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Spontaneous Adult-Onset Pulmonary Arterial Hypertension Attributable to Increased Endothelial Oxidative Stress in a Murine Model of Hereditary Hemorrhagic Telangiectasia

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Objective—Loss-of-function mutations in genes coding for transforming growth factor-β/bone morphogenetic protein receptors and changes in nitric oxide* (NO*) bioavailability are associated with hereditary hemorrhagic telangiectasia and some forms of pulmonary arterial hypertension. How these abnormalities lead to seemingly disparate pulmonary pathologies remains unknown. Endoglin (Eng), a transforming growth factor-β coreceptor, is mutated in hereditary hemorrhagic telangiectasia and involved in regulating endothelial NO* synthase (eNOS)-derived NO* production and oxidative stress. Because some patients with pulmonary arterial hypertension harbor ENG mutations leading to haploinsufficiency, we investigated the pulmonary vasculature of Eng+/− mice and the potential contribution of abnormal eNOS activation to pulmonary arterial hypertension.

Methods and Results—Hemodynamic, histological, and biochemical assessments and x-ray micro-CT imaging of adult Eng+/− mice indicated signs of pulmonary arterial hypertension including increased right ventricular systolic pressure, degeneration of the distal pulmonary vasculature, and muscularization of small arteries. These findings were absent in 3-week-old Eng+/− mice and were attributable to constitutively uncoupled eNOS activity in the pulmonary circulation, as evidenced by reduced eNOS/heat shock protein 90 association and increased eNOS-derived superoxide (*O2−) production in a BH4-independent manner. These changes render eNOS unresponsive to regulation by transforming growth factor-β/bone morphogenetic protein and underlie the signs of pulmonary arterial hypertension that were prevented by Tempol.

Conclusion—Adult Eng+/− mice acquire signs of pulmonary arterial hypertension that are attributable to uncoupled eNOS activity and increased *O2− production, which can be prevented by antioxidant treatment. Eng links transforming growth factor/bone morphogenetic protein receptors to the eNOS activation complex, and its reduction in the pulmonary vasculature leads to increased oxidative stress and pulmonary arterial hypertension. (Arterioscler Thromb Vasc Biol. 2010;30:509-517.)

Key Words: Alk-1 ● endoglin ● free radicals ● nitric oxide ● pulmonary arterial hypertension ● transforming growth factor

Endoglin (Eng; CD105) is an ancillary receptor for several transforming growth factor (TGF)-β superfamily ligands, including bone morphogenetic proteins (BMP).1 It is predominately expressed on vascular endothelial cells2 and found in both TGF-β and BMP receptor complexes,1,3 where it modulates TGF-β1/β3 and BMP9/10 effects,3 respectively, via its physical association with the activin-like kinase receptor-1 (ACVLR1) gene product, ALK1. Eng-null mice die at mid-gestation with impaired angiogenesis and severe cardiac defects.5,7 Whereas Eng+/− mice have a normal lifespan, they display abnormal systemic vascular autoregulatory functions related to endothelial nitric oxide synthase (eNOS) activity.8

Mutations in the endoglin (ENG) and activin-like kinase 1 receptor (ACVLR1) genes lead to haploinsufficiency and are the underlying cause of hereditary hemorrhagic telangiectasia type 1 (HHT1)9 and type 2 (HHT2),10 respectively. This disease is characterized by multiple focal telangiectases and arteriovenous malformations (AVMs) in the pulmonary, hepatic, and cerebral microcirculations.11 These fragile structures are low-pressure conduits that can affect local tissue blood flow, and their...
potential rupture in vital organs may lead to internal hemorrhage, anemia, and death. Patients with HHT1 and HHT2 display phenotypically similar vascular lesions but diverge with respect to organ involvement with a higher prevalence of pulmonary arteriovenous malformations (PAVMs) in HHT1.12

Mutations in ACVRL1 have also been reported in patients presenting with pulmonary arterial hypertension (PAH),13–15 suggesting that HHT and PAH may share defects in related signaling pathways. PAH is characterized by a sustained elevation in mean pulmonary arterial pressure causing progressive right ventricular hypertrophy and leading to heart failure and death.16 The increase in pulmonary arterial pressure is attributable to increased pulmonary vascular resistance (PVR) caused by progressive loss/pruning of the peripheral lung vasculature attributable to increased oxidative stress in the pulmonary vasculature.16–18 eNOS activity is reduced in hypoxia-induced pulmonary hypertension as a result of impaired association of eNOS with its allosteric regulator, heat shock protein 90 (Hsp90).19 Such changes in eNOS activation were shown to lead to increased eNOS-derived reactive oxygen species (ROS) generation instead of NO+ bioavailability contributable to an animal model of persistent pulmonary hypertension of the newborn.21,22 Whereas loss-of-function mutations in TGF-β/BMP receptors have been associated with familial forms of PAH, the contributions of abnormal eNOS biology and endothelial oxidative stress in these forms of PAH remain to be elucidated.

We report that adult Eng+/− mice spontaneously develop signs of PAH that are attributable to uncoupled eNOS activity and ROS production causing progressive loss in pulmonary vasculature and increased muscularization of arterioles. Eng links TGF-β/BMP receptors to the eNOS activation complex, where its reduction leads to constitutive endothelial eNOS-derived oxidative stress, rendering the enzyme unresponsive to regulation by TGF-β/BMP signaling. These changes underlie PAH in Eng+/− mice, which can be prevented by treatment with Tempol.

**Materials and Methods**

**Animal Studies**

All experimental protocols were performed in accordance with the Canadian Council on Animal Care and approved by the Animal Care Committee of the Hospital for Sick Children. N17-N19 Eng+/− and Eng+/− C57BL/6 mice were generated by Dr. Letarte at the Hospital for Sick Children by successive backcrosses. Mice ranging from 3 to 18 weeks old were used; 8- to 12-week-old mice were exposed to 1 mmol/L Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; Fluka) in the drinking water for 6 weeks. Mice were anesthetized with 1.5% isoflurane for hemodynamic measurements or euthanized for lung histology, x-ray micro-CT, and biochemical studies.

**Cardiac Measurements**

Peak right ventricular systolic pressure (RVSP) was measured in mice by Millar Mikro-tip pressure transducer catheterization of the right ventricle (RV) via the external jugular vein (AD Instruments). The RV was dissected from the left ventricle and septum, and the Fulton index (RV/left ventricle+septum weight ratio) was calculated.

**Ultrasound Biomicroscopy**

Using a Vevo 770 ultrasound biomicroscope (VisuaSonic), observations of the mouse heart and great vessels were conducted as described.23 Dynamic changes in chamber/lumen dimensions were recorded using M-mode. RV stroke volume was calculated by multiplying the velocity–time integral by the main pulmonary artery cross-sectional area at peak systole. The isovolumetric contraction, relaxation, and ejection times were measured to calculate the Tei index = (isovolumetric contraction + isovolumetric relaxation IVRT)/ejection time.

**Lung X-Ray Micro-CT**

Anesthetized mice were intubated by tracheotomy and breathing was monitored using a pressure-controlled ventilator. Mice were perfused at 20 mm Hg via the RV with warm heparinized phosphate-buffered saline, followed by Microfil (Flow Tech) at 40 mm Hg using a pressure Servo System PS/200 (Living Systems Instrumentation). For 3-week-old mice, lungs were perfused at 10 mm Hg and with 3-fold diluted Microfil at 25 mm Hg. Specimens were scanned at 29 μm using a Micro-CT scanner (GE Healthcare). Three-dimensional volume data were reconstructed using the Feldkamp algorithm for cone-beam CT geometry. Three-dimensional iso-surface rendering of the pulmonary vasculature was accomplished using MicroView software (GE Healthcare).

**Morphometric Analysis of Small Arteries in Lungs**

Paraffin-embedded transverse lung sections of 8- to 12-week-old and 18-week-old mice were stained with Movat’s pentachrome, and 5 independent fields were quantified for the number of small arteries per 100 alveoli. Average wall areas of arteries were determined from the difference between outer and inner wall areas for 20 vessels per section and averaged for each mouse.

