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Inhibition of NOX1 Mitigates Blood Pressure Increases in Elastin Insufficiency

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Abstract

Elastin (ELN) insufficiency leads to the cardiovascular hallmarks of the contiguous gene deletion disorder, Williams–Beuren syndrome, including hypertension and vascular stiffness. Previous studies showed that Williams–Beuren syndrome deletions, which extended to include the NCF1 gene, were associated with lower blood pressure (BP) and reduced vascular stiffness. NCF1 encodes for p47phox, the regulatory component of the NOX1 NADPH oxidase complex that generates reactive oxygen species (ROS) in the vascular wall. Dihydroethidium and 8-hydroxyguanosine staining of mouse aortas confirmed that Eln heterozygotes (Eln+/−) had greater ROS levels than the wild-types (Eln+/+), a finding that was negated in vessels cultured without hemodynamic stressors. To analyze the Nox effect on ELN insufficiency, we used both genetic and chemical manipulations. Both Ncf1 haploinsufficiency (Ncf1+/−) and Nox1 insufficiency (Nox1−/−) decreased oxidative stress and systolic BP in Eln−/− without modifying vascular structure. Chronic treatment with apocynin, a p47phox inhibitor, lowered systolic BP in Eln−/− but had no impact on Eln+/+ controls. In vivo dosing with phenylephrine (PE) produced an augmented BP response in Eln−/− relative to Eln+/+, and genetic modifications or drug-based interventions that lower Nox1 expression reduced the hypercontractile response to PE in Eln−/− mice to Eln+/+ levels. These results indicate that the mechanical and structural differences caused by ELN insufficiency leading to oscillatory flow can perpetuate oxidative stress conditions, which are linked to hypertension, and that by lowering the Nox1-mediated capacity for vascular ROS production, BP differences can be normalized.
Introduction

Vascular reactive oxygen species (ROS) are signaling molecules that play an important physiological role in controlling endothelial function and vascular tone, factors that can affect vessel mechanics associated with arterial stiffening. Arterial stiffness is a hallmark of hypertension, and a common feature of aging. People with Williams–Beuren syndrome (Williams syndrome, WS) have a congenital vasculopathy as a result of a deletion of 25–27 genes from chromosome 7, including the elastin (ELN) gene. ELN encodes for the extracellular matrix protein, ELN, an important component of the vascular wall that provides recoil to elastic vessels. Arteries with reduced ELN content are less compliant and develop structural modifications that include increased numbers of smooth muscle and elastic lamellae. Consequently, individuals with ELN insufficiency have developmentally, rather than environmentally, induced vascular stiffness. They also have anatomical differences in branching and arterial tortuosity. Recent work by our group and others revealed that deletions, which reduce copy number for NCF1, are associated with lower risk of hypertension than those deletions in which the individual retained both copies. Remarkably, an unbiased genetic screen for modifiers of ELN-mediated vascular disease using quantitative trait locus analysis in more than 600 Eln−/− mice also suggested Ncf1 as a likely modifier.

The Ncf1 gene encodes for p47phox, the regulatory subunit of NOX1 and NOX2 NADPH oxidase complexes that generate ROS in the vascular wall and have been implicated in hypertension and vascular disease. Each NOX complex is made up of 6 subunits including a catalytic domain Nox1 (Nox1, gp91-2) in the NOX1 complex and Nox2 (Cybb, gb91phox) in the NOX2 complex. These complexes catalyze the conversion of NADPH and oxygen to NADP−, superoxide, and hydrogen. Nox1 and Nox2 are differentially expressed and vary in ROS-generating activity. The NOX1 complex is produced by endothelial and smooth muscle cells (SMCs), whereas NOX2 is made by endothelial cells, adventitial fibroblasts, and inflammatory cells. ELN insufficiency outcomes are not thought to be primarily mediated by inflammation, making a NOX1-mediated mechanism more attractive. Reduction in blood pressure (BP) has been seen for Nox1−/− mice in angiotensin infusion-induced hypertension models; however, the Nox1 complex has not been studied in genetic, or specifically structural, models of hypertension. Eln−/−
mice have stiff, tortuous, and narrow vasculature and recapitulate much of the human disease of WS, manifesting hypertension, arterial stiffness, and having increased numbers of elastic lamellae in their arteries. Given that the WS vascular phenotype appears to be modified by NC1, it is important to understand how this complex, and the ROS it produces, modifies disease severity. Determining whether the NOX1 complex can be implicated in the vascular pathology associated with WS and ELN insufficiency may guide therapy for patients with this condition.

Materials and Methods

Study Approval and Animal Lines

Experiments were approved by the animal studies committees at Washington University School of Medicine or the National Heart, Lung and Blood Institute of the National Institutes of Health. All animals studied in a particular experiment were housed similarly in accordance with institutional guidelines. Littermates were used when available. Not all studies were performed on all animals.

Eln**+/−** mice were first described by Li et al. This animal was additionally backcrossed into C57Bl6/J, and Single nucleotide polymorphism (SNP) genotyping was performed to assure a majority C57Bl6/J background.9 To generate the two mutant lines, the Eln**+/−** was crossed to Ncf1**+/−** (B6.Cg-Ncf1tm1J/J; JAX #004742), Nox1**−/−** (B6.129X1-Nox1tm1Kkr/J; JAX #018787), or Nox2**−/−** (Nox2 B6.129S-Cybbtm1Din/J; JAX #002365). Because Nox2 and Nox1 are on the X chromosome, all studies were performed in male mice to avoid the complex impact of lyonization.

