NADPH Oxidase 4 Is Expressed in Pulmonary Artery Adventitia and Contributes to Hypertensive Vascular Remodeling


Objective—Pulmonary hypertension (PH) is a progressive disease arising from remodeling and narrowing of pulmonary arteries (PAs) resulting in high pulmonary blood pressure and ultimately right ventricular failure. Elevated production of reactive oxygen species by NADPH oxidase 4 (Nox4) is associated with increased pressure in PH. However, the cellular location of Nox4 and its contribution to aberrant vascular remodeling in PH remains poorly understood. Therefore, we sought to identify the vascular cells expressing Nox4 in PAs and determine the functional relevance of Nox4 in PH.

Approach and Results—Elevated expression of Nox4 was detected in hypertensive PAs from 3 rat PH models and human PH using qualitative real-time reverse transcription polymerase chain reaction, Western blot, and immunofluorescence. In the vascular wall, Nox4 was detected in both endothelium and adventitia, and perivascular staining was prominently increased in hypertensive lung sections, colocalizing with cells expressing fibroblast and monocyte markers and matching the adventitial location of reactive oxygen species production. Small-molecule inhibitors of Nox4 reduced adventitial reactive oxygen species generation and vascular remodeling as well as ameliorating right ventricular hypertrophy and noninvasive indices of PA stiffness in monocrotaline-treated rats as determined by morphometric analysis and high-resolution digital ultrasound. Nox4 inhibitors improved PH in both prevention and reversal protocols and reduced the expression of fibroblast markers in isolated PAs. In fibroblasts, Nox4 overexpression stimulated migration and proliferation and was necessary for matrix gene expression.

Conclusion—These findings indicate that Nox4 is prominently expressed in the adventitia and contributes to altered fibroblast behavior, hypertensive vascular remodeling, and development of PH. (Arterioscler Thromb Vasc Biol. 2014;34:1704-1715.)

Key Words: adventitia • fibroblast • NADPH oxidase • pulmonary artery

Pulmonary hypertension (PH) is a progressive disease resulting from increased pulmonary vascular resistance. PH is resistant to current therapies and is characterized by excessive vascular cell proliferation, inward remodeling, rarefaction, and a loss of compliance of pulmonary blood vessels.1-3 Increased resistance to blood flow and more rigid blood vessels leads to failure of the right ventricle (RV) and eventual death. Furthermore, PH is more frequent in women than men and, if untreated, has a survival time of <5 years postdiagnosis.4,5

Reactive oxygen species (ROS) have been proposed as a pathogenic mechanism underlying the vascular remodeling observed in PH. However, the source, cellular origin, and functional significance of ROS in PH remain poorly defined. Elevated levels of ROS in PH are the net result of increased production and decreased degradation, and there is evidence for both mechanisms in the cause of elevated pulmonary pressure.6-10 The major intracellular sources of ROS include the mitochondria, aberrant oxygenase activity, and the NADPH oxidase family of oxidases (Nox).11,12 The human genome encodes 5 Nox isoforms and 4 of these, Nox1, Nox2, Nox4, and Nox5, are expressed in vascular cells (although Nox5 is not present in the genomes of rats and mice). In comparison with other sources of ROS, Nox enzymes are regarded as
Remodeled blood vessels in PH are characterized by increased stiffness, secondary to collagen and elastin deposition, a process regulated by the adventitial fibroblast. The fibroblast, a primary cell type of the adventitia, contributes to the continual reorganization of the extracellular matrix via matrix deposition and secretion of growth factors, chemokines, and inflammatory cytokines. Fibroblasts also influence and promote the inflammatory response by manipulating leukocyte recruitment, survival, and behavior. In addition, a subset of circulating bone marrow-derived cells, termed fibrocytes, that possess genetic markers and behaviors consistent with both fibroblasts and macrophages can also be found in the adventitia. Nox enzymes and elevated ROS stimulate fibroblast proliferation; however, the contribution of specific Nox isoforms to adventitial proliferation and the development of PH is poorly defined.

The goal of the current study was to address the above deficiencies in our understanding of PH and provide new data on the regulation and functional significance of Nox4 in the pulmonary circulation. Collectively, our data support a significant and novel role for Nox4 in the pathogenesis of PH.

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

Results
Real-time reverse transcription polymerase chain reaction was used to determine relative expression levels of Nox enzymes and associated subunits in isolated PAs. In all 3 rat models of PH (fawn hooded rat [FHR], monocrotaline, and Sugen-hypoxia), Nox4 mRNA was significantly increased compared with the normotensive Sprague-Dawley rats (Figure 1A–1C). In addition, Western blot analysis revealed that Nox4 protein expression was significantly upregulated in PA from monocrotaline-treated rats (Figure 1D and 1E). Nox1 and Nox2 gene expressions were also increased in PA from monocrotaline-treated rats with PH (Figures 1A and 1B in the online-only Data Supplement). However, Nox1 mRNA expression was not increased in PA from the FHR or the Sugen-hypoxia rat or in hypoxic mouse lung (Figure IB–ID in the online-only Data Supplement). Screening of p22phox, p47phox, and p67phox subunits revealed no significant differences in mRNA expression between the normotensive (Sprague-Dawley rats) and hypertensive (monocrotaline) PA (Figure IIC–IIE in the online-only Data Supplement). However, there was a significant downregulation of p47phox and p67phox and NOX1 and NOXO1 in Sugen-hypoxia PA (Figure IID–IIG in the online-only Data Supplement). There was no change in the expression of p22phox, p47phox, p67phox, NOX1, and NOX1A between control rats and FHR (Figure IIH–III in the online-only Data Supplement).