**Lung ROS Measurements**

Lungs were homogenized in phosphate-buffered Krebs containing 1 mmol/L NaHCO3, and ROS levels were assessed using 10 μmol/L S-(and-β)-caboxy-2,7-dichloro dihydrofluorescein diacetate (carboxy-H2DCFDA; Molecular Probes) at 37°C. Fluorescence was quantified on a SpectraMax spectrofluorometer (Molecular Devices) using 488 nm excitation and 525 nm emission wavelengths and was normalized for protein content.

**Lung NO+ Measurements**

Excised lung tissue was incubated in Krebs-Henseleit buffer for 2 hours at 37°C in the presence and absence of l-NAME. NO+ production was quantified using an NO+ microsensor ISO-NOP700 attached to an Apollo 4000 Free Radical Analyzer. The microsensor was calibrated using S-Nitroso-N-acetylpenicillamine in the presence of copper sulfate.

**eNOS/Hsp90 Association and Immunoblotting**

Lung segments were stimulated with vehicle or 1 μmol/L ionomycin for 15 minutes. Mouse Eng+/− and Eng+/+ endothelial cells derived from the yolk sac of E8.5 embryos24 were stimulated with increasing concentrations of TGF-β1. Extracts were prepared in 10 mmol/L Tris-HCl (pH=7.4; 1% Triton X-100 with protease/phosphatase inhibitors) and preclinared with protein A/G mixture. eNOS homodimer formation in lung extracts were performed by low-temperature SDS-PAGE. Proteins were immunoprecipitated or immunoblotted with antibodies to phospho-eNOS Thr95, eNOS, Hsp90, h-actin (BD Biosciences), and Eng (MJ7/18, Southern Biotech) or Alk1 (Santa Cruz Biotech). Bands were visualized by chemiluminescence and quantified.

**NOS-Derived O2− Measurements**

eNOS-derived ROS levels were assessed by dihydroethidium (DHE; Molecular Probes) staining in isolated main pulmonary arterioles.
arteries. After preincubation with and without L-NAME (10^{-5} mol/L), vessels were incubated with acetylcholine (ACh, 10^{-5} mol/L) and 2 μmol/L DHE at 37°C. Vessels were washed, mounted en face on glass slides exposing the endothelial layer, and analyzed by fluorescence microscopy.

Eng^{+/+} and Eng^{+/−} embryonic endothelial cells were grown in Optilux 96-well black-clear-bottom plates and serum-starved for 3 hours. Cells were incubated with 5 μmol/L DHE in the presence and absence of L-NAME and stimulated with 1 μmol/L ionomycin. Live cells were observed through the CY3 fluorescence channel and photographed using a TE2000 inverted microscope (Nikon) equipped with an environmental chamber set to 37°C and 5% CO₂. The number of positive nuclei/field was quantified based on size and intensity using 3-dimensional imaging software (Volocity).

Lung BH4 and BH2 Measurements
Lungs were homogenized in 50 mmol/L phosphate buffer (pH 2.6) containing 0.1 mmol/L DTPA and 1 mmol/L DTE. Samples were loaded onto a high-performance liquid chromatography system (Cou- lArray system, Model 582 and 542; ESA Biosciences) with a Synergi Polar-RP column (4 μm; 250×4.6 mm; Phenomenex) and eluted with argon-saturated 50 mmol/L phosphate buffer (pH 2.6). Calibration curves were made by summing up the peak areas collected at 0 and 150 mV for BH₄ and 280 and 365 mV for BH₂. Intracellular concentrations were calculated using authentic BH₄ and BH₂ (10 to 100 nmol/L) as standards and normalized to protein content.

eNOS Activity
Cultured Eng^{+/+} and Eng^{+/−} endothelial cells were preincubated with and without L-NAME and NOS activity was assayed by monitoring the conversion of ^3H-l-arginine to ^3H-l-citrulline in response to TGF-β1 (250 pmol/L), as previously described.²

Statistical Analysis
Comparisons were performed by 1- or 2-way ANOVA, and significant overall differences were evaluated posthoc using the Bonferroni procedure. Results are expressed as the mean±SEM, with P<0.05 representing significance.

Results
Eng^{+/−} Mice Have Elevated RVSP and RV Hypertrophy
The 8- to 12-week-old Eng^{+/−} mice had elevated RVSP reaching 33.9±2.6 mm Hg vs 27.6±2.6 mm Hg in control Eng^{+/+} mice (Figure 1A; P<0.05). In 18-week-old mice, this parameter was unchanged in the Eng^{+/+} group (27.1±0.5 mm Hg) but significantly increased to 36.1±1.8 in the Eng^{+/−} group. Exposure to the pulmonary hypertension-inducing stimulus, 12% O₂ for 3 weeks, resulted in similarly elevated RVSP (P<0.01 vs normoxic mice) in Eng^{+/−} (40.1±2.1 mm Hg) and Eng^{+/−} mice (45.1±2.9 mm Hg). Eng^{+/−} mice had normal heart rates (412±42 vs 442±31 bpm), mean systemic arterial pressures (87.4±2.8 vs 90.4±3.9 mm Hg), body weights, and activity levels. RV hypertrophy was not observed in 8- to 12-week-old mice but was seen in 18-week-old normoxic Eng^{+/−} mice (Figure 1B), suggesting that a longer exposure to elevated RV load was necessary for this manifestation. Similar levels of RV hypertrophy were noted in Eng^{+/−} and Eng^{+/−} mice after hypoxia (P>0.05), consistent with the increased RVSP. Left ventricle plus septum weights did not vary between groups and the RV-to-body weight ratios confirmed the RV hypertrophy determined by the Fulton index. Whereas absolute Eng levels were increased after hypoxia (P<0.01), relative levels between Eng^{+/+} and Eng^{+/−} mice remained unchanged (Supplemental Figure IA, available online at http://atvb.ahajournals.org).

Doppler flow velocity spectra in the main pulmonary artery were recorded using ultrasound biomicroscopy (Figure 1C), and reduced RV output (Figure 1D) was determined for Eng^{+/−} mice (P<0.05 vs Eng^{+/+}). Hypoxia reduced RV
output in $\text{Eng}^{+/+}$ mice but did not further affect this parameter in $\text{Eng}^{+/−}$ mice. The calculated RV Tei index and RV fractional shortening were unchanged in normoxic $\text{Eng}^{+/−}$ mice, suggesting normal RV function (Supplemental Figure II A, IIB, available online at http://atvb.ahajournals.org). Moreover, left ventricle function was normal in $\text{Eng}^{+/−}$ mice, as evidenced by unchanged percent fractional shortening (27.9 ± 1.3 vs 25.9 ± 1.9), isovolumetric contraction time-to-ejection time ratio (0.22 ± 0.01 vs 0.22 ± 0.02), and isovolumetric relaxation time-to-filling time ratio (0.28 ± 0.01 vs 0.27 ± 0.01).

**Adult $\text{Eng}^{+/−}$ Mice Show Reduced Pulmonary Vascular Density**

Pulmonary circulation of 3-week-old $\text{Eng}^{+/+}$ mice was analyzed by x-ray micro-CT and found to be normal (Figure 2A). In contrast, 8- to 12-week-old $\text{Eng}^{+/−}$ mice displayed enlarged main pulmonary arteries and pruning of peripheral vessels (Figure 2B), suggesting that these features were acquired in adult $\text{Eng}^{+/−}$ mice. The absence of Microfil in pulmonary veins of $\text{Eng}^{+/−}$ mice suggested increased resistance to flow in distal pulmonary arterioles and lack of large arteriovenous shunts. The number of pulmonary arteries (<60 μm) per 100 alveoli was reduced in normoxic 8- to 12-week-old and 18-week-old $\text{Eng}^{+/−}$ mice compared to age-matched controls (Figure 2C; $P<0.05$). Exposure to hypoxia reduced arterial density in both $\text{Eng}^{+/+}$ and $\text{Eng}^{+/−}$ mice to the same extent, whereas alveolar density remained unchanged (Figure 2C). Lung sections of 7-day-old $\text{Eng}^{+/+}$ and $\text{Eng}^{+/−}$ mice showed similar vessel and alveoli densities, indicating no detectable congenital defects in lung size, angiogenesis, or alveolarization (data not shown).