Organ Culture

Mice were sacrificed as above. Ascending aortas (Eln**+/−** and Eln**−/−**) were dissected free, rinsed with PBS, and cut into 2 pieces using surgical scissors. The 2 halves were cultured for 16 h at 37°C, 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM, Gibco 10566032) supplemented with 10% fetal bovine serum (Cytiva SH30071.03H), 1 mM nonessential amino acid (Gibco 11140050), and 1 mM sodium pyruvate (Gibco 11360070). Before evaluation with DHE as defined above, 1 of the 2 pieces of aorta was treated with a ROS inducer Antimycin A (AMA 20 mM, Abcam, San Francisco, ab141994) for 60 min at 37°C in DMEM media in a CO2 hood to confirm viability and the retained ability to produce ROS after culture. After completion of treatments, aortas were flash frozen in OCT, sectioned, and stained with DHE as described above. Fresh (real-time) aortas were processed on the day of DHE treatment for comparison to the organ culture samples. DHE was quantified as above.

Vascular Ex vivo Viability

Mice were sacrificed as above. Aortas were then flushed with physiologic salt solution (PSS; NaCl 130 mM, KCl 4.7 mM, KH2PO4 1.18 mM, MgSO4·7H2O 1.17 mM, NaHCO3 14.9 mM, dextrose 5.5 mM, EDTA 0.026 mM, CaCl2 1.6 mM, pH 7.4—all components from Sigma Aldrich, St Louis, MO), via the left ventricle, and a single 2-mm segment of ascending aorta was isolated. Aortic segments were either immediately mounted on a wire myograph (Model 620M, Danish Myotechnology, Denmark) in cold PSS and equilibrated at 37°C for 20 min with continuous oxygenation (95% O2, 5% CO2) or placed in tissue culture media for 16 h (as in the organ culture section above) and mounted on the myograph after the no-flow period. Viability was confirmed with potassium PSS (KPSS; NaCl 74.7 mM, KCl...
and placed in prone position (see Halabi et al. for implantation). Mice were anesthetized with 1.5% isoflurane (Isoflurane florane, Baxter, Louis, MO). To implant the osmotic micropumps, mice were subcutaneously implanted with ALZET microosmotic pumps (model 1004; DURECT, Cupertino, CA) as previously described. Pumps delivered a continuous dose of 50% dimethyl sulfoxide (DMSO) in water, or 3/mg/kg/day of apocynin dissolved in 50% DMSO (n = 5 each). All reagents were purchased from Sigma-Aldrich (St Louis, MO). To implant the osmotic micropumps, mice were anesthetized with 1.5% isoflurane (Isoflurane florane, Baxter), and placed in prone position (see Halabi et al. for implantation procedure). As each osmotic pump is designed to deliver the agent for 28 days, on day 28, the first pump was removed and replaced by a new pump using the same surgical technique. Physiological studies were done on day 56, when mice were euthanized. At replacement and death, pumps were removed, and delivery of the drug was confirmed by assessing the volume of solution remaining in each pump.

Chronic Apocynin Treatment

At 4–6 weeks of age, male Eln−/− and Eln+/− mice were subcutaneously implanted with ALZET microosmotic pumps (model 1004; DURECT, Cupertino, CA) as previously described. Pumps delivered a continuous dose of 50% dimethyl sulfoxide (DMSO) in water, or 3/mg/kg/day of apocynin dissolved in 50% DMSO (n = 5 each). All reagents were purchased from Sigma-Aldrich (St Louis, MO). To implant the osmotic micropumps, mice were anesthetized with 1.5% isoflurane (Isoflurane florane, Baxter), and placed in prone position (see Halabi et al. for implantation procedure). As each osmotic pump is designed to deliver the agent for 28 days, on day 28, the first pump was removed and replaced by a new pump using the same surgical technique. Physiological studies were done on day 56, when mice were euthanized. At replacement and death, pumps were removed, and delivery of the drug was confirmed by assessing the volume of solution remaining in each pump.

Acute Apocynin Treatment

To determine the effect of acute apocynin treatment, 3-month-old male Eln−/− mice were treated with 3 doses of 12 mg/kg apocynin. Baseline BP was measured by placing a Millar pressure-transducing catheter (Millar Instruments, Houston, TX) in the right carotid artery under 1.5% isoflurane, as previously described. The left jugular vein was then isolated and cannulated with PE-10 tubing (Instech, Plymouth Meeting, PA) preloaded with either 3 20 μL doses of vehicle (20% DMSO) or 3 20 μL doses of 12 mg/kg drug, apocynin. Isoflurane was decreased to 1% and a baseline BP established. Bolus injections of either drug or vehicle were then administered allowing BP to recover between doses. Systolic BP (SBP) was measured, preinjection and 30 s postinjection. SBP changes resulting from either apocynin or vehicle injection are represented as SBP percent reduction which was calculated by: ([SBP at 30 s – baseline SBP before injection]/baseline SBP before injection) × 100. SBP reduction was compared between the acute apocynin treatment group and vehicle/control.

SBP and Heart Rate Measurement

The narrow diameter and tortuous nature of Eln−/− carotid arteries limit the placement and continued function of indwelling radionucleotemporellers. Consequently, this study uses a smaller, acutely placed angiocatheter to measure BP. To achieve this measurement, mice were restrained on a heated holder to maintain body temperature, anesthesia induced with 2.5% inhaled isoflurane, then reduced to 1.5% to achieve a level plane of anesthesia. A 1.4F pressure catheter (Ncut 1/14 experiments), model SPR671 or 1.0F (Ncut 1/12 experiments), model SPR1000, Millar Instruments, Houston, TX) was introduced into the right carotid artery and advanced to the ascending aorta. Each animal was allowed to acclimate for 5 min, at which time isoflurane was reduced to 1%. Pressures were recorded using Chart 5 software (ADInstruments, Colorado Springs, CO) and analyzed from 1 to 4 min post reduction. Animals were monitored for discomfort or over-sedation.

Assessment of Aortic Stiffness (Pulse-Wave Velocity)

Aortic-arch pulse-wave velocity (PWV) was performed as previously described using a modification of the transit-time method on a Vevo 770 ultrasound system with a MS-400 transducer (VisualSonics Inc., Toronto, Canada). In some cases (typically the Eln−/− mice, due to their longer and more tortuous aortas), vessels could not be imaged in a single plane. For these animals, the pulse-wave Doppler was performed in 2 segments. First, a proximal measurement was taken near the aortic valve and then the probe was quickly moved to the adjacent descending aorta without changing the imaging plane. The curvilinear distance between the proximal and distal points of the aortic velocity interrogation (D2–D1) was measured, as well as the time delay between the onset of flow velocity in the distal and proximal portions of the aorta (T2–T1) relative to the simultaneously recorded electrocardiogram signal. PWV = (D2–D1)/(T2–T1).

Histological Analysis

Mice were flushed with PBS via the left ventricle and then, using a syringe pump (Model 200, Cole-Parmer, Vernon Hills, IL), the vasculature was fixed with 10% buffered formalin (Sigma) at a constant flow rate of 1.5 mL/min. The ascending aorta was excised, placed in 10% formalin overnight, transferred to 70% ethanol, embedded in paraffin, and then serially sectioned at 5 μm. For mesenteric samples, a segment of the first-order mesenteric artery branch was freed of fat and connective tissue under a dissection microscope before being processed. Verhoff-van Gieson (VVG) stain was used to assess ELN, whereas Masson’s trichrome stain was used to assess collagen. Movat stain can simultaneously detect collagen and ELN. All slides were performed according to the product instructions. Stained slides were scanned using a Nanozoom RS digital slide scanner (Hamamatsu) and analyzed using NDP Viewer software (Hamamatsu Photonics, Hamamatsu, Japan). Wall thickness, lumen area, and lamellar number were determined using methods previously described.

Pressure–Diameter Testing

Ascending aortas were dissected from mice post euthanasia. Vessels were mounted on a pressure arteriograph (Danish Myotechnology, Copenhagen, Denmark) in PSS at 37°C. Vessels were then pressurized and longitudinally stretched 3 times to in vivo length before data capture. Arteries were transilluminated under a microscope connected to a camera and computerized measurement system (Myoview, Danish Myotechnology). Intravascular pressure was increased from 0 to 175 mmHg in 25 mmHg steps. At each step, the outer diameter of the vessel was measured and manually recorded. Functional distensibility (FD) was calculated from the pressure diameter curves as the...
distensibility at average working pressure of the vessel: 
FD = (ODSBP − ODDBP)/(ODBP − DBP), where OD = outer diameter at either SBP or DBP. For pressures that fall between diameter measurement intervals, the working diameter at the specific pressure was calculated using the known slope of the line between the preceding and subsequent pressure for that animal.

Measurement of Oxidative Modification of DNA: 8-Hydroxyguanosine Immunohistochemistry

Ascending aorta and first-order branch mesenteric arteries were dissected and fixed in 10% buffered formalin overnight, before transfer to 70% ethanol. Fixed samples were then embedded in paraffin and 10-µm-thick sections were cut and placed on glass slides. Before immunofluorescence analysis, sections were deparaffinized, rehydrated, and boiled in 10 mM citrate buffer (pH 6.0) for 10 min for antigen retrieval. Sections were then blocked for 1 h in 10% normal donkey serum in PBS, and subsequently incubated with goat polyclonal antibody against 8-hydroxyguanosine (8OH, a DNA oxidative marker) (50 µL, 1:200, Abcam, San Francisco, ab10802) in 2% donkey serum in PBS overnight in a humidified chamber at 4°C. After 3 washes in PBS, sections were incubated with Alexa-fluor-594 donkey anti-goat antibody (2 mg/mL, 1:500, Abcam, San Francisco, ab150132) for 60 min at room temperature in the dark. Sections were then rinsed in PBS and sealed with mounting medium containing DAPI (Vectashield, Burlingame, H-1200). Images were captured from each section using a Zeiss 780 inverted confocal microscope at ×40 magnification (ExcEm = 594 nm/588–695 nm). To quantify immunofluorescence, each aortic sample was imaged in 4 quadrants and quantifications were done in each quadrant and averaged together. Mesenteric artery samples were imaged and quantified using a single image. Staining intensity was measured in the endothelium and media for aortic and mesenteric artery samples. This region was defined by manually outlining the area within and the external elastic lamina and using the lumen as the internal boundary. Average corrected fluorescence intensity of the 8OH was measured in defined regions of interest using ImageJ software (National Institutes of Health, Bethesda, MD) and data are expressed as fluorescence signal per area, as previously described.25,26 To account for variability, the imaging was done using identical conditions of magnification, illumination, and exposure time.

Response to Vasoactive Medications

To evaluate the impact of various vasoactive drugs on animal physiology, a central venous catheter (PE-10 tubing, Intrtech, Plymouth Meeting, PA) was placed in the jugular vein of Eln−/−, Eln+/−, and Eln−/+; Nox1−/− mice sedated with isoflurane. An arterial pressure catheter (Millar Instruments, Houston, TX) was also placed as noted above to measure continuous pressure and heart rate. To assess physiological response to vasoconstriction, baseline BP was established, and mice were then injected with increasing bolus concentrations (10 µg/kg, 100 µg/kg, 1 mg/kg) of PE diluted in PBS. In subsequent experiments, Eln−/− and Eln+/− mice were injected with either tempol (Sigma, St Louis, 176141, 50 mg/kg), PE (100 µg/kg), or consecutive doses of tempol/PE (50 mg/kg tempol and 100 µg/kg of PE). Similarly, to assess physiological response to acetylcholine, baseline BP was established, and mice were then injected with increasing bolus concentrations (1, 10, and 100 µg/kg) of Ach (Sigma, St Louis, MO) diluted in PBS. BP and heart rate were monitored throughout the procedure, and the animal’s BP was allowed to return to baseline before administration of the next dose. Response to vasoactive drugs was calculated as percent change in SBP: ([SBP − SBPi]/SBPi) × 100, where SBPi is the initial SBP before injection of the drug(s), and SBP maximal deviation from baseline after drug administration(s) before return to baseline. Each drug combination (PE alone, tempol alone and tempol plus PE, or Ach alone in the Ach experiment) was given twice during the 50-min experiment and the mean value of the 2 doses was used for statistical assessment.

Statistics

All statistics were performed using Prism 7.0 statistical software. BPs, FD, histological quantification, and PWV were compared in Eln+/− and untreated and treated (drug or Nox mutant) 3-month-old Eln+/− mice using one-way analysis of variance (ANOVA). For pressure–diameter, distensibility and vasoactive drug testing, two-way ANOVA with repeated measures were used. When appropriate, multiple comparisons testing were performed, and test type is reported in the legend for each figure. Multiple t-tests were performed for the acute apocynin and tempol experiments with an False discovery rate (FDR) of 0.05 used for discovery phase.

Results

Increased Oxidative Stress in Eln+/− Relative to Eln+/− Mouse Aorta

Oxidative stress is known to be implicated in the pathophysiology of hypertension.27 Recent studies have shown a correlation between ELN insufficiency and increased ROS production, suggesting that oxidative stress may play a role in hypertension in the Eln+/− mouse.8,9,28 To confirm the effect of ELN insufficiency on vascular ROS production, we stained sections of Eln+/− and Eln−/− aorta with the superoxide sensitive stain, DHE. In the presence of superoxide, DHE is oxidized to a fluorescent adduct that intercalates into DNA, producing a nuclear staining pattern. The degree of staining correlates with superoxide production by the tissue. In agreement with previously published studies,9,29,30 DHE fluorescence was visibly higher in the endothelium/media of the Eln+/− aorta relative to the Eln−/− (Figure 1A and B).

Eln+/− Vascular Anomalies Result in Turbulent Flow: Loss of Flow Reduces ROS

It has previously been suggested that hemodynamic forces, such as shear stress resulting from turbulent blood flow, can either directly or indirectly activate vascular NADPH oxidase-derived ROS production.30–33 Previous reports of the Eln+/−/− mouse have noted a decrease in vascular caliber and an increase in wall thickness with increased small and large vessel tortuosity.14–16 To assess for changes in flow parameters in this animal model, we made use of high-resolution, high-frame rate 2D echocardiogram to visualize flow in the aortic arch. The images show fine anatomical details of the Eln+/− arch (Figure 2A) and demonstrate marked tortuosity of the Eln+/− aorta (Figure 2B), with elongation of the ascending aorta and abnormal orientation of its proximal branches. Video S1 (Eln+/−) shows normal biphasic pulsatile laminar flow with predominant systolic but also lower velocity diastolic antegrade flow. Alternatively, Video S2 (Eln−/−) shows rapid cessation of antegrade flow in end-systole with short duration retrograde flow in early diastole and static flow during midlate diastole,
implying turbulence and oscillatory flow. Doppler interrogation of the aortic valve showed no aortic regurgitation. Oscillatory (disturbed) flow has been linked to increased ROS production.32,34,35

To determine whether hemodynamic differences present in Eln−/− mice are responsible for increased ROS production, we cultured sections of ascending aortas from these mice for 16 h under flow-free conditions before evaluating for ROS. When cultured in the absence of flow for 16 h, both Eln+/+ and Eln−/− aortas revealed low-level superoxide production and there was no obvious difference between the 2 (Figure 1C and D). The average DHE intensity per pixel in each endothelial and medial nucleus was determined (Figure 1E). Two-way ANOVA found a significant Eln effect (P < 0.05), a significant treatment effect (P < 0.01), and a borderline interactive effect (P = 0.05). DHE fluorescence intensity was found to be significantly greater in the real time Eln−/− group relative to all other subgroups (P < 0.05 or better). Antimycin A added after the 16-h culture period to a second section of aortic tissue confirmed that the cells from the cultured vessels were capable of producing ROS at similar levels (Figure S2A–C). Likewise, real time and flow-free vessels were evaluated for response to PE on a ring myograph. All vessels

![Figure 1. Hemodynamic Forces Influence ROS Production in Eln−/−.](image)

Figure 1. Hemodynamic Forces Influence ROS Production in Eln−/−. (A) [Eln+/+] and (B) [Eln−/−] Confocal images of aortic tissue stained with DHE immediately after dissection. (C) [Eln+/+] and (D) [Eln−/−] Confocal images of aortic tissue sections stained with DHE after a 16-h incubation in culture media. Average DHE mean fluorescence intensity per nuclei (E). Tukey’s multiple comparisons test: *P < 0.05 and **P < 0.01 between indicated groups.

