Molecular Medicine

Genetic Deletion of NADPH Oxidase 1 Rescues Microvascular Function in Mice With Metabolic Disease

Jennifer A. Thompson, Sebastian Larion, James D. Mintz, Eric J. Belin de Chantemèle, David J. Fulton, David W. Stepp

Rationale: Early vascular changes in metabolic disease that precipitate the development of cardiovascular complications are largely driven by reactive oxygen species accumulation, yet the extent to which excess reactive oxygen species derive from specific NADPH oxidase isoforms remains ill defined.

Objective: Identify the role of Nox1 in the development of microvascular dysfunction in metabolic disease.

Methods and Results: Four genotypes were generated by breeding Nox1 knockout mice with db/db mice: lean (HdbWnox1), lean Nox1 knockout (HdbKnox1), obese (KdbWnox1), and obese KK (KdbKnox1). The degree of adiposity, insulin resistance, and dyslipidemia in KW mice was not influenced by Nox1 deletion as determined by nuclear magnetic resonance spectroscopy, glucose tolerance tests, and plasma analyses. Endothelium-dependent responses to acetylcholine in pressurized mesenteric arteries were reduced in KW versus HW (P<0.01), whereas deletion of Nox1 in KW mice normalized dilation. Vasodilator responses after inhibition of NO synthase blunted acetylcholine responses in KK and lean controls, but had no impact in KW, attributing recovered dilatory capacity in KK to normalization of NO. Acetylcholine responses were improved (P<0.05) with Tempol, and histochemistry revealed oxidative stress in KW animals, whereas Tempol had no impact and reactive oxygen species staining was negligible in KK. Blunted dilatory responses to an NO donor and loss of myogenic tone in KW animals were also rescued with Nox1 deletion.

Conclusions: Nox1 deletion reduces oxidant load and restores microvascular health in db/db mice without influencing the degree of metabolic dysfunction. Therefore, targeted Nox1 inhibition may be effective in the prevention of vascular complications. (Circ Res. 2017;121:502-511. DOI: 10.1161/CIRCRESAHA.116.309965.)

Key Words: acetylcholine ▪ animals ▪ insulin resistance ▪ obesity ▪ protein isoforms

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Overproduction of reactive oxygen species (ROS) is a key mechanism by which insulin resistance, obesity, and other components of the metabolic syndrome (MetS) lead to the development of vascular disease. For this reason, restoring redox balance has long been hailed as a promising therapeutic strategy; however, scavenging of ROS with antioxidants has proven ineffective for the prevention of cardiovascular death in the majority of large randomized clinical trials. Several factors may account for this unexpected failure of antioxidants, including the indiscriminate scavenging of ROS, which have both physiological and pathophysiological roles. Specific targeting of pathophysiological ROS may offer a more efficacious approach; however, this will depend on a better understanding of the source of ROS under conditions of metabolic disease.

Members of the NADPH oxidase (Nox) family of enzymes function to generate physiological levels of ROS, but under various pathological conditions are significant contributors to the surplus of ROS characteristic of oxidative stress. Excess ROS exert deleterious effects on the vasculature through a variety of mechanisms. One such mechanism is the quenching of NO by superoxide (O2−) and consequent blunting of endothelium-mediated vasodilation. Loss of endothelial function in the microvasculature is a significant predictor of end-organ damage and cardiovascular mortality in metabolically compromised patients. Although Nox enzymes have been implicated in the pathogenesis of metabolic disease by a handful of studies, relative contributions of the different Nox isoforms in animal models of vascular disease have not been sufficiently delineated because the majority of studies use pharmacological agents that lack isoform specificity, and conflicting results defer definitive conclusions. Further, there is currently a lack of data with

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From the Vascular Biology Center (J.A.T., S.L., J.D.M., E.J.B.d.C., D.J.F., D.W.S.), Department of Physiology (D.W.S.), Department of Pharmacology (D.J.F.), and Department of Medicine (S.L., E.J.B.d.C.), Augusta University, GA.

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Correspondence to David W. Stepp, PhD, Vascular Biology Center, Augusta University, C.B. Room 3211B, 1120 15th St, Augusta, GA 30912. E-mail dstepp@augusta.edu

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**What Is Known?**

- ROS are key pathogenic factors in metabolic disease.
- Nox is expressed in the vasculature and produces ROS.
- In an in vitro setting, glucose and other metabolic stimuli induce Nox1 activity.

**What New Information Does This Article Contribute?**

- Nox1 contributes importantly to oxidant load in an in vivo setting of metabolic disease.
- Nox1-derived ROS play a critical role in the development of microvascular dysfunction in metabolic disease.
- Inhibition of Nox1 may be an effective therapeutic strategy in the prevention of vascular complications.

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>L-NAME</td>
<td>L-N^6^-nitroarginine methyl ester</td>
</tr>
<tr>
<td>MetS</td>
<td>metabolic syndrome</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Nox</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>O_2^-</td>
<td>superoxide</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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Although ROS have been implicated in the pathogenesis of metabolic disease and its sequelae, clinically tested approaches to mitigating ROS have proven ineffective in preventing cardiovascular complications. Directly targeting enzymatic sources of ROS represents a potential alternative strategy that has not been sufficiently tested in animal models of metabolic disease. We found that loss of Nox1 through genetic deletion reduced oxidant load and prevented the loss of myogenic tone and NO-mediated microvascular dilation in the db/db (leptin receptor deficient) model, without impacting the degree of metabolic dysfunction. These findings link Nox1 to microvascular dysfunction in an in vivo setting of metabolic disease, and implicate oxidative stress in the autoregulatory dysfunction that occurs in abnormal metabolic states.