Our inability to effectively perfuse the venous system via the pulmonary artery in adult $\text{Eng}^{+/−}$ mice suggested increased resistance to flow in distal pulmonary arteries. Movat’s pentachrome staining revealed significant muscularization of small arterioles in adult $\text{Eng}^{+/−}$ vs $\text{Eng}^{+/+}$ mice. This phenotype was observed in both groups of mice after hypoxia. Muscularization of arteries was confined to the <30-μm group in the 8- to 12-week-old normoxic $\text{Eng}^{+/−}$ group (Figure 2D; $P<0.05$ vs control). However, in 18-week-old $\text{Eng}^{+/−}$ mice, wall thickening was observed in <30-μm and >40-μm arteries, indicating more widespread remodeling with age. After hypoxia, no difference in vessel wall area was observed between $\text{Eng}^{+/+}$ and control mice, and all vessels displayed increased wall area compared to those from normoxic mice. For each vessel caliber, internal diameters were unchanged (data not shown), indicating outward remodeling of $\text{Eng}^{+/−}$ blood vessels and suggesting that increased PVR was contributed by reduced vascularity and changes in local vasomotor tone. Some vessels harbored fibrin clots/thrombi, a feature observed in both PAH and HHT (Supplemental Figure IIIA, available online at http://atvb.ahajournals.org). Some older $\text{Eng}^{+/−}$ mice also displayed AVM-like structures with notable arterioalization of veins (Supplemental Figure IIB).

**Increased ROS Production and Uncoupled eNOS Activity in $\text{Eng}^{+/−}$ Pulmonary Vasculature**

Total ROS production in lungs of mice was assessed and shown to be significantly elevated in $\text{Eng}^{+/−}$ lungs compared

![Figure 2](image-url)
arteries (PA) by eNOS-derived ROS production was examined in pulmonary activation and potential uncoupling (Figure 3B). Increased inhibitable NO• generation, which was abolished by L-NAME treatment of 3-week-old Eng+/− mice did not display any l-NAME–inhibitable NO• production, suggesting abnormal eNOS activity. Excitation of pulmonary arteries, probably by inhibiting the normal "O2•− scavenging by NO•.

Representative immunoprecipitation/Western blot and graph showing increased Hsp90 levels in eNOS immunoprecipitates of Eng+/− lungs, but not Eng−/− lungs, stimulated with ionomycin (P<0.05 vs untreated). Basal eNOS/Hsp90 association was also reduced in Eng+/− lung samples (P<0.05 vs untreated). Basal eNOS/Hsp90 association was also reduced in Eng+/− lungs, but not Eng−/− lungs, stimulated with ionomycin (P<0.05 vs untreated). Basal eNOS/Hsp90 association was also reduced in Eng+/− lungs, but not Eng−/− lungs, stimulated with ionomycin (P<0.05 vs untreated). Basal eNOS/Hsp90 association was also reduced in Eng+/− lungs, but not Eng−/− lungs, stimulated with ionomycin (P<0.05 vs untreated).

Figure 3. ROS production and uncoupled eNOS activity in Eng+/− lung vasculature. A, Eng+/− lungs produced significantly greater amount of ROS (*P<0.05 vs Eng−/−). B, Lack of l-NAME–inhibitable NO• production in Eng+/− lungs (**P<0.01 vs untreated Eng+/−; n=5/group). C, Representative images showing increased acetylcholine-induced DHE staining in Eng−/− vs Eng+/− pulmonary arteries. L-NAME abrogated this response in Eng+/− arteries but enhanced DHE staining in Eng−/− arteries, probably by inhibiting the normal "O2•− scavenging by NO•.

