Hypoxia as a therapy for mitochondrial disease

Isha H. Jain,1,2,3 Luca Zazzero,4 Rahul Goli,5,2,3 Kristen Alexa,5 Stephanie Schatzman-Bone,6 Harveen Dhillon,1,2,3 Olga Goldberger,1,2,3 Yun Peng,1,2,3 Ophir Shalem,3,6,7 Neville E. Sanjana,3,6,7 Feng Zhang,3,6,7 Wolfram Goessling,3,5,8,9 Warren M. Zapol,4 Vamsi K. Mootha1,2,3*

Defects in the mitochondrial respiratory chain (RC) underlie a spectrum of human conditions, ranging from devastating inborn errors of metabolism to aging. We performed a genome-wide Cas9-mediated screen to identify factors that are protective during RC inhibition. Our results highlight the hypoxia response, an endogenous program evolved to adapt to limited oxygen availability. Genetic or small-molecule activation of the hypoxia response is protective against mitochondrial toxicity in cultured cells and zebrafish models. Chronic hypoxia leads to a marked improvement in survival, body weight, body temperature, behavior, neuropathology, and disease biomarkers in a genetic mouse model of Leigh syndrome, the most common pediatric manifestation of mitochondrial disease. Further preclinical studies are required to assess whether hypoxic exposure can be developed into a safe and effective treatment for human diseases associated with mitochondrial dysfunction.

Mitochondria are ancient organelