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HIF2α–arginase axis is essential for the development of pulmonary hypertension

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Hypoxic pulmonary vasoconstriction is correlated with pulmonary vascular remodeling. The hypoxia-inducible transcription factors (HIFs) HIF-1α and HIF-2α are known to contribute to the process of hypoxic pulmonary vascular remodeling; however, the specific role of pulmonary endothelial HIF expression in this process, and in the physiological process of vasoconstriction in response to hypoxia, remains unclear. Here we show that pulmonary endothelial HIF-2α is a critical regulator of hypoxia-induced pulmonary arterial hypertension. The rise in right ventricular systolic pressure (RVSP) normally observed following chronic hypoxic exposure was absent in mice with pulmonary endothelial HIF-2α deletion. The RVSP of mice lacking HIF-2α in pulmonary endothelium after exposure to hypoxia was not significantly different from normoxic WT mice and much lower than the RVSP values seen in WT littermate controls and mice with pulmonary endothelial deletion of HIF-1α exposed to hypoxia. Endothelial HIF-2α deletion also protected mice from hypoxia remodeling. Pulmonary endothelial deletion of arginase-1, a downstream target of HIF-2α, likewise attenuated many of the pathophysiological symptoms associated with hypoxic pulmonary hypertension. We propose a mechanism whereby chronic hypoxia enhances HIF-2α stability, which causes increased arginase expression and dysregulates normal vascular NO homeostasis. These data offer new insight into the role of pulmonary endothelial HIF-2α in regulating the pulmonary vascular response to hypoxia.

### Results

#### Deletion of HIF\(\alpha\) Isoforms in Pulmonary Endothelium

Genetically manipulated mouse strains with conditional alleles of either the HIF-1α or -2α isoforms (18, 19) were crossed to mouse strains or -2 in pulmonary endothelium. The RVSP of mice lacking HIF-2α isoforms in Pulmonary Endothelium. Genetically manipulated mouse strains with conditional alleles of either the HIF-1α or -2α isoforms (18, 19) were crossed to mouse strains or -2 in pulmonary endothelium. The RVSP of mice lacking HIF-2α isoforms in conditions were significantly lower than in littermate controls (22.4 ± 1.1 mmHg, n = 9, P = 0.03) mice. However, RVSPs from normoxic L1cre-HIF-1α mice (24.7 ± 1.7 mmHg, n = 6) did not differ from those of WT controls (Fig. 1A).

**Figure 1**

### Significance

The expression of hypoxia-inducible factor (HIF)-2α in pulmonary endothelium of mice influences pulmonary vascular resistance and development of hypoxic pulmonary hypertension (PH) via an arginase-1-dependent mechanism. The HIF-2α:arginase-1 axis influences the homeostatic regulation of nitric oxide synthesis in the lung. Impaired generation of this vasoactive agent contributes to the initial development and vascular remodeling process of PH.


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**Significance**

The expression of hypoxia-inducible factor (HIF)-2α in pulmonary endothelium of mice influences pulmonary vascular resistance and development of hypoxic pulmonary hypertension (PH) via an arginase-1-dependent mechanism. The HIF-2α:arginase-1 axis influences the homeostatic regulation of nitric oxide synthesis in the lung. Impaired generation of this vasoactive agent contributes to the initial development and vascular remodeling process of PH.

**Author contributions:** A.S.C., C.B., N.W.M., E.R.C., and R.S.J. analyzed data; and A.S.C., E.R.C., and R.S.J. wrote the paper.

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The RVSPs of L1cre-HIF-2α mice following hypoxic exposure (26.1 ± 1.6 mmHg, n = 7) were not significantly different from those of untreated WT mice (22.48 ± 1.19, n = 9) and were much lower than the elevated values seen in WT littermates controls (41.9 ± 1.8 mmHg, n = 12, P < 0.0001) and L1cre-HIF-1α mice (36.25 ± 2.37 mmHg, n = 7, P < 0.005) (Fig. 1A).

