Pulmonary hypertension in adult Alk1 heterozygous mice due to oxidative stress

Mirjana Jerkic1,2, Mohammed G. Kabir2, Adrienne Davies1, Lisa X. Yu3,4, Brendan A.S. McIntyre5, Nasir W. Husain1, Masahiro Enomoto5, Valentin Sotov1,6, Mansoor Husain2,7, Mark Henkelman3,4, Jaques Belik2,5,8, and Michelle Letarte1,2,4,6,8*

1Molecular Structure and Function Program, Hospital for Sick Children, 555 University Ave., Toronto, ON, Canada M5G 1X8; 2Heart and Stroke Richard Lewar Center of Excellence, University of Toronto, Toronto, Ontario, Canada; 3Mouse Imaging Centre, Hospital for Sick Children, Toronto, Ontario, Canada; 4Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada; 5Physiology and Experimental Medicine Program, Hospital for Sick Children, Toronto, Ontario, Canada; 6Department of Immunology, University of Toronto, Toronto, Ontario, Canada; 7Toronto General Hospital Research Institute, Toronto, Ontario, Canada; and 8Department of Pediatrics, University of Toronto, Toronto, Ontario, Canada

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1. Introduction

Mutations in the ALK1 gene, coding for an endothelial-specific receptor of the transforming growth factor-β superfamily, are the underlying cause of hereditary haemorrhagic telangiectasia type 2, but are also associated with familial pulmonary hypertension (PH). We assessed the lung vasculature of mice with a heterozygous deletion of Alk1 (Alk1+/-) for disease manifestations and levels of reactive O₂ species (ROS) implicated in both disorders.

Methods and results

Several signs of PH, including elevated right ventricular (RV) systolic pressure leading to RV hypertrophy, reduced vascular density, and increased thickness and outward remodelling of pulmonary arterioles, were observed in 8- to 18-week-old Alk1+/− mice relative to wild-type littermate controls. Higher ROS lung levels were also documented. At 3 weeks, Alk1+/− mice were indistinguishable from controls and were prevented from subsequently developing PH when treated with the anti-oxidant Tempol for 6 weeks, confirming a role for ROS in pathogenesis. Levels of NADPH oxidases and superoxide dismutases were higher in adults than newborns, but unchanged in Alk1−/− mice vs. controls. Prostaglandin metabolites were also normal in adult Alk1+/− lungs. In contrast, NO production was reduced, while endothelial NO synthase (eNOS)-dependent ROS production was increased in adult Alk1+/- mice. Pulmonary near resistance arteries from adult Alk1+/- mice showed less agonist-induced force and greater acetylcholine-induced relaxation; the latter was normalized by catalase or Tempol treatment.

Conclusion

The increased pulmonary vascular remodelling in Alk1+/− mice leads to signs of PH and is associated with eNOS-dependent ROS production, which is preventable by anti-oxidant treatment.

Keywords

Endothelial receptors ● Pulmonary hypertension ● Oxygen radicals ● Nitric oxide ● Anti-oxidant

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* Corresponding author. Tel: +1 416 813 6258; fax: +1 416 813 7877; Email: michelle.letarte@sickkids.ca

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1. Introduction

Mutations in the ALK1 gene are the underlying cause of hereditary haemorrhagic telangiectasia type 2 (HHT2) and occur in 1 in 8000 people. A similar number of individuals have endoglin (ENG) mutations and HHT1. Clinical features of HHT include recurrent nosebleeds, mucocutaneous telangiectases, and pulmonary, cerebral, and hepatic arteriovenous malformations (AVM). Both diseases are autosomal dominant and associated with haploinsufficiency, with a functional reduction in transforming growth factor (TGF-β) superfamily-mediated responses. ALK1 is an endothelial cell receptor primarily mediating bone morphogenic protein (BMP)-9 effects, but also signalling for TGF-β.