D, Representative immunoprecipitation/Western blot and graph showing increased Hsp90 levels in eNOS immunoprecipitates of Eng+/− lungs, but not Eng−/− lungs, stimulated with ionomycin (P<0.05 vs untreated). Basal eNOS/Hsp90 association was also reduced in Eng+/− lung samples (P<0.05 vs untreated Eng−/−; n=4/group). E, Representative Western blot and graph showing increased ratio of eNOS monomer to dimer levels in lung extracts of Eng−/− (P<0.05 vs Eng+/− mice; n=6/group). F, Unchanged BH4 levels and BH4-to-BH2 ratio in Eng+/− lungs.

to Eng+/+ littermates (Figure 3A; P<0.05). Unlike Eng+/+ mice, lungs of Eng+/− mice did not display any l-NAME–inhibitable NO• production, suggesting abnormal eNOS activation and potential uncoupling (Figure 3B). Increased eNOS-derived ROS production was examined in pulmonary arteries (PA) by en face DHE staining of the endothelium in response to ACh. Compared to Eng+/+ PA, those of Eng+/− mice displayed increased endothelial DHE staining, confirming ROS generation, which was abolished by l-NAME (Figure 3C). Very little DHE staining was observed in ACh-stimulated Eng+/+ arteries, and l-NAME had an enhancing effect, likely attributable to the loss of ROS scavenging activity of NO•. These results were substantiated by reduced eNOS/Hsp90 association in Eng−/− vs Eng+/+ lungs (Figure 3D) under basal conditions and after stimulation with ionomycin. Total eNOS and Hsp90 levels remained unchanged in Eng−/− vs Eng+/+ lungs (Supplemental Figure IB, IC). However, the ratio of eNOS monomer to dimer levels was significantly higher in lung extracts of Eng−/− vs Eng+/+

mouse (Figure 3E), whereas lung BH4 levels or the BH4-to-BH2 ratio remained unchanged (Figure 3F). Taken together, these data suggest that eNOS does not produce NO• in the lungs of Eng−/− mice and instead predominantly contributes to ROS production via uncoupling of its dimeric form in a BH4-independent manner.

Tempol Prevents Onset of PAH in Adult Eng+/− Mice

Treatment of 3-week-old Eng+/+ and Eng+/− mice with Tempol prevented the expected increase in RVSP (Figure 4A) in adult Eng+/− mice, as well as the outward arterial remodeling (Figure 4B), the rarefaction of distal arterioles (Figure 4C), and the increase in vessel wall area (Figure 4D).

Eng Links TGF-β/BMP Signaling to eNOS

Eng immunoprecipitates with eNOS, suggesting that eNOS activation may be regulated by TGF-β/BMP receptor signaling. We now report that ALK1 can also be immunoprecipi-
suggested that Eng is critical for eNOS activation by TGF-β1 and possibly other ligands of the TGF-β superfamily.

Constitutive eNOS-Derived ROS Production in Eng-Deficient Endothelial Cells

Our finding that Eng was required for both TGF-β-induced and ionomycin-induced stimulation of eNOS suggested constitutive eNOS uncoupling in Eng-deficient cells, independent of TGF-β/BMP receptors. Compared to mouse Eng+/− endothelial cells, a significantly greater number of Eng−/− cells showed nuclear DHE staining that was inhibitable with 1-NAME under basal conditions, suggesting constitutive eNOS-derived ROS production (Figure 5E). Ionomycin significantly increased ROS production in Eng+/+ but not Eng−/− cells. Pretreatment with 1-NAME attenuated the ionomycin effect in both groups of cells (Figure 5E).

Figure 4. Tempol prevents onset of PAH in Eng+/− mice. A, Elevated RVSP in 9-week-old Eng+/− mice (P<0.05 vs Eng+/+) was prevented by Tempol (P<0.05 vs untreated Eng−/−). B, Representative Movat’s pentachrome—stained lung sections from untreated and Tempol-treated Eng+/+ and Eng−/− mice. Tempol caused normalization (C) of pulmonary artery (<60 μm) density and attenuation (D) of pulmonary vessel wall area (P<0.05 vs untreated Eng+/+). n=7–9 mice per group.