The ratio of right ventricular weights to those of the left ventricle plus septum (RV/LS+S), an indicator of RVH, was likewise significantly higher in WT (0.316 ± 0.01, n = 9, P < 0.0001) and L1cre-HIF-1α (0.323 ± 0.02, n = 8, P < 0.001) mice exposed to hypoxia compared with the ratios found in L1cre-HIF-2α (0.209 ± 0.008, n = 8) mice (Fig. 1B). WT and mutant mice reacted to hypoxia normally in other respects (Fig. S2 A–C).

**Pulmonary Endothelial HIF-2α Is Essential for Vascular Remodeling.**

Medial thickening of pulmonary vessels was calculated. Both WT littermates and L1cre-HIF-1α mice showed a large relative reduction in medial thickness relative to WT animals. Normoxic animals showed no changes in vessel structure (Fig. S3).

Serial lung sections were immunostained to mark endothelial cells. Lungs from WT and L1cre-HIF-1α mice had typical tissue remodeling following CH (Fig. 1D), with an increase in α-smooth muscle actin (α-SMA) associated with pulmonary arteries (Fig. 1E). However, little to no remodeling was observed in lung sections from L1cre-HIF-2α mice (Fig. 1F). Lung sections showed increases in elastin in both WT controls and L1cre-HIF-1α mice, but only minimal staining in the L1cre-HIF-2α mice (Fig. 1D) (20). Collagen was also significantly higher in hypoxia-conditioned WT and L1cre-HIF-1α mice relative to L1cre-HIF-2α mice (Fig. S4A). Pulmonary endothelial deletion of HIF-2α resulted in an increase in α-SMA-positive muscle cell coverage after hypoxic exposure (Fig. 1F). In comparison, both WT littermate control and L1cre-HIF-1α mice developed full and partial rings of α-SMA-positive cells around vessels in hypoxia-conditioned animals (Fig. 1F).

**Reduced Arginase Expression in HIF2α Mutant Mice.** Previous work from our laboratory and others has demonstrated that the two HIFα isoforms act to control NO homeostasis during hypoxia. This occurs through HIF1α regulation of the NOS2/iNOS gene, and HIF-2α regulation of the Arg-1 and Arg-2 genes (21–25). The enzyme Arg-2 in particular has been implicated in reducing airway NO and promoting remodeling and collagen deposition in pulmonary arterial hypertension (PAH) patients (26, 27). We found that hypoxic up-regulation of arg-1 and -2 was reduced in hypoxia-conditioned isolated murine primary pulmonary endothelial cells (Fig. S5A) and whole lung samples from L1cre-HIF-2α mice, relative to WT littermate controls following hypoxic conditioning (Fig. S4).

Consistent with these data, we found that plasma NOx concentrations were significantly reduced in HIF-1α mice and elevated in HIF-2α mutant mice compared with WT control mice (Fig. 2B); this was mirrored in an increase in NO metabolites detected in whole lung extracts of hypoxically treated animals (Fig. 2C). There was also an increase in exhaled NO detected in HIF-2α mutant mice (Fig. 2D). Although several recent studies have highlighted the role of endothelin-1 (ET-1) in pulmonary hypertension and indicated its regulation by the HIF pathway, purified lung endothelial cells showed little change in ET-1 expression in this model (Fig. S5 B–D). There was a significant increase in PDGF-β expression in WT mice during the acute hypoxic phase (days 1–3) before levels returned to near baseline at the chronic phase (day 21). However, there seems to be little separation between WT and L1cre-HIF2α mice (Fig. S6 A–D). We further analyzed a number of stem cell markers known to be up-regulated in PAH (28). We found enhanced whole lung gene expression of...
Changes in Acute Response to Hypoxia Caused by Loss of Endothelial HIF-2α. Hypoxia rapidly stimulates pulmonary vascular resistance (HPV) and increases the pressure needed to maintain normal output from the right ventricle of the heart. We compared HPV in WT control and mutant animals by measuring RVSPs, both immediately before and during hypoxic challenge (method shown in Fig. 3A). L1cre-HIF-2α mice have a lower resting RVSP (18.99 ± 1.00 mmHg, n = 11) than WT control animals (22.47 ± 1.19 mmHg, n = 9) (Fig. 1A); nonetheless, the magnitude of pressure changes induced by hypoxia were still significantly lower in mice lacking pulmonary endothelial HIF-2α (3.10 ± 0.60 mmHg, n = 13) relative to that seen in WT animals (5.45 ± 0.76 mmHg, n = 13, P = 0.023). RVSP recorded from L1cre-HIF-1α (4.56 ± 1.53 mmHg, n = 7) mice, however, did not significantly deviate from WT controls (Fig. 3B) during acute hypoxic exposure. Thus, acute pressure changes in the pulmonary circulation induced by hypoxia are significantly lower in animals that lack HIF-2α in the pulmonary endothelium. Baseline arterial saturations for L1cre-HIF-2α mice did not deviate from WT controls (Fig. 3C). However, during the acute hypoxic challenge, there was a strong trend toward greater desaturation in the L1cre-HIF-2α compared with WT mice (Fig. 3C, P = 0.06, n = 7).

Whole-body plethysmography showed that resting ventilation rates in normoxia are similar in the HIFα mutant animals relative to WT controls [WT control 168 ± 3.0 breaths per minute (BrPM), L1cre-HIF-1α 174 ± 2.8 BrPM, and L1cre-HIF-2α 170 ± 2.7 BrPM] (Fig. 3D). All mice responded to acute hypoxia by increasing ventilation rates. L1cre-HIF-1α and WT littermate control mice increased to 258 and 250 BrPM, respectively, for the initial 5 min before reducing their ventilation rates to 187 and 174 BrPM during the first hour of exposure to hypoxia. However, L1cre-HIF-2α mice increased respiratory rate to 308 BrPM, then reduced their ventilation rates to 233 BrPM in the same time period (Fig. 3D). Of note, both tidal volume and peak inflation/exhalation flow rates are comparable between the three groups (Fig. S8 A–C), indicating that there is no anatomic difference in pulmonary capacity. The carotid bodies in all animals were histologically normal (Fig. S8 D–H).
attenuated the development of hypoxic pulmonary hypertension. Given the role of HIF-2α in regulating arginases specifically, this indicates that a key aspect of the function of HIF-2α in PAH is its regulation of arginase expression.

Endothelial Progenitor Cells from Human PAH Patients Have Altered HIF-2α-Dependent Arginase Expression. Blood outgrowth endothelial cells (BOECs) have been extensively used as a model for studying in vitro endothelial function in vascular disorders (29, 30) with close functional and gene expression similarity to pulmonary artery endothelial cells (31).

Previous work has shown enhanced expression and activity of Arg-2 in PAH patients (27, 32). Fig. 4.4 demonstrates that these cells have no deficiencies in HIF expression, and that Arg-2 expression is increased in the PAH patient-derived cells (Fig. 4.4 and Fig. S9 A and B). Hypoxia further increases the expression of Arg-2 in these BOECs from PAH patients.

BOECs from control volunteers produce significantly more NO than BOECs from PAH patients following 48 h in culture (Fig. S9C). NO production was restored to near that seen in control BOECs following arginase inhibition with S-(2-boronoethyl)-L-cysteine (BEC) (Fig. S9C). shRNA technology was used to specifically knock down HIF-1α, HIF-2α, and Arg-2 in these BOECs. These data show that knockdown of HIF-2α suppresses the expression of Arg-2 (Fig. 4B) and decreases Arg-2 enzyme activity in these cells from patients with PAH (Fig. 4C).