To address the functional contribution of elevated Nox4 expression in the development of PH, we treated control and monocrotaline rats with 3 different Nox4 inhibitors. To confirm efficacy, we measured ROS in a cell type expressing Nox4. Inhibitor VCC588646 (A), VCC202273 (C), and GKT136901 (G) decreased Nox4 activity in a concentration-dependent manner (Figure 2A–2C). ROS production was completely inhibited by the flavoprotein inhibitor, diphenyleneiodonium, and by catalase, a scavenger of hydrogen peroxide (Figure 2C). Morphometric analysis of PA revealed significant medial remodeling by 4 weeks in monocrotaline-treated rats that was abrogated by both Nox4 inhibitor VCC588646 (A) and VCC202273 (C; Figure 2D and E). The relative activity of Nox4 inhibitors VCC588646 (A) and VCC202273 (C) against Nox1 activity was tested in a human embryonic kidney cell line expressing Nox1, NOX1O1, and NOX1A as previously described. Both inhibitors reduced Nox1 activity but at concentrations higher than for Nox4 (Figure III in the online-only Data Supplement).

The effect of Nox4 inhibition on cardiac remodeling and indices of cardiopulmonary function is shown in Figure 3 and extended in Figure IV in the online-only Data Supplement. Monocrotaline significantly increased RV hypertrophy (RV/LV+S), which was inhibited with VCC202273 (C; Figure 3A) and also VCC588646 (A) and GKT136901 (G; Figure IVA and IVB in the online-only Data Supplement). Changes in RV function (RV systolic pressure, $RV_{max} \Delta P/\Delta t$), which were elevated in the monocrotaline-treated lungs, were also abrogated by Nox4 inhibition (Figure 3B and 3C; Figure IVC in the online-only Data Supplement). Noninvasive assessment of RV remodeling using digital ultrasound also revealed significant time-dependent increases in RV thickness in professional ROS generators and are capable of synthesizing high levels of ROS in a spatial and temporal manner. Nox1 to Nox4 are bound to p22phox, and Nox1 and Nox2 are activated by binding numerous cytosolic subunits, including p47phox, p67phox or NOXO1, and NOXA1. In contrast, Nox4 is regarded as a constitutively active enzyme with ROS levels primarily controlled by changes in gene expression. In mice, genetic deletion of Nox2 has been shown to reverse hypoxia-initiated PH, and Nox1 has been shown to be important for systemic hypertension. Increased expression of Nox4 has been reported in both mouse models of PH and human PH. Despite this knowledge, the functional significance of Nox4 in the development of PH is poorly understood. A recent publication by Green et al demonstrated the ability of combined Nox4/Nox1 inhibitors to ameliorate PH in mice. Although that study supports the importance of Nox4 in the development and functional significance of Nox4 in the pulmonary arteries (PAs).

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>cellFN1</td>
<td>cellular fibronectin</td>
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<tr>
<td>FHR</td>
<td>fawn hooded rat</td>
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<tr>
<td>HPAVSMC</td>
<td>human pulmonary arterial vascular smooth muscle cell</td>
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<tr>
<td>Nox</td>
<td>NADPH oxidase</td>
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<tr>
<td>PA</td>
<td>pulmonary artery</td>
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<td>PH</td>
<td>pulmonary hypertension</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>RV</td>
<td>right ventricle</td>
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<td>TGF–β1</td>
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monocrotaline-treated rats, which were reduced by Nox4 inhibitors (Figure 3D and 3E; Figure IVD–IVE in the online-only Data Supplement). Cardiac output was decreased at 4 weeks post–monocrotaline treatment and improved with Nox4 inhibition (Figure 3F). There was evidence of PA remodeling as determined by analysis of PA hemodynamics,
including a reduction in the velocity time integral, pulmonary ejection time, and PA acceleration time (Figure 3G–3I; Figure IVD–IVI in the online-only Data Supplement), which collectively suggest a reduction in vessel stiffness (remodeling) and improved cardiac performance. Importantly, in all of the end points measured, all 3 inhibitors of Nox4 demonstrated significant efficacy in ameliorating the structural and functional changes with PH.
To assess whether inhibition of Nox4 can reverse, attenuate, or halt the progression of experimental PH in monocrotaline-treated rats with established PH, we measured time-dependent changes in RV and PA remodeling via ultrasound. Reversal protocols were used because they are more relevant to the clinical predicament and also can determine whether the target of interest remains functionally important in the later stages of disease and whether pre-existing end points of PH can be improved. The Nox4 inhibitor VCC202273 (C) was administered to monocrotaline-treated rats on the first detectable increase in RV thickness, which occurred at 3 weeks post–monocrotaline treatment (Figure 3E). As shown in Figure 4, VCC202273 (C; 1 mg/kg per day) significantly attenuated the progression of compensatory RV hypertrophy (Figure 4A), velocity time integral (Figure 4B), and PA acceleration time (Figure 4C) in monocrotaline-treated rats by weeks 5 and 6 post–monocrotaline treatment. At the conclusion of the experiment (6 weeks), invasive and postmortem end points were recorded (RV systolic pressure and Fulton index). Nox4 inhibition attenuated the monocrotaline-induced increases in RV systolic pressure and Fulton index at 6 weeks postinitiation of monocrotaline treatment (Figure 4D and 4E). Although there was no indication that Nox4 inhibition promoted reversal of PH beyond pretreatment levels, there was significant evidence for the attenuation of progression of the disease.

To identify the regions of the blood vessel wall and cell types expressing Nox4 in hypertensive PA, we performed immunofluorescence staining of lung sections from monocrotaline-treated rats and in lungs from normal (control) and human PH. In control rat PA, Nox4 was detected in the intima (endothelial cells) and cells of the adventitia (Figure 5, top panel). In 4-week monocrotaline-treated rats, using 2 different antibodies that are selective for Nox4 (Epitomics and Abcam), there was a dramatic increase in Nox4-positive cells in the adventitia (monocrotaline; Figure 5, left panels). Nox4 exhibited a staining pattern in sections of human lung from individuals with normal and elevated pulmonary blood pressure (Figure 5, lower panels) that was consistent with the monocrotaline rat model. In lung sections from animals treated with the Nox4 inhibitor VCC202273 (C), monocrotaline-stimulated vascular remodeling and Nox4 expression were significantly attenuated (Figure 5; monocrotaline plus C). Furthermore, a time course of Nox4 expression in monocrotaline-treated rats revealed increased Nox4 expression and progressive medial remodeling at 2 and 3 weeks post–monocrotaline treatment (Figure VI in the online-only Data Supplement). Negative controls for the fluorescent secondary antibodies were performed using nonimmune immunoglobulin G in human lung sections and were without significant staining as shown in Figure VII in the online-only Data Supplement.

**Figure 4.** Inhibition of NADPH oxidase 4 (Nox4) slows the progression of established pulmonary hypertension (PH). Rats with established monocrotaline (MCT)-induced PH were treated with VCC202273 (C; 1 mg/kg per day) starting from wk 3 through wk 6. Inhibition of Nox4 reduced the thickness of the right ventricle (RV; A) and improved velocity time integral (VTI; B) and pulmonary artery acceleration time (PAAT; C) in wk 4 to 6 post-MCT treatment. End point measurements of RV systolic pressure (RVSP) and Fulton index show a reduction in RV hypertrophy in rats treated with VCC202273 (C; D and E). *Significantly different from vehicle/control; #significantly different from MCT; $P<0.05$ (n=6 for each group).
The location of ROS production in hypertensive PA was performed by immunofluorescence imaging for 8-hydroxydeoxyguanosine, a DNA nucleoside that is generated by ROS. 8-Hydroxydeoxyguanosine is used as an in vivo footprint of ROS production, and increased staining was detected in PA from 4-week monocrotaline-treated rats. The highest signal was observed in the adventitia (endothelial cells) and cells of the adventitia in 4-wk MCT-treated rats and human PH PAs. There is an abundance of Nox4-expressed cells in the remodeled medial layer but devoid of α-actin expression in the MCT and human PH PAs. In the presence of Nox4 VCC202273 (C), MCT plus C, Nox4 expression is similar to vehicle-treated PAs in the MCT-treated group.

Figure 5. NADPH oxidase 4 (Nox4) expression and reactive oxygen species (ROS) production is localized in the adventitia. A, Sections of control and 4-wk monocrotaline (MCT)-treated rat lungs costained for fibroblast activating protein (FAP), 8 hydroxy-2′-deoxyguanosine (ROS marker), and DAPI. B, Costaining for Nox4 and cellular fibronectin [cellFN1], CD90, and CD11b ROS production is elevated in pulmonary artery (PA) adventitia from 4-wk MCT-treated rats, which overlapped significantly with the fibroblast marker FAP (A; MCT). ROS are decreased to control (vehicle) levels by the Nox4 inhibitor VCC202273 (C; A; MCT + C). In MCT-treated PAs, there is significant overlap between Nox4-positive cells in the adventitia and cells expressing fibroblast markers (cellFN1, CD90) as well as the monocytic cell marker CD11B (B). SDR indicates Sprague-Dawley rats.

Figure 6. NADPH oxidase 4 (Nox4) expression and reactive oxygen species (ROS) production is localized in the adventitia. A, Sections of control and 4-wk monocrotaline (MCT)-treated rat lungs costained for fibroblast activating protein (FAP), 8 hydroxy-2′-deoxyguanosine (ROS marker), and DAPI. B, Costaining for Nox4 and cellular fibronectin [cellFN1], CD90, and CD11b ROS production is elevated in pulmonary artery (PA) adventitia from 4-wk MCT-treated rats, which overlapped significantly with the fibroblast marker FAP (A; MCT). ROS are decreased to control (vehicle) levels by the Nox4 inhibitor VCC202273 (C; A; MCT + C). In MCT-treated PAs, there is significant overlap between Nox4-positive cells in the adventitia and cells expressing fibroblast markers (cellFN1, CD90) as well as the monocytic cell marker CD11B (B). SDR indicates Sprague-Dawley rats.

in the media compared with the adventitia. ROS levels in both the medial and adventitial layers were decreased in sections from rats treated with the Nox4 inhibitor VCC202273 (C; Figure 6A; monocrotaline plus C), suggesting that the elevated ROS production in hypertensive PA derives from increased Nox4 expression. In monocrotaline-treated PA, there was significant overlap between Nox4-positive cells in the adventitia and cells expressing fibroblast markers (cellular fibronectin [cellFN1], CD90) as well as the monocytic cell marker CD11B.
Increased expression of fibroblast markers in hypertensive pulmonary arteries (PAs) and effect of NADPH oxidase 4 (Nox4) inhibition in established pulmonary hypertension (PH). Western blots of PA isolated from control and 4-wk monocrotaline (MCT) rats (A). MCT rats were treated with Nox4 inhibitor VCC202273 (C) starting at wk 3 post-MCT and continuing through wk 6 in a reversal protocol (see Materials and Methods; B). n=3–4 per group. NG2 indicates chondroitin sulfate proteoglycan; and TSP4, thrombospondin-4. cellFN1 indicates cellular fibronectin.

(Figure 7B). These data are supported by Western blot analysis of PA from control and 4-week monocrotaline-treated rats revealing increased protein expression of fibroblast markers such as CD90, cellFN1, periositin, tenascin N, and vimentin (Figure 7A and 7B; densitometry shown in Figure VA in the online-only Data Supplement). The effect of Nox4 inhibition on fibroblast and inflammatory markers was also determined in PA isolated from control, monocrotaline, and monocrotaline-treated rats treated with VCC202273 (C) using the reversal protocol (see Materials and Methods). Three-week exposure to a Nox4 inhibitor that was initiated after 3 weeks of monocrotaline resulted in prominent decreases in fibroblast markers (CD90, cellFN1, vimentin, periositin, and thrombospondin-4) as well as a marker of pericytes (chondroitin sulfate proteoglycan) compared with monocrotaline treatment alone (Figure 7B; densitometry shown in Figure VB in the online-only Data Supplement). A time course of Nox4 expression in isolated lung segments is shown in Figure VI in the online-only Data Supplement. These data, in conjunction with the functional indices of PA hypertension (Figure 3; Figure IV in the online-only Data Supplement), provide strong evidence for the pharmacological efficacy of Nox4 inhibitors and the importance of this pathway in the progression of PH.

To assess whether Nox4 modifies fibroblast function, we transduced human lung fibroblasts with an adenovirus expressing Nox4. Nox4-transduced fibroblasts exhibited a robust increase in cellular proliferation as demonstrated by real-time changes in electric impedance using electric cell–substrate impedance sensing arrays, as shown in Figure 8A. In addition, fibroblasts transduced with the Nox4 adenovirus displayed increased cell proliferation (total cell number; Figure 8B) and the number of viable cells as measured via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 8C). Furthermore, using anti-CD90-immunomagnetic isolation, fibroblasts were isolated from control or 4-week monocrotaline-treated rat lungs. Western blot analysis revealed the expected enrichment of CD90-positive cells along with increased expression of Nox4 relative to the loading control, heat shock protein 90 (Figure 8D). Fibroblasts isolated from PA of monocrotaline-treated rats displayed a high level of proliferation that was decreased by the Nox4 inhibitor VCC202273 (C; Figure 8E). Adenoviral delivery of Nox4 also stimulated an increase in fibroblast motility (Figure 8F) compared with cells treated with a control virus. These data show that increased Nox4 expression alters fibroblast behavior, favoring a proproliferatory and promigratory phenotype. Lung fibroblasts exposed to the profibrotic growth factor transforming growth factor-β1 (TGF-β1) robustly increased Nox4 mRNA and protein levels as well as cellFN1 in a time-dependent manner (Figure 8G). To determine the relative ability of vascular cell types to express Nox4 in response to TGF-β1, we exposed human PA endothelial cells, human PA smooth muscle cells (HPASMCs), and human lung fibroblasts to TGF-β1 for 24 hours. The phenotype of each cell type was confirmed using relevant cell-specific markers. We found the greatest induction of Nox4 in fibroblasts > human PA endothelial cells >> HPASMCs (Figure 8H). Next we determined if Nox4 was important for the ability of TGF-β1 to induce profibrotic changes. In human fibroblasts, Nox4 small interfering RNA decreased Nox4 expression and subsequently prevented the expression of the TGF-β1–dependent genes, fibronectin, and ACTA (smooth muscle actin; Figure 8I).

Discussion

A positive correlation between increased PA ROS production and elevated blood pressure of PH was observed >2 decades ago and has since been confirmed by numerous independent groups. Although the consensus is for elevated ROS levels in PH, the source of ROS and its functional contribution to pathological remodeling of pulmonary blood vessels remain less well defined. The major goal of the current study was to determine the relative importance of Nox isoforms, a major source of cellular ROS, to the cause of PH.

Given the inability of any single animal model of PH to faithfully emulate the complexity of human PH, we first determined the expression of individual Nox isoforms in multiple rat models, including 2 inducible and 1 genetic model. Our studies were conducted primarily in rat models because they exhibit robust, irreversible pulmonary vascular remodeling, which is a defining characteristic of the human disease. Changes in vascular gene expression were selectively measured in isolated PAs (down to the fourth branch). In PA, we found significantly increased expression of Nox4 mRNA in all 3 rat models. This correlated with increased protein expression in the monocrotaline model and suggests that Nox4 is a potential source of elevated ROS in hypertensive PAs. These data are consistent with prior studies in mice, rats, and in humans with PH. In the monocrotaline model, we observed increased expression of Nox1 and Nox2 mRNA, which is also consistent with previous reports. However, Nox1 levels were not elevated in PA from the FHR, a genetic model of PH, or in the rat and mouse hypoxia models. Furthermore, in all 3 rat PH models, no increase in the expression of multiple accessory proteins that support the post-translational activation of Nox1 to Nox3 was observed. The significance of this is not known because the activity of Nox1 to Nox3 is dependent on stimulus-dependent assembly of a functional
oxidase. In contrast, the elevated expression of Nox4 has additional significance because it is the only Nox isoform that is constitutively active. This means that increases in Nox4 gene expression translates to increased ROS. Collectively, these findings support elevated Nox4 expression in hypertensive PAs as a common variable in multiple rodent models of PH and in humans.

A major obstacle in the identification of functional roles for Nox isoforms in PH has been the lack of appropriate tools, particularly in the rat. To investigate the significance