Discussion

Loss-of-function mutations in TGF-β/BMP receptors and endothelial oxidative stress have been associated with PAH. However, a functional inter-relationship between these alterations in PAH pathogenesis remains unknown. We have found that adult Eng+/− mice spontaneously develop signs of PAH because of endothelial dysfunction leading to progressive pruning of the pulmonary vasculature and muscularization of small arteries. Both in vivo and in vitro biochemical data suggest that PAH in Eng+/− mice is associated with an intrinsic defect in eNOS activation leading to constitutively increased eNOS-derived ROS production while rendering this enzyme refractory to regulation by members of the TGF-β/BMP superfamily. The onset of PAH in adult Eng+/− mice is prevented by treatment with the O2− dismutase mimetic, Tempol, underscoring the importance of eNOS uncoupling in this model of PAH.

Balancing eNOS-derived NO* vs O2− production is an important determinant in numerous cardiovascular diseases, including PAH.25–28 Interestingly, we observed increased eNOS-derived O2− production and were unable to detect any 1-NAME–inhibitable NO* generation in lungs of Eng+/− mice. Increased monomerization of eNOS was observed in Eng−/− lungs, which may partly account for the reduced NO* production. Whereas it has been proposed that monomeric eNOS can produce O2−, it has been recently shown that this is unlikely and that this form of the enzyme is inactive.29 Interestingly, our finding that BH4 levels remained unchanged in lung extracts of Eng−/− mice suggests that the observed increase in NOS-dependent O2− production occurs via the uncoupling of dimeric eNOS activity in a BH4-independent manner. Taken together, these findings ascribe to Eng a critical role in modulating the eNOS activation complex.

Caveolin-1 null mice display signs of PAH attributable to persistent activation of eNOS leading to PKG nitration, a mechanism that necessitates both oxidative stress and heightened NO* production.30 Eng is enriched in caveolar lipid rafts, communoprecipitates with caveolin-1 in endothelial cells, and regulates eNOS activation and NO* bioavailability.8 However, it is unlikely that PKG nitration plays a role in the PAH phenotype seen in Eng−/− mice, because we could not detect any 1-NAME–inhibitable NO production. Perhaps
the difference between the 2 models is related to the balance of eNOS-derived \( \cdot \text{NO} \) vs \( \cdot \text{O}_2^- \) and that the significant production of eNOS-derived \( \cdot \text{O}_2^- \) in Eng\(^{-/-}\)/H11002 mice is sufficient to induce PAH.

In adult Eng\(^{-/-}\) mice, eNOS-derived ROS production and reduced NO\(^\cdot\) bioavailability likely reduced endothelial cell survival, leading to pruning of the distal pulmonary microvasculature and contributing to the muscularization of small arteries, effects that were prevented by the antioxidant, Tempol. We contend that the salutary effects of Tempol reported in the current study are likely attributable to its role in preserving NO\(^\cdot\) bioavailability by the dismutation of \( \cdot \text{O}_2^- \) to H\(_2\)O\(_2\). NO\(^\cdot\) normally prevents excessive smooth muscle proliferation/remodeling and coordinates arterial repair.\(^{31}\) Moreover, H\(_2\)O\(_2\) likely served as an endothelium-derived hyperpolarizing factor of vascular smooth muscle, thus attenuating the increase in pulmonary vascular resistance and pressure. We believe excess \( \cdot \text{O}_2^- \), and not H\(_2\)O\(_2\), leads to PAH in Eng\(^{-/-}\) lungs and that endogenous \( \cdot \text{O}_2^- \) dismutase activities are relatively insufficient to prevent disease. Inter-
estingly, PAH is acquired in adult Eng+/− mice, suggesting an increased predisposition of these mice to age-related uncoupling of eNOS, a developmentally regulated phenomenon recently reported in piglets.32

