Impaired hypoxic pulmonary vasoconstriction in a mouse model of Leigh syndrome

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Hypoxic pulmonary vasoconstriction (HPV) is a physiological vasomotor response of the small pulmonary resistance arteries to alveolar hypoxia. HPV contributes to the maintenance of normal systemic oxygenation by redistributing blood flow away from poorly ventilated lung regions, thereby matching alveolar ventilation with perfusion (22, 31).

Mitochondrial complex I (CI) is a major component of the electron transport chain (ETC) and contributes both to the oxidation of NADH and to the generation of a proton gradient across the mitochondrial inner membrane to drive ATP production (37). Mutations in either nuclear or mitochondrial genes that encode proteins involved in the assembly and/or the electron transport function of CI cause mitochondrial dysfunction (33).

The signals that trigger HPV are incompletely understood. It is hypothesized that CI “senses” alveolar hypoxia and initiates the depolarization of pulmonary arterial smooth muscle cells, which results in calcium entry and subsequent vasoconstriction. Previous studies showed that pharmacological inhibition of CI impaired the ability of pulmonary smooth muscle cells to induce vasoconstriction in isolated lung models of HPV (2, 36). However, to our knowledge, no study has previously evaluated HPV in a genetic model of complex I deficiency in vivo.

CI deficiency, caused by a deletion in the nuclear encoded gene Ndufs4, results in Leigh syndrome, which is the most common mitochondrial disease in children (8, 30). To date, mutations in more than 75 different genes can cause Leigh syndrome, which is a devastating neurodegenerative disease leading to death typically within the first years of life (17). Mice lacking Ndufs4, a protein which is responsible for the assembly and normal function of CI, develop a progressive neurodegenerative disease and die ~60 days after birth (18). We previously reported that continuously breathing normobaric 11% O2 prevents neurological disease and improves survival in Ndufs4−/− mice (14). The objective of this study was to investigate the effect of Ndufs4 deficiency and phar-
macological inhibition of CI on HPV in vivo. We hypothesized that congenital deficiency and pharmacological inhibition of CI impair HPV and worsen arterial systemic oxygenation. To characterize the beneficial effects of chronic hypoxia in Ndufs4 deficient mice better, we also investigated whether exposure to breathing 11% O₂ for 3 wk would restore HPV and improve systemic oxygenation in Ndufs4−/− mice.

MATERIALS AND METHODS

Animals. All animal experiments outlined in this study were approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital. We studied Ndufs4-deficient (Ndufs4−/−) mice and their wild-type (Ndufs4+/+) littermates on a C57BL/6J genetic background of both sexes with an age range of 4–5 wk and a weight range between 12 and 17 g. Ndufs4−/− mice were kindly provided by the Palmiter laboratory at the University of Washington. Ndufs4−/− and their Ndufs4+/+ littermates were housed and bred in the hospital’s animal facility. Animals for each experiment were matched for sex and age. Measurement of HPV in mice. To measure HPV, left lung pulmonary vascular resistance index (LPVRI) was calculated before and during alveolar hypoxia. Surgical preparation was performed as described previously (4, 12, 39). Briefly, general anesthesia was induced by intraperitoneal injection of ketamine (120 mg/kg) and fentanyl (0.09 mg/kg). Mice were placed on a heating pad to maintain core temperature at 37°C. Following a tracheostomy, muscle relaxation was induced by intraperitoneal injection of rocuronium (1 mg/kg), and volume-controlled ventilation was initiated at a respiratory rate of 110 breaths/min and a tidal volume of 8 ml/kg. Inspired oxygen fraction of 1.0 and a positive end-expiratory pressure of 1 cm H₂O were provided throughout the measurements using MiniVent 845 (Harvard Apparatus, Holliston, MA). A polyethylene catheter was placed in the right carotid artery and the right jugular vein for hemodynamic monitoring and infusion of 0.06 ml·h⁻¹·g⁻¹·lactated Ringer solution. After left parasternal thoracotomy, a precision perivascular flow probe (MA-0.5PSB, Transonic Systems, Ithaca, NY) was placed around the left parasternal thoracotomy, a precision perivascular flow probe (MA-0.5PSB, Transonic Systems, Ithaca, NY) was placed around the left parasternal thoracotomy. A fluid-filled polyethylene 10 catheter was inserted into the main pulmonary artery to measure pulmonary blood flow (Q̇P₂,PA). A fluid-filled polyethylene 10 catheter was inserted into the main pulmonary artery for measurement of pulmonary arterial pressure (PAP), Q̇l₂,PA, heart rate (HR), mean arterial pressure (MAP), and PAP were continuously measured and recorded with a digital data software (Chart 5, AD Instruments, Colorado Springs, CO). To calculate LPVRI, transient reduction of Q₂,PA was carried out by three partial occlusions of the inferior vena cavae described previously (13). LPVRI was calculated from the slopes of the regression line representing the PAP/Q₂,PA relationship. HPV was expressed as the percentage in