ALK1 mutations have been reported in patients with pulmonary hypertension (PH). The gene commonly associated with familial PH is BMPR2, coding for BMP type 2 receptor, which mediates signalling for several BMPs. The observations that both ALK1 and BMPR2 mutations can lead to PH argue in favour of defective BMP9 signalling being involved in PH. TGF-β has also been implicated; a recent study revealed that continuous administration of a TGF-β
receptor blocking antibody at an early stage of disease attenuated experimental PH.17

We previously showed impaired vasomotor function in isolated vessels of Endoglin heterozygous (Eng+/−) mice and a stabilizing role of endoglin in endothelial NO synthase (eNOS) activation.18 Furthermore, we showed that adult Eng+/− mice spontaneously develop signs of PH.19 The haemodynamic properties of Alk1+/− mice not having been described, we evaluated their pulmonary vascular function over time. We report that these mice develop signs of PH, associated with reduced NO bioavailability and increased production of eNOS-dependent reactive oxygen species (ROS) that can be prevented by systemic anti-oxidant treatment.

2. Methods

See Supplementary material online for additional information.

2.1 Mouse studies

All protocols were approved by the Hospital for Sick Children and the Toronto Centre for Phenogenomics Animal Care Committees (AUP#0067) in accordance with the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996; A5047-01). Alk1+/− mice were generated and genotyped as described.20 In one experimental series, 3-week-old Alk1+/− and control littermates drank water containing 1 mM Tempol for 6 weeks.

For arterial pressure and X-ray micro-computed tomographic (CT) measurements, mice were anaesthetized with 1.5% isoflurane and maintained with 1% throughout the experiments. Efficiency of anaesthesia was monitored by lack of withdrawal reflex upon hind toe pinching, regular respiratory rate 30% below normal, and no reaction to skin pinch over the area to be incised. Peak right ventricular systolic pressure (RVSP) was measured by Millar Mikro-tip pressure transducer catheterization via the external jugular vein. Left ventricular (LV) systolic and diastolic pressures were measured by catheterization via the right carotid artery, and stroke volume and cardiac output were estimated. RV was dissected from LV and septum, and RV/(LV + septum) weight ratios were calculated. Vessel measurements by X-ray micro-CT were done by perfusion with microfil under control pressure, scanning at 29 μm resolution and performing three-dimensional volume data reconstruction as described.21

Mice were also anaesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), perfused with phosphate-buffered saline, and heart and lungs were collected. Paraffin-embedded transverse mouse lung sections were stained with Movat’s pentachrome and pulmonary arterial density (number of arteries <60 mm diameter per 100 alveoli) quantified in five independent fields. The mean wall area was calculated by subtracting the internal luminal area from the external (luminal plus mural) area for 20 vessels/section and averaged per mouse.

2.2 Organ bath studies of isolated pulmonary arteries

Third-generation intralobar pulmonary artery ring segments were dissected and mounted in a wire myograph as described.21 Muscle contraction was induced with U46619 in newborns or phenylephrine in adults while relaxation was induced with acetylcholine, with and without the NOS inhibitor N-(G)-nitro-L-arginine methyl ester (l-NAME, 100 nM). Effects of PEG catalase (200 U/mL) and 1 mM Tempol were tested by pre-incubating adult pulmonary arteries for 20 min prior to pre-constriction and acetylcholine addition.

2.3 ROS, NO, and arachidonic acid metabolite determination

Nuclear dihydroethidium (DHE) staining was assessed by fluorescence microscopy on sections from cryopreserved lungs by co-staining with FITC-conjugated antibody to CD31 and 5 μM DHE. H2DCFDA was measured in lung homogenate supernatants using a spectrofluorometer. Levels were normalized for protein content by Bradford assay. A calibrated H2O2 microsensor attached to an Apollo 4000 Free Radical Analyzer was used to measure H2O2 levels in lung homogenate supernatants and plasma. Amplex Red assay was used to estimate H2O2 levels in lung homogenates according to the manufacturer’s instructions. The effects of 1 mM l-NAME and 0.05 mM antimycin (respiratory chain reaction inhibitor) were tested. Fluorescence was quantified and H2O2 levels normalized for protein content.

NO was quantified on freshly excised lung fragments, with and without l-NAME, using a calibrated NO microsensor attached to an Apollo 4000 Free Radical.

The lipid peroxidation product B-isopGF2α and eicosanoid concentrations in lung tissues were determined using liquid chromatography–tandem mass spectrometry (LC–MS/MS) as described.22

2.4 eNOS phosphorylation and western blot analysis

Small fragments of fresh lung tissues were pre-stimulated with ionomycin, with and without l-NAME, prior to tissue homogenization, for eNOS phosphorylation assays. For all western blots, lung tissues were homogenized in 1% Triton X-100 and protease/phosphatase inhibitors and equivalent amounts of lysate proteins were fractionated on 4–12% gradient SDS/PAGE and immunoblotted using the antibodies and methods described in Supplementary material online.

2.5 Statistical analysis

Comparisons were performed by one- or two-way ANOVA and significant differences were evaluated post hoc using either the Bonferroni or Newman–Keuls tests. Results are expressed as mean ± SEM with P < 0.05 representing significance.

3. Results

3.1 Adult Alk1+/− mice develop PH

Alk1+/− mice exhibit normal development and initially show no apparent signs of PH. First, they begin to manifest increased RVSP by 9 weeks of age (Figure 1A). Individual values are shown to indicate that most but not all Alk1+/− mice have higher RVSP than wild-type littermates at 9 and 12 weeks. By 18 weeks, all Alk1+/− mice had increased RVSP. Overall, there was a significant increase in mean RVSP values observed between Alk1+/− and control mice at 9, 12, and 18 weeks (Figure 1). Secondly, Alk1+/− mice developed RV hypertrophy, as measured by RV/LV + S weight ratios, which are normal in 3-week-old Alk1+/− mice but increased by 9 weeks and onwards (Figure 1B). Importantly, changes in this ratio were exclusively attributable to higher RV weights, as LV weights were unchanged (see Supplementary material online, Figure S1). Thirdly, stroke volume and cardiac output were significantly reduced in 12-week-old Alk1+/− mice, suggesting compromised right heart function and/or increased pulmonary vascular resistance, as systemic haemodynamic parameters were not altered in Alk1+/− mice (see Supplementary material online, Table S1). Taken together, these findings suggest that adult Alk1+/− mice develop significant signs of PH by 9 weeks of age.
Figure 1  Signs of PH in adult Alk1+/− mice. (A) The peak RVSP values are shown for individual Alk1+/− (filled circle) and littermate control (open triangle) mice at 9, 12, and 18 weeks. Mean values ± SEM were 36.5 ± 2.3* vs. 26.1 ± 1.4 mm Hg at 9 weeks, 38.1 ± 2.9* vs. 27.5 ± 1.1 at 12 weeks, and 38.4 ± 2.2* vs. 25.9 ± 0.9 at 18 weeks for Alk1+/− vs. controls (n = 6–7/group). (B) RV hypertrophy was similar in 3-week-old Alk1+/− and Alk1−/− littermates but increased in 9-, 12-, and 18-week-old Alk1+/− mice (n = 6–12/group). (C) Quantification of vessel wall area for peripheral arteries <60 μm in diameter showed no difference between groups at 3 weeks (n = 4–5/group) but increased values in older Alk1+/− mice (n = 6–7/group). (D) Pulmonary arterial density was unchanged in Alk1+/− mice at 3 weeks (n = 4–5/group) but significantly reduced at 9 and 18 weeks (n = 6–7/group). *P < 0.05 vs. Alk1+/* controls at corresponding age; † P < 0.05 vs. Alk1+/− controls at 3 weeks; P < 0.05 vs. Alk1+/− controls at 9 weeks. (E and F) Representative X-ray micro-CT images of pulmonary vasculature in 9-week-old Alk1+/− vs. Alk1+/− mice showing the main right and left pulmonary arteries (PA) and veins (V). PA were enlarged in Alk1+/− vs. Alk1+/− mice (mean internal diameters: 1.06 ± 0.06 vs. 0.83 ± 0.04 mm for first generation (P = 0.02); 0.73 ± 0.07 vs. 0.58 ± 0.05 mm for second generation (P = 0.1); and 0.56 ± 0.04 vs. 0.47 ± 0.03 mm for third generation (P = 0.08)). The Alk1+/− peripheral vessels were less numerous, dilated, and tortuous (small arrows), compared with Alk1+/− vessels.
3.2 Adult Alk1+/− lungs demonstrate signs of vascular remodelling

