Correction

MEDICAL SCIENCES

The authors note that Fig. 2 and its legend appeared incorrectly. Additionally, the legends for Figs. 8 and 9 appeared incorrectly. The corrected figures and their corrected legends appear below. The online version has been corrected.

Fig. 2. Absence of neurodegenerative pathology in 250-d-old hypoxia-treated Ndufs4 KO mice. (A) Representative images with staining for the microglial activation marker Iba-1 (n = 3). Normoxic KO mice at 50 d show a significant inflammatory response in the cerebellum and OB. Analogous images in 250-d-old hypoxic KO mice and WT mice do not show brain inflammation. (B) Axial head MRI images showing bilateral, symmetrical hyperintense lesions in the OB (Left) and brainstem (Right) of normoxic-breathing KO mice at 55 d. These lesions were not present in hypoxic KO mice at 250 d.
Breathing 11% O₂ in late-stage neurological disease reverses the radiographic lesions of Ndufs4 mice seen on MRI. Four Ndufs4 mice were breathing 21% O₂ until they developed late-stage neurological disease (55 d). They underwent MRI to document bilateral lesions in the vestibular nuclei (Upper, white arrow). Subsequently they commenced breathing 11% oxygen. The mice were scanned again at 2 wk and 4 wk of hypoxic breathing (Middle and Lower, respectively). Neurologic lesions disappeared by 4 wk of hypoxic breathing. The section of the fourth ventricle shown at the center of the brainstem appeared enlarged in the early scans, suggesting parenchymal atrophy. After 4 wk of treatment, this area appeared to be reduced in size, and morphological relationships were restored.
Breathing 11% O₂ in late-stage neurological disease reverses pathological inflammation in the brains of Ndufs4 KO mice. Representative images with Iba-1 staining of the OB and cerebellum (CB) in KO mice and WT controls (n = 3 per group). Iba-1 is a marker of inflammation in the brain, indicative of microglial activation. Images demonstrate the reversibility of the neuropathological pattern by breathing 11% O₂ at the late stage of disease (55 d). (A) KO mice breathing 21% O₂. (B) KO mice breathing 21% O₂ up to 55d and then breathing 11% O₂ (to 160d). MRI-demonstrated reversal of the lesions reported in Fig. 8 was observed in these same mice. (C) Normoxic WT controls. (D) Hypoxic WT controls.

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Hypoxia treatment reverses neurodegenerative disease in a mouse model of Leigh syndrome

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Contributed by Vamsi K. Mootha, March 27, 2017 (sent for review January 5, 2017; reviewed by Marni J. Falk and Matt Kaeberlein)

The most common pediatric mitochondrial disease is Leigh syndrome, an episodic, subacute neurodegeneration that can lead to death within the first few years of life, for which there are no proven general therapies. Mice lacking the complex I subunit, Ndufs4, develop a fatal progressive encephalopathy resembling Leigh syndrome and die at ~60 d of age. We previously reported that continuously breathing normobaric 11% O2 from an early age prevents neurological disease and dramatically improves survival in these mice. Here, we report three advances. First, we report updated survival curves and organ pathology in Ndufs4 KO mice exposed to hypoxia or hyperoxia. Whereas normoxia-treated KO mice die from neurodegeneration at about 60 d, hypoxia-treated mice eventually die at about 270 d, likely from cardiac disease, and hyperoxia-treated mice die within days from acute pulmonary edema. Second, we report that more conservative hypoxia regimens, such as continuous normobaric 17% O2 or intermittent hypoxia, are ineffective in preventing neuropathology. Finally, we show that breathing normobaric 11% O2 in mice with late-stage encephalopathy reverses their established neurological disease, evidenced by improved behavior, circulating disease biomarkers, and survival rates. Importantly, the pathognomonic MRI brain lesions and neurohistopathologic findings are reversed after 4 wk of hypoxia. Upon return to normoxia, Ndufs4 KO mice die within days. Future work is required to determine if hypoxia can be used to prevent and reverse neurodegeneration in other animal models, and to determine if it can be provided in a safe and practical manner to allow in-hospital human therapeutic trials.


Significance

Inherited or acquired defects in mitochondria lead to devastating disorders for which we have no effective general therapies. We recently reported that breathing normobaric 11% O2 prevents neurodegeneration in a mouse model of a pediatric mitochondrial disease, Leigh syndrome. Here we provide updated survival curves of mice treated with varying doses of oxygen and explore eventual causes of death. We explore alternative hypoxia regimens and report that neither intermittent nor moderate hypoxia regimens suffice to prevent neurological disease. Finally, we show that hypoxia can not only prevent, but also reverses the brain lesions in mice with advanced neuropathology. Our preclinical studies will help guide future clinical studies aimed at harnessing hypoxia as a safe and practical therapy.