Measurement of lower thoracic aortic flow. Cardiac output was estimated by measuring lower thoracic aortic flow (Q̇l₁,TAF) before and during LMBO using the MA-0.5PSB flow probe connected to a flowmeter (TS-420 module, Transonic Systems, Ithaca, NY). To estimate total systemic vascular resistance index (TSVRI), Q̇l₂,TAF was transiently reduced three times by partial occlusions of the inferior vena cava as described previously (12, 38). TSVRI was calculated from the slopes of the regression line representing the MAP/Q̇l₂,TAF relationship.

Administration of piericidin A or angiotensin II to mice. Piericidin A, an NADH/ubiquinone oxidoreductase complex I inhibitor, was purchased from VWR International (Radnor, PA). Piericidin A dissolved in 100% DMSO, or DMSO alone (vehicle) was administered intraperitoneally in Ndufs4−/− mice 30 min before HPV measurement. The amount of DMSO used in these studies did not exceed 1 ml/kg. Angiotensin II, a potent vasoconstrictor, was obtained from VWR International and dissolved in saline solution. A programmable syringe pump (BS-300, Braintree Scientific, Braintree, MA) was used to continuously infuse 0.5 μg·kg⁻¹·min⁻¹ angiotensin II via right jugular vein. Before and during infusion of angiotensin II, LPVRI was measured as described above. The dose and timing of angiotensin II administration was selected based on a previous study (9).

Mice breathing 11% O₂. Thirty-five-day-old mice were exposed to either air or 11% hypoxia and housed in 80-ml transparent acrylic boxes for 21 days. A membrane technology nitrogen generator (MAG-20, Higher Peak) was used to adjust relative concentration of nitrogen and oxygen in the chamber to obtain a continuous inspired oxygen concentration of 11% or air. The oxygen concentration was monitored daily using an O₂ sensor (MiniOx 1, OhioMedical). CO₂ levels were continuously monitored inside the chambers (CO200, Extech), and the CO₂ concentration was maintained below 0.4% by adjusting the gas flow between 5 and 10 l/min. Soda lime was used as a CO₂ scavenger inside the chambers. Mice were housed in cages with standard bedding, and a 12-h/12-h light-dark cycle was used throughout the experiment.

Measurement of succinate, fumarate, and cytokine mRNA levels in lung tissue. To characterize the function of complex II in mice with congenital deficiency of the Ndufs4 gene better, we measured the levels of succinate and fumarate in lung tissues of Ndufs4−/− and Ndufs4+/+ mice treated either with air or 11% O₂. Following HPV measurements, the ventilated lungs were excised and snap-frozen in liquid nitrogen. Liquid chromatography mass spectrometry was used to measure metabolites in the lungs. Metabolites were extracted from lung samples on dry ice with 1.5 ml of 80% methanol. The tissue samples were homogenized with a Qiagen TissueLyser for 4 min. Samples were then centrifuged at 21,100 g for 20 min, and 750 μl of supernatant was collected, diluted to 30% in methanol, and lyophilized overnight. The lyophilized powder was resuspended in 70% acetonitrile. Liquid chromatography mass spectrometry measurements were performed on a Q-Exactive Plus Orbitrap mass spectrometer coupled with a Dionex UltiMate 3000 ultra-high performance liquid chromatography system (Thermo Fisher Scientific, Waltham, MA). Metabolites were separated on an Xbridge amide HILIC column (2.1 x 100 mm, 2.5 μM particle size; Waters, 186060901). Mobile phase A was 20 mM ammonium acetate and 0.25% ammonium hydroxide (pH 9) with 5% acetonitrile as described previously (15). Mobile phase B was 100% acetonitrile. Data acquisition was performed in full scan mode, with a range of 70–1,000 mass-to-charge ratio, resolving power of 140,000 at 1,000 mass-to-charge ratio, an automatic gain control target of 3e6, and a maximum injection time of 400 ms.

