plates. Cells were cultured in endothelial basal medium (EBM) containing FCS (1%). The monolayer was wounded with the aid of a sterile pipette tip and endothelial cell migration was monitored by taking images directly after the scratch and after cells were allowed to migrate for 8 hours. The distance migrated was calculated using ImageJ software.

**Tube formation**

For the matrigel assay, LEC were seeded onto matrigel (1.5x10^4 cells/cm^2) in EBM medium with or without H_2O_2 or PEG-catalase in the concentrations indicated. Tube formation was assessed after 8 hours and quantified by calculating the relative tube length.

**Spheroid sprouting assay**

Spheroids were generated as described for human umbelical vein endothelial cells in^3. Images were acquired with an Axiovert135 microscope (Zeiss). For quantification of the cumulative sprout number, ten spheroids per condition were analyzed with the help of the AxioVision software (Zeiss). In some experiments, cell were pre-stained with cell tracker green (WT) or red (NoxO1-/-)

**Real-Time PCR**

Total RNA from the muscle tissue was extracted with TRizol (Qiagen) or from cells with the Bio&Cell Kit according to the manufacturer's instructions. From 1 μg of RNA cDNA synthesis was carried out with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers; semiquantitative real-time PCR was performed with Fast Plus EvaGreen Master Mix for qPCR w/Low ROX (2x, 100 rxn) (Biotium, Hayward, CA, USA) in a Mx3005 cycler (Stratagene) with the indicated primers. Relative expressions of target genes were normalized using B2M as housekeeping gene, analyzed by the delta-delta-CT method and given as ratio compared to control experiments. The following primers were used:

<table>
<thead>
<tr>
<th>Gene</th>
<th>forward 3'-5'</th>
<th>reverse 3'-5'</th>
</tr>
</thead>
<tbody>
<tr>
<td>h,m,rEEF2</td>
<td>GACATCACCAAGGGTGTGCAG</td>
<td>GCGGTCACACACTGGCATA</td>
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<tr>
<td>mBeta-actin</td>
<td>TGACAGGATGCAGAAGGAGA</td>
<td>GCTGGAGGTGGACAGTGAG</td>
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<tr>
<td>mADAM17</td>
<td>AGAAAGCGAGTACAGCGTGA</td>
<td>GTATGCACCTCAGATGCTCT</td>
</tr>
<tr>
<td>mAngiopoietin1</td>
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<td>GCCACAGGCACTGAACCACC</td>
</tr>
<tr>
<td>mAngiopoietin2</td>
<td>GGGAGGCAACGAGGCGATT</td>
<td>CGCGGTCCCCGTGAGTCTG</td>
</tr>
<tr>
<td>mEGFR</td>
<td>TAAAGACCATCCAGGAGG</td>
<td>TGG</td>
</tr>
<tr>
<td>mHey1</td>
<td>GCC AGC ATG AAG CGA GCT C</td>
<td>GGGTCAGGACATCTGATGCTC</td>
</tr>
<tr>
<td>mMMP2</td>
<td>GCCAACATACAGAGGACTATG</td>
<td>CAGGCCTTGTTTCTCACAG</td>
</tr>
<tr>
<td>mMMP9</td>
<td>AGACCAAGGGTGACAGCTGTTC</td>
<td>GCCACGCTGGAATGATCTAAG</td>
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<tr>
<td>mNOS3</td>
<td>CTCACCATAAGCTGTGGCTTAC</td>
<td>GATGCAGGCAAGTGGATCAGG</td>
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<td>mNotch1</td>
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<td>CAGGCCTTGTTTCTCACAG</td>
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<tr>
<td>mNoxO1</td>
<td>ACTTAAACGCCTGTGCCATC</td>
<td>CCCAAACACTGCCCTAAAGTA</td>
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<tr>
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<td>CTTGAGCACTGCTGGAACCT</td>
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<td>CTTCCATCTCTGATCAGG</td>
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<tr>
<td>mVEGFR2</td>
<td>TGGGAGCAGGAGATGCAAAAGAC</td>
<td>CTTGAGGACATCTGCTCATCT</td>
</tr>
</tbody>
</table>