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**Ex Vivo Vascular Function**

In ice-cold Krebs solution, 2nd order (≈150 μm internal diameter) mesenteric arteries were isolated and mounted in a small vessel myograph. After equilibration, cumulative concentration-response curves were generated in preconstricted vessels by adding increasing concentrations of acetylcholine and sodium nitroprusside. In some experiments, vessels were preincubated with L-NG-nitroarginine methyl ester (L-NAME) or Tempol for 30 minutes before vasodilator was added. Contractile responses to increasing doses of phenylephrine were also measured in some vessels. Myogenic tone was determined by measuring the active diameter in the presence of Ca^2+ and passive diameter in the absence of Ca^2+, at 20 mm Hg increments.

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**Oxidative Stress**

Cross-sections of mesenteric arteries were embedded in optimal cutting tissue compound (Tissue-Tek) and slow frozen while suspended in isopentane cooled to −80°C. After blocking, cryosections were incubated in rabbit antinitrotyrosine antibody (Life Technologies, Inc) overnight at 4°C. The next day, slides were washed and incubated with secondary antibody Alexa Fluor 594 (Life Technologies), measured ex vivo in mesenteric resistance arteries, and extensive metabolic profiling was performed, providing the first comprehensive investigation into the role of Nox1 in microvascular disease under conditions of metabolic dysfunction.

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**Animal Model and Metabolic Profiling**

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Augusta University and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female C57BL6 mice heterozygous for the leptin receptor mutation (db/db−) were crossed with male C57BL6 Nox1 knockout mice. Male mice of the following genotypes were studied: HdbWnox1 (Leprdb/db.Nox1+/y), HdbKnox1 (Leprdb/db.Nox1−/y), KdbWnox1 (Leprdb/db.Nox1−/−), KdbKnox1 (Leprdb/db.Nox1−/−). At ≈12 weeks of age, whole-body composition of fat and lean mass was determined by nuclear magnetic resonance, and intraperitoneal glucose tolerance tests were performed to examine glucose homeostasis. A subset of mice were monitored for 4 days with a comprehensive laboratory animal monitoring system for noninvasive measurement of energy expenditure, locomotor activity, and food intake. At sacrifice, animals were anesthetized in an induction chamber and subsequently decapitated with a guillotine. Plasma was isolated and later analyzed for fasting insulin and lipid levels. A subset of lean HW and HK mice were treated with low-dose streptozotocin (50 mg/kg) for 5 consecutive days and euthanized 5 weeks post-treatment after confirmation of hyperglycemia.

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**Methods**

**Deletion of Nox1 Rescues Microvascular Function**

Although ROS have been implicated in the pathogenesis of metabolic disease and its sequelae, clinically tested approaches to mitigating ROS have proven ineffective in preventing cardiovascular complications. Directly targeting enzymatic sources of ROS represents a potential alternative strategy that has not been sufficiently tested in animal models of metabolic disease. We found that loss of Nox1 through genetic deletion reduced oxidant load and prevented the loss of myogenic tone and NO-mediated microvascular dilation in the db/db (leptin receptor deficient) model, without impacting the degree of metabolic dysfunction. These findings link Nox1 to microvascular dysfunction in an in vivo setting of metabolic disease, and implicate oxidative stress in the autoregulatory dysfunction that occurs in abnormal metabolic states.

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at room temperature for 30 minutes in a covered humidity chamber. Replacement of the primary antibody with PBS was used as negative control. Nuclei were stained with Sytox Green (Life Technologies). A second experiment was performed wherein freshly isolated vessels were incubated with dihydroethidium, subsequently washed and slow frozen in optimal cutting tissue compound (Tissue-Tek). A subset of vessels was simultaneously treated with superoxide dismutase (SOD) polyethylene glycol (PEG-SOD), to confirm the specificity of dihydroethidium to superoxide. All images were captured on a Zeiss LSM780 confocal microscope at ×40 magnification and analyzed with ImageJ. Additionally, plasma levels of thiobarbituric acid-reactive substances—a by-product of lipid peroxidation—was measured using a colorimetric assay according to the manufacturer’s instructions (Cayman Chemicals, Ann Arbor, MI).

**Expression of Nox and Its Subunit P22^phox**

For determination of gene or protein expression, vessels were pooled from 3 to 5 animals for each sample included in the analysis. Complementary DNA synthesized from isolated RNA was analyzed for the following genes using polymerase chain reaction: Nox1, Nox4, P22^phox, and endothelial NO synthase (eNOS). For quantitative polymerase chain reaction, the expression of individual target genes is reported as fold change relative to the control group using the 2^ΔΔct method, with RPL7 (ribosomal protein L7) as the housekeeping gene. Primers used for analysis are shown in Online Table I and a representative blot is shown in Online Figure I. The phosphorylation status of eNOS was determined by Western blot. Briefly, extracted protein was separated by gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked and incubated overnight with antibodies against phospho-eNOS and total eNOS (Cell Signaling Technology, Danvers, MA).

**Results**

**Nox1 Deletion Does Not Impact the Degree of Obesity in db/db Mice**

Metabolic profiling of the 4 mouse genotypes was performed to determine the impact of Nox1 deletion on metabolic function. At 12 weeks of age, body weight in the KW and KK groups was 68% (P<0.001) and 57% (P<0.001) higher than lean HW and HK animals, respectively (Table 1). Nuclear magnetic resonance spectroscopy determined that whole-body percentage fat was 270% higher in KW versus HW (P<0.001) and 250% higher in KK versus HK (P<0.001) groups (Online Figure II). In obese mice, absolute fat mass (%×body weight) was 34±3.1 g in KW versus 5±0.8 g in HW (P<0.001) and 24±2.7 g in KK versus 5±0.5 g in HK (P<0.001). Whole-body percentage lean mass was 60% (P<0.001) and 50% (P<0.001) lower in KW and KK mice compared with their respective controls (Online Figure II). Absolute lean mass (%×body weight) was 12±0.9 g in KW versus 18±0.5 g in HW (P<0.001) and 15±0.8 g in KK versus 18±0.4 g in HK (P<0.05). No differences in body weight or composition were present in obese KW versus KK or lean HW versus HK.