Reviews: M.I.F., University of Pennsylvania; and M.K.K., University of Washington.

Conflict of interest statement: V.K.M., W.M.Z., and I.H.J. are listed as coinventors on a patent application submitted by Massachusetts General Hospital on the therapeutic uses of hypoxia for mitochondrial disease.

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Effects in mitochondrial function, owing to mutations either in the nuclear genome or in the mitochondrial DNA, result in severe diseases that can present at any point from infancy through adulthood (1). The most common biochemical class of mitochondrial disorders arises from genetic mutations affecting the mitochondrial respiratory chain, with an incidence of ~1 in 4,300 live births (2). The management of these disorders is challenging in part because of their clinical and genetic heterogeneity. Virtually any organ system can be impacted, and more than 250 genes encoding mitochondrial proteins are known to be disease-causing (3). Of these, at least 150 can underlie disorders of oxidative phosphorylation. The mainstay of therapy involves using vitamin mixtures with little or no proven efficacy, as well as avoiding drugs known to be mitochondrial toxins.

Leigh syndrome, the most common pediatric manifestation of mitochondrial disease, is characterized by bilaterally symmetric lesions in the gray matter of the brainstem, basal ganglia, or cerebellum. It affects ~1 in 40,000 live births and can be due to mutations in any of 75 different genes (4). Children with this disorder often become hypotonic and may develop vision and hearing loss. Although the course of disease can vary widely among individuals, severely affected children can experience developmental delay and ultimately succumb to respiratory failure within the first few years of life. These patients are typically healthy at birth but then experience a neurometabolic crisis, often in the context of a febrile illness, resulting in psychomotor regression and death within the first few years of life. Diagnosis is based on clinical presentation and classic findings on T2-weighted brain magnetic resonance imaging (MRI). A small subset of these disorders are related to defects in vitamin transport and alleviated with dietary supplementation, such as in the case of riboflavin, thiamine, and biotin deficiencies (5, 6); however, there are no proven therapies for the vast majority of these disorders. Several new experimental strategies are currently under investigation in preclinical and clinical settings, including agents that target oxidative stress, the mechanistic target of rapamycin pathway, NAD+ pool sizes, and complex I bypass (idebenone) (7–10).

We recently demonstrated the therapeutic potential of hypoxia in preventing mitochondrial disease. We reported that chronically exposing the Ndufs4 mouse model of Leigh syndrome (11, 12) to breathing 11% O2 at normobaria before disease onset markedly extended the lifespan, improved behavior, and prevented neurodegeneration (13). Furthermore, we demonstrated that breathing 55% O2, a level that is well tolerated by WT mice, led to rapid death of the diseased mice.
The goal of our present study was to build on this initial work (13) to better understand the disease course and pathophysiology of these mice when breathing different 
O_2_ levels, and to explore alternative hypoxia regimens. We provide an update of the survival

Results

Breathing 11% O_2_ Prevents the Development of Brain Lesions Throughout the Life of Ndufs4 KO Mice. To investigate the therapeutic potential of hypoxia treatment on mitochondrial dysfunction, we studied whether hypoxia is capable of preventing the neurodegenerative disease rather than simply delaying its onset (Fig. 1A–E). KO and WT controls were randomized to breathe either 21% or 11% O_2_ starting at 30 d of age. As reported previously (13), our initial cohort of control KO mice breathing 21% O_2_ all died between 42 and 75 d (median, 58 d). In contrast, KO mice breathing 11% O_2_ were still alive at 170 d (13). Here we report an updated survival curve, showing that KO mice breathing 11% O_2_ had an overall median survival duration of 270 d (log-rank, P = 0.0001 vs. 21% O_2_ controls) (Fig. 1A). To investigate the chronic physiological adaptation to hypoxia, we measured the venous hematocrit of mice after 9 mo of breathing 11% O_2_. Venous hematocrit was elevated, albeit to a lesser extent, in KO mice compared with WT controls (47 ± 2% vs. 55 ± 5%, respectively; P < 0.05; n = 8) (Fig. 1B). The circulating plasma disease biomarkers lactate and α-hydroxybutyrate are elevated in humans with Leigh syndrome and in the Ndufs4 mouse model (14). In our original report (13), both markers were partially normalized in KO mice breathing 11% oxygen. At 250 d, α-hydroxybutyrate trended toward a partial rescue in KO mice breathing 11% oxygen (39 ± 8 μM; n = 7) relative to normoxic-breathing KO mice at day 50 (15), although the difference was not statistically significant (Fig. 1D). At 250 d, plasma lactate levels in KO mice breathing 11% O_2_ were similarly ameliorated (Fig. 1E) relative to normoxic-breathing KO mice at 50 d (15).

To study whether hypoxic breathing delays death by delaying the onset of CNS lesions, we performed T2-weighted brain MRI studies of lightly anesthetized mice chronically breathing either 21% or 11% O_2_ (n = 4). KO mice breathing 21% O_2_ developed lesions in the posterolateral portion of the brainstem, localized to the vestibular nuclei. Lesions became evident at 55 d of age (Fig. 2B). In contrast, brain MRI scans performed in KO mice breathing 11% O_2_ showed no signs of neural lesions even at 250 d.

We next assessed neuropathology in the same cohort of mice. Brains were stained for the inflammatory activation marker Iba-1 (Fig. 2A). KO mice breathing 21% O_2_ displayed significant microglial activation in both the cerebellum and olfactory bulb (OB) by 50 d. In contrast, KO mice breathing 11% O_2_ displayed no accumulation of Iba-1 in the cerebellum, OB, or brainstem at 250 d. In the latter group, brain sections were comparable to those of hypoxic, age-matched WT mice (n = 4). These findings suggest that the cause of death in older, hypoxia-treated KO mice might differ from the progressive neurodegenerative disease of normoxia-treated KO mice. This led us to explore the functional alterations of other organs known to be involved in mitochondrial disease pathology.

Ndufs4 KO Mice Breathing 11% O_2_ for >150 d Exhibit Mild Left Ventricular Dysfunction. A common presentation of pediatric mitochondrial disease is cardiomyopathy, with left ventricular (LV) dysfunction negatively affecting prognosis in up to 20% of cases (15). The pathogenesis of cardiac pathology is incompletely understood. The sudden death of some of our >170-d-old KO mice breathing 11% O_2_ led us to investigate their cardiac function (Fig. 3A–F). To do so, we obtained echocardiograms in mildly sedated mice at 200 d (Fig. 3A and C). Using ultrasound, we detected decreased LV fractional shortening in KO mice compared with WT mice breathing 11% O_2_ at 200 d (37 ± 4% vs. 57 ± 9%; P < 0.05; n = 6 in each group) (Fig. 3A and C). We also examined ventricular function by MRI at 9.4 T with a gradient echo cine sequence (Fig. 3F) (16). The age-matched WT mice were anesthetized with 1–2% isoflurane, whereas the KO mice were anesthetized using 0.3% isoflurane owing to their increased sensitivity to anesthetic agents (17). Scans were performed while the mice were breathing 21% O_2_. Body temperature was maintained in the physiological range with a warm air blower. Heart rate averaged ∼400 bpm in WT mice and 300 bpm in KO mice. LV function assessed by cardiac MRI demonstrated a reduced LV ejection fraction in KO mice treated by breathing 11% O_2_ at >200 d of age, compared with WT controls breathing 11% O_2_ at the same age (56 ± 7% vs. 69 ± 2%; P < 0.05; n = 3 in each group) (Fig. 3D), confirming the echocardiographic findings. Assessment of right
ventricular ejection fraction also revealed no significant differences between KO and WT mice (58 ± 1% vs. 58 ± 1%; P = 0.97; n = 3 in each group) (Fig. 3E). Of note, ECG sporadically revealed intermittent atrioventricular blockade in hypoxia-treated KO mice under isoflurane anesthesia. Furthermore, no differences in LV fractional shortening were detected between hypoxia-treated KO mice at 50 d and age-matched KO controls breathing 21% O₂ (Fig. 3B).

Breathing 55% O₂ Causes Acute Lung Edema and Isolated OB Lesions in Ndufs4 KO Mice. To further understand the critical role of inhaled oxygen concentration on mitochondrial disease progression, we investigated the physiological effects of breathing 55% O₂. We previously reported the remarkable sensitivity of KO mice to mild hypoxic breathing (13). On exposure to breathing 55% O₂ KO mice died between 48 h and 10 d, with a median survival of 5 d. To assess whether supplemental O₂ exacerbates the original neurologic disease, we obtained brain MRI scans in KO mice that had been breathing 55% O₂ for 24 h. Imaging showed hyperintense lesions in the OB, but no lesions in the brainstem (Fig. 4D), suggesting augmented O₂ sensitivity of the olfactory cells of mice exposed to hyperoxia via the nasal epithelium, but not in the brainstem via the arterial circulation.

We then focused our attention on the lung, an organ known to be sensitive to supplemental O₂ toxicity. It is known that breathing 100% O₂ results in pulmonary edema within several days in WT mice (18). Thus, we wondered whether similar pathology would ensue at a more moderate hyperoxia exposure in KO mice. To estimate pulmonary edema, we assessed the wet-to-dry lung weight (WD) ratio (19). Mice were exposed to 55% O₂ for 24 h starting at 30 d of age. The WD ratio was elevated, at 5.63 ± 0.6 in KO mice and 4.38 ± 0.02 in WT controls (P < 0.001; n = 7) (Fig. 4A), indicating significant acute pulmonary edema. Furthermore, lung myeloperoxidase activity at 24 h was 4.6-fold greater than that of WT controls (23 ± 8 vs. 5 ± 4 U/g; P < 0.001; n = 7) (Fig. 4B), suggesting significant neutrophil infiltration.

To further investigate lung pathology, we performed H&E staining at 48 h in mice breathing 55% O₂. We observed the presence of alveolocapillary membrane swelling, inflammatory cell infiltration, extensive capillary disruption, and hemorrhagic extravasation of red cells into alveoli (n = 4). These findings were specific to the KO mice breathing 55% O₂ (Fig. 4C).

Intermittent 11% O₂ Breathing Is Not Effective. Intermittent hypoxic exposure is currently used in sports training, aviation, and clinical studies of sleep apnea (20–22). Because nighttime hypoxia therapy would allow unhindered patient mobility during the day, we tested whether such a regimen would have a therapeutic effect on KO mice. Starting at 30 d, Ndufs4 KO mice breathed 11% O₂ for 10 h each day from 9 AM to 7 PM (while sleeping), and 21% O₂ for the remaining 14 h (while awake nocturnally). Daily intermittent exposure to breathing 11% O₂ increased the venous hematocrit to 56 ± 4% within 3 wk (n = 4) (Fig. 5E), indicating a systemic physiological response to hypoxia. However, the survival time of these mice was not increased relative to that of mice continuously breathing 21% O₂ (58.5 d vs. 58.5 d; log-rank P = 0.77; HR, 1.15; 95% CI, 0.45–2.96; n = 8) (Fig. 5A). During exposure to intermittent hypoxia, body weight decreased until humane euthanasia criteria were eventually met. Of note, KO mice undergoing the intermittent hypoxic protocol had a significantly lower body weight at 60 d compared with control mice breathing 21% O₂ (10.6 ± 0.8 g vs. 12.1 ± 1.1 g; P < 0.05; n = 6) (Fig. 5B). Core body temperature at 50 d was low in both groups (32.4 ± 0.9 °C vs. 35.0 ± 1.5 °C; P < 0.06; n = 8) (Fig. 5C). We tested the ability of KO mice to remain on an accelerating, rotating rod, and found no significant difference between KO mice after 3 wk of intermittent hypoxia treatment and control KO mice breathing 21% O₂ (Fig. 5D).

We further studied whether brain lesions in the Leigh syndrome model were ameliorated by intermittent 11% O₂ hypoxic breathing. All four MRI scans of KO mice receiving intermittent treatment revealed hyperintense lesions in the brainstem and OB by 60 d, resembling the neurologic lesions seen in mice breathing 21% O₂ at the same age (Fig. 5F). Thus, although intermittent 11% O₂ hypoxia was sufficient to trigger certain aspects of hypoxia adaptation, such as hematocrit elevation, intermittent hypoxia did not prevent the progression of KO neuropathology.

Milder Hypoxic Breathing Regimens Are Not Effective. We previously reported the results of KO mice breathing normobaric 11% O₂, equivalent to the partial pressure of oxygen found at an altitude of 4,500 m. In the present study, we investigated whether chronic exposure to a milder level of hypoxia would be sufficient to prevent encephalopathic disease. Breathing 17% O₂ for 3 wk, equivalent to living at 1,800 m, starting at 30 d of age did not prevent Leigh syndrome in KO mice (Fig. 6A). After 3 wk of treatment, the core temperature of mice breathing 17% O₂ and normoxic controls was
hematocrit in KO mice was 51 ± 3%, compared with 60 ± 6% at 11% O₂ (normoxic control level, 44 ± 6%), (P < 0.05 between groups; n = 6) (Fig. 6D). These findings suggest that breathing 17% O₂ triggered an intermediate physiological hematocrit response, but did not prevent the manifestations of neurologic disease.

To test the durability of the effects of hypoxia on neurodegenerative disease, we transitioned three KO mice that were breathing
11% O\textsubscript{2} for >100 d back to breathing 21% O\textsubscript{2} air. These KO mice lost weight and died within 4, 5, and 9 d after transitioning to normoxia. This finding highlights the importance of maintaining hypoxic exposure throughout the lifetime of the murine disease model.

Breathing 11% O\textsubscript{2} Reverses Clinical Manifestations of Leigh Syndrome. Patients with Leigh syndrome are typically diagnosed after an acute metabolic crisis, with the concomitant presence of bilateral lesions detectable by brain MRI (25). Thus, the degenerative brain disease usually manifests before diagnosis. We previously demonstrated that starting hypoxic breathing at an early age (30 d) can prevent the onset of neuropathological disease in the KO mouse model (13). An even more clinically relevant question is whether neuropathological disease can be reversed after its onset. To assess this possibility, we initiated therapeutic hypoxia exposure just before death in untreated KO mice breathing 21% O\textsubscript{2} (55 d). We randomly assigned the KO mice to one of three treatment groups:

1. Kontrol (KO) hypoxia (11% O\textsubscript{2})
2. Kontrol mild hypoxia (17% O\textsubscript{2})
3. Kontrol normoxia (21% O\textsubscript{2})

Fig. 5. Intermittent hypoxic (11%) breathing (10 h/d) does not alleviate mitochondrial disease in Ndufs4 KO mice. (A) Survival rates for Ndufs4 KO mice with intermittent hypoxic breathing (Int) vs. those breathing 21% O\textsubscript{2} (log-rank P = 0.77; HR, 1.13; 95% CI, 0.47–2.73; n = 8). (B) Body weights after breathing at various oxygen levels and during intermittent hypoxic breathing starting at age 30 d (n = 8). (C and D) Core temperature (C) and falling latency (D) from an accelerating, rotating rod for KO mice breathing various oxygen levels or subjected to intermittent hypoxic breathing starting at 30 d of age (n = 8). (E) Hematocrit levels in WT and KO mice following 3 wk of exposure to normoxic, hypoxic, or intermittent hypoxic breathing (n = 4). (F) Representative MRI in a 60-d-old KO mouse exposed to intermittent hypoxic breathing. Arrows denote lesions in vestibular nuclei. Data are mean ± SD. *P < 0.05 vs. KO breathing 11% O\textsubscript{2}; #P < 0.05 vs. KO breathing 21% O\textsubscript{2}.

Fig. 6. Breathing at moderate hypoxia (17% O\textsubscript{2}) does not alleviate mitochondrial disease. (A) Survival rates for Ndufs4 mice breathing various oxygen levels starting at age 30 d. (B and C) Time course of body weight (B) and body temperature (C) for mice breathing 17% O\textsubscript{2} and those breathing breathing 11% or 21% O\textsubscript{2} for 30, 40, and 50 d (n = 6). (D) Venous hematocrit values after 3 wk of exposure to various oxygen levels (n = 6). Data are mean ± SD. *P < 0.05 vs. 11% oxygen, #P < 0.05 vs. 17% oxygen.

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Table 1. SpO₂, SaO₂, and PaO₂ values in healthy adult mice and adult human subjects breathing at F\textsubscript{O\textsubscript{2}} of 11%, 17%, 21%, and 55% \hspace{1cm} F\textsubscript{O\textsubscript{2}}

<table>
<thead>
<tr>
<th>Variable</th>
<th>11%</th>
<th>17%</th>
<th>21%</th>
<th>55%</th>
</tr>
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<tbody>
<tr>
<td>Mouse SpO₂, %</td>
<td>61.3±2.5</td>
<td>88.5±3.1</td>
<td>96.8±1.6</td>
<td>99.8±0.3</td>
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<tr>
<td>Mouse SaO₂, %</td>
<td>60.4±8.1</td>
<td>85.4±2.8</td>
<td>92.3±1.1</td>
<td>100±0</td>
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<tr>
<td>Mouse PaO₂, mmHg</td>
<td>45±4</td>
<td>77±8</td>
<td>111±14</td>
<td>318±20</td>
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<tr>
<td>Human SpO₂, %*</td>
<td>83±5</td>
<td>93±2</td>
<td>98±1</td>
<td>100±0</td>
</tr>
<tr>
<td>Human PaO₂, mmHg*</td>
<td>47±8</td>
<td>71±12</td>
<td>110±3</td>
<td>299±4</td>
</tr>
</tbody>
</table>

SpO₂ and SaO₂ in mice were measured using two different techniques (Methods) \( n = 5 \).

*Human data from refs. 23, 24.

Discussion

We previously demonstrated that early-onset chronic exposure of the Ndufs4 KO mouse model of Leigh syndrome to breathing 11% O₂ prevented the onset of neurodegenerative disease and extended the duration of KO survival. The goal of our present study was to explore the disease course and pathophysiology of these mice when exposed to breathing different oxygen tensions, and to determine whether alternative hypoxia exposure regimens could be useful. To our knowledge, no other therapeutic approach has demonstrated such high levels of efficacy in mouse models of mitochondrial encephalopathy.

We previously reported that hypoxia extends, and hypoxia reduces, the lifespan of mice with Ndufs4 deficiency, but eventual lifespan and organ pathologies were not yet known. Here we have shown that whereas Ndufs4 KO mice breathing 21% O₂ develop subacute neurodegeneration, those breathing 11% O₂ do not show signs of neurodegeneration at age >200 d, but rather develop mild LV dysfunction and eventually die at a median age of 270 d. Previous studies have shown that cardiac-specific Ndufs4 KO mice breathing 21% O₂ developed mild cardiac dysfunction (26, 27). Although we cannot conclusively define the cause of death in our hypoxia-treated KO mice, we did observe cardiac dysfunction at age >200 d. In contrast, untreated KO mice breathing 21% O₂ did not exhibit cardiac dysfunction at 50 d, just before their death. One interpretation of these findings is that hypoxic breathing at 11% O₂ is able to prevent neurodegeneration, but not eventual cardiac dysfunction.