Discussion
In this study, we have shown that the endothelial cell is a necessary element in the changes that result in PAH, and that the HIF isoform HIF-2α is in turn required for that endothelial response. We also observed the down-regulated expression of arginase in isolated pulmonary endothelial cells and whole lungs from L1cre-HIF2a mice following CH and demonstrate here that deletion of arginase-1 specifically in the pulmonary endothelium

Fig. 3. Acute HPV is significantly blunt in L1cre-HIF-2α mutants. (A) Line diagram showing the time line and gas composition used to determine the acute HPV response. (B) Acute HPV was determined by measuring RVSP before and during acute hypoxic challenge (10% O₂). The delta between the two pressures was determined as the hypoxic vasoconstriction response. Data shown in bar graph as mean ± SEM from WT (n = 13), L1cre-HIF-1α (n = 7), and L1cre-HIF-2α (n = 13). (C) Percentage arterial oxygen saturation was recorded during the acute hypoxic challenge. Data recorded at 5-s intervals mean ± SEM of WT (n = 7) and L1cre-HIF-2α (n = 7). (D) Ventilation rate in response to acute hypoxia was determined by whole-body plethysmography. Resting/normoxic ventilation was determined 60 min before acute hypoxic stimulus. Data shown as mean Breaths per minute ± SEM for WT (n = 10), L1cre-HIF-1α (n = 5), and L1cre-HIF-2α (n = 6). *P < 0.05.

Fig. 4. Analysis of human BOECs as a model for studying in vitro endothelial function in PAH. (A) Western blot analysis of HIF1α and HIF2α stability and arginase-2 expression in normoxia and 16 h posthypoxia. Data shown in bar graph as a ratio of target gene to β-actin, mean ± SEM of control (closed bar, n = 3) and PAH (red bar, n = 4). (B) A lentiviral shRNA strategy was used to target HIF1α, HIF2α, and Arg-2 expression. Three shRNAs were used to knock down each gene of interest. A scramble short hairpin and GFP-tagged lentivirus and no treatment were included as controls. Data shown (mean ± SEM qPCR fold change compared with no-treatment control) for Arg-2 from control (black bar, n = 3) and PAH (red bar, n = 3) 16 h posthypoxia. (C) Cell lysates were analyzed for arginase activity following the shRNA strategy to knock down HIF1α, HIF2α, and Arg-2. Data shown are mean ± SD of urea produced corrected per milligram of protein lysate for control (black bar n = 2) and PAH (red bar n = 2). *P < 0.05, **P < 0.001 (control) and *P < 0.05, **P < 0.001 (PAH).
The HIF pathway was implicated in PH initially through demonstrations showing that mice hemizygous for HIF-1α or HIF-2α have diminished levels of pulmonary hypertension (15, 33); subsequent work was able to show that hemizygosity for HIF-1α resulted in changes in myocyte hypertrophy and polarization (14). In contrast, hemizygosity of HIF-2α revealed that endothelial changes resulting in PAH were partially blocked when HIF-2α was diminished (15). Recent studies have also shown that PHD2 loss, which gives rise to increased HIF protein stability, promotes an HIF-2α–dependent increase in pulmonary hypertension (34, 35). These studies further demonstrate the importance of the HIF pathway in the etiology of PAH.

The causal link between pulmonary hypertension and NO homeostasis has been extensively documented (26, 36), and this is reflected clinically in the finding that intrapulmonary nitrates, biochemical reaction products of NO in bronchoalveolar fluid, and exhaled NO are all diminished in human pulmonary hypertension (37, 38). Interestingly, primary pulmonary endothelial cells isolated from PAH patients have substantially increased expression of arg-2 (27), which would be predicted to decrease available L-arginine and reduce NOS-derived NO formation. We have previously shown that both Arg1 and Arg2 are HIF-2α–dependent genes, and we show here that their expression in pulmonary endothelium is decreased in HIF-2α pulmonary endothelium mutants. This should result in an increase in pulmonary endothelial NO, which itself has been shown to alleviate PAH experimentally (39). Consistent with this hypothesis, genetic deletion of arg-1 resulted in a marked attenuation in the pathologies associated with PAH. Given a mechanistic link between these findings and the etiology of PAH, future therapies to manipulate the control of NO homeostasis by the HIFα pathway should certainly be explored.