**Blood Glucose and Lipid Control Are Similarly Compromised in db/db Mice With and Without Nox1 Deletion**

Indices of glycemic control are shown in Online Figure II and Table 1. Blood glucose was similarly increased in KW (386±49.1 mg/dL) and KK (385±50.9 mg/dL) mice compared with HW (205±16.8 mg/dL) and HK (184±17.0 mg/dL), respectively (both P<0.01). A similar pattern was observed for HbA1c, where both obese groups exhibited markedly higher levels (KW, 8.6±0.68%; KK, 10.1±0.97%) relative to lean controls (HW, 6.0±0.36%; HK, 5.7±0.34%). Likewise, fasting plasma insulin levels were increased in KW (1.30±0.173 ng/mL) versus HW (0.22±0.06 ng/mL) and KK (1.53±0.07 ng/mL) versus HK (0.06±0.06 ng/mL; both P<0.001). Glucose clearance after a glucose challenge was impaired to the same degree in obese KW and KK mice (Online Figure II). Area under the curve was increased in KW versus HW (P<0.05) and not different between KW and KK (KW, 14289±3104 mg·DL·min; KK, 19718±2248 mg·DL·min; HW, 34392±5842 mg·DL·min; HK, 31848±2872 mg·DL·min). No differences were found when comparing the 2 lean groups or the 2 obese groups in any of the aforementioned measurements of glycemic control. Fasting plasma triglyceride, cholesterol, and nonesterified fatty acid levels are shown in Table 1. Total plasma triglycerides were higher in KW (83.6±6.94 mg/dL) versus HW (289±3204 mg/dL·min) and not different between KW and KK (KW, 99.2±17.86 mg/dL·min; KK, 76.2±20.37 mg/dL·min). Differences were found when comparing the 2 lean groups (both P<0.05). Although there were no significant differences in cholesterol and nonesterified fatty acid between KW and KK groups, increases in the KW group were 270% higher (KW, 99.2±17.86 mg/dL·min) versus HK (KK, 76.2±20.37 mg/dL·min).

**Table 1. Basal Metabolic Parameters for All Four Mouse Genotypes**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>HW (Leprdb++, Nox1+/y)</th>
<th>HK (Leprdb++, Nox1+/y)</th>
<th>KW (Leprdb++, Nox1−/y)</th>
<th>KK (Leprdb++, Nox1−/y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>29±0.6</td>
<td>28±0.8</td>
<td>48±3.4*</td>
<td>44±3.2†</td>
</tr>
<tr>
<td>Fasting blood glucose, mg/dL</td>
<td>205±16.8</td>
<td>184±16.9</td>
<td>386±49.1*</td>
<td>385±50.9†</td>
</tr>
<tr>
<td>Fasting plasma insulin, ng/mL</td>
<td>0.2±0.02</td>
<td>0.1±0.01</td>
<td>1.3±0.17*</td>
<td>1.5±0.07†</td>
</tr>
<tr>
<td>Fasting plasma triglyceride, mg/dL</td>
<td>27.5±5.61</td>
<td>34.9±4.52</td>
<td>83.6±6.94*</td>
<td>99.2±17.86†</td>
</tr>
<tr>
<td>Fasting plasma cholesterol, mg/dL</td>
<td>81.7±6.51</td>
<td>88.8±10.33</td>
<td>165.1±6.51*</td>
<td>128.7±11.93</td>
</tr>
<tr>
<td>Food consumption, g/d</td>
<td>4.2±0.19</td>
<td>3.7±0.06</td>
<td>5.4±0.29*</td>
<td>5.3±0.29†</td>
</tr>
<tr>
<td>VCO₂, mL·kg·h</td>
<td>3446±55.4</td>
<td>3412±27.3</td>
<td>1892±36.1*</td>
<td>1941±24.7†</td>
</tr>
<tr>
<td>VO₂, mL·kg·h</td>
<td>3458±7.9</td>
<td>3583±24.4</td>
<td>2034±22.6*</td>
<td>1996±45.8†</td>
</tr>
</tbody>
</table>

NEFA indicates nonesterified fatty acid.

*P<0.05 KW vs. HW. †P<0.05 KK vs. HK.

**Nox1 Deletion Does Not Influence the Degree of Energy Expenditure Reduction in db/db Mice**

Food intake, locomotor activity, and energy expenditure were monitored for 4 days; data are shown in Table 1. VO₂ and VCO₂ were reduced by 44% to 45% in KW and KK mice relative to lean controls (all P<0.001). Respiratory exchange ratio and heat production were comparable among the 4 groups.
Daily food intake for 4 days was similarly increased in KW ($P<0.05$) and KK ($P<0.001$) versus HW and HK, respectively. There were no differences in water accumulation among the 4 groups. Locomotor activity was also markedly decreased in obese groups compared with lean, with no differences in obese mice with and without Nox1 (KW, 142±64.1 wheel counts versus HW, 11274±2602 wheel counts; $P<0.05$; KK, 47±24.5 wheel counts versus HK, 17151±5381 wheel counts; $P<0.01$).

Microvascular Endothelial Function and NO Bioavailability Are Restored in db/db Mice With Nox1 Deletion

Microvascular function was evaluated by measuring dilatory responses of pressurized mesenteric arteries to vasoactive agents. Dilatatory responses to acetylcholine in preconstricted mesenteric arteries are shown in Figure 1. A 50% reduction in maximal dilatory responses to acetylcholine was observed in KW mice, indicating endothelial dysfunction. Maximal acetylcholine responses in KK animals were comparable with lean controls (HK). There were no differences in logEC$_{50}$ for acetylcholine responses among the 4 groups. Nox1 deletion also improved endothelium-dependent responses to acetylcholine in microvessels from lean mice made diabetic by streptozotocin treatment (Figure 2). Next, the mechanism by which Nox1 deletion impacts endothelium-independent vasodilation was assessed. Vessels were preincubated with L-NAME—an eNOS inhibitor—to assess the contribution of NO to endothelial dysfunction. L-NAME treatment reduced maximal dilatory responses to acetylcholine by 60% to 70% in lean HW and HK groups. L-NAME had no impact on responses in KW mice, consistent with impaired NO-dependent signaling; yet reduced maximal dilation by 64% in KK animals (Figure 3). The contribution of superoxide ($O_2^-$) to endothelium-dependent responses was determined by preincubating the vessels with Tempol—an $O_2^-$ dismutase mimetic. Tempol improved acetylcholine responses ≈60% in KW mice but had no effect on dilation in KK and lean controls (Figure 3).

Vascular Smooth Muscle Cell Function Is Rescued in db/db Mice With Nox1 Deletion

Vascular smooth muscle cell function was assessed by measuring dilatory responses to sodium nitroprusside—an NO donor—and myogenic reactivity to incremental increases in intraluminal pressure. Sodium nitroprusside responses are shown in Figure 4. Maximal responses to sodium nitroprusside were reduced by ≈50% in mesenteric arteries from KW animals compared with HW control ($P<0.0001$), yet comparable between KK and HK mice. Figure 5 shows active and passive diameters at intraluminal pressures between 20 and 120 mm Hg in the 4 groups. In lean animals, myogenic tone developed at 80 mm Hg, whereas tone development is both delayed and blunted in KW animals. The percentage of myogenic tone at each pressure was calculated and shown in Online Figure III. At each pressure, the percentage of myogenic tone was decreased in the KW mice compared with HW. This impairment in myogenic reactivity seems independent of changes in vascular wall stiffness, as measured by diameter changes...
in response to incremental increases in pressure in Ca^{2+}-free Krebs solution (Online Figure III).

**Nox1 Deletion Abolishes Oxidative Stress in the Microvasculature of db/db Mice**

Nitrotyrosine is a product of ROS and reactive nitrogen species-mediated modification of tyrosine residues and has been widely used as a marker of oxidative stress. Antinitrotyrosine antibody immunoreactivity was high in mesenteric arteries from KW mice but markedly attenuated in KK arteries (Figure 6). Staining in HW and HK arteries was comparable with negative control. Total oxidant load was inferred from the quantification of thiobarbituric acid–reactive substances in the plasma. There was a significantly higher circulating level of thiobarbituric acid–reactive substances in KW mice (*P*<0.001), which was significantly attenuated with Nox1 deletion (KW versus KK *P*<0.001; Figure 6). Superoxide production was measured in vessels stained with dihydroethidium (Figure 7). Dihydroethidium fluorescence was negligible in HW vessels and increased in KW. Staining for dihydroethidium in KW sections was abolished when simultaneously incubated with SOD (PEG-SOD).

**Nicotinamide Adenine Dinucleotide Phosphate Oxidase Subunit (P22phox) Expression Is Increased and Nox4 Expression Unchanged in Mesenteric Arteries of db/db Mice**

Expression of Nox1 and its subunit is shown in Figure 8. Expression of Nox1 in the vasculature was negligible in HW, HK, and KK mice, such that there was no detectable signal in real-time polymerase chain reaction. Amplicons for Nox1 were separated on an agarose gel, showing a strong band for the KW vessels, whereas there were no detectable bands in the Nox1-null animals and in the lean controls. Real-time polymerase chain reaction revealed that the mRNA expression of P22phox—a subunit required for the activation of Nox1, Nox2, and Nox4 isoforms of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase—was increased in db/db (*P*<0.05), but unchanged in KK mice, relative to control (HK, 1.32±0.23-fold change versus HW; KW, 1.72±0.20-fold change versus HW; KK, 1.44±0.19-fold change versus HW). There were no changes in the mRNA levels of Nox4 or eNOS in mesenteric arteries between the 4 groups. The ratio of phosphorylated eNOS relative to total eNOS was also unchanged between groups, as determined by Western blot.

**Discussion**

Despite compelling evidence implicating oxidative stress as a key mechanism underlying the vascular complications of MetS, there is yet no approved therapeutic specifically designed to temper ROS accumulation. In this regard, progress necessitates a deeper understanding of the chief sources of ROS in the setting of metabolic dysfunction and their contribution to the development of vasculopathy. The distinct role...
of Nox1 was examined in the present study by using a novel transgenic approach whereby Nox1 is selectively deleted in the db/db mouse. Endothelial and smooth muscle dysfunction present in the microvessels of db/db mice were rescued with Nox1 deletion, identifying Nox1 as a critical source of ROS and mediator of the early vascular changes that characterize incipient cardiovascular disease. Importantly, we performed comprehensive metabolic profiling that showed Nox1 deletion to have no impact on the degree of obesity, glucose intolerance, and dyslipidemia in db/db mice, thus, eliminating the possibility that the improvement in vascular function is because of an off-target effect of Nox1 deletion on metabolic homeostasis. Metabolic stimuli abundant under such conditions impart deleterious effects on the vasculature largely through stimulation of local ROS production, as evinced by a considerable body of in vitro data in the literature. Herein, our

Figure 5. Nox1 deletion improves myogenic tone in db/db mice. Active and passive diameters at increments of 20 mm Hg between 20 and 120 mm Hg in mesenteric arteries isolated from HW (A), HK (B), KW (C), and KK (D) groups. Groups were compared using ANOVA with a Bonferroni post hoc test. (n=5–7 per group). *P<0.05 active vs passive diameter.

Figure 6. Oxidative stress is abolished in microvessels of db/db mice with Nox1 deletion. Representative cross-sections of mesenteric arteries stained with an antinitrotyrosine antibody (red) and the nuclear stain, sytox green (green) in lean and obese mice with Nox1 intact (Nox1+/y) and Nox1 deletion (Nox1−/y; A). Quantification of nitrotyrosine immunoreactivity in the 4 groups (B), n=4 to 5 per group. Plasma levels of thiobarbituric acid reactive substances was used as a measure of total oxidant load (C). *P<0.05, **P<0.001: KW vs HW or KW vs HK.
findings suggest that in the context of in vivo metabolic disease, the vascular consequences of injurious metabolic stimuli are primarily transduced through Nox1-derived ROS.

Nox1 is among the 4 isoforms of the Nox family of enzymes expressed in the vasculature and a major source of $\text{O}_2^-$ in endothelial cells and vascular smooth muscle cells under both physiological and pathological conditions. Existing data supporting a role of Nox1 in vascular disease are overwhelmingly drawn from models of hypertension. For instance, studies report a blunting of the hypertensive response to angiotensin II infusion in mice with genetic loss of Nox1 and reduction of $\text{O}_2^-$ generation in vessels from Dahl salt-sensitive and deoxycorticosterone acetate salt-induced hypertensive rats after pharmacological inhibition of Nox enzymes. These studies along with in vitro data and data in Nox1 smooth muscle overexpressing mice demonstrate that Nox1 mediates the pressor and hypertrophic responses to angiotensin II. In contrast, there remains a scarcity of information on the contribution of Nox1 to the development of microvascular pathology in the setting of metabolic disease. To date, the majority of evidence relies heavily on in vitro data revealing Nox1 as a mediator of the cellular responses to metabolic stimuli that precipitate atherogenesis. For instance, Gray et al. reports that silencing of Nox1 abrogates the elevation in $\text{O}_2^-$ production and inflammatory gene expression in human endothelial cells treated with high glucose, whereas the influence of Nox4 silencing is limited to $\text{H}_2\text{O}_2$. Another study shows Nox1, but not Nox2 or Nox4 expression, to be upregulated in response to high glucose in a microvascular endothelial cell line. Further, gene silencing techniques reveal cellular events involved in neointima formation to be driven by Nox1 activation in cell lines of vascular smooth muscle cells, namely, phenotypic modulation and proliferation in response to cyclic stretch and low-density lipoprotein. This putative role of Nox1 in diabetes mellitus-induced atherosclerosis holds true in the intact animal, as demonstrated by the seminal article by Gray et al. In this study, genetic deletion of Nox1, but not Nox4, abolished atherosclerotic lesion development in aortae of ApoE−/− mice made diabetic by daily injections of streptozotocin. Herein, we provide the first direct evidence linking Nox1 to the early microvascular changes that precede the development of hypertension and end-organ damage. Together, these findings generate an impetus to substantiate the value of targeted Nox1 inhibition in the prevention of macrovascular and microvascular complications in metabolic disease.

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The present study focused on the mesenteric artery, which represents a peripheral vascular bed that undergoes vasomotor changes underling resistance component of abnormal hemodynamic patterns in MetS. Reduced vasodilatory capacity is a highly documented finding in metabolic disease, which manifests during the subclinical stages in both atherosclerotic-resistant arteries, such as the mesenteric artery and atherosclerotic-prone arteries, and is an independent predictor of cardiovascular death. Three recent studies provide indirect evidence implicating Nox1 as a causative factor in the reduced vasodilatory responses exhibited by the db/db model. The first, published by Liang et al., reports a reduction in Nox1 mRNA and protein levels to accompany improved endothelium-dependent dilatory responses in aortae of db/db mice with toll-like receptor 4 mutation. The authors of the second study also attributed impaired dilatory capacity to Nox-derived ROS on the grounds that endothelium-dependent and independent dilatory responses were restored along with decreases in ROS production and Nox1/2/4 expression in mesenteric arteries of db/db mice treated with atorvastatin.

Correlative changes in protein expression do not necessarily establish Nox1 as the primary agent in pathogenesis given the many sources of ROS and the lack of specificity and sufficient validation of currently available antibodies against Nox1. The third study, published by our group, showed an improvement in endothelium-dependent dilatation in db/db mice after in vitro treatment with GTK136901—a Nox1/4 inhibitor. Direct evidence specifically linking Nox1 to microvascular dysfunction is provided by the present study with the finding that blunted endothelium-dependent and independent dilatation in the mesenteric arteries of db/db mice are recovered with loss of Nox1 in the absence of changes in body composition or metabolism. Endothelial function was assessed by measuring responses to acetylcholine—an activator of eNOS. Although there was no difference in eNOS mRNA expression between groups, acute inhibition of eNOS with L-NAME had differential effects on acetylcholine responses, with no impact on db/db mice and a drastic blunting effect in double knockout animals. Furthermore, incubation of vessels with Tempol—a SOD mimetic—improved acetylcholine responses in obese mice with Nox1 intact but had no influence on lean and double knockout animals. Together, these results suggest that the beneficial effect of Nox1 deletion on endothelial function is, at least in part, because of a restoration of NO bioavailability via normalization of $\cdot O_2^-$ production—the most well-characterized means by which ROS mediate vascular dysfunction. Reduced staining of dihydroethidium and nitrotyrosine immunoreactivity in double knockout animals lends further support to the conclusion that Nox1 deletion prevents excess $\cdot O_2^-$ production and oxidative stress in MetS. Therefore, Nox1 is a principal source of ROS and mediator of NO-related endothelial dysfunction under conditions of metabolic compromise.

There are multiple mechanisms in addition to NO depletion by which Nox1-derived ROS may promote development of vascular dysfunction, including the modulation of redox-sensitive signaling pathways involved in vasomotor function and structural changes because of abnormal extracellular matrix turnover or cytoskeleton reorganization. One or more of these mechanisms may participate in the abnormal vascular smooth muscle cell (VSMC) behavior observed in db/db mice of the current study, evident by loss of myogenic regulation and impairment of vasodilatory responses to an NO donor. Nox1 is expressed in VSMC of microvessels; therefore, improvement of VSMC function in mice lacking Nox1 suggests that the VSMC responses to adverse metabolic stimuli are predominately driven by local ROS generation via Nox1.

Myogenic responsiveness is an intrinsic property of VSMC that autoregulates organ perfusion amid fluctuations in upstream pressure. Heightened myogenic contraction in response to elevations in intraluminal pressure has been reported in hypertensive and diabetic patients and in animal models of these disorders. However, with the persistence of hypertension or metabolic dysfunction, myogenic control is lost and with it, the ability to protect downstream organs from excessive increases in perfusion pressure. Thus, impairment of myogenic responsiveness, as observed in db/db mice of the present study, contributes to the end-organ damage that accompanies complications of MetS. This transition from compensatory increases in myogenic tone to autoregulatory failure has been observed in the type 2 diabetic Goto-Kakizaki rat, in that exaggerated pressure-induced contraction precedes loss of myogenic responses with aging. Impaired myogenic reactivity contributes to renal injury in the Dahl salt-sensitive rat, whereas enhanced myogenic contractions and preserved renal function are found in the spontaneously hypertensive rat. Similarly, in diabetic patients, loss of myogenic control is associated with the development of retinopathy. Last, Schofield et al. reveals that when essential hypertension is compounded by type 2 diabetes mellitus, myogenic responsiveness weakens in small arteries harvested from subcutaneous fat. The present study is the first to identify Nox1-derived ROS as critical in mediating autoregulatory failure in the setting of severe metabolic disease.

Little investigation has been directed toward understanding the link between ROS and autoregulatory abnormalities in states of metabolic compromise. In one study, transgenic overexpression of SOD, SOD1, prevented the blunting of myogenic reactivity in cerebral vessels that resulted from high glucose exposure before ischemia-reperfusion injury. Similarly, eliminating ROS with antioxidants restored myogenic responses in rats fed a high-sugar diet. Still, less is known with respect to the distinct roles of the Nox isoforms. Herein, we present novel data showing that transgenic loss of Nox1 in db/db mice virtually normalizes myogenic regulation. To our knowledge, the only data providing insight into a potential mechanism by which Nox1 impairs myogenic tone is that published by Howit et al., linking Nox enzymes to the modulation of calcium channel function after acute NO depletion. There are multiple other levels along the cascade of events orchestrating myogenic responses that may be affected by Nox1-derived ROS, the identification thereof is beyond the scope of this study. Nevertheless, our data reveal a causal role of Nox1 in the loss of myogenic regulation in metabolic disease.

It is possible that the profound impact of targeted Nox1 inhibition on microvascular function is, in part, attributable...
to a secondary effect on other enzymatic sources of ROS. Excess ROS by one source can trigger ROS release by another, thereby triggering a cycle of unfettered ROS accumulation. The Nox are increasingly viewed as drivers of this feed-forward cycle. Indeed, uncoupled eNOS is a significant source of vascular O$_2^-$ and cause of NO depletion in the diabetic state, and there exist data implicate Nox1 as a mediator of eNOS uncoupling under such conditions. Specifically, a study performed by Yoon et al$^{12}$ demonstrates that eNOS uncoupling is reversed with genetic deletion of Nox1 or transfection of Nox1 siRNA, but preserved with loss of Nox2, in a model of type 1 diabetes mellitus. Further, mitochondrial enzymes release ROS as by-products of cellular respiration, but contribute to ROS overabundance in pathological states, such as diabetes mellitus. Mitochondria-derived ROS were reduced in endothelial cells after blocking of Nox activation via deletion of the P22^top subunit, as shown by Doughan et al.$^{43}$ Data presented in the current article show that Nox1 deletion attenuates total oxidant load as measured by circulating levels of lipid peroxidation products, providing evidence that the detrimental effects of Nox1 are owing to its synergistic effects with other ROS-producing enzymes.

There are several caveats to the present study that should be noted. First, our model of obesity is the db/db mouse, chosen because it consistently produces metabolic dysfunctions consequent to excess caloric intake and accompanying endothelial dysfunction, much like human obesity. It should be noted, however, that obesity manifests in different individuals for various reasons and risk factors such as elevated low-density lipoprotein cholesterol that are not mimicked in our model may impact application of these findings in other forms of obesity. Second, although we provide functional evidence suggesting that Nox1 contributes to both endothelial and VSMC dysfunction in the setting of metabolic disease, the global knockout approach does not reveal whether endothelial cells or smooth muscle cells are primarily affected or whether both are equally involved. The results of these experiments should provide a substantial basis for cell-specific deletion studies of NOX1 in obesity and other models of metabolic vascular disease.

In summary, this study provides novel data showing that selective deletion of Nox1 is sufficient to reduce oxidant load and restore vasomotor responses of mesenteric arteries in the face of severe metabolic compromise, thus, highlighting Nox1 as critical in mediating the injurious effects of adverse metabolic stimuli on the microvasculature. Loss of hemodynamic control consequent to microvascular dysfunction is an important antecedent to the end-organ damage underlying cardiovascular complications of MetS. Therefore, selective inhibition of Nox1 may be effective in preventing cardiovascular death in MetS, which remains a significant clinical challenge, despite current therapies effective in controlling blood glucose and lipid levels.

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**Disclosures**

None.

**References**


Genetic Deletion of NADPH Oxidase 1 Rescues Microvascular Function in Mice With Metabolic Disease
Jennifer A. Thompson, Sebastian Larion, James D. Mintz, Eric J. Belin de Chantemèle, David J. Fulton and David W. Stepp

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SUPPLEMENTARY MATERIAL

Detailed Methods

Animal model

Female C57BL6 mice heterozygous for the leptin receptor mutation (db+/db-, The Jackson Laboratory, strain 000697) were crossed with male C57BL6 Nox1 knockout mice (The Jackson Laboratory, strain 018787) for two generations to produce double knockout animals. Male mice of the following genotypes generated over the next five generations were studied: H dbWnox1 (Leprdb/+ .Nox1+/y), H dbKnox1 (Leprdb/+ .Nox1−/y), K dbWnox1 (Leprdb/db .Nox1+/y) and K dbKnox1 (Leprdb/db .Nox1−/y). All mice were genotyped with DNA that was isolated and purified from toe clippings obtained at the time of weaning (3 weeks of age) using a DNA extraction kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Gene products were amplified by PCR with Taq DNA polymerase on a Bio-rad C1000 Touch Thermal Cycler and separated on a 2% agarose gel. The following primers were used to genotype the Nox1−/y and Nox1+/y alleles: primer for wild-type Nox1 allele: GGGACAGCTTCCTGCATCCCTCTGT; primer for disrupted Nox1 allele: TCGGATCGAGCGCTCTGAAGTTCCT; common primer: TAGCCTGGCTGTTCCCTCACCCAAA. Genotyping of the leptin receptor mutation was performed with the following primers: forward: CCCAACAGTCCATACATATTAGAAGATTATTTACATTTTGA; reverse: GTCCAAACTGAACCATACATCAACCTAC. Representative PCR products stained with ethidium bromide are shown in Online Figure I. At approximately 12-weeks of age, animals were anaesthetized in an induction chamber (5% isoflurane with 1 L/min O2) and subsequently decapitated with a guillotine. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Augusta University and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Body composition

After weaning biweekly measurements of body weight were recorded. Immediately prior to sacrifice, whole-body fat and lean tissue content were determined by Nuclear Magnetic Resonance (NMR) with a Bruker’s Minispec Body Composition Analyzer LF90ll (Bruker, Billerica, MA). Total visceral fat, comprising the retroperitoneal, epididymal and perirenal depots, was excised and weighed during necropsy.

Metabolic phenotype

After an overnight fast, the clearance of an intraperitoneally injected glucose load (2g glucose/kg body mass) was determined in conscious mice by measuring blood glucose sampled from the surface of the tail at baseline and at 5, 10, 15, 30, 60 and 120 minutes after glucose injection. AUC was calculated after subtracting the baseline. Trunk blood collected immediately after decapitation with a heparinized syringe was analyzed for fasting blood glucose using a glucometer (Zoetis Inc., Kalamazoo, MI) and HbA1c levels with an A1C multi-test AK System kit (PD Diagnostics). Plasma was isolated by centrifugation from the remaining sample and frozen for later analysis. Colorimetric assays (Wako Diagnostics, Mountain View, CA) were used to measure plasma levels of triglycerides, total cholesterol and non-esterified fatty acids, while fasting plasma insulin levels were determined with ELISA (ALPCO, Salem, NH). A sub-set of mice were individually housed in clear respiratory chambers and continuously monitored over 4 days with an open-circuit Oxymax apparatus that is part of a Comprehensive Lab Animal Monitoring System.
(CLAMS) (Columbus Instruments, Columbus, OH), for non-invasive measurement of energy expenditure, locomotor activity and food intake. The volume of O2 consumed and volume of CO2 produced were normalized to body mass and the respiratory exchange ratio was calculated as the ratio of CO2 produced to O2 consumed (VCO2 /VO2).

**Vasodilatory capacity**

At necropsy, the entire intestine was grossly dissected and placed in ice-cold Krebs solution (118 mM NaCl; 25 mM NaHCO3; 11.1 mM d-glucose; 4.71 mM KCl; 2.56 mM CaCl2 · 2H2O; 1.13 mM NaH2PO4 · 2H2O; MgCl2 · 6H2O; 0.114 mM ascorbic acid; 0.0297 mM disodium EDTA). Under a dissecting microscope, 2nd order mesenteric arteries (~150 µm i.d.) carefully cleared of adipose tissue were cannulated and secured with sutures onto two diametrically opposed glass pipettes suspended in the Krebs-filled chamber of a small vessel arteriograph (LSI, Burlington, Vermont). The arteriograph was connected to a camera mounted on a Nikon inverted light microscope (Melville, NY) for visualization of the arterial lumen. Temperature control was afforded by a heating element and thermostat (LSI, Burlington, Vermont). Once mounted, the chamber was aerated with 95% O2 and 5% CO2 and maintained at a temperature of 37°C, while the vessel equilibrated at an intraluminal pressure of 60 mmHg for 30 min. After equilibration, arterial viability was assessed by stimulation with a dose of saturated KCL solution. Following washing and stabilization to baseline, basal internal diameter (BD) was recorded and cumulative concentration-response curves were generated by adding increasing concentrations (10⁻¹¹ – 10⁻⁴ mol/L) of acetylcholine (Ach) or sodium nitroprusside (SNP) after pre-constriction with phenylephrine hydrochloride. In some experiments, vessels were pre-incubated with 10⁻⁴ mol/L L-NAME or 10⁻³ mol/L Tempol for 30 min before vasodilator was added. The percent vasodilation (%VD) was calculated from the pre-constricted internal diameter (PD) and the dilated diameter (DD) at a given concentration such that %VD = [(DD-PD)/(BD-PD)]*100. All drugs were purchased from Sigma-Aldrich Co. (St. Louis, MO).

**Myogenic tone**

Myogenic tone was determined in 2nd order mesenteric arteries mounted in a pressure myograph apparatus. After a 30 min equilibration period, active diameter in the presence of Ca²⁺ was measured at 20 mmHg increments from 20 to 120 mmHg. Subsequently, the vessel bath was replaced with Ca²⁺-free Krebs solution. After a 45 min equilibration period, passive diameter was noted over the same pressure range as that used for active diameter. The percent myogenic tone at a given pressure was calculated from the active diameter (AD) and passive diameters (PD) with the following equation: [(PD – AD)] * 100%.