Table 2. Experimental Ndufs4 cohorts exposed to 11% O₂ breathing at different time points

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype</th>
<th>Age at start of 11% O₂ breathing, d</th>
<th>Description of group</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Ndufs4\textsuperscript{+/−}</td>
<td>30</td>
<td>Breathing 11% O₂ begins before disease onset. Behavior, temperature, and neurologic findings are indistinguishable from those seen in WT mice at this stage.</td>
</tr>
<tr>
<td>B</td>
<td>Ndufs4\textsuperscript{+/−}</td>
<td>55</td>
<td>Breathing 11% O₂ begins after disease onset. KO mice have already lost body weight and body temperature and have neurologic lesions detectable by MRI.</td>
</tr>
<tr>
<td>C</td>
<td>Ndufs4\textsuperscript{−/−}</td>
<td>—</td>
<td>Normoxic Ndufs4 KO controls.</td>
</tr>
</tbody>
</table>
We found that breathing 55% $O_2$ resulted in rapid death of Ndufs4 KO mice from acute pulmonary edema resulting from acute pulmonary $O_2$ toxicity, with no MRI evidence of brainstem lesions that were responsible for the death of the untreated mice. The lungs of WT mice exposed to breathing similar $O_2$ levels for similar periods were normal. This observation is consistent with hyperoxic pulmonary pathology in WT mice at much higher $O_2$ levels (e.g., 100% $FiO_2$ or rebo above) (18). It is notable that the respiratory epithelium and the primary olfactory neurons are in direct contact with extremely high inhaled oxygen tensions (not requiring blood oxygen delivery). Our pathophysiological findings highlight the variable tissue-specific oxygen sensitivities of the nose, lung, brain, and heart of Ndufs4 KO mice with diffusely impaired oxidative metabolism across all of their tissues. We speculate that these differences arise from variations in metabolic flexibility, such as the ability to rely on glycolytic ATP production (28–30), or, alternatively, differences in the ability of these tissues to compensate for oxidative stress (31).

Perhaps the most exciting finding of the present study is that hypoxia can reverse brain lesions of established Leigh syndrome. Most mitochondrial disorders are diagnosed after the first metabolic crisis, and often it is the T2-intense lesions detected on brain MRI that are indicative of mitochondrial disease and help define the diagnosis. Of note, inherited deficiencies of vitamins, such as thiamine, riboflavin, and biotin, also can lead to Leigh syndrome. Such a deficiency in a mitochondrial metabolite transporter is one of the few truly treatable forms of Leigh disease (5, 6). Our finding that body weight, body temperature, behavior, and even neuroradiographic and histopathological lesions can be reversed within a few weeks by 11% $O_2$ breathing offers hope more generally for diverse genetic etiologies of established Leigh disease. We hypothesize two means by which this could take place: (i) either hypoxia is removing the proximal cause of the damage, and thus endogenous repair mechanisms can occur, or (ii) hypoxia actively triggers repair mechanisms that restore damaged tissue. Cardiomyocytes are known to be capable of regeneration and proliferation when exposed to very low oxygen levels even after completion of fetal development (32). Furthermore, hypoxia recently has been shown to improve adult neurogenesis (33–35).

An important question is whether the doses of hypoxia used in mice in the present study can be extrapolated to humans. We are optimistic about this prospect. Our healthy hypoxic mice breathing 11% oxygen had an average $PaO_2$ of 45 mmHg (Table 1). Human volunteers at the Mont Blanc research station (4,559 m), equivalent to breathing 11% oxygen at sea level, had an average $PaO_2$ of 46 mmHg (36). Of course, the ability of hemoglobin to bind $O_2$ is...
lower in mice than in humans, which is why murine arterial oxygen saturation (SaO$_2$) is lower despite a comparable PaO$_2$ (Table 1). Nonetheless, if PaO$_2$ is the key parameter in alleviating mitochondrial pathology, an 11% FiO$_2$ in humans ought to be comparable to that in mice. Furthermore, in murine models of chronic hypoxia (37), breathing 11% oxygen at sea level produces pulmonary hypertension like that seen in humans, suggesting that the pulmonary vasoconstrictor response to breathing 11% oxygen is conserved across both species. Thus, several major aspects of oxygen transport and physiology show comparable dose responses in humans and mice.