Figure 8. NADPH oxidase 4 (Nox4) stimulates fibroblast proliferation and migration and transforming growth factor-β1 (TGF-β1) expression. A, Electric cell-substrate impedance sensing (ECIS) of human lung fibroblasts in the presence/absence of Nox4 adenovirus. Nox4-transduced fibroblasts exhibit a robust increase in cellular proliferation in real time using ECIS. B, Nox4 increases fibroblast cell number. C, Number of viable cells using 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyletrazolium bromide (MTT) assay. D, Magnetic bead isolation of CD90-positive fibroblasts from lungs of control and monocrotaline (MCT)-treated rats. Western blot analysis revealed enrichment of CD90-positive cells with increased expression of Nox4 relative to the loading control, Hsp90. E, Fibroblasts (p1) isolated from pulmonary arteries (PAs) of MCT-treated rats displayed robust proliferation that is decreased by Nox4 inhibitor VCC202273 (C). F, Adenoviral delivery of Nox4 stimulated an increase in fibroblast motility. G, Twenty-four-hour exposure to TGF-β1 robustly increased both Nox4 mRNA and protein levels in human lung fibroblasts while contemporaneously increasing the synthesis of cellular fibronectin. H, Expression of cell specific markers (eNOS, H-caldesmon, CD90) in cultured human PA endothelial cells (HPAECs), human PA vascular smooth muscle cells (HPAVSMCs), and fibroblasts; TGF-β1 increased CD90 and Nox4 expression in fibroblasts. I, Transfection of fibroblasts with Nox4 small interfering RNA decreased Nox4 expression and subsequently prevented the expression of TGF-β1-dependent genes such as fibronectin and ACTA. *Significantly different from Lac Z, 0, control, 0 h, and Cont siRNA; +significantly different from Cont siRNA plus TGF-β1; P<0.05; n=3–6 per group.
of Nox4 in PH, we used 3 structurally distinct Nox4 inhibitors.36,37 This was done to ensure greater selectivity toward a common target (Nox4) and to minimize any possible non-specific effects of individual inhibitors. The efficacy of these inhibitors against Nox4 activity was first confirmed in vitro and then in vivo in the monocrotaline rat model of PH. This model was selected based on the expeditious time course of PH (3–4 weeks versus 14 and 24 weeks for Sugen-hypoxia and FHR). We initially determined if inhibiting Nox4 would prevent the development of PH in monocrotaline-treated rats (day 1 treatment). Our objective in these studies was to determine if the increased Nox4 expression contributes to the induction of PH. We found that Nox4 inhibitors were effective at preventing PH as determined by indices of RV remodeling and noninvasive in vivo measurements of RV thickness, cardiac output, and remodeling of PA (velocity time integral, pulmonary ejection time, PA acceleration time). Our data are consistent with those reported recently by Green et al20 in a mouse hypoxia model of PH. In that study, a related Nox4 inhibitor (GKT137831) improved indices of ventricular remodeling and PA wall thickening, but did not decrease RV systolic pressure. However, a major limitation of the mouse model is the absence of significant vascular remodeling. Our study revealed that Nox4 inhibitors were effective at preventing the robust hypertrophic remodeling of PA in monocrotaline-treated rats with PH. A further distinction of our study is that we also used Nox4 inhibitors in protocols to assess the ability to reverse established PH. This approach is more clinically relevant because the early stages of PH are generally silent, difficult to detect, and rarely treated. We found that Nox4 inhibitors were effective at halting the progression of PH. However, at the dose of inhibitor used and the time points studied, we did not observe an ability to fully halt the progression of the measured parameters of PH to levels below that of pretreatment in 3-week monocrotaline-treated rats. The reason for this is not yet known. In isolated PAs from rats with PH, we observed near-complete reversal of some indices such as the expression of CD90 and vimentin with Nox4 inhibition. However, incomplete reversal was seen with others including cellFN1, thrombospondin-4, and periostin. The reasons for diversity in the ability of Nox4 inhibitors to reverse changes in the expression of specific genes in PA and the inability to completely stop or reverse the progression of PH await further investigation. It remains possible that higher doses of Nox4 inhibitors or combination therapy with established therapeutics for PH (endothelin-1 antagonists, PDE5 inhibitors, etc) may enable more complete reversal of established PH. Germaine to the overall thesis of this study, we found that inhibition of Nox4 was able to decrease the expression of genes in hypertensive PAs that are considered to be cellular markers of fibroblasts.

To determine a cellular mechanism by which Nox4 influences the progression of PH, we first performed immunofluorescence imaging in isolated lung sections. Nox4 expression was detected in both endothelium and adventitia of PA, and perivascular staining was prominently increased in animals and humans with PH. In the adventitia, Nox4 was detected in cell types expressing markers consistent with fibroblasts and monocytes. The perivascular expression of Nox4 also matched the location of ROS production and the markers of fibroblasts and monocytic cells. A surprising observation was the relative absence of Nox4 in PA vascular smooth muscle. Adventitial staining was confirmed using 2 distinct Nox4 antibodies, and in hypertensive PAs, the cell types expressing Nox4 in the medial layer did not overlap with those staining for smooth muscle actin. These results are supported by the higher levels of Nox4 detected in fibroblasts immunosolated from monocrotaline-treated rat lungs and are consistent with previous reports showing elevated Nox4 in perivascular fibroblasts from individuals with idiopathic PA hypertension.17 Nox4 has been detected in most cell types with higher levels of expression seen in fibroblasts, particularly in the setting of pulmonary fibrosis.37,26,38,39 Also consistent with previous studies, we observed that HPAVSMCs in culture have detectable levels of Nox4, which are increased in the presence of TGF-β1. However, these levels are less robust than those observed in fibroblasts.