To analyze the inflammatory response in the lung tissue of mice with the Ndufs4 deficiency, we measured cytokine levels in lung tissue obtained from Ndufs4−/− and Ndufs4+/+ mice breathing air. Real-time qPCR was used to measure the levels of IL-6, TNFα, and IL-1β blood oxygen was assumed to be 1.0. Qs/Qt was calculated with the Berggren equation (5).

Measurement of lower thoracic aortic flow. Cardiac output was estimated by measuring lower thoracic aortic flow (Q̇l₁,TAF) before and during LMBO using the MA-0.5PSB flow probe connected to a flowmeter (TS-420 module, Transonic Systems, Ithaca, NY). To estimate total systemic vascular resistance index (TSVRI), Q̇l₂,TAF was transiently reduced three times by partial occlusions of the inferior vena cava as described previously (12, 38). TSVRI was calculated from the slopes of the regression line representing the MAP/Q̇l₂,TAF relationship.
mRNAs as described previously (3). Briefly, total RNA was extracted by the phenol/guanidinium method and reverse transcription was performed using MultiScribe MuLV Reverse Transcriptase (Thermo Fisher Scientific). A Mastercycler ep realplex (Eppendorf, Hamburg, Germany) was used for real-time amplification and quantification of mRNA transcripts. All mRNA levels were determined by the relative CT method normalized to 18S ribosomal RNA. TaqMan gene expression assays were used to quantify mRNA levels encoding IL-6, TNFα, and IL-1β, as well as the level of 18S RNA.

Measurement of NADH/NAD⁺ ratio in lung tissues. Total NAD (NADH and NAD⁺) and NADH were measured to determine the NADH/NAD⁺ ratio in lung tissue using a NAD/NADH quantitation colorimetric kit (K337-100, BioVision, Inc.) in accordance with manufacturer’s protocol (24). Lung tissue was homogenized in extraction buffer and centrifuged at 14,000 g for 5 min. The amount of NAD and NADH was measured in the supernatant, and NAD⁺ was calculated by subtracting NADH from total NAD.

Statistical analysis. Statistical analyses were performed using Prism 7 (GraphPad Software, La Jolla, CA) and RStudio Version 1.0.136 (RStudio, Boston, MA). The Shapiro-Wilk test was used to determine the normality of data variables. Multiple group differences in normally distributed data were determined with the one-way ANOVA with a post hoc Bonferroni correction. A Kruskal-Wallis test was used for multiple comparisons. Group differences of the same parameter in the same treatment group were compared by a paired t-test. Data are expressed as means ± SE. HPV, hypoxic pulmonary vasoconstriction; LMBO, left mainstem bronchial occlusion; LPVRI, left pulmonary vascular resistance index; MAP, mean arterial pressure; PAP, pulmonary arterial pressure; QqPA, left pulmonary arterial blood flow.

RESULTS

Effect of Ndufs4 deficiency or pharmacological inhibition of CI on HPV. To investigate the effect of Ndufs4 deficiency on HPV, the LPVRI was measured at baseline and during LMBO in Ndufs4−/− and Ndufs4+/− mice. At baseline (before LMBO), with mice breathing 100% oxygen, hemodynamic parameters and LPVRI did not differ between the two genotypes (Table 1). In Ndufs4−/− mice, LMBO induced a smaller increase in LPVRI, compared with Ndufs4+/− mice (% increase in LPVRI in Ndufs4−/− vs. Ndufs4+/− mice: 70 ± 3 vs. 147 ± 7%, P < 0.05, Fig. 1A). During LMBO, there was no difference in hemodynamic parameters, including HR, MAP, and PAP between the two genotypes (Table 1). To investigate whether changes in systemic vascular resistance might explain the differences in HPV between wildtype and Ndufs4−/− mice, the QqTAF and MAP were measured before and during LMBO. QqTAF and MAP did not differ between Ndufs4−/− and Ndufs4+/+ mice at baseline or during LMBO (Table 2). The TSVRI, obtained from the relationship between QqTAF and MAP, was also similar at baseline between the two genotypes and was not altered by LMBO (Table 2). Taken together, these results show that the absence of CI subunit Ndufs4, and the resulting decreased CI activity, leads to impaired HPV but does not alter systemic vascular resistance either at baseline or during LMBO.