Although chronic, continuous 11% O$_2$ breathing has a dramatic effect in Ndufs4 KO mice, more practical regimens are desirable. In the present study, however, we found that neither more moderate, chronic hypoxic breathing of 17% O$_2$ nor intermittent 11% O$_2$ breathing for a duration of 10 h each day was effective. We also found that the therapeutic effects of chronic hypoxia in mice do not appear to be durable; when hypoxia-treated mice were returned to normoxic breathing, they died after 1 wk of air breathing. Thus, the only currently available regimen that prevents and reverses Leigh syndrome in Ndufs4 KO mice is continuously breathing 11% O$_2$.

Although the Ndufs4 KO mouse is perhaps the most accurate mouse model of Leigh syndrome, its disease trajectory is distinct from that of humans. In particular, this mouse exhibits very uniform, downward trajectories of body temperature, body weight, and activity, typically dying at around day 55. The disease course in humans tends to be more variable and episodic, and in fact, more practical hypoxia regimens may be intermittently effective in helping repair neurodegenerative lesions in humans. Future investigations are needed to determine whether hypoxia can be delivered safely and effectively as therapy for mitochondrial diseases.

**Methods**

**Animal Care.** Ndufs4$^{-/-}$ mice were generously provided by the Palmiter laboratory at the University of Washington. We continuously bred heterozygous mice to provide sufficient Ndufs4$^{-/-}$ and control (Ndufs4$^{+/+}$ and WT) mice for our experiments. Because Ndufs4$^{-/-}$ and WT mice are identical in all reported assays performed by us and others, we treated both groups as controls. Pups were weaned and genotyped at ~25 d after birth. All cages were provided with daily food, water, and hydrated gel (Hydrogel). Food...
Mice were housed in 80-L transparent acrylic boxes. The body weights were recorded regularly, and mice were humanely euthanized when they had lost 20% of peak body weight, in accordance with the most recent American Veterinary Medical Association guidelines. Animals were randomized on a 1:1 basis, balanced by age and sex. All animal studies were approved by the Subcommittee on Research Animal Care and the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

**Hypoxic Chambers.** Mice were housed in 80-L transparent acrylic boxes. The desired FiO2 was obtained by mixing a constant flow of medical air with pure nitrogen from a liquid nitrogen tank (Airgas) or by direct separation of nitrogen and oxygen present in room air by a nitrogen generator (MAG-20; Higher Peak). The total gas flow through the chamber was measured using a flowmeter (Cole Parmer) and adjusted to 5–10 L/min to maintain chamber CO2 concentrations below 0.4% (CO200; Extech). Soda lime was added to the chambers as a CO2 scavenger. The oxygen concentration was measured at the outlet port of the chamber with an O2 sensor (Mini Ox 1; Ohio Medical), which was calibrated weekly using an 8.55% O2 reference target (Airgas). Oxygen levels inside the chambers were tolerated within a 0.4% offset from the target concentration by adjusting nitrogen flow as needed. Temperature was maintained at 24–26 °C, and humidity was maintained at 30–70%. A standard light-dark cycle of ~12 h light exposure was used. Mice were housed in cages with standard bedding and given unlimited access to food and water.

**MRI.** MRI scans of the brain were performed with the mice under general anesthesia with isoflurane 0.5–1.5% in 21% O2. T2-weighted RARE (rapid acquisition of refocused echoes) MRI images were acquired on a 4.7-T small animal scanner (Pharmascan; Bruker) with a transmit-receive volume coil and the following parameters: repetition time (TR), 6,000 ms; echo time (TE), 60 ms; rare factor, 10; 24 slices; 192 × 192 matrix; 0.130 × 0.130 × 0.7 mm voxels; eight averages. The data were converted from the RAW Bruker format into DICOM images and visualized with a freeware DICOM reader (Osirix; University of Geneva).

Cardiac MRI was performed with a 9.4-T horizontal-bore small animal MRI scanner (BioSpec; Bruker) equipped with a 1,500-mT/m gradient system. Images were acquired with an MRI-compatible cardiorespiratory gating system (SA Instruments) and a transmit-receive surface coil. Cine imaging was performed on the left ventricle and the left atrium in short-axis cine sequence and the following parameters: field of view, 25 × 25 mm2; slice thickness, 1 mm with no gaps; matrix, 200 × 200; 20 frames per cycle; TR, RR interval/20; TE, 1 ms; flip angle, 30°; four averages. The data were converted in MATLAB (Mathworks) into the DICOM format and analyzed with the Osirix freeware DICOM reader.

**Brain Histology.** Mice were placed under deep anesthesia with ketamine and fentanyl, and a needle was inserted into the left ventricle. The whole body was perfused with ice-cold PBS, followed immediately by 4% PFA. The brain was removed, stored in 4% PFA for 24–48 h, and then placed in 30% sucrose (in PBS) for 48 h. Fixed brains were sectioned parasagittally. In addition, two transverse sections of cerebellum were prepared: a rostral section with subjacent pons and a more caudal section with medulla oblongata. Immunohistochemistry was performed using an antibody against the microglial marker Iba-1 (Wako) at 2 µg/mL.

