Paradoxical Activation of Endothelial Nitric Oxide Synthase by NADPH Oxidase

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Objectives—Increased formation of reactive oxygen species (ROS) has been identified as a causative factor in endothelial dysfunction by reducing NO bioavailability and uncoupling endothelial nitric oxide synthase (eNOS). However, the specific contribution of ROS to endothelial function is not well understood.

Methods and Results—A major source of intracellular ROS is the NADPH oxidase (Nox) family of enzymes. The goal of the current study was to directly assess the contribution of NADPH oxidase derived superoxide to eNOS function by expressing Nox5, a single gene product that constitutively produces superoxide within cells. Paradoxically, we found that instead of inhibiting eNOS, coexpression of Nox5 increased NO release from both bovine and human endothelial cells. To establish the functional significance of this observation in intact blood vessels, the endothelium of mouse aorta was transduced with Nox5 or control adenoviruses. Nox5 potently inhibited Ach-induced relaxation and potentiated contractile responses to phenylephrine. In precontracted aortae, acute exposure to superoxide dismutase induced significant vascular relaxation in vessels exposed to Nox5 versus control and unmasked the ability of Nox5 to activate eNOS in blood vessel endothelium.

Conclusions—These findings suggest that ROS inhibit eNOS function via consumption of NO rather than direct inhibition of enzymatic activity. (Arterioscler Thromb Vasc Biol. 2008;28:1627-1633)

Key Words: nitric oxide • endothelial nitric oxide synthase • NADPH oxidase • endothelial cells • superoxide dismutase

Reactive oxygen species (ROS) are leading candidates in the etiology of endothelial dysfunction and ensuing cardiovascular disease.1 Increased superoxide formation in endothelial cells (EC) has been identified as a causative factor in this process by reducing NO bioavailability, uncoupling eNOS via BH4 depletion or homodimer disruption, and also by altering redox-sensitive signaling cascades. However, elevated superoxide production is frequently accompanied by increases in superoxide formation.2–4 However, whether increased superoxide is the causative factor, a participant, or requires the cooperation of other factors present in the extracellular milieu is not yet known.

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Within vascular cells, there are several sources of ROS including the mitochondrial electron transport chain, xanthine oxidase, arachidonic acid oxygenases (lipogenase, cyclooxygenase, cytochrome P450), uncoupled eNOS, and NADPH oxidases (Noxs).5 However, superoxide production is an unintended secondary product in all of these enzymes except for the Nox isoforms, which have acquired the unique capacity to exclusively synthesize superoxide. Endothelial cells primarily express Nox2 and Nox4,6,7 and changes in their expression or activity of these enzymes have been reported in diabetes,8,9 hypertension, and in response to angiotensin II.10–12 The functional consequences of superoxide production from NADPH oxidases versus other cellular sources such as the mitochondria are not well defined. Increased superoxide anion and other reactive oxygen species have been proposed to contribute to endothelial dysfunction,3 elevated blood pressure,13,14 and increased cellular proliferation and hypertrophy.15,16 The restricted intracellular distribution of Noxs to specific locations such as the endoplasmic reticulum (ER) suggests that they may have important roles in modulating discrete aspects of intracellular signaling. However, the individual role of NADPH oxidases in these processes remains uncertain because of the presence of multiple Nox isoforms and their binding partners and the lack of specific inhibitors.17

Thus, the goal of our current study is to identify the functional consequences of increased Nox activity in the...
vascular endothelium as a functional correlate to the elevated Nox activity seen in cardiovascular disease. To achieve this we have adopted a novel genetic approach of using the Nox5 gene to produce superoxide at a time and place of our choosing. This strategy has several advantages. First, the ability of Nox5 to produce superoxide is contained within a single gene. This is important, as the protein-binding partners required to activate the other Nox isoforms are not necessary and thus their absence or inactivity cannot impede superoxide formation. For example, Nox2 requires the coexpression of at least 4 different gene products just to have the capacity to produce superoxide and then requires a stimulus, such as PMA, to induce activity. Second, Nox5 is a calcium-activated enzyme and produces low levels of superoxide constitutively and therefore is active in the vast majority of vascular cells. Third, the intracellular distribution of Nox5 is consistent with that described for Nox4 and Nox2 in endothelium, and consequently the location of superoxide production from Nox5 should reliably replicate that derived from other Nox(s).