Piericidin A blocks mitochondrial electron transfer by potently inhibiting CI (10, 40). To investigate whether pharmacological inhibition of CI impairs HPV, we measured the effect of piericidin A on LMBO-induced changes in pulmonary vascular resistance. Mice treated with piericidin A (dissolved in DMSO) had a smaller increase in LPVRI in response to LMBO than Ndufs4−/− mice treated with DMSO alone (% increase in LPVRI in piericidin A- vs. DMSO-treated Ndufs4−/− mice: 49 ± 12 vs. 144 ± 11%, P < 0.05, Fig. 1B). Hemodynamic parameters, including HR and LPVRI, did not differ between piericidin A- and DMSO-treated mice before LMBO (Table 1). Mice treated with piericidin A had a lower MAP at baseline than Ndufs4−/− mice treated with DMSO (MAP in Ndufs4−/− mice: 70 ± 3 vs. 87 ± 7%, P < 0.05 vs. saline-treated Ndufs4−/−; P < 0.05 vs. saline-treated Ndufs4−/− mice in the same treatment group.

Values are means ± SE. The effect of LMBO on each parameter was analyzed in each group by ANOVA or Kruskal-Wallis test with a post hoc correction for multiple comparisons. Group differences of the same parameter in the same treatment group were compared by a paired t-test. HR, heart rate; LMBO, left mainstem bronchial occlusion; LPVRI, left pulmonary vascular resistance index; MAP, mean arterial pressure; PAP, pulmonary arterial pressure; QqPA, left pulmonary arterial blood flow. *P < 0.05 vs. saline-treated Ndufs4−/−; **P < 0.05 vs. saline-treated Ndufs4−/− mice in the same treatment group.
piericidin A- vs. DMSO-treated Ndufs4+/+ mice: 66 ± 6 vs. 85 ± 5, P < 0.05, Table 1). When compared with DMSO-treated Ndufs4+/+ mice, piericidin A did not alter QLTAF or TSVRI in response to LMBO (Table 2). Taken together, these results indicate that acute pharmacological inhibition of CI, similar to genetic deficiency of Ndufs4, impairs LMBO-induced HPV. The LMBO-related changes in LPVRI caused by piericidin A are not a result of alterations in systemic vascular resistance.

Ndufs4 deficiency does not induce pulmonary inflammation. Previous studies showed that pulmonary inflammation may result in loss of HPV (25, 26, 38). To consider the possibility that Ndufs4 deficiency causes impaired HPV by inducing inflammation, we measured cytokine levels in lung tissue from air breathing Ndufs4+/− and Ndufs4+/+ mice breathing air. The levels of IL-6, TNFα, and IL-1β mRNA in the lung tissue of Ndufs4+/− mice were not significantly increased compared with the levels of these cytokine mRNAs in the lungs of Ndufs4+/+ mice (Fig. 2, A–C).

Effect of chronic hypoxia on HPV and hemodynamic parameters in Ndufs4−/− mice. Continuous exposure of Ndufs4−/− mice to normobaric 11% O2 prevents the development of neurological disease and markedly improves survival (9, 14). To investigate the effects of chronic hypoxia on LMBO-induced HPV in Ndufs4-deficient mice, Ndufs4−/− and Ndufs4+/+ mice breathed 11% O2 for 3 wk. At baseline (before LMBO), breathing 11% O2 increased PAP and LPVRI in both Ndufs4−/− and Ndufs4+/+ mice to a similar extent (Table 1). The increase in LPVRI induced by left bronchus occlusion was similar in Ndufs4−/− and Ndufs4+/+ mice (%increase in LPVRI in Ndufs4−/− and Ndufs4+/+ mice: 118 ± 10 vs. 121 ± 12%, P = not significant, Fig. 3). Hemodynamic parameters, including MAP and HR, did not differ between the two groups either before or during LMBO (Table 1). To assess the effect of chronic hypoxia on systemic vascular resistance, we measured QLTAF in both genotypes. When compared with Ndufs4+/+ mice, breathing 11% O2 did not alter QLTAF or TSVRI in Ndufs4−/− mice (Table 2). These results show that in Ndufs4-deficient mice the impairment of LMBO-induced HPV can be reversed by breathing 11% O2 for 3 wk and that there are no differences in systemic vascular resistance at baseline or during LMBO between chronically hypoxic Ndufs4-deficient and Ndufs4+/+ mice. Effects of congenital Ndufs4 deficiency or pharmacological CI inhibition on arterial oxygenation and intrapulmonary shunt in air- or 11% O2-treated mice. To assess the impact of impaired HPV on systemic oxygenation in Ndufs4−/− and Ndufs4+/+ mice breathing air, arterial blood-gas analysis was performed during LMBO. In mice breathing air, the PaO2 during LMBO was lower in Ndufs4−/− than in Ndufs4+/+ mice (PaO2 in piericidin A− vs. DMSO-treated Ndufs4+/+ mice: 156 ± 20 vs. 335 ± 33 mmHg, P < 0.05; Fig. 4A and Table 3), consistent with impaired HPV in the former. To investigate whether pharmacological inhibition of CI also impairs systemic oxygenation during LMBO, arterial blood-gas analysis was performed in piericidin A-treated Ndufs4−/+ mice. When compared with DMSO-treated Ndufs4+/+ mice, piericidin A decreased arterial oxygenation during LMBO (PaO2 in piericidin A− vs. DMSO-treated Ndufs4+/+ mice: 163 ± 32 vs. 352 ± 38 mmHg, P < 0.05; Fig. 4C and Table 3). An assessment of the intrapulmonary shunt (Qs/Qt) was used to investigate further the effect of disrupting normal CI function on the matching of pulmonary ventilation and perfusion. Ndufs4−/− mice breathing air had a marked increase in Qs/Qt compared with Ndufs4+/+ mice (Qs/Qt in Ndufs4−/− vs. Ndufs4+/+ mice: 26 ± 3 vs. 16 ± 1%, P < 0.05; Fig. 4B). Similarly, the Qs/Qt ratio in