Our findings that eNOS co-immunoprecipitates with both Eng and ALK1 further supports a central role for Eng in linking TGF-β/BMP receptors to eNOS. TGF-β1 caused increased eNOS/Hsp90 association, eNOS Thr495 diphosphorylation, and 1H1-targinine to 1H1-citrulline conversion in Eng+/+, but not in Eng−/− endothelial cells. However, failure of the receptor-independent eNOS agonist, ionomycin, and of TGF-β1 to induce eNOS/Hsp90 association in Eng-deficient cells suggests an intrinsic defect in eNOS activation. This is supported by our findings of significantly elevated eNOS-derived ROS production in unstimulated Eng−/− cells, indicating constitutive uncoupling and thus rendering eNOS refractory to regulation by TGF-β1/BMP and other ligands in this superfamily.

Our study supports clinical observations in which HHT patients manifest PAVMs and varying degrees of pulmonary hypertension,15,33,34 suggesting a common defective pathway involving Eng/ALK1 that predisposes to lesions associated with these diseases. We have found abnormal vascular structures suggestive of PAVMs in Eng+/− mice that also display PAH (Supplemental Figure III). We have previously reported that Eng+/− systemic arteries have a reduced ability to contract in response to elevations in transmural pressure, thus failing to effectively reduce vessel wall tension. Elevated parietal wall tension attributable to increased pulmonary perfusion pressure would increase the probability of PAVMs and their subsequent risk of rupture. Perhaps the observed enlargement of large feeding pulmonary arteries in Eng−/− may be a prelude to abnormal vascular structures reminiscent of HHT. We propose that small, low-resistance AVM-like structures may arise from elevated pulmonary vascular pressure and account for the relatively mild increase in RVSP and PAH progression in adult Eng+/− mice. Manifestations of PAH or HHT may be influenced by genetic and environmental modifying factors that specifically affect the integrity of the Eng+/− pulmonary vasculature and its ability for normal remodeling/repair undergoing elevated intravascular pressure. Interestingly, shRNA-mediated silencing of BMP receptor-2 in mice resulted in signs of HHT instead of the expected PAH.35 More recently, conditional ablation of BMP receptor-2 in the pulmonary endothelium was sufficient to elicit signs of PAH only in a subset of mice.36 It remains to be determined if the appearance of small PAVM-like structures may have counteracted the increase in RVSP and progression of PAH in the seemingly asymptomatic group of BMP receptor-2−/− deficient mice.

PAVMs are more prevalent in HHT1 than in HHT2 patients (48% vs 5%).12 These low-resistance structures could alleviate overall PVR, providing a partial explanation for the higher prevalence of PAH in patients with ACVRL1 vs ENG mutations.15 Whereas a current study suggests that embolization of PAVMs does not lead to a sustained elevation in pulmonary arterial pressure in HHT patients,34 this does not necessarily preclude an increased PVR because embolization can also be associated with reduced cardiac output. Patients with ENG mutations presenting with PAH typically have lower PVR than those with ACVRL1 mutations.4 Moreover, in some of these cases, the increased pulmonary arterial pressure is eventually normalized with the appearance of PAVMs.13,37 Our findings suggest that ENG mutations leading to haploinsufficiency are a predisposing factor to PAH, and that PAVMs in HHT1 may result from abnormal vascular remodeling under high local pressure conditions that may, in turn, serve to alleviate PVR.

Our study ascribes to Eng a critical role in the maintenance of the mature lung vasculature. In adult Eng+/− mice, alterations in eNOS activation leading to uncoupling renders the enzyme refractory to regulation by TGF-β/BMP signaling and constitutes a critical event leading to excessive oxidative stress and PAH pathogenesis. The absence of disease in younger mice may be related to the prolonged time course required for PAH manifestations, or mechanisms surrounding eNOS uncoupling are developmentally regulated. Our study suggests a close association between PAH and HHT1, and our experimental system may provide a means to define the specific determinants in the pathogenesis of both diseases. Future genetic screening of families with PAH may unravel more individuals with ENG mutations.

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Disclosures
None.

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