![Figure 2. High-resolution, High-frame Rate 2D Echocardiogram of the Eln+/+ and Eln−/− Aortic Arch.](image)

Figure 2. High-resolution, High-frame Rate 2D Echocardiogram of the Eln+/+ and Eln−/− Aortic Arch. Still images from the ECG-gated Kilohertz Visualization image acquisition show a normal appearing aortic arch in the Eln+/+ (A) and marked tortuosity of the Eln−/− (B) aorta, with elongation of the ascending portion and abnormal orientation of the proximal branches. The bright regions in the arch lumen represent jets of blood. Videos S1 (Eln+/+) and S2 (Eln−/−) show the movement of blood through the arch, highlighting the turbulence apparent in the Eln−/−.
showed the expected PE dose effect (Figure S2D; \(P < 0.01\)) and there was no contraction difference between the real time and flow-free vessels.

**Chronic, but not Acute, Apocynin Treatment Lowers SBP in Eln\(^{-/-}\) Mice**

To determine the source of ROS in Eln\(^{-/-}\) vessels, we treated mice with apocynin, an NADPH oxidase inhibitor. Apocynin blocks the phosphorylation of p47phox, the regulatory subunit of the NOX1 and 2 and thus prevents their activation. Chronically inhibiting NOX activation through long-term administration of apocynin (3 mg/kg/day from weaning to 3 months of life) leads to lower SBP in Eln\(^{-/-}\). Two-way ANOVA analysis of the chronic apocynin-treated groups showed an Eln effect (\(P < 0.001\)) and an interactive effect (\(P < 0.05\)), with a significantly lower SBP in the Eln\(^{-/-}\) treated with apocynin than in carrier-treated Eln\(^{-/-}\) (\(P < 0.05\)), whereas the Eln\(^{-/-}\) was unaffected by chronic apocynin treatment (Figure 3A). Because of the mechanism of action, acute treatment with apocynin would be expected to block the production of new ROS by NOX but not scaveng/reduce existing ROS. Unlike the chronic treatments, 3 consecutive 20 \(\mu\)L doses of 12 mg/kg of apocynin over 30 min, in 3-month-old mice yielded no change in SBP in Eln\(^{-/-}\) by unpaired \(t\)-test (Figure 3B).

**Decreased Ncf1 expression reduces BP and improves vascular stiffness in Eln\(^{-/-}\) mice**

Previous studies in humans and mice with the WS deletion showed that larger deletions that included the nearby NCF1 gene (individuals were left with 1 rather than 2 functional copies of the gene) were associated with lower BP.\(^7,8,28\) NCF1 is the gene that encodes for p47phox, the molecule inhibited by apocynin. Focusing on the specific interaction between Eln and Ncf1 haploinsufficiency, we measured BP and vascular stiffness in \(~3\)-month-old progeny from an Eln\(^{-/-}\) \(\times\) Ncf1\(^{-/-}\) cross. Invasive BP monitoring (Figure 4A) showed an expected ELN effect on SBP (\(P < 0.0001\), 2-way ANOVA), an Ncf1 effect (\(P < 0.05\)), and an interactive effect (\(P < 0.05\)). As expected, a significant difference between Eln\(^{-/-}\) and Eln\(^{-/-}\) mice, regardless of Ncf1 genotype, was noted (\(P < 0.0001\)). Just as inhibition of p47phox by apocynin reduced SBP in the Eln\(^{-/-}\) mice, Ncf1 heterozygosity also led to a significant decrease in SBP in Eln\(^{-/-}\) (\(P < 0.05\)), while it had no effect on Eln\(^{-/-}\) BP. In addition to BP differences, previous studies also showed that patients with the WS deletion that included NCF1 also had reduced PWV, an in vivo measure of vascular stiffness. Similarly, PWV evaluation in mice revealed a significant Eln effect (\(P < 0.0001\), 2-way ANOVA) with Eln\(^{-/-}\) mice having higher PWV and stiffer aortas (Figure 4B). The 2-way ANOVA also showed an Ncf1 effect (\(P < 0.05\)) as well as an interactive one (\(P < 0.01\)). A robust reduction in PWV was found in Eln\(^{-/-}\); Ncf1\(^{-/-}\) mice as compared with those Eln\(^{-/-}\); Ncf1\(^{-/-}\) (\(P < 0.01\)), whereas Ncf1 dosage had no significant impact on PWV in Eln\(^{-/-}\) mice.

**Biomechanical Properties and ELN Composition of Eln\(^{-/-}\); Aortas are not Modified by Ncf1 Expression**

Aortic tissue was assessed using VVG (ELN) and Masson’s Trichrome (collagen) stains to determine the impact of Ncf1 dosage on vessel structure (Figure 5A–H). No obvious differences in collagen composition (blue) were noted between Eln\(^{-/-}\) and Ncf1\(^{-/-}\) samples, regardless of Ncf1 mutant status (Figure 5E–H). Lamellar number is higher in Eln\(^{-/-}\) ascending aorta (\(P < 0.0001\), 2-way ANOVA), this difference is not impacted by Ncf1 genotype (Figure 5I). Neither Eln nor Ncf1 genotype had an effect on medial area (Figure 5J). To investigate how Ncf1 insufficiency impacts stiffness, we evaluated the biomechanical properties of the aorta by generating pressure–diameter curves (Figure 5K). Although the test showed the expected difference between Eln\(^{-/-}\) and Eln\(^{-/-}\) mice (2-way ANOVA, genotype effect \(P < 0.0001\)), with Eln\(^{-/-}\) mice being narrower and stiffer, the addition of Ncf1 insufficiency did not influence the pressure–diameter relationship in either ELN genotype.

**Improved distensibility in Eln\(^{-/-}\); Ncf1\(^{-/-}\) aorta**

Medications and conditions that lower SBP have the potential to alter functional stiffness by shifting the working pressure of a vessel to a more compliant portion of the pressure–diameter curve.\(^6,27,36\) Consequently, we hypothesized that lower SBP in the Eln\(^{-/-}\); Ncf1\(^{-/-}\) may have the same effect. To test this, we calculated FD for each animal using working BP (Figure 5L). FD is a measure of the change in diameter a vessel sees when measured at its systolic and diastolic BP, normalized to the change in pressure (see “Materials and Methods” for full description). A 2-way ANOVA found in FD is significantly lower in the Eln\(^{-/-}\) mouse relative to Eln\(^{-/-}\) (Eln effect <0.0001, Ncf1 effect \(P < 0.001\), and interactive effect <0.0001), with a significant difference between the Eln\(^{-/-}\) groups depending on Ncf1 genotype (\(P < 0.0001\)). However, Eln\(^{-/-}\); Ncf1\(^{-/-}\) mice have an FD that is more similar to
Eln\(^{+/+}\)/; Ncf1\(^{+/+}\) mice. Because Eln\(^{+/+}\)/; Ncf1\(^{+/+}\) mice experience no SBP reduction with the addition of Ncf1 heterozygosity, the Eln\(^{+/+}\)/; Ncf1\(^{+/+}\) F2 remains similar to the Eln\(^{+/+}\)/; Ncf1\(^{+/+}\).

**NOX1 plays the Dominant Role in Modulating Vascular Phenotypes of Eln\(^{+/+}\)**

Ncf1\(^{p47phox}\) is a regulatory subunit of NOX1 and NOX2 NADPH oxidase complexes. The NOX1 complex is detectable in endothelial and SMCs and NOX2 in endothelial and inflammatory cells associated with the adventitia. Both genes are present on the X chromosome in mice and humans and both have been implicated in BP differences in angiotensin infusion models. However, none of them has been evaluated in genetic/developmental models of hypertension, such as the mental models of hypertension, such as the...
To determine if ROS play a role in causing the significant response difference at 100 μg/kg PE, we used a known ROS scavenger, tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) in Eln+/+ and Eln−/−. Tempol was injected alone 30 s prior to the 100 μg/kg PE injection (Figure 9B). SBP change from baseline after 50 mg/kg of Tempol was significantly larger in the Eln−/− relative to the Eln+/+ (P < 0.01). As shown previously, the Eln−/− had an augmented response to the 100 μg/kg PE dose (P < 0.0001) when no tempol was administered; however, injecting tempol prior to PE removed the difference in percent SBP change between the Eln+/+ and Eln−/−. The response to PE in the presence of tempol was found to be similar in the Eln+/+ and Eln−/−.

Discussion

ELN insufficiency produces blood vessels with reduced lumen size, tortuosity, and altered vascular mechanics. People with WS have a contiguous deletion on chromosome 7 that removes the ELN gene in combination with approximately 24 other coding genes. In some cases, the deletion extends to include the NCF1 gene on the telomeric end of the typical deletion. Patients with deletions that cover this gene, and hence retain only 1 functional copy of NCF1, have lower BP and PWV, a measure of vascular stiffness, than those with 2 copies. To determine if ROS play a role in causing the significant response difference at 100 μg/kg PE, we used a known ROS scavenger, tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) in Eln+/+ and Eln−/−. Tempol was injected alone 30 s prior to the 100 μg/kg PE injection (Figure 9B). SBP change from baseline after 50 mg/kg of Tempol was significantly larger in the Eln−/− relative to the Eln+/+ (P < 0.01). As shown previously, the Eln−/− had an augmented response to the 100 μg/kg PE dose (P < 0.0001) when no tempol was administered; however, injecting tempol prior to PE removed the difference in percent SBP change between the Eln+/+ and Eln−/−. The response to PE in the presence of tempol was found to be similar in the Eln+/+ and Eln−/−.

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Discussion

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Much of the work in ROS-mediated hypertension published to date has focused on the ROS produced by inflammatory cells in the adventitia and perivascular fat in response to acute angiotensin infusion. The genetic model here is distinct from the acute induction of hypertension used in those experiments. In ELN insufficiency, the vascular defect is chronic as blood vessels exhibit obvious structural differences early in the postnatal period, with higher BPs noted by as early as postnatal day 1. Consequently, in ELN insufficiency, there is no abrupt physiological change prompting acute immune response at the age we are studying, rather the BP differences are chronic and likely mediated by other mechanisms.