### Table 2. Lower thoracic aortic flow and total systemic vascular resistance index measurements before (baseline) and 5 min after LMBO

| Genotype | Treatment                  | QLTAF, μl·min⁻¹·g⁻¹ | TSVRI, mmHg·min·g·ml⁻¹ |
|----------|----------------------------|----------------------|------------------------|
|          |                            | Baseline             | LMBO                  | Baseline             | LMBO                  |
| Ndufs4+/+ | Saline                     | 197 ± 13             | 197 ± 12              | 153 ± 11             | 161 ± 16              |
| Ndufs4+/+ | DMSO 100% (0.5 ml/kg)      | 233 ± 11             | 230 ± 8               | 154 ± 12             | 143 ± 7               |
| Ndufs4+/+ | Piericidin A (0.5 mg/kg)   | 224 ± 7              | 229 ± 8               | 142 ± 10             | 154 ± 22              |
| Ndufs4+/+ | After 3 wk at FlO2 0.11    | 212 ± 10             | 202 ± 11              | 140 ± 16             | 151 ± 24              |
| Ndufs4+/− | After 3 wk at FlO2 0.11    | 187 ± 20             | 185 ± 22              | 167 ± 26             | 168 ± 17              |
|          |                            | 218 ± 20             | 205 ± 18              | 147 ± 14             | 148 ± 8               |

Values are means ± SE. The effect of LMBO on each parameter was analyzed in each group by ANOVA with a post hoc correction for multiple comparisons. F1O2, fraction of inspired oxygen; LMBO, left mainstem bronchial occlusion; QLTAF, lower thoracic aortic blood flow; TSVRI, total systemic vascular resistance index.

Fig. 2. Relative levels of IL-6, TNFα, and IL-1β mRNA in lung tissue from air breathing Ndufs4−/− and Ndufs4+/+ mice. Levels of IL-6 (A), TNFα (B), and IL-1β (C) mRNA were similar between the two genotypes [n = 3 (male), n = 3 (female), P = not significant for each pair]. Group differences were compared by Student’s t-test. Data are expressed as means ± SE.
piericidin A-treated Ndufs4+/+ mice was greater than that in DMSO-treated control mice (Qs/Qt in piericidin A- vs. DMSO-treated Ndufs4+/+ mice: 36 ± 7 vs. 17 ± 1%, P < 0.05; Fig. 4D).

To characterize the effects of chronic hypoxia on systemic oxygenation in Ndufs4-deficient mice better, we also measured PaO2 after breathing 11% O2 for 3 wk. Ndufs4−/− mice exposed to 11% O2 showed a significant improvement in arterial oxygenation during LMBO compared with Ndufs4+/− mice breathing air (PaO2 in Ndufs4+/− mice breathing 11% O2: 336 ± 26 vs. 156 ± 20 mmHg, P < 0.05, Table 3). After chronic exposure to 11% O2, arterial PaO2 in Ndufs4−/− mice was similar to that in Ndufs4+/− mice breathing 11% O2 (PaO2 in Ndufs4+/− mice breathing 11% O2: 336 ± 26 vs. 269 ± 36 mmHg, P = not significant; Fig. 5A and Table 3). Ndufs4-deficient mice breathing 11% O2 had a marked decrease in the intrapulmonary shunt (Qs/Qt) compared with Ndufs4+/− mice breathing air. Qs/Qt ratio during LMBO was reduced to the levels of Ndufs4+/+ mice breathing 11% O2 (Qs/Qt in Ndufs4−/− mice breathing 11% O2: 22 ± 2 vs. 20 ± 1%, P = not significant; Fig. 5B). Taken together, these results show that intact CI function is required to maintain systemic oxygenation and increased oxidation of succinate and increased production of fumarate by CI (Fig. 6B).

Effect of chronic hypoxia on lung NADH/NAD+ ratio in Ndufs4−/− and Ndufs4+/+ mice. In addition to maintaining the normal cellular energy state, mitochondria are essential for regulating cellular redox balance (32). To investigate whether alterations in redox balance might contribute to the loss of HPV, we measured NADH and NAD+ levels and calculated the ratio of NADH/NAD+ in lungs obtained from Ndufs4−/− and Ndufs4+/+ mice breathing either air or 11% O2 for 3 wk. In mice breathing air, the lungs of Ndufs4−/− mice had a higher NADH/NAD+ ratio than the lungs of Ndufs4+/+ mice (whole lung NADH/NAD+ ratio in Ndufs4−/− vs. Ndufs4+/+ mice breathing air: 0.160 ± 0.008 vs. 0.126 ± 0.004, P < 0.05; Fig. 7). Breathing 11% O2 for 3 wk decreased the ratio of NADH/NAD+ in the lungs of Ndufs4-deficient mice but had no effect on the ratio of NADH/NAD+ in the lungs of Ndufs4+/+ mice (whole lung NADH/NAD+ ratio in Ndufs4−/− vs. Ndufs4+/+ mice breathing 11% O2: 0.111 ± 0.005 vs. 0.124 ± 0.003, P = not significant; Fig. 7). Taken together, the results show that the lack of functional CI increases the ratio of NADH to NAD+ and chronic hypoxia (breathing 11% O2) improves redox homeostasis.

Effect of angiotensin II on constriction of the pulmonary vasculature in Ndufs4−/− mice. To learn whether impairment of LMBO-induced HPV in Ndufs4−/− mice is caused by increased levels of succinate and fumarate in air- and 11% O2-breathing Ndufs4+/− mice. Changes in the function of CI have an impact on the overall activity of the ETC (11). Mitochondrial complex II (CII) is able to pass electrons, released by the oxidation of succinate, to coenzyme Q of the ETC thereby bypassing CI (4). To characterize better whether changes in the function of CII, induced by dysfunction of CI, might explain the observed improvement of HPV in Ndufs4−/− mice breathing 11% O2, we measured whole lung levels of succinate and fumarate in air- and 11% O2-breathing Ndufs4+/− mice. Succinate levels did not differ between the two groups either during normoxia or during hypoxia (Fig. 6A). Ndufs4+/− mice breathing 11% O2 had higher fumarate levels compared with 11% O2-breathing control mice, consistent with
nonspecific dysfunction of the pulmonary contractile apparatus, Ndufs4<sup>−/−</sup> and Ndufs4<sup>+/+</sup> mice were treated with intravenous angiotensin II. In Ndufs4<sup>−/−</sup> mice, LPVRI increased from 107 ± 23 to 259 ± 57 mmHg·min·g<sup>-1</sup>·ml<sup>-1</sup> (Table 4) during continuous infusion of angiotensin II (5 μg·kg<sup>-1</sup>·min<sup>-1</sup>). Ndufs4<sup>+/+</sup> mice showed a similar increase in LPVRI from 88 ± 5 at baseline to 216 ± 12 mmHg·min·g<sup>-1</sup>·ml<sup>-1</sup> during infusion of angiotensin II (%increase in LPVRI in angiotensin II-treated Ndufs4<sup>−/−</sup> vs. Ndufs4<sup>+/+</sup> mice: 142 ± 9 versus 145 ± 11, P = not significant, Fig. 8). These results demonstrate that impairment of LMBO-induced HPV in Ndufs4<sup>−/−</sup> mice is not a result of nonspecific dysfunction of the pulmonary vascular contractile apparatus.