Methods

Animals. All animals were housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. All protocols and surgical procedures were approved by the local and national animal care committees. Targeted deletion of HIF-1α, HIF-2α, and arginase-1 in pulmonary endothelial cells was created by crossing (C57Bl6/j) homozygous for Cre recombinase expression drive by the L1 (alk-1) promoter kindly donated by Paul Oh, University of Florida, Gainesville, FL (17). Mice characterized as WT were in all cases littermates of respective mutant mice, homozygous for conventional alleles but without the cre recombinase transgene.

Measurement of RVSP. For induction of PAH due to CH, groups of male mice (8–12 wk) were maintained in a normobaric hypoxic chamber (FiO2 10%) for up to 21 d. Mice were weighed then anesthetized (isoflurane) and right-sided heart catheterization was performed through the right jugular using a pressure-volume loop catheter (Millar) (40–42). Blood was taken for hemodynamic assessment.

RVH. To measure the extent of RVH, the heart was removed and the right ventricle (RV) free wall was dissected from the left ventricle plus septum (LV+S), and weighed separately (43). The degree of RVH was determined from the ratio RV/LV+S.

Tissue Preparation. In all animals the left lung was fixed in situ in the dis tended state by the infusion of 0.8% agarose into the trachea and then placed in 10% (wt/vol) paraformaldehyde before paraffin embedding. The right lung was frozen in liquid nitrogen for mRNA extraction.

Pulmonary Vascular Morphometry. Detailed methods are given in Methods.

Hematological Analysis. Anticoagulated blood was analyzed using Vet abc hematology analyzer (Horiba) according to the manufacturer’s instructions.

Nitrite/Nitrate Analysis. Blood samples were centrifuged to separate plasma and were passed through a column with a 10-kDa cutoff filter. All samples were analyzed for total NOx content using a NOA 280i (Sievers; GE Healthcare) according to the manufacturer’s instructions.

RNA Analysis. Detailed methods are given in Methods.

BOEC isolation culture. BOECs were prepared in lysis buffer for an arginase pathway experiment to promote cell-cycle progression. Detailed methods are given in Methods.

Arginase activity assay. BOECs were prepared in lysis buffer for an arginase activity assay as previously described (45). Detailed methods are given in Methods.

Carotid Body Histology. Carotid body histology was performed as previously reported (46). Carotid body volume and cell numbers were quantified on a microscope images (Leica DM-RE) using ImageJ software. Detailed methods are given in Methods.

Whole-Body Unrestrained Plethysmography. A single-chamber plethysmograph (Data Science) was used in conjunction with a pressure transducer. Non-anesthetized mice were randomly placed into the plethysmograph and allowed to acclimate. Once they were acclimated to the chamber, the composition of the flow gas was switched from 21% O2 to 10% O2 using a PEGAS mixer (Columbus Instruments). Detailed methods are given in Methods.

Exhaled NO analysis. Exhaled NO was measured noninvasively in non-anesthetized mice using a closed chamber system as previously described (47). Briefly, gas phase NO was measured by a chemiluminescence-based NO analyzer sensitive to 0.1 ppb NO (NOA 280i; Sievers). Detailed methods are given in Methods.

Primary lung endothelial cell isolation. Primary endothelial cells were isolated and cultured from lungs of L1cre-HIF2α and WT mice, as previously described (25). Detailed methods are given in Methods.

Knockdown experiments. Human BOECs derived from both control and PAH donors were transduced using lentiviral particles containing three different shRNAs targeting human HIF-1α, HIF-2α, and Arg-2 mRNA, respectively. Detailed methods are given in Methods.

Statistical Analysis. All data represents the mean ± SEM of n separate experiments unless otherwise stated. Difference between groups was assessed using t test unless otherwise stated. A P value of <0.05 was considered significant.

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