**Echocardiography.** For LV studies, mice were scanned using a 13-MHz probe (Vivid 7; GE Healthcare). All scans were performed by an experienced echocardiographer who was blinded to the study group assignments. During the procedure, all mice were breathing 21% O2 and were anesthetized with isoflurane (0.5–1.5%) titrated to maintain a heart rate between 500 and 600 bpm.

**Pulmonary Studies.** The WD ratio was calculated by dividing the wet lung tissue weight by the dry lung tissue weight. Organs were left to dry for 24 h at 60 °C to obtain dry lung tissue. Frozen lung samples were stored at ~80 °C, and myeloperoxidase activity was assessed as described previously (38). Lungs were perfused for H&E staining using a wash solution of Ringer’s lactate followed by 4% PFA. Perfusion was performed during deep anesthesia, followed by open chest puncture of the cardiac apex using a 20-gauge needle. A 1-mm incision was made in the right atrium to allow outflow of blood from the venous return. After 1 min, the lungs were inflated with 4% PFA through the trachea, allowing 1 min for proper filling of alveoli. A pressure of ~30 cmH2O was obtained by gravity flow. Organs were stored overnight in PFA and processed on slides by the Histopathology Core at Massachusetts General Hospital.

**Behavior.** Behavioral experiments were performed using a Rotarod (Ugo Basile) machine with and acceleration of 5 rpm/min and a maximum speed of 40 rpm. Mice were placed on the accelerating, rotating rod while breathing room air. Latency to fall was measured up to a maximum time of 300 s. The test was performed three times, with a minimum of 10 min between sessions to allow for recovery. The median latency to fall was reported to avoid the incorporation of aberrant behavior trials. If mice latched onto a rotarod rather than walking on the rod for more than 10 s, this time was recorded as the latency to fall.

**Biomarker Measurements.** Isotope-labeled standards (CDN isotope) of lactate and 1-13C-hydroxybutyrate were used to generate a standard curve. This allowed for absolute quantification of metabolites in mouse plasma. For this, 30 µL of the mouse plasma sample was combined with 20 µL of isotope-labeled internal standard, and 70% acetonitrile was used for metabolite extraction. LC-MS was
performed on a Q Exactive Plus Orbitrap mass spectrometer coupled to a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific). The Xbridge amide HILIC column (2.1 × 100 mm, 2.5 μm particle size; Waters, 18600091) was used for separate tests in the negative ionization mode. Mobile phase A was 20 mM ammonium acetate and 0.25% ammonium hydroxide (pH 9). Mobile phase B was 100% acetonitrile. Data acquisition was performed in full scan mode, selected for a range of 70–1000 m/z, with resolution of 140,000, an automatic gain control target of 3e6, and a maximum injection time of 400 ms.

**Blood and Tissue Collection.** For collection of plasma and tissues, mice were anesthetized with an i.p. injection of ketamine (120 mg/kg) and fentanyl (0.09 mg/kg). After tracheostomy, volume-controlled ventilation was maintained at a respiratory rate of 90 breaths/min, a tidal volume of 10 ml/kg, a positive end-expiratory pressure of 1 cmH₂O, and an inspired O₂ of 21% (Mini Vent 845; Harvard Apparatus). Whole blood was collected by cardiac puncture and placed in an EDTA-containing tube. Plasma was obtained by centrifuging whole blood at 2,000 × g for ~20 min. Tissues were harvested and immediately flash-frozen in liquid nitrogen. When repeat samples were required, a small tail nick was performed, and blood was collected in heparinized glass capillary tubes. Blood samples were then centrifuged as described above.

**Transcutaneous Awake SpO2 and Arterial Blood Gas Tensions at Various FiO₂ Values.** Healthy, unanesthetized adult C57Bl/6 mice were placed inside a 5-L chamber, and gas flow was set to maintain chamber CO₂ concentration below 0.1% (CO200, Extech). Oxygen concentrations were measured at the chamber inlet and outlet using an O₂ analyzer (MiniOx 1). A murine transcutaneous oximeter collar probe was placed on the mice to allow for unrestrained measurements (MouseOx Plus; Starr Life Sciences). The chamber F₂O₂ was adjusted to obtain 11%, 17%, and 21% O₂. If the transcutaneous oximeter reading was below the measured values were considered to indicate statistical significance.

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**Statistics.** Data are reported as mean ± SD. Analyses were performed using GraphPad Prism 6.0 software. The two-sample Student t test was used for two-group comparisons. One-way ANOVA with Bonferroni’s correction was used for multiple comparisons. The log-rank test was performed, and HRs with 95% CIs were calculated to compare survival rates. A P value < 0.05 was considered to indicate statistical significance.