Materials and Methods

Materials
All chemicals and reagents were purchased from Sigma. Antibodies: GAPDH (Ambion), HA (Roche), hsp90, phosphorylated eNOS, (Cell Signaling, Millipore).

Animals
C57b6 mice (Jackson Laboratories Bar Harbor, Maine) were used in accordance with the guidelines for animal use of the Institutional Animal Care and Use Committee of the Medical College of Georgia.

Cell Culture and Transfection

COS-7 cells, bovine aortic endothelial cells (BAECs), human aortic endothelial cells (HAEC) were grown as previously described. Replication-deficient adenoviruses encoding the control viruses GFP, β-gal or HA-Nox5 were generated as described previously. Adenoviral gene delivery into blood vessel endothelium has been described in detail elsewhere.

NO Release
Thirty-six hours after transfection or adenoviral transduction, net NO release was calculated by NO specific chemiluminescence after subtracting background levels from cells treated with L-NMMA, cells without eNOS, and unstimulated basal release as described previously.

cGMP Reporter Assay

HAECs were grown on glass coverslips and acutely placed over the top of HAVSMCs, a source of soluble guanylyl cyclase (sGC). The HAECs were stimulated with 1 μmol/L ionomycin in the presence of 100U/mL SOD and 300 μmol/L IBMX for 10 minutes. cGMP content was measured in HVSMCs using a cGMP specific EIA (Cayman).

Isometric Measurements of Aortic Tone

Aortic rings (1 mm in length) were mounted on 2 wires in a 6-mL chamber vessel myograph (Danish Myo Technology) with 1 g basal tension. After equilibration, rings were tested for reproducible contraction using 32 mmol/L KCl. Concentration-response curves were then constructed to phenylephrine (PE) and subsequently to ACh in vessels precontracted with a submaximal concentration of PE.

Statistical Analysis

Data were expressed as means±SEM. Comparisons were made using 2-tailed Student t test or analysis of variance with a posthoc test where appropriate. Differences were considered significant at P<0.05.

Results

Nox5 Increases eNOS Activity in Both Transfected COS-7 Cells and Endothelial Cells

To evaluate the direct interaction between eNOS and NADPH oxidase, we transfected COS-7 cells with cDNAs encoding eNOS, with or without the epitope tagged, HA-Nox5. In contrast to our expectations, we found that Nox5 increased eNOS activity as determined by the detection of nitrite (NO₂⁻) in the extracellular media. This was apparent under both basal and 100 nmol/L thapsigargin stimulated conditions (data not shown). We next extended these studies to bovine aortic endothelial cells (BAECs), which were transduced with control (β-gal) or Nox5 expressing adenoviruses. Increased expression of Nox5 (MOI ranging from 4 to 200) resulted in the dose-dependent increases in superoxide production (Figure 1A) and corresponding dose-dependent increases in NO release (Figure 1B). The Nox5-dependent increase in superoxide production in BAECs was sensitive to DPI and SOD inhibition, but was not inhibited by L-NAME, catalase, or allopurinol (supplemental Figure 1, available online at http://atvb.ahajournals.org). The increase in eNOS activity occurred without variation in total eNOS expression (Figure 1B lower panel) suggesting that the mechanisms responsible for the increase in NO release are posttranslational. Furthermore, coexpression of active Nox1 was also capable of increasing eNOS activity (supplemental Figure II). Our next goal was to assess the effect of increased ROS on the release of biologically active NO. To achieve this we used a coculture cGMP bioassay in which glass coverslips coated with human aortic endothelial cells (HAECs) are placed over the top of human aortic vascular smooth muscle cells (HAVSMCs, a source of soluble guanylyl cyclase [sGC] that functions as a bio-detector). cGMP content in HAVSMCs was then measured using a cGMP specific EIA. In the presence of extracellular SOD, selective expression of Nox5 in HAECs increased cGMP production under both basal conditions and following ionomycin stimulation (Figure 1C), data consistent with that obtained in BAECs and COS cells. eNOS expression in HAECs was unchanged (data not shown). These findings demonstrate that the increase in cGMP levels in vascular smooth muscle cell (VSMCs) derive exclusively from Nox5 modulation of endothelial eNOS activity. In the absence of extracellular SOD, basal cGMP production dropped significantly to levels equivalent to L-NAME treatment (11.47±1.1357 versus 5.4924±0.8834pmol/mL). Collectively, these data show that increased superoxide via expression of Nox5 activates, rather than inhibits, eNOS activity and that the major inhibitory action of superoxide anion is to consume biologically active NO.

Nox5 Induces Endothelial Dysfunction in Isolated Blood Vessels

To establish the vascular significance of this relationship in the intact blood vessel, the endothelium of mouse aorta was
transduced with control or Nox5 adenovirus. Adenovirus was delivered into the lumen of the aorta as previously described.24 This procedure facilitates the selective uptake of virus into the endothelium as shown in Figure 2A. To further confirm the validity of this approach, we first determined whether we could detect the expression and activity of Nox5 in the aorta and also determine whether it affected eNOS expression. As shown in Figure 2C, expression of Nox5 can be detected in blood vessels transduced with the Nox5 adenovirus. This correlated with an increase in ROS production (Figure 2B) but did not significantly modify the level of eNOS expression or phosphorylation compared to control virus (Figure 2C). We next examined the functional responses of the transduced blood vessels using a myograph to quantify changes in isometric tension. Nox5 potentiated contractile responses to phenylephrine (Figure 2D) and potently inhibited endothelium-dependent relaxation in response to acetylcholine (Figure 2E). In contrast, endothelial expression of Nox5 did not modify responses to the NO donor, sodium nitroprusside (SNP) (Figure 2F). These are well established characteristics of a dysfunctional endothelium and importantly, they occur without changes in the expression level of eNOS (Figure 2C).

Mechanism of Endothelial Dysfunction Induced by Nox5
To determine whether Nox5-derived ROS competes with NO in a stoichiometric manner or simply “stuns” the endothelium to produce an all or none effect, we next performed a titration experiment. In phenylephrine preconstricted aortic rings, progressively lower (logarithmic) titer of Nox5 adenovirus impaired Ach-induced relaxation in a dose-dependent manner (Figure 3A through 3C). The highest concentration of Nox5 (3-fold lower versus that used in Figure 2) completely inhibited Ach-dependent vasorelaxation versus control (Figure 3A), whereas a further 3-fold lower concentration of Nox5 resulted in approximately 50% impairment of relaxation (Figure 3B). Eventually a concentration was reached that was without effect (Figure 3C). The extent of Nox5-inhibition is summarized in Figure 3D. In the next experiment we determined whether inhibition could be reversed with SOD. Blood vessels were transduced with Nox5 or β-gal control virus (1.8 × 10¹¹ particles/mL) and then pretreated with either SOD (100U/mL) or vehicle. Consistent with that shown previously, Nox5 potently inhibited endothelium-dependent relaxation to Ach (Figure 3E), and this deficit was reversed with SOD pretreatment (Figure 3E through 3F).

Extracellular SOD Reveals eNOS Activation in Blood Vessels Expressing Nox5
To evaluate whether Nox5 has a direct effect on eNOS function in blood vessel endothelium, we exposed aortae to β-gal (control) or Nox5 adenovirus (6 × 10¹¹ particles/mL). Blood vessels were preconstricted with PE as described previously and at the plateau of contraction SOD was administered acutely. This is shown graphically by representative traces of isometric tension in Figure 4A. In blood vessels expressing Nox5, SOD induced an immediate and striking relaxation (45%, Figure 4B and 4C) that was much greater than that observed in β-gal–treated vessels (5%, Figure 4A and 4C).

Nox5 Does Not Modify the Phosphorylation of eNOS or Induce Uncoupling
The multi-site phosphorylation of eNOS is an important posttranslational mechanism regulating its activity and...
calcium-sensitivity. To further our understanding of the mechanisms by which Nox5 activates eNOS, we first examined whether the level of eNOS phosphorylation is modified by coexpression of Nox5. There was no significant difference in the level of phosphorylated bovine eNOS (S116, T-497, S-617, S-635, and S-1179) between Nox5 and control cDNA (GFP) expressing COS-7 cells (data not shown). Similarly, as shown in Figure 5A, Nox5 did not modify eNOS expression or phosphorylation versus control (lacZ) virus in BAECs.

Increased superoxide levels have been proposed to reduce tetrahydrobiopterin (BH4) levels and uncouple eNOS. To assess whether depletion of BH4 further restrains the ability of Nox5 to activate eNOS, we administered the BH4 donor sepiapterin to cells expressing Nox5 and measured NO release. There was no significant difference in the ability of sepiapterin to enhance NO release from cells regardless of ROS production (Figure 5B). We next investigated whether the ability of Nox5 to activate eNOS derives from extra or intracellular superoxide production. NO release was measured from populations of COS cells expressing either eNOS or Nox5 in separate cells or both proteins coexpressed within the same cell. Cells expressing both Nox5 and eNOS produced more NO compared to cells expressing equivalent amounts of eNOS only or those in which Nox5 and eNOS are expressed in separate cells. These findings suggest that the extracellular release of superoxide is insufficient to activate eNOS (supplemental Figure III).