In this study, we further drill down on the hypertension phenotype in this genetic model by reducing the potential genetic interaction from the larger WS deletion to a specific interaction between Eln and Ncf1. Although a more directly quantitative...
method may have been improved our ability to measure tissue ROS, our 2 semi-quantitative methods confirmed evidence of increased ROS in ELN insufficient tissues, similar to previously published findings. In addition, our studies also show that ROS are not generated in excess the absence of flow. Oscillatory shear stress is a known inducer of ROS production and the anatomical differences associated with ELN insufficiency (narrow, stiff arteries with abnormal branching patterns) predispose vessels to increased shear as evidenced by the turbulent aortic arch flow detected in our high-resolution, high-frame rate 2D echocardiogram studies. Consequently, it is the abnormal vascular anatomy itself that predisposes to increased ROS and thus leads to hypertension.

Although additional computational or in vivo modeling (partial carotid ligation) could be done to further evaluate the impact of shear, the role of increased ROS in ELN insufficiency mediated vasculopathy is validated by the finding that decreasing Ncf1 function by genetic (Ncf1<sup>−/−</sup>) or chemical (chronic apocynin) means fails to reduce the BP in Eln<sup>−/−</sup> mice have BP that is equivalent to that of Eln<sup>−/−</sup>. Consequently, it is possible that the increased pathogenicity could be related to the higher number of cells impacted by its loss (endothelial and SMC) or because of differences in regulation or basal activity of the 2 complexes. The decreased impact of Nox2 insufficiency may be related to the chronic nature of the condition. Most of the studies on this mutant have implicated T-cell activity in the pathogenesis. The inflammatory response may be more relevant to the acute hypertension models than chronic deficiency may be related to the chronic nature of the condition. Most of the studies on this mutant have implicated T-cell activity in the pathogenesis. The inflammatory response may be more relevant to the acute hypertension models than chronic development/structural ones such as the Eln<sup>−/−</sup> mouse.

Previous investigations of Nox1 have suggested that it functions in long-term regulation of BP by altering the composition of the vascular extracellular matrix. In our assessment, although ROS differences could be detected in both large and small Eln<sup>−/−</sup> vessels, no obvious differences in matrix

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**Figure 6.** Nox1 deficiency reduces SBP and oxidative stress in Eln<sup>−/−</sup>. SBP data for (A) Eln<sup>−/−</sup> × Nox2<sup>−/−</sup> and (C) Eln<sup>−/−</sup> × Nox1<sup>−/−</sup> progeny. Tukey’s multiple comparisons test: *P < 0.01 and **P < 0.001, ***P < 0.0001 between indicated groups. Nox2 (B) and Nox1 (D) do not alter the pressure-diameter relationship in Eln<sup>−/−</sup> or Eln<sup>−/−</sup> aorta. Tukey’s multiple comparison (B) *P < 0.05, **P < 0.01, and ***P < 0.0001 for comparisons of Eln<sup>−/−</sup> versus Eln<sup>−/−</sup> comparisons regardless of Ncf genotype. (D) *P < 0.05, **P < 0.01, and ***P < 0.0001 for comparisons of Eln<sup>−/−</sup> versus Eln<sup>−/−</sup> comparisons regardless of Ncf genotype. *P < 0.01 for comparison between Eln<sup>−/−</sup>, Nox1<sup>−/−</sup> versus Eln<sup>−/−</sup>, Nox1<sup>−/−</sup> and Eln<sup>−/−</sup>, Nox1<sup>−/−</sup> versus Eln<sup>−/−</sup>, Nox1<sup>−/−</sup>, **P < 0.01 for comparison between Eln<sup>−/−</sup>, Nox1<sup>−/−</sup> versus Eln<sup>−/−</sup>, Nox1<sup>−/−</sup>.
composition were detected histologically. Likewise, pressure diameter curves were not altered in Eln\textsuperscript{+/+}; Ncf1\textsuperscript{-/-}, Eln\textsuperscript{+-}; Nox1\textsuperscript{-/-}, or Eln\textsuperscript{+-}; Nox2\textsuperscript{-/-} mutants. Consequently, previous differences noted in arterial stiffness in WS patients with reduced dosage of NCF1\textsuperscript{+} are likely secondary to the change in BP which alters the FD by moving the vessel to a more compliant portion of its pressure diameter curve.

Instead, we see that Eln\textsuperscript{+-} mice have more dramatically increased BP in response to vasoconstrictors like PE. This general finding has been described previously by Osei-Owusu et al., but our studies carry the finding further, showing that Eln\textsuperscript{+-}; Nox\textsuperscript{-/-} mutants no longer have this amplified response. Tempol, a ROS scavenger, mitigates the response. Interestingly, treatment with apocynin, a drug that stops production of new ROS rather than scavenges them, has no effect when used acutely in this model. This finding may suggest that the impact of ROS is more chronic by modifying the production, stability, or activity of receptors to vasoconstrictors such as