We next examined the pulmonary vasculature. At 3 weeks of age, pulmonary blood vessels appeared normal and calculated wall areas did not differ between Alk1+/− and control mice. However, at 9 weeks, small peripheral artery walls were significantly thicker in Alk1+/− mice and further thickening by 18 weeks suggested progressive remodelling (Figure 1C). Of interest, mean luminal diameters of these peripheral arteries were identical in all groups at 3, 9, and 18 weeks of age (data not shown), indicating that mural thickening observed in Alk1+/− mice was accommodated by outward vessel remodelling. As such, we anticipated that the PH observed in these animals might be due to loss of vessel density or rarefaction. Indeed, vascular density as quantified by the number of small peripheral arteries per 100 alveoli was significantly reduced in 9- and 18-week-old Alk1+/− mice vs. age-matched control mice, and vs. 1-week-old pups. Bronchi and blood vessels (CD31+/−; green fluorescence) show positive nuclei with more intense red fluorescence in adult Alk1+/− lungs (Figure 2A) than littermate controls. (D) H2O2 levels, estimated with a microsensor, were increased in the lungs of adult Alk1+/− vs. control mice. *p < 0.05 vs. Alk1+/− samples; †p < 0.05 vs. Alk1+/+ pups; and #p < 0.05 vs. Alk1+/− pups; n = 6–14/group.

3.3 Increased ROS production in adult Alk1+/− mice

To assess the potential role of oxidative stress in PH development, lung ROS production was measured by multiple approaches. First, DHE-positive nuclei were identified in lung sections also stained for CD31. Figure 2A reveals similar DHE staining in the lungs of 3-week-old Alk1+/− and Alk1+/+ mice; however, in 12-week-old mice, greater DHE staining was observed in Alk1+/− lungs, suggesting higher O2− production. Next, we estimated the amount of ROS in lung homogenates using the H2DCFDA dye, which detects more species of ROS than DHE. This method revealed significantly higher ROS levels in adults compared with pups, and in adult Alk1+/− vs. Alk1+/+ lungs (Figure 2B). As a third metric of ROS, we used LC−MS/MS to estimate the concentration of 8-iso PGF2α, a product of lipid peroxidation. Consistent with the above findings, Alk1+/− adult mice manifested higher ROS production than controls (Figure 2C). Finally, we estimated H2O2 levels (Figure 2D) using a specific microsensor and noted significant increases in adult Alk1+/− vs. control lungs. Together, these independent methods confirmed higher ROS levels in adult Alk1+/− lungs.
3.4 Treatment with anti-oxidant prevents the development of PH in Alk1$^{+/−}$ mice

To determine whether the observed ROS increases were contributing to PH development in adult Alk1$^{+/−}$ mice, we subjected 3-week-old mice displaying normal lungs and ROS levels, to systemic treatment with the orally active anti-oxidant Tempol for a period of 6 weeks. Remarkably, anti-oxidant treatment prevented all changes associated with the onset of PH in Alk1$^{+/−}$ mice, including the rise in RVSP, RV hypertrophy, mural thickening, and pulmonary vascular rarefaction (Figure 3A–D). While Tempol corrected all abnormalities observed in untreated Alk1$^{+/−}$ mice, it had no effect on control littermates. The efficiency of this superoxide dismutase (SOD) mimetic was demonstrated by the presence of higher levels of H$_2$O$_2$ in the plasma of the Tempol-treated mice (Figure 3E).

3.5 Levels of NADPH oxidases, SODs, catalase, and eNOS do not differ in Alk1$^{+/−}$ mice

We next attempted to address the molecular basis of increased ROS production in Alk1$^{+/−}$ mice. First, we assessed levels of the two most abundant NADPH oxidases expressed in lungs. While levels of Nox2 did not change with age (data not shown), Nox4 levels were significantly increased in the lungs of older mice (Figure 4A). However, there was no difference in Nox2 or Nox4 levels in Alk1$^{+/−}$ vs. Alk1$^{+/+}$ lungs, suggesting that these enzymes do not mediate the increased ROS observed in Alk1$^{+/−}$ mice. We then measured levels of SODs, which convert O$_2^−$ to H$_2$O$_2$, to determine whether deficiencies in these scavengers might underlie the higher ROS levels observed in Alk1$^{+/−}$ mice. While lung levels of SOD1, SOD2,
and SOD3 increased with age, they did not differ between Alk1\(^{+/-}\) and control mice (Figure 4A). Catalase levels were also similar in all groups examined.

After confirming that ALK1 expression was reduced in adult Alk1\(^{+/-}\) lungs relative to controls (Figure 4B), we tested endoglin expression as we previously reported that Eng\(^{+/-}\) mice develop PH. Levels of endoglin were normal in Alk1\(^{+/-}\) mice and could not contribute to PH in these animals. eNOS being implicated in HHT1 and in Eng\(^{+/-}\) mice with PH, we measured its level in Alk1\(^{+/-}\) lungs and found no difference with control lungs (Figure 4B).

### 3.6 The cyclooxygenase pathway is not altered in adult Alk1\(^{+/-}\) mice

Increases in pulmonary resistance can be associated with changes in prostaglandins, which have potent vasomotor effects and are generated from endogenous arachidonic acid through the cyclooxygenase (COX) pathway. For example, the potent vasodilator prostaglandin I\(_2\) (prostacyclin) is an effective treatment for PH, and deficiency of PGI\(_2\) and PGI2-synthase and excess thromboxane B2 have been reported in PH. Moreover, PGI2 receptor-deficient mice develop more severe hypoxia-induced PH, while PGI2-overexpressing mice are protected against hypoxia-induced PH.

Accordingly, we tested levels of COX-2 and various arachidonic acid metabolites in adult Alk1\(^{+/-}\) lungs. Western blot analysis indicated that COX-2 levels were unchanged in adult Alk1\(^{+/-}\) vs. control mice (Figure 4B). Furthermore, no differences in arachidonic acid metabolites including prostaglandins D2, E2, F2, and I\(_2\) and thromboxane B2 were detected (see Supplementary material online, Table S2). Products of the lipoxygenation of arachidonic acid, 5- and 15-HETE, which can induce pulmonary vasoconstriction and oedema, were also unchanged in Alk1\(^{+/-}\) lungs (see Supplementary material online, Table S2). In addition, cytochrome P-450 arachidonic acid metabolite, 5,6-epoxyeicosatrienoic acid, which acts as a vasodilator in the pulmonary circulation, was similar in Alk1\(^{+/-}\) and control mice (see Supplementary material online, Table S2). We concluded that neither the COX pathway nor the arachidonic acid metabolites with known vasoactive effects are involved in the onset of PH in adult Alk1\(^{+/-}\) mice.

### 3.7 Decreased NO production and increased eNOS-derived ROS are associated with PH development in adult Alk1\(^{+/-}\) mice

The observation that increased ROS production was not due to changes in the levels of NOX, SOD, catalase, eNOS, COX-2, or...
metabolites of the COX pathway suggested a different mechanism for this unique model of PH. Uncoupling of eNOS is an alternate mechanism underlying ROS production that we demonstrated in Eng+/− mice.18,19 We therefore tested NO production. It was similar in 3-week-old Alk1+/− and control mice and inhibited by L-NAME (Figure 5A). In contrast, there was significantly less NO produced in the lungs of adult Alk1+/− vs. control mice and no L-NAME inhibition (Figure 5A). The residual amount of NO in the presence of L-NAME likely corresponds to the endogenous tissue level, contributing to the background of the microprobe assay. If one calculates L-NAME inhibitable NO produced, it is unchanged in newborn mice and equivalent to control adult levels (≏220 pmol/g) but reduced to 37 pmol/g in adult Alk1+/− mice.

Figure 5 Involvement of eNOS in ROS generation in Alk1+/− lungs. (A) NO production is similar in the lungs of 3-week-old Alk1+/− and Alk1+/+ mice and inhibited normally by L-NAME in both groups. NO levels are reduced in 12-week-old Alk1+/− lungs and no longer inhibited by L-NAME (n = 6–10/group). (B) There was significantly more H2O2 generated in lung homogenates of Alk1+/− vs. control mice, as measured by Amplex Red assay. L-NAME significantly inhibited H2O2 production in Alk1+/− but not control mice. Antimycin inhibited mitochondrial H2O2 generation by a similar percentage in both groups (n = 10–11/group). (C) Higher levels of eNOS Ser-177 phosphorylation at baseline (C) or after stimulation with ionomycin (Io) were observed in the lungs of Alk1+/− vs. control mice; inhibition was seen with L-NAME plus ionomycin (L-N+Io) in both groups. A representative gel shows phospho- (p-eNOS) and total eNOS; the graph shows the mean of six gels, with values expressed relative to basal eNOS levels in control mice. †P, 0.05 vs. corresponding control group; *P, 0.05 vs. adult Alk1+/− lungs; #P, 0.05 vs. Io-treated Alk1+/− lungs. (D) Model illustrating how eNOS uncoupling in adult Alk1+/− mice results in O2•− triggering vascular remodelling, the dismutation of O2•− by SOD or Tempol and the vasodilatory effect of H2O2.

The Amplex Red assay revealed a substantial increase in H2O2 production in Alk1+/− lungs, with a significant inhibition (28%) by L-NAME (Figure 5B). Antimycin, which blocks mitochondrial-derived H2O2, also inhibited production of this particular ROS but at a similar extent (~50%) in both Alk1+/+ and control lungs. Even in the presence of both L-NAME and antimycin, the lungs of adult Alk1+/− mice produced more H2O2 than wild-type controls (3.36 ± 0.65 vs. 1.76 ± 0.31, P = 0.039). These data clearly indicate that the lungs of adult Alk1+/− mice produce less NO and more ROS than controls.

It was shown that phosphorylation of eNOS Ser-177 is pivotal in regulating ROS generation, whereas eNOS Thr-495 phosphorylation had a minimal effect.28 We observed that the basal level of phospho-eNOS Ser-177 was 38% higher in the lungs of Alk1+/− vs. control mice; stimulation with ionomycin (25–30%) and its inhibition by L-NAME were similar in both groups (Figure 5C). Levels of phospho-eNOS Thr-495 were unchanged (data not shown). Thus, phosphorylation of eNOS at Ser-177 may contribute to higher ROS levels in the lungs of Alk1+/− mice.

Figure 5D illustrates how eNOS uncoupling may affect vascular remodelling through superoxide generation.
3.8 Changes in vasomotor tone in adult pulmonary vessels from Alk1+/− mice

Given the evidence for increased ROS generation in Alk1+/− vs. control lungs and the fact that O₂− induces vasoconstriction29 while H₂O₂ can cause pulmonary vasodilation,21,30 we assessed the balance between these opposing forces in Alk1+/− pulmonary arteries. First, the phenylephrine dose–response was significantly blunted in the Alk1+/− vs. littermate controls (Figure 6A). Secondly, the pulmonary arteries from adult Alk1+/− mice showed a greater L-NAME-sensitive relaxation in response to acetylcholine (Figure 6B). No such difference was observed in newborn mice (see Supplementary material online, Figure S2). Together, these data suggest an NOS-dependent shift from NO to H₂O₂ generation in Alk1+/− pulmonary arteries (Figure 6D). These data are confirmed by the inhibition of excess vasodilatation in Alk1+/− pulmonary arteries in the presence of PEG catalase (Figure 6C) or the superoxide scavenger Tempol (Figure 6D).

4. Discussion

Alk1 heterozygous mice spontaneously develop signs of PH by 9 weeks of age, including a significant increase in RVSP accompanied by RV hypertrophy, and muscularization/rarefaction of peripheral arteries. All Alk1+/− mice showed signs of PH at 18 weeks; by 36 weeks, further pulmonary vascular remodelling and more occluded vessels were observed, indicating disease progression.

Arteriolar remodelling together with lower cardiac output suggests that increased pulmonary arterial pressure in Alk1+/− mice is attributable to higher vascular resistance. Reduced cardiac output in Alk1+/− mice (see Supplementary material online, Table S1) rules out secondary PH in these mice. Hepatic high-flow AVM, frequent in individuals with ALK1 mutations and HHT2, can lead to high output heart failure and PH associated with normal pulmonary vascular resistance.31 A lower cardiac output could be caused by increased systemic vascular resistance under normal systemic blood pressure, leading to LV dysfunction and hypertrophy.
However, our results show that Alk1+/− mice have normal LV pressure (see Supplementary material online, Table S1), normal LV dp/dt max and dp/dt min (data not shown), and unchanged LV mass (see Supplementary material online, Figure S1).