The functional relevance of Nox4 in adventitial cells is not well described. The tunica externa or adventitia is a loosely defined collection of cells, including fibroblasts and immune cells, collagen, and elastic fibers that encircle the tunica media and intima layers of the blood vessel.40 The adventitia orchestrates inflammation and vascular proliferation in response to injury, atherosclerosis, and pulmonary and systemic hypertension.41 The fibroblast is a primary cell type of the adventitia, responsible for the continual reorganization of the extracellular matrix via matrix deposition and secretion of growth factors, chemokines, and inflammatory cytokines.42 Aberrant vascular remodeling in PH occurs through increased inflammation, proliferation, and fibrosis, processes that collectively yield more muscular and less compliant PA.1 Fibroblasts have key roles in these actions and actively secrete matrix, growth factors, and promote the inflammatory response by manipulating leukocyte recruitment and behavior.43,44 We observed that increased expression of Nox4, in the absence of other stimuli, was sufficient to increase fibroblast migration and proliferation, and treatment of fibroblasts isolated from PAs from monocrotaline-treated rats with Nox4 inhibitors reduced cellular proliferation. Similarly, silencing Nox4 in fibroblasts decreased the ability of TGF-β to increase matrix and induce contractile gene expression, which is consistent with other reports.36,39,45 A subset of bone marrow–derived cells, termed fibrocytes, can also be found in the adventitia, which have characteristics of both fibroblasts and macrophages.25,46 Although we observed significant overlap of Nox4 staining with fibroblast and macrophage markers, it is not yet known if Nox4 is expressed in fibrocytes. In isolated PAs from monocrotaline-treated rats, we found increased expression of numerous markers that have been used to identify fibroblasts, including CD90, cellFN1, periostin, vimentin, and fibroblast activation protein. A role for Nox4 in regulating perivascular fibroblast behavior in PH is supported by results showing that Nox4 inhibition decreases the expression of fibroblast markers in isolated PAs from rats with established PH. These data suggest an ability of Nox4 to regulate the number and behavior of adventitial fibroblasts in animals with PH.
TGF-β1, an autocrine growth factor implicated in the pathophysiological vascular remodeling in PH, robustly increased both Nox4 mRNA and protein levels in human lung fibroblasts. When comparing the ability of TGF-β1 to drive Nox4 expression in PA endothelial cells, PAVSMCs, and lung fibroblasts, we observed the greatest upregulation of Nox4 in fibroblasts, with less in VSMCs and none in PA endothelial cells. These data suggest that other mechanisms regulate Nox4 expression in endothelial cells. Indeed, numerous stimuli have been shown to modulate Nox4 expression, including hypoxia, angiotensin II, cyclic adenosine monophosphate, and protein kinase C, and it is possible that these signaling pathways contribute in varying degrees to the elevated Nox4 expression observed in PH. Our data are in agreement with previous studies, and others have shown that TGF-β1 can upregulate Nox4 expression in other cell types, including human cardiac fibroblasts, airway smooth muscle, and vascular smooth muscle. The functional effects of Nox4 in vascular smooth muscle are similar to those reported in fibroblasts and include altered signaling, increased ability to proliferate and migrate, and may reflect the altered phenotype of smooth muscle cells in culture. A role for fibroblasts in pathological remodeling in PH is supported by studies in transgenic mice with fibroblast-specific activation of TGF-β1 signaling. These mice develop mild PH with medial hypertrophy, inflammation, and fibrosis. Although this study strongly supports a role for fibroblast TGF-β1 signaling in the development of PH, it has also been shown that fibroblasts, we observed the greatest upregulation of Nox4 in adventitial fibroblasts isolated from monocrotaline-treated rats. They did not observe increased expression of these subunits in hypertensive PA, but it is not known whether this is important for assembly of a functional oxidase in PH. In our study, a functional role for Nox4 can be deduced from the use of Nox4/1 inhibitor GKT136901, which is not selective for Nox2, and also from other studies using GKT137831, which has been shown to be effective in a mouse model of PH.

Nox4 has gained considerable attention as a primary source of ROS and cellular proliferation in the pathogenesis of both idiopathic pulmonary fibrosis and PH. The results of the current study are in agreement with both concepts and connect Nox4 as a common variable in fibroblasts (and other perivascular cells) that contributes to the remodeling of hypertensive pulmonary arterioles. The remodeling of blood vessels requires the participation of all 3 layers, and although numerous studies have proposed a central role for endothelial cells (inside-out remodeling), it has also been shown that vascular remodeling can be driven by changes in the adventitia (ie, outside-in). In both human and animal models of PH, prominent inflammation, activation, and restructuring of the adventitia are observed. The adventitial location of Nox4 is, therefore, highly suited to orchestrate the changes in vascular inflammation and matrix deposition that are widely observed in PH. Current therapies for PH are ineffective in the long term, and new therapeutic strategies are needed. Our study supports the effectiveness of Nox4 inhibitors in halting the progression of experimental PH, although (as previously addressed) this strategy was unable to completely reverse the cardiopulmonary functional and morphometric changes that have already occurred. It remains to be determined whether this is a limitation of modalities that target Nox4 or whether a combination of therapeutic approaches will have superior efficacy.

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Disclosures

None
References


This study is novel in that we address the importance of NADPH oxidase 4 (Nox4) in pulmonary hypertension using a comprehensive pharmacological approach in vivo with novel Nox4 inhibitors, identifying previously understudied perivascular cell types expressing Nox4 in multiple animal models and in hypertensive human pulmonary arteries. We also address the functional significance of Nox4 in adventitial fibroblasts. The traditional approach to study vascular changes in pulmonary hypertension has been to focus on endothelial and smooth muscle cells (ie, inside-out). Our studies diverge from this theme and suggest an outside-in (adventitial) process of vascular remodeling that is mediated by Nox4, which although has physiological roles in the endothelium may also have pathological importance in fibroblasts. Nox4 has been shown to be important in the pathogenesis of other fibrotic conditions such as pulmonary fibrosis, but our study reveals a role for Nox4 in vascular fibrosis associated with pulmonary hypertension.
NADPH Oxidase 4 Is Expressed in Pulmonary Artery Adventitia and Contributes to Hypertensive Vascular Remodeling

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Supplemental Figure I. Nox1 expression is increased only in MCT-treated rat hypertensive pulmonary arteries (PA). Nox1 mRNA (measured by qRT-PCR (ΔΔCt) normalized to GAPDH) is significantly increased in PA from 4week MCT-treated rats (A). In contrast, Nox1 expression is not increased in PA from SU/HYP treated rats (14week; B) or FHR (24week; C) relative to control or in hypoxic mouse lung (3week; D). * Significantly different from Vehicle, p < 0.05 (n = 4-5).
Supplemental Figure II. Relative expression of Nox2 and Nox subunits in isolated pulmonary arteries (PA) from rats with pulmonary hypertension. Nox2 mRNA (measured by qRT-PCR (ΔΔCt) normalized to GAPDH) is significantly increased in PA from MCT-treated rats (4 week; A) and unchanged in FHR rats (24 week; B). Expression levels of p22phox (C), p67phox (D), and p67phox (E) and NOXA1 (F) and NOXO1 (G) in PA from normotensive (SDR), SUGEN/hypoxia (SU/HYP; 14 week) and MCT-treated (4 week) hypertensive rats. Expression levels of p22phox (H), p47phox (I), and p67phox (J) and NOXA1 (K) and NOXO1 (L) in PA from normotensive (SDR) and 24 week FHR.