**Nitrotyrosine immunoreactivity**

Cross-sections of mesenteric arteries were embedded in OCT and slow-frozen while suspended in isopentane cooled to -80°C. Cryosections were dried for 10 min on a 45°C heat block and subsequently post-fixed in AAF fixative for 1 min. Background SNIPER (Biocare Medical, Concord CA) was applied for 8 min to reduce non-specific background staining. Sections were then incubated in rabbit anti-nitrotyrosine antibody (Life Technologies Inc.) overnight at 4°C. After washing in phosphate-buffered saline, slides were incubated with secondary antibody, Alexa Fluor 594 (Life Technologies), at room temperature for 30 min in a covered humidity chamber. Replacement of the primary antibody with PBS was used as negative control. After washing of
the secondary antibody, nuclei were stained with Sytox Green (Life Technologies) for 10 min. Following washing, slides were mounted with Fluorescence VectaShield mounting medium (Vector Laboratories, Burlingame, CA). Slides were stained simultaneously to minimize variation in staining intensity. Sections were imaged on a Zeiss LSM780 confocal microscope and captured at 40x magnification using a camera and software (Zen) for image capture. To account for differing vessel size, fluorescence was quantified in ImageJ using raw integrated density divided by percent area stained.

**Dihydroethidium staining for superoxide**

Dihydroethidium (DHE) dye (Molecular Probes, ThermoFisher Scientific) was reconstituted in anhydrous DMSO and diluted with Krebs to a concentration of 5 µmol/L before use. Mesenteric arteries were isolated and immediately incubated with the diluted DHE at 37°C for 30 min in a dark room, followed by washing with phosphate-buffered saline. Some vessels were simultaneously treated with superoxide dismutase polyethylene glycol (PEG-SOD) for negative control. After washing, vessels were slow-frozen in OCT while suspended in isopentane cooled to -80°C. Cryosections were then mounted with Fluorescence VectaShield (Vector Laboratories, Burlingame, CA) containing DAPI for visualization of nuclei. Images were captured with a Zeiss LSM780 confocal microscope at excitation and emission wavelengths of 518 and 605 nm, respectively.

**Gene expression**

Total RNA was extracted from fast-frozen vessels using Trizol (Invitrogen Life Technologies, Carlsbad CA) and assessed for RNA integrity using 1.2% agarose electrophoresis with ethidium bromide staining. Complementary DNA was synthesized from 1µg purified RNA using oligo(dT) primers and the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad CA). Primers used for analysis (Supplementary Table 1) were chosen among 3 primer pairs designed with BLAST and tested for efficiency and specificity. Quantitative real-time PCR (qRT-PCR) was performed with SYBR green (Bio-Rad Laboratories, Hercules, CA) on a Bio-Rad CFX Connect Realtime PCR detector. Amplification was performed in triplicate at 95°C for 3 min, followed by 40 cycles at 95°C for 15s, 58.5°C for 15s and 72°C for 15s. The expression of individual target genes is reported as fold changes relative to the control group using the 2^ΔΔct method, with ribosomal protein L7 (RPL7) used for normalization. Amplification of Nox1 gene products was performed with Taq DNA polymerase (New England BioLabs) and amplicons visualized on a 1.8% Agarose gel, showing the absence of bands in HK and KK animals.

**Protein expression**

Aliquots of protein extract were added to the NuPAGE lithium dodecyl sulfate (LDS) sample buffer and sample reducing agent (Invitrogen) and heated for 10 min at 85°C. Ten µg of protein was resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred to PVDF Immobilon membranes (Millipore) at 100V for 2 hours. Following transfer, membranes were washed and blocked for 1 hour and then probed with primary antibodies. Following incubation with the secondary antibody, immunoreactivity was visualized by chemiluminescence and densitometry performed.
Statistical analysis

Statistical analyses for metabolic parameters, maximal dilatory responses, immunoreactivity and gene expression were performed using a one-way ANOVA and Bonferroni post hoc test for comparison between the 4 groups. For calculation of the half maximal effective concentration (log EC$_{50}$), concentrations were log-transformed, normalized to percent maximal response and fitted to a four-parameter logistic curve. The pressures at which myogenic tone developed in each of the 4 groups were determined by two-way ANOVA comparing active and passive diameters at each pressure with Bonferroni post-hoc test. The % myogenic response at each pressure was compared between the 4 groups using two-way ANOVA with Bonferroni post-hoc test. All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc.). Values of p < 0.05 were considered statistically significant, and data expressed as mean ± SEM.
<table>
<thead>
<tr>
<th>Gene</th>
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**Online Figure I:** Representative PCR products for genotyping Nox1^{+/y} and Nox1^{−/y}
Online Figure II: Nox1 deletion does not influence the degree of obesity or hyperglycemia in db/db mice. Whole-body percent fat mass (A) and lean body mass (B) were determined by NMR spectroscopy. After an overnight fast, hemoglobin A1c (HbA1c) was measured and glucose tolerance tested by measuring blood glucose with a glucometer in conscious mice at baseline and selected time points after an IP injection of a glucose load (2g glucose/kg body mass). Groups were compared using ANOVA with a Bonferonni post hoc test. (n = 9-15/group).

*** p < 0.001 KW (Lepr db/db .Nox1 y/y) vs. HW (Lepr db/+ .Nox1 y/y) or KK (Lepr db/db .Nox1 y/y) vs. HK (Lepr db/+ .Nox1 y/y)
Online Figure III: Improvement of myogenic responsiveness in db/db mice with Nox1 deletion is independent of passive wall mechanics. Percentage of myogenic tone (A) and passive diameter in Ca$^{2+}$-free Krebs over incremental increases in pressure (B) in mesenteric arteries from lean HW (Lepr$^{db/+}$.Nox1$^{+/y}$); HK (Lepr$^{db/+}$.Nox1$^{-/-y}$) and obese KW (Lepr$^{db/db}$.Nox1$^{+/y}$); KK (Lepr$^{db/db}$.Nox1$^{-/-y}$) mice. Groups were compared using two-way ANOVA with a Bonferroni post hoc test. (n = 5-7/group). * p < 0.05 KW vs. HW