**Immunoblotting**

Western blot analyses were performed with an infrared-based detection system (Odyssey, Licor, Bad Homburg, Germany). Dll4 primary antibodies were from abcam, Hes1 from Santa Cruz and all others were purchased from Cell signaling and infrared-fluorescent-dye-conjugated secondary antibodies were obtained from Licor (Bad Homburg). The following lysis buffer was used (pH 7.4, concentrations in mmol/L): Tris-HCl (50), NaCl (150), sodium pyrophosphate (10), sodium fluoride (20), nonidet P40 (1%), sodium desoxycholate (0.5%), proteinase inhibitor mix, phenylmethylsulfonyl fluoride (1), orthovanadate (2), okadaic acid (0.00001).

**Biotin labeling assay of oxidized thiols followed by streptavidine immunoprecipitation**

Cells were incubated with 50mM NEM, washed and the reaction was stopped by adding 20% TCA into the dish. After 15 min on ice cells were scraped of, centrifuged and the pellet was resuspended in the following buffer: (pH 8.5, concentrations in mmol/L): Tris-HCl (50), EDTA (5), Urea (8000), SDS (20%). For reduction and labeling of oxidized thiols DTT (50 mM) and BIAM (10mg/ml) were added to the buffer. Labeled proteins were precipitated with acetone in order to remove unbound BIAM. Proteins then were resuspended and further processed directly for immunoblotting or immunoprecipitation with streptavidin coupled Dynabeads (Merk, Darmstadt, Germany). Eventually the initial loading (pre-IP), the immunoprecipitation (IP) and the remaining fraction (post-IP) were subjected to an immunoblot and ADAM17 abundance was detected.

**Gelatinase assay**
Standard gelatinase assay to determine the proteolytic activity was performed from conditioned medium with gelatin (0.1%) as substrate. 50,000 cells were seeded in a 12-well plate (Greiner Bio-One, Frickenhausen, Germany) in Dulbecco's MEM containing 2% FCS. Cells cultured in Dulbecco's MEM containing 0.1% BSA served as controls. Cells were then scratched as described in the wound-healing assay, and supernatant was collected 12 h after placing the scratch. Supernatant was then diluted with sample buffer (5% SDS, 20% glycerol in 0.4 mol/l Tris, pH 6.8 containing 0.02% bromophenol blue). Samples were electrophoresed through an 10% polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, gels were washed twice in 2.5% Triton X-100 for 15 min and subsequently incubated for 24 h at 37C in 0.2 mol/l NaCl, 5 mmol/l CaCl2, 0.02% Brij 35, and 50 mmol/l Tris, pH 7.5. The gel was then stained with Coomassie blue G in 30% methanol and 10% acetic acid for 10 min and then destained for 30 min in 10% acetic acid.

**ROS measurements**

ROS production was assessed by chemiluminescence with luminol (100 μmol/L)/horseradish peroxidase (HRP at 1U/ml), or lucigenin (5 μmol/L) in a Berthold six-channel luminometer (LB9505; Berthold). All measurements were performed in the HEPES-Tyrode (HT) buffer containing in mmol/L the following: 137 NaCl, 2.7 KCl, 0.5 MgCl2, 1.8 CaCl2, 5 glucose, 0.36 NaH2PO4, and 10 HEPES.

**Lipid peroxidation rate**

For analysis of lipid peroxidation rate was performed with the aid of the Click-iT® Lipid Peroxidation Imaging Kit (ThermoFischer, Darmstadt, Germany) following the manufacturers instruction. Briefly cells were plated on a 8 well μ-slide (ibidi, Martinsried, Germany) and fed with 50 μM Click-it® LAA in complete growth medium at 37 °C for 30 minutes followed by fixation with 4% formaldehyde for 15 mins at room temperature. After washing 3 times with PBS, cells were for 10 minutes permeabilized with 0.05% Triton X-100, washed again and blocked with 1% BSA in PBS for 30 minutes. The cells were washed and the click reaction was performed with 5 μM Alexa Fluor® 488 azide for 30 mins. After another washing step, cells were stained with Hoechst 33342 and then imaged on a Zeiss Axiovert inverted microscope using a 40x objective.