* Significantly different from SDR/vehicle controls, p < 0.05 (n = 5-6).
Supplemental Figure III. Relative potency of Nox4 inhibitor A (VCC588646) and C (VCC202273) against ROS production in cells expressing Nox1. HEK293 cells expressing Nox1, NOXO1, NOXA1 were exposed to the indicated concentrations of inhibitor VCC202273 (C) and VCC588646 (A) (A-B) for 1h and the Nox1-dependent production of reactive oxygen species determined by L-012 chemiluminescence. * Significantly different from Vehicle p < 0.05 (n = 7).
Supplemental Figure IV. Effect of Nox4 inhibition on cardiac remodeling and indices of cardiopulmonary function in MCT-treated rats. MCT-treatment significantly increases right ventricular (RV) hypertrophy as measured by the increased Fulton Index (RV/LV+S; A-B), right ventricular systolic pressure (RVSP; C) and right ventricle (RV) thickness (D-E). These variables are decreased in rats treated with the Nox4 inhibitors VCC588646 (A) (A, C, D) and GKT136901 (G) (B, E). MCT-treatment significantly decreased pulmonary artery acceleration time (PAAT; F, G), velocity time integral (VTI; H, I) and these were increased with Nox4 inhibitor VCC588646 (A) and also with GKT136901 (G) (F-I). * Significantly different from Vehicle, # significantly different from MCT, p < 0.05 (n = 5-6).
Supplemental Figure V. Densitometric analysis of changes in gene expression in hypertensive PA and reversal by Nox4 inhibition. Analysis of selected markers of inflammatory cells and fibroblasts in PA isolated from control and hypertensive (MCT-treated) rats (CD45, CD90, cellFN1, periostin, tenascin N, and vimentin) (A). Expression of markers of fibroblasts in PA isolated from MCT-treated rats in the presence and absence of the Nox4 inhibitor VCC202273 (C) in a reversal protocol (see Methods) (B). (cell FN1 = cellular fibronectin, TSP4 = thrombospondin-4; NG2 = chondroitin sulfate proteoglycan/ Cspg4) * Significantly different from Vehicle, # significantly different from MCT, p < 0.05 (n = 3-4).
Supplemental Figure VI. Time course of Nox4 expression in PA from MCT-treated rats. Confocal images of lung sections from control, and experimental PH (2-week; 3-week MCT). Sections were stained with Nox4 and α-actin antibodies (SMA).
Supplemental Figure VII. Confocal imaging of IgG staining of human lung sections. Sections were stained first with non-immune rabbit IgG and non-immune mouse IgG and then with goat anti-rabbit IgG and goat anti-mouse IgG.
Materials and methods:

Cell culture and Reagents. HEK cells and human lung fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) containing L-glutamine, penicillin, streptomycin, and 10% (v/v) fetal bovine serum. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) as described previously. Human pulmonary artery endothelial cells (HPAEC), human pulmonary artery vascular smooth muscle cells (HPAVSMC) were purchased from Lonza, and were grown in Endothelial Growth Medium-2-Microvessel (EGM-2MV) or smooth muscle growth media (SmGM) consisting of defined growth factors and supplemented with additional FBS up to 5% final concentration (Lonza). Cells were grown at 37 °C in 5% CO2 incubator and used from passage 2–6. All chemicals were purchased from Sigma unless indicated otherwise.

DNA/adenoviral constructs. Plasmid DNA encoding Nox5β (AF325189), Nox1 and Nox4 have been described previously. A Nox4 adenovirus was generated using the Invitrogen Virapower system as described previously for Nox5.

Cell Proliferation and Migration. Human lung fibroblasts were cultured in a 8W10E array. Control (LacZ) or Nox4 adenovirus (MOI 30) were added at 20h and resistance was measured using the ECIS Z® (Applied Biophysics) and normalized to the value of each well at 0h. Alternatively, fibroblasts were exposed to the indicated amounts of adenovirus and cell number determined at 48h by manual count or MMT assay. In brief, cells were incubated with CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent (Promega) for 1 h at 37°C. Absorbance at 495nm was measured BMG Polarstar plate reader. Cell migration was determined in fibroblasts exposed to control or Nox4 adenovirus (MOI 30) and determined using the Oris™ Cell Migration Assay (Platypus Technologies).

Rat models of PH. Three rat models of pulmonary hypertension (PH) were employed. The monocrotaline (MCT)-model was induced by a single i.p. injection of MCT (60/mg/kg), which produces a progressive and severe PH after 4 weeks of MCT exposure. The Sugen/Hypoxia (SU/HYP) model of PH results from injection of the VEGF receptor antagonist SU-5416 (20mg/kg,SQ) followed by 3 weeks of hypoxia (10% O2) and 11 weeks of normoxia (21% O2) as previously described. The Fawn-hooded rat (FHR), a genetic model of PH spontaneously develops PH after 20 weeks of age. Adult age-matched male Sprague-Dawley (SDR, 250-300g) rats were used as controls for all rat models of PH. The Animal Care and Use Committee at Georgia Regents University approved all procedures and protocols, and this study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All groups of rats were housed under temperature-controlled conditions (21-23°C), maintained on standard rat chow, allowed free access to food and water, and exposed to a 12:12-h light-dark cycle.

Murine model of PH. C57BJ6 mice were exposed to room air (normoxia) or 10% oxygen for 3 weeks as previously described.