To determine whether increased ROS uncouples eNOS we next measured the eNOS monomer/dimer ratio and the relative association of hsp90 in endothelial cells expressing Nox5. Increased production of ROS did not influence the ratio of eNOS monomer/dimer, suggesting that the low level ROS production from NADPH oxidase is not sufficient in itself to disrupt the eNOS dimer (Figure 5C). We next immunoprecipitated eNOS from cells expressing Nox5 or a control gene and the relative amount of hsp90 bound to eNOS was determined by immunoblotting. As shown in Figure 5D, increased expression of Nox5 and attendant ROS production resulted in an increased association of eNOS with hsp90. These data argue in favor of increased coupling of eNOS and accordingly, increased NO production.

**Discussion**

The goal of the present study was to directly assess the contribution of elevated superoxide anion to endothelial cell function in vitro and in isolated blood vessels. Elevated ROS resulting from increased expression or activity of Nox enzymes has been identified as a key mechanism contributing to endothelial and vascular dysfunction. However, because disease states result in many accompanying changes to the extracellular milieu, the direct causative effects of Nox-derived superoxide on vascular cells are poorly understood. A common approach in the past has been to acutely apply...
supraphysiological concentrations of ROS outside the cell and assess the cellular response. Other approaches using redox cycling agents that elevate intracellular superoxide may not faithfully mimic the amount or location of Nox derived superoxide. Therefore, we took the approach of expressing a novel Nox isoform, Nox5, in the vascular endothelium. This approach allows for the production of more biologically relevant concentrations of superoxide from a defined intracellular area. We found that in contrast to what we had expected, elevated Nox expression and ROS production dose-dependently increased eNOS activity. However, the increased production of superoxide from Nox5 consumed NO and abrogated the ability of both cultured endothelial cells and blood vessel to deliver biologically active NO. These findings suggest that increased intracellular production of superoxide-derived from NADPH oxidase does not inhibit eNOS activity directly, but instead prevents the extracellular actions of NO.

We originally hypothesized that Nox5 would decrease eNOS activity because numerous studies have shown that ROS decrease eNOS activity by reducing BH4 bioavailability, uncoupling eNOS and disrupting the eNOS homodimer. However, we found increased activation of eNOS in a range of cells in vitro and also in intact blood vessels. Many of these previous studies may not have detected the increased activity of eNOS as they directly measured intact NO (which is consumed by superoxide) or vascular function. However, in the current study, we measured the level of NO.

Figure 3. Endothelial dysfunction in blood vessels expressing Nox5 is dose-dependent and SOD sensitive. Murine aortae were transduced with 3-fold progressively lower (1.8 x 10^11 to 2 x 10^10) concentrations of Nox5 or control (GFP) adenovirus (A–C). Blood vessels were precontracted with a submaximal concentration of PE (10^-6 mol/L) and endothelium-dependent relaxation initiated with acetylcholine (Ach, 10^-8 to 10^-5 mol/L). Data are presented as the means±SE of the percentage of PE-induced tone. *P<0.05 vs the control (n=4). A summary of the degree of inhibition is shown in D. E–F, Mouse aortae were transduced with control or Nox5 adenovirus (1.8 x 10^11 particles/mL). Aortic rings were exposed to SOD 100 U/mL before preconstriction with PE (10^-6 mol/L). Endothelium-dependent relaxation was recorded in response to acetylcholine (Ach, 10^-8 to 10^-5 mol/L) as described. Data are presented as the means±SE of the percentage of PE-induced tone. *P<0.05 vs the control (n=4).