The increase in pulmonary pressure in Alk1+/− mice was associated with higher ROS production assessed by four independent methods (Figure 2). Increased oxidative stress has been demonstrated in the lungs and RV of several animal models of PH.9 Excess ROS in the vasculature is known to reduce NO levels, cause tissue injury, and induce proinflammatory responses. However, ROS also affect redox-sensitive cellular pathways and modulate cell migration, growth/apoptosis, and extracellular matrix protein turnover, which all contribute to vascular remodelling.32 Our data show that increased ROS production is associated with a loss of L-NAME inhibitable NO production and a significant increase in NOS-dependent H2O2 generation in adult Alk1+/− lungs (Figure 5). Together, these findings suggest reduced NO bioavailability and an uncoupled state of eNOS activation in the pulmonary circulation. As neuronal NOS was not observed and inducible NOS was barely detectable in lung tissues (data not shown), we conclude that the observed increase in L-NAME inhibitable H2O2 is eNOS-derived. Antimycin significantly inhibited H2O2 but similarly in Alk1+/− and Alk1+/+; however, the absolute amount of mitochondria-derived ROS was higher in Alk1+/− lungs. This might be explained by NO reduction indirectly inhibiting mitochondrial KATP channel opening, further increasing oxidative stress caused by mitochondrial ROS release.33

No differences were observed in several enzymes generating and scavenging ROS, nor in the COX pathway, leaving eNOS-dependent ROS generation to account for vascular remodelling and changes in vasomotor tone in adult Alk1+/− lungs. The observed decrease in phenylephrine-induced vasoconstriction and increase in acetylcholine-induced relaxation (i.e. NOS-mediated relaxation) suggest a switch from NO- to H2O2-dependent vasodilation in isolated pulmonary arteries of adult Alk1+/− mice (Figure 5D). We reported similar findings for adult Eng−/− mice where H2O2 had a vasorelaxant effect on pulmonary arteries.27 Thus, Alk1+/− mice appear to have a similar defect in terms of eNOS uncoupling associated with PH.19 We previously observed impaired eNOS—Hsp90 association and uncoupling of dimeric eNOS in a BHy4-independent mechanism in Eng−/− mice and demonstrated that endoglin and ALK1 associate with and stabilize the eNOS activation complex.18,19 Here, we show that the eNOS Ser-1177, previously implicated in the generation of ROS, 28 is higher under basal conditions in the lungs of Alk1+/− vs. control mice and further increased upon eNOS stimulation.

The systemic administration of Tempol from the age of 3 weeks prevented the onset of PH in adult Alk1+/− mice (Figure 3). Apart from its main action as a SOD mimetic, Tempol was shown to enhance endogenous NO generation/bioactivity and restore NO-mediated vasodilatation.34 Tempol had an minimal effect on the vasomotor function of isolated lung arteries of Alk1+/− mice, except at the highest Ach concentrations tested; this may be caused by superoxide being quickly converted to H2O2 in vitro. However, Tempol did increase the vasodilation of normal arteries, likely through stimulation of NO production by normal eNOS.

PH is a complex disease with initial stages likely involving the interaction between genetic predisposition and environmental risk factors.35 The observations that BMP9, ALK1, and ENG haploinsufficiency predispose to PH infer that their downstream pathways are critical. The finding of circulating BMP9 as the true physiological ligand for ALK1, acting in combination with BMPR2,7,8 supports a role for BMP9 in PH pathobiology. Endoglin potentiates the effects of BMP9 and can therefore affect these pathways.7,8 While BMP2+/− mice had no or mildly elevated RVSP,36,37 the infusion of serotonin caused several signs of PH, further increased by hypoxic conditions.38 A transgenic dominant-negative form of BMP2 specifically expressed in smooth muscle cells also led to increased RVSP.39 More recently, BMP2 deletion in pulmonary endothelial cells was shown to predispose mice to elevated RVSP, RV hypertrophy, and thickening of peripheral arteries.40 However, none of these models progress to intimal fibrosis or arterial occlusion. The extent of PH observed in this study in the Alk1+/− mice and previously in the Eng+/− mice19 is also consistent with an early stage of disease that does not progress to the fibrotic hyperplastic stage.

Our results suggest that eNOS-dependent O2− induces vascular remodelling and rarefaction of peripheral arteries in Alk1+/− mice leading to an increase in pulmonary vascular resistance. The lack of progression to fibrotic disease may be due to the intrinsic defect in vasomotor tone, characteristic of Alk1+/− and Eng+/− mice. This may explain why only a small fraction of patients with ALK1 and ENG mutations develop PH.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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