Nox4 Inhibitor treatments for MCT-induced PH. Three different small molecule Nox4 inhibitors were administered to MCT-treated rats: VCC588646 (2-[N’-(3,4-Dihydroxy-benzylidene)-hydrazino]-N-(3-nitro-phenyl)-2-oxo-acetamide; Inhibitor ‘A’), VCC202273 (N4-(4-Amino-phenyl)-1[benzothieno[3,2-d]pyrimidine-4,8-diamine; Inhibitor ‘C’), both from Vichem-Chemie, LTD or GKT136901 (2-(2-Chlorophenyl)-4-methyl-5-(pyridin-2-ylmethyl)-1H-pyrazolo-[4,3-c]pyridine-3,6(2H,5H)-dione; Inhibitor ‘G’). To assess prevention, the Nox4 inhibitors (‘A’, ‘C’,
or ‘G’), were dissolved in DMSO and administered via i.v. injection daily at a dose of 1mg/kg/day, commencing on the day of the single MCT injection (day 1), for a duration of 28 days. To assess whether Nox4 inhibition could halt the progression of PH (reversal protocol), Nox4 inhibitor VCC202273 (C) was administered (1mg/kg/day) daily to MCT-treated rats upon the first detectable increase in right ventricle (RV) thickness, which occurred at week 3 post MCT administration. Inhibitors or vehicle (DMSO) were given daily for 21 days and cardiopulmonary indices were monitored on a weekly basis for three weeks corresponding to weeks 4, 5, and 6 of MCT exposure.

Assessment of RV function. Rats were anesthetized (pentobarbital, 50 mg/kg, i.p.) and the trachea intubated. The diaphragm was surgically exposed through the abdomen, and a 25 gauge needle connected to a pressure transducer (AD instruments) was inserted into the right ventricle (RV) through the diaphragm, and RV pressure was continuously monitored for 10-15 minutes. Indices of RV function (RVSP; RV max dp/dt) were recorded using a PowerLab data acquisition system (AD Instruments). With this approach, the diaphragm remains intact without opening the chest. We have previously established that measurements of right ventricular systolic pressure (RVSP) are comparable to measurements obtained using the right jugular vein.

Non-invasive measurement of cardiopulmonary parameters. Rats were temporally anesthetized (1-4% inhaled isoflurane), and RV hypertrophy and functional parameters of PA remodeling (velocity time integral (VTI), pulmonary ejection time (PET) and the pulmonary artery acceleration time (PAAT) were measured using the VEVO 2100 digital ultrasound micro-imaging system (VisualSonics).

Histological analysis. Post hemodynamic measurements, rats were euthanized by thoracotomy. Blood in the pulmonary vasculature was removed by PBS infusion through the pulmonary artery and the heart and lungs removed en bloc. The free wall of the RV, left ventricle (LV), and septum (S) were carefully dissected free and weighed individually to calculate the RV/LV+S ratio (Fulton index) as an index of RV hypertrophy. The right lungs were removed and snap frozen in liquid nitrogen for preparation of homogenates, and the left lungs were filled with 4% PFA solution with 0.5% agarose at 25 cm H₂O and fixed in 4% PFA for 24 hours. The fixed lungs were then sliced mid-sagittally and embedded in paraffin. The slides (7μm thickness) were stained with hematoxylin and eosin for morphometric analysis and were examined with an Olympus BX41 microscope. An Olympus DP72 digital camera and ImageJ software (http://rsbweb.nih.gov/ij/) were used to analyze slides. A minimum of 10 microscopic fields were examined for each slide. To quantitate pulmonary arterial wall thickness, the lumen area at the level of the basement membrane and total vascular area at the adventitial border in 20 muscular arteries with diameters of 50–100 μm per lung section were outlined, and area sizes were measured using ImageJ. The vascular wall thickness was calculated as follows: wall thickness = (total vascular area − lumen area)/ total vascular area.

Confocal microscopy. To determine the location of specific cellular markers in blood vessels, both normotensive and PH lung sections were stained with α-actin (Abcam; 1:700 dilution) for 30 minutes before being double-stained with antibodies against either Nox4 (Abcam (ab116534); or Epitomics; 1:1000 dilution), CD90 (Abgent; 1:1000 dilution), FAP (Santa Cruz; 1:500 dilution), cellFN1 (SCBT; 1:500 dilution), CD11b (BD Pharmingen; 1:1000 dilution), Tenascin-N (Santa Cruz; 1:250 dilution), Periostin (Novus; 1:500 dilution), Vimentin (Oncogene; 1:1000 dilution) and the marker of ROS production, 8-hydroxydeoxyguanosine, (Thermo Scientific; 1:200 dilution) for 30 minutes. For (negative) control IgG images, human lung sections were incubated first with non-immune rabbit IgG and mouse IgG (dilution 1:100).
overnight and then with goat anti-rabbit IgG Alexa Fluor 488 and goat anti-mouse IgG Alexa Fluor 594 for 2 hrs. All fluorescence-labeled lung sections were examined using a Zeiss LSM 510 laser scanning confocal microscope.

**Analysis of Gene expression** - Pulmonary arteries (down to 4th order) were dissected from the surrounding pulmonary parenchyma, snap frozen in liquid nitrogen, pulverized and RNA extracted using TRIZOL or proteins solubilized in 2x Laemmli buffer. cDNA was synthesized using the iScript cDNA Synthesis Kit (Biorad) and used to assess relative gene expression using real time RT-PCR (Bio Rad iQ SYBR Green). Western blot experiments were performed as described\(^2\) and relative densitometry determined using ImageJ software (NIH).

Nox activity – Nox4 activity was determined by the Amplex Red assay in HEK293 cells stably expressing Nox4 as described\(^1\). The relative activity of Nox1 and Nox5 was determined using L-012 as described\(^1,2\).

**Statistical analysis** - Statistical analysis was performed using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA). The mean ± SEM was calculated in all experiments. Data sets were assessed for normal distribution and statistical significance determined either by the unpaired t-test (for 2 groups) or ANOVA (for ≥ 3 groups). For the ANOVA analyses, Newman-Kuels post-hoc testing was employed. A value of p <0.05 was considered significant.

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