**Statistics**

Unless otherwise indicated, data are given as mean ± standard error of mean (SEM). “n” refers to the number of animals, where tissue/animal data were shown, and to the number of independent experiments where cell culture data were reported. Calculations were performed with GraphPad Prism 5.0 or BiAS.10.12. The latter was also used to test for
normal distribution and similarity of variance. In case of multiple testing, Bonferroni correction was applied. For multiple group comparisons, ANOVA followed by post hoc testing was performed, in case of time series, ANOVA for repeated measurements was used. Individual statistics of unpaired samples was performed by the t-test or, if not normally distributed by the Mann–Whitney test. p-Value of p<0.05 was considered significant. Unless otherwise indicated, n indicates the number of individual experiments.

References


**Supplemental Figure I A:** Expression of NoxO1 mRNA (exon 2-7) and eNOS mRNA in carotid arteries from WT and NoxO1-/- mice as determined by qRT-PCR. The endothelial fraction was analyzed separately from the remaining carotid artery tissue. n.d.: not detected. n=3

**Supplemental Figure I B:** Validation of the rabbit anti-NoxO1 antibody. HEK cells were left untransfected (control) or were transfected with plasmids coding for murine NoxO1 (mNoxO1) or human NoxO1 (hNoxO1). Triton soluble (TL) and insoluble extras (TI) as well as cells directly lysed in Laemmli buffer (LB) were subjected to Western blot.

**Supplemental Figure II:** Representative microscopic images and statistical analysis of matrigel tube formation assays of WT and NoxO1-/- LECs stimulated with (VEGF) or without (CTL) VEGF (50ng, 8hours). n=7-9, *p<0.05 CTL vs. VEGF.
Supplemental figure III:
Effect of Menadione (30µmol/L) on spheroid sprouting of HUVECs in the presence of VEGF to induce sprouting and of catalase (100U/ml) to prevent positive angiogenic effects by H2O2. n=15; *p<0.05 treatment vs. control
Supplemental figure IV:
(A) Number of mononuclear cells in the bone marrow and (B) FACS analyses of CD45/Flk-1 positive circulating angiogenic myeloid cells and lineage negative/Sca-1/flk-1/c-kit positive angiogenic cells the peripheral blood of wildtype (WT) and NoxO1-/- mice 14 days after femoral artery ligation. n=7-9; *p<0.05 WT vs. NoxO1-/-
Supplemental figure V A: qRT-PCR for the mRNAs indicated in LECs of wildtype (WT) and NoxO1-/- (KO) mice. *p<0.05 WT vs. KO n>3.

Supplemental figure V B&C: Representative image and statistics of gelatinase assays for MMP2 and 9 expression (B) and activity (C) in the culture medium of wildtype (WT) and NoxO1-/- (KO) lung endothelial cells with or w/o 50ng/ml VEGF as indicated. *p<0.05 WT vs. KO n>3.
Supplemental figure VI:
Redox immunoprecipitation of ADAM17 in HUVECs treated with or without H2O2 and DPI. Reduced thiols were alkylated with NEM and oxidized thiols were labeled with biotinylated iodoacetamide after DTT-mediated reduction. Subsequently, streptavidin immunoprecipitation was carried out. Initial loading (pre-IP), the immunoprecipitation (IP).
Supplemental figure VII:
Relative \( \cdot \) production as measured by LO12 chemiluminescence and ADAM17/TACE activity as measured by conversion of an artificial substrate in not transfected HUVECs (ctl) or those transfected with green fluorescent protein (GFP) or NoxO1. \( n=7; \) *\( p<0.05 \)