Figure 7. Increased Oxidative Stress in Eln\textsuperscript{+-} is Modestly Reduced in the Presence of Ncf1 Insufficiency. Sections of ascending thoracic aorta from Eln\textsuperscript{+-} \times Ncf1\textsuperscript{-/-} progeny (A-C) and Eln\textsuperscript{+-} \times Nox1\textsuperscript{-/-} (D-F) progeny were evaluated for the oxidative stress marker 8OH \times 40. Red fluorescent signal (8OH) was assessed in the media (outlined by white hash marks), as defined by the area in between the internal and external elastic lamellae. (G) Comparison of average fluorescence intensity/area of 8OH in the Ncf1 (G) and Nox1 (H) experiments are shown. Tukey's multiple comparison test, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Figure 8. Increased ROS are Apparent in the Eln\textsuperscript{+-} Mesenteric; Matrix Stain is Typical for Eln Genotype. (A–C) Representative Movat-stained histological sections of mesenteric artery showed no difference in ELN lamellar number between samples regardless of genotype. There is a visibly apparent difference in internal elastic lamellae (IEL) and external elastic lamellae (EEL) thickness. The Eln\textsuperscript{+-} variant has a thicker IEL and EEL relative to the Eln\textsuperscript{+-} and Eln\textsuperscript{+-}; Nox1\textsuperscript{-/-}. NOX expression has no obvious impact on IEL or EEL thickness, with samples resembling Eln\textsuperscript{+-}. A middle elastic lamella, MEL, was noted in all mesenteric samples, regardless of ELN or NOX expression differences. (D) Sections of primary branch mesenteric artery were sectioned and stained for oxidative stress using 8OH. 8OH fluorescence intensity measurements from the medial area. Tukey's multiple comparison test, ***P < 0.001 and ****P < 0.0001.
Indeed, several groups have shown the interaction between Nox activity and PE response, suggesting that both the endothelial and smooth muscle response to ROS are important in the pathophysiology of hypertension. Of particular note was the absence of a differential response of the $Eln^{+/+}/C0$ mouse to Ach. NOX-generated ROS are typically thought to limit endothelium-dependent relaxation. Normally, eNOS-produced NO diffuses across the endothelial layer and initiates a chain of events which ultimately decrease intracellular calcium levels, causing vasodilation. In the setting of increased NOX-generated ROS, however, superoxide anions generated by NOX react with NO to form peroxynitrite and reduce the availability of NO, therefore inhibiting relaxation. Previous studies have shown that overexpression of vascular smooth muscle NOX1 induces endothelial dysfunction and hypertension, whereas reduction of Nox1 is associated with resistance to AngII-induced hypertension and decreased endothelial dysfunction. Consequently, one may have predicted the $Eln^{+/+}$ mouse, with its increased ROS, to display evidence of endothelial dysfunction and reduced dilation to Ach. Indeed, that is exactly what was shown after ex vivo interrogation of mesenteric and cerebral $Eln^{+/+}$ arteries using myography techniques. A similar vascular bed-specific response was seen in $Eln^{+/+}$ vessels treated with AngII. The in vivo technique shown here differs from the ex vivo ones used in the majority of the NOX and $Eln^{+/+}$ published studies in that it reflects the response of the entire vascular tree (including vessels too small to sample on a myograph) to the vasoreactive stimulus and therefore may yield different results than the ex vivo measures.

Taken together, this study confirms that hypertension in $Eln^{+/+}$ mice is mediated through NADPH-produced ROS and that reduction in those ROS through chemical or genetic means normalizes BP. Of the 2 Nox complexes with which p47phox (Ncf1) affiliates, inhibition of Nox1 has the more profound effect; its loss leading to complete normalization of the BP. This model differs from the Nox2-dominated effects seen in acute

Figure 9. Augmented Phenylephrine Response in $Eln^{+/+}$ is Mitigated by Nox Deficiency or Contemporaneous Tempol Treatment. (A) Percent SBP change after injection of increasing doses of PE. Tukey's multiple comparison test: "P < 0.01, ""P < 0.001 for comparison between $Eln^{+/+}$ and $Eln^{-/-}$. ""P < 0.05, """"P < 0.0001 for comparison between $Eln^{-/-}$ and $Eln^{+/+}$, Nox1". (B) Percent SBP change after treatment with 50 mg/kg tempol, 100 μg/kg PE, or 50mg/kg tempol plus 100 μg/kg PE. Tukey's multiple t-test comparison test: "P < 0.05 and ""P < 0.01.

Figure 10. Loss of NOX1 Activity Reduces Blood Pressure and Functional Stiffness in $Eln^{+/+}$ Mice. $Eln^{+/+}$/Nox1$^{+/+}$ have normal caliber and experience laminar flow with relatively low shear. This combination results in low ROS production at baseline, yielding normal blood pressure and PWV. When Nox1 insufficiency is introduced in this background, the relative reduction of ROS is minimal and the change in blood pressure and PWV is negligible. In the $Eln^{+/+}$ background, the narrow caliber and altered anatomy lead to oscillatory flow and increased shear, which produces a marked increase in ROS. In the high ROS $Eln^{-/-}$ background, introduction of Nox1 insufficiency has the potential to dramatically reduce ROS, leading to altered vasoconstrictor response and lower blood pressure, which secondarily reduces FD and therefore PWV.
hypothesis that the chronicity of the perturbation that likely minimizes any impact of inflammation.

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Authors’ Contributions
A.T. and R.H.K. conducted experiments, acquired and analyzed data, and wrote the manuscript. C.M.H., A.W., E.K.K., D.M., Z.X.Y., K.M.T., A.K., and R.P.M. conducted experiments, acquired, and analyzed data. B.A.K. designed the study, analyzed data, and wrote the manuscript. All authors edited the manuscript.

Supplementary material
Supplementary material is available at the APS Function online.

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Conflict of Interest Statement
The authors state that they have no conflicts of interest to this study.

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