Figure 4. Acute exposure of extracellular SOD reveals eNOS activation in blood vessels expressing Nox5. Aortic endothelium was transduced with control or Nox5 adenovirus (6 x 10^11 particles/mL). 36 hours later, aortic rings were precontracted with PE and acutely exposed to 100 U/mL SOD and the degree of relaxation recorded. Representative traces of the changes in isometric tension are shown in A–B. C, Data are summarized as the percentage of PE-induced tone in response to SOD in control and Nox5 transduced blood vessels. *P<0.05 vs the control (n=5).
nitrite (NO\textsuperscript{−}) which results from the spontaneous breakdown of NO and peroxynitrite in aqueous media.\textsuperscript{37} Therefore, even if the majority of the NO produced is scavenged by O\textsubscript{2}−, the elevated production of NO can be efficiently recorded via detection of nitrite. Indeed, increased formation of nitrite in blood vessels from atherosclerotic animals has been shown, and this occurs despite impaired endothelium-dependent relaxation.\textsuperscript{38} Increased ROS have also been shown to increase NO release in blood vessels and cultured endothelial cells.\textsuperscript{39,40} The ability of increased Nox expression and accompanying superoxide production to activate eNOS provides a mechanism to account for this phenomenon.

eNOS is acutely regulated by a number of posttranslational mechanisms including phosphorylation, protein–protein interactions, and subcellular location.\textsuperscript{41,42} To address the mechanisms by which Nox5 stimulates eNOS activity, we first examined whether Nox5 affects eNOS phosphorylation at different sites and no changes were observed. The elevated intracellular superoxide and NO that accompanies Nox5 expression in endothelial cells also did not disrupt the eNOS homodimer suggesting that factors beyond NADPH oxidase derived superoxide must contribute to monomer formation seen in disease states. ROS have also been shown to reduce cellular BH4 levels, but cells expressing Nox5 had sufficient BH4 levels to support increased eNOS activity and supplementation with a BH4 donor did not reveal a significant deficit of BH4. Decreased association of hsp90 with eNOS is known to induce what has been termed “uncoupling” and results in increased superoxide formation from eNOS at the expense of NO.\textsuperscript{43} However, the converse relationship is poorly understood. In this study we found that elevated superoxide production increases the association of hsp90 with eNOS and thereby facilitates rather than inhibits NO synthesis.

In summary, we found that increased superoxide production via expression of Nox5 paradoxically enhanced overall eNOS activity in a variety of different cell types and in intact blood vessels. The increase in activity occurred despite a decrease in the amount of biologically available NO reaching adjacent cells. The functional significance of this is not yet clear but may represent a feedback system that enhances NOS enzymatic activity to counterbalance a decrease in the amount biologically available NO. Importantly, the ability of Nox-derived superoxide to increase eNOS activity may accelerate the formation of peroxynitrite and have deleterious effects on vascular cells.

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Disclosures

None.

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Figure Legends for Supplemental data

Supplemental Figure 1. Effect of inhibitors on Nox5-induced chemiluminescence. BAEC were transduced with Nox5 adenovirus (100MOI). 36hrs post transduction, ROS production was monitored by chemiluminescence in the presence and absence of (A) SOD (100U/ml), (B) L-NAME (1mM), (C) Allopurinol (1mM) and (D) Catalase (200U/ml). The data are presented as mean±SE (n=5). *P<0.05 vs control.

Supplemental Figure 2. Nox1 increases eNOS activity. COS-7 cells were transfected with cDNAs encoding eNOS with or without RFP(control) and Nox1/NoxO1, NoxA1. Basal NO release was measured using NO-specific chemiluminescence and the data were presented as mean ± SE (n=5). * P < 0.05 vs control.

Supplemental Figure 3. Extracellular superoxide does not increase eNOS activity. COS-7 cells were transfected in 10cm dishes with cDNAs encoding eNOS, RFP, Nox5, or eNOS together with Nox5 at ratios of (1000:100, 1000:300 and 1000:1000). Where needed, RFP was used a control to balance total DNA transfected. 24hrs after transfection, the cells were split and replated into 12well plates for NO release. Cells transfected individually with eNOS, RFP or Nox5 were combined to maintain equivalent eNOS expression. Basal NO release was measured using NO-specific chemiluminescence and the data were presented as mean ± SE (n=6). * P < 0.05 vs control.
Supplemental Figure 1
Supplemental Figure 2

The figure shows a bar graph comparing NO\textsubscript{2} accumulation (pmol/well) between eNOS and eNOS+Nox1 conditions. The graph indicates a significant increase in NO\textsubscript{2} accumulation when Nox1 is co-expressed with eNOS, as denoted by the asterisk (*) above the bar for eNOS+Nox1. Below the graph, Western blots are displayed showing protein expression levels for eNOS and GAPDH, with no significant difference observed between the two conditions.
Supplemental Figure 3