**DISCUSSION**

The objective of this study was to investigate the role of CI in the regulation of HPV. Mice lacking the Ndufs4 gene (Ndufs4<sup>−/−</sup>) and Ndufs4<sup>+/+</sup> mice with pharmacological inhibition of CI had impaired HPV, which resulted in impairment of systemic oxygenation. The levels of cytokines in lung tissue obtained from Ndufs4<sup>−/−</sup> mice breathing air were not significantly different from those in control mice, suggesting that impairment of HPV was not a result of pulmonary inflammation. Intravenous administration of angiotensin II to Ndufs4-deficient mice increased pulmonary resistance, showing that impairment of HPV was not a result of a dysfunctional pulmonary vascular contractile apparatus. In Ndufs4<sup>−/−</sup> mice, breathing 11% O<sub>2</sub> for 3 wk restored HPV and improved systemic oxygenation during LMBO. When compared with Ndufs4<sup>+/+</sup> mice, Ndufs4<sup>−/−</sup> mice breathing air had an increased whole lung ratio of NADH to NAD<sup>+</sup>. Restoration of HPV by breathing 11% O<sub>2</sub> for 3 wk was associated with a normalized NADH/NAD<sup>+</sup> ratio.

Children with Leigh syndrome usually die within the first years of life as a consequence of pneumonia and respiratory failure (8, 17). To our knowledge, no previous studies in humans or mice have examined whether HPV is impaired in patients with Leigh syndrome. The effects of complex I deficiency on systemic and pulmonary hemodynamics in Ndufs4<sup>−/−</sup> mice has not been studied extensively. In the present study, we report that in young (35–40 days old) Ndufs4<sup>−/−</sup> mice, complex I deficiency does not affect pulmonary hemodynamics under normoxic conditions. Previous studies showed that Ndufs4<sup>−/−</sup> mice have altered cardiac function that progressively worsens with age (7, 28). However, in the present study, HR, blood pressure, and QLTAF did not differ between Ndufs4<sup>−/−</sup> and Ndufs4<sup>+/+</sup> mice before or during LMBO. The observed differences between the results in these studies may be attributable to the different ages of the mice that were studied and the different types of anesthesia used. Chouchani and colleagues (7) studied older mice (10–20 wk) and used isoflurane anesthesia to study cardiac function using MRI. In contrast, in this study we used...
the NADH/NAD$^+$ pH associated with lactic acidosis, compared with control 
while assessing cardiac function. Of note, Karamanlidis and 
anesthetics, we used a combination of fentanyl and ketamine

Ndufs4

[52x356]Ndufs4

Ndufs4

[52x508]Ndufs4

Ndufs4

[52x54]pressure; QLPA, left pulmonary arterial blood flow.a

Increased pyruvate conversion to lactate with restoration of
creased oxidation of pyruvate inside mitochondria, resulting in

The observed increase in whole lung NADH/NAD$^+$ ratio compared with

controls. The percentage increase in LPVRI in angiotensin II-treated Ndufs4-
deficient mice breathing air that lead to the impairment of HPV.

Mitochondrial dysfunction in Ndufs4$^{-/-}$ mice was previously reported by our group and other investigators (9, 14, 18, 27, 28). Calvaroso and colleagues (6) measured the complex I activity in multiple organs of Ndufs4$^{-/-}$ mice and observed the lowest activity in lung tissue (9% of the control mice), compared with other organs, including brain, liver, and heart. Although measures of oxidative stress in the lung were not performed in the current study, previous investigators showed increased oxidative damage in the brain and fibroblasts of Ndufs4-deficient mice (15, 27, 34). Reactive oxygen species

Table 4. Hemodynamic measurements before (baseline) and 5 min after the start of angiotensin II infusion in Ndufs4$^{-/-}$ and Ndufs4$^{+/+}$ mice breathing air

| Genotype | Treatment   | HR, beats/min | MAP, mmHg | PAP, mmHg | Qe,pA, ml·min$^{-1}$·g·ml$^{-1}$ | LPVRI, mmHg·min·g⁻¹·ml⁻¹ |
|----------|-------------|---------------|-----------|-----------|---------------------------------|---------------------------|
|          |             | n             | Baseline  | ANG II    | Baseline | ANG II | Baseline | ANG II | Baseline | ANG II | Baseline | ANG II |
| Ndufs4$^{+/+}$ | ANG II (0.5 μg·kg$^{-1}$·min$^{-1}$) | 5 | 542 ± 36 | 620 ± 23$^a$ | 82 ± 8 | 134 ± 5$^a$ | 18 ± 1 | 26 ± 2$^a$ | 88 ± 6 | 67 ± 6$^a$ | 88 ± 5 | 216 ± 12$^a$ |
| Ndufs4$^{-/-}$ | ANG II (0.5 μg·kg$^{-1}$·min$^{-1}$) | 4 | 506 ± 23 | 617 ± 9$^a$ | 74 ± 5 | 138 ± 8$^a$ | 19 ± 2 | 29 ± 3$^a$ | 104 ± 17 | 61 ± 9$^a$ | 107 ± 23 | 259 ± 57$^a$ |

Values are means ± SE. Group differences were compared by Student’s t-test. The effect of angiotensin II (ANG II) on each parameter was analyzed in each group by a paired Student’s t-test. HR, heart rate; LPVRI, left pulmonary vascular resistance index; MAP, mean arterial pressure; PAP, pulmonary arterial pressure; Qe,pA, left pulmonary arterial blood flow. *P < 0.05 vs. baseline value of the same parameter in the same treatment group.
release has also been reported in the muscle and skin fibroblasts of Ndufs4-deficient patients (35). The level of superoxide production by fibroblasts from Ndufs4-deficient patients was inversely related to CI activity (35). Increased levels of reactive oxygen species in pulmonary smooth muscle cells may alter the function of potassium channels and thereby impair a signaling mechanism that is important for HPV.

We previously reported that hypoxia improves survival and reverses neurodegenerative disease in Ndufs4+/− mice (9, 14). In these earlier studies, circulating lactate and α-hydroxybutyrate, both markers of tissue NADH/NAD+ ratios, increased with worsening disease and decreased with chronic breathing of 11% O2. In this study, breathing 11% O2 for 3 wk was found to restore HPV in Ndufs4+/− mice and normalized the lung NADH/NAD+ ratio. The enhancement of HPV in Ndufs4+/− mice breathing 11% O2 was associated with increased PaO2 and reduced shunt fraction during LMBO.

Krusse and colleagues reported that CII activity was increased in the liver and skeletal muscle of Ndufs4+/− mice (18). Supplemental succinate was also shown to reverse the effect of pharmacological CI inhibition and restore HPV (19). In the present study, the fumurate levels of the whole lung were higher in 11% O2 breathing Ndufs4+/− mice compared with the controls, suggesting an increase in succinate oxidation as a possible compensatory mechanism in the setting of dysfunctional CI.

In conclusion, in the present study we demonstrated that mice congenitally lacking CI subunit Ndufs4 have impaired HPV and decreased systemic arterial oxygenation during LMBO. Similar results were found when CI was pharmacologically inhibited using piericidin A. The impaired HPV associated with Ndufs4 deficiency was not accompanied by either pulmonary inflammation or nonspecific vasomotor dysfunction. Breathing 11% O2 for 3 wk restored HPV in Ndufs4+/− mice and improved systemic arterial oxygenation during LMBO. Restoration of HPV was accompanied by normalization of the whole lung NADH/NAD+ ratio. The murine results predict that if patients with Leigh syndrome have impaired HPV, they may be at risk to develop systemic hypoxemia because of ventilation/perfusion mismatch during episodes of pneumonia and respiratory failure. Our work motivates future investigations in patients with inherited mitochondrial disease to evaluate whether HPV is impaired and to what extent it may contribute to pathology in these patients.

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DISCLOSURES

V. K. Mootha and W. M. Zapol are coinventors on a patent application submitted by Massachusetts General Hospital on the use of hypoxia as a therapy. V. K. Mootha owns equity stake in Raze Therapeutics and is a paid consultant for Janssen Pharmaceuticals and 5AM Ventures.

AUTHOR CONTRIBUTIONS

W.M.Z. conceived and designed of research; G.S., E.M., M.F., R.S., O.S., O.G., R.M.H.G., and K.P. performed experiments; G.S., E.M., M.F., R.S., O.S., R.M.H.G., K.P., M.W., and F.I. analyzed data; G.S., E.M., M.F., R.S., O.G., R.M.H.G., K.P., M.W., F.I., D.B.B., V.K.M., and W.M.Z. interpreted results of experiments; G.S., E.M., R.S., O.S., K.P., F.I., and W.M.Z. prepared figures; G.S., E.M., M.F., R.S., O.S., R.M.H.G., K.P., M.W., F.I., D.B.B., V.K.M., and W.M.Z. edited and revised manuscript; G.S., E.M., M.F., R.S., O.S., R.M.H.G., K.P., M.W., F.I., D.B.B., V.K.M., and W.M.Z. approved final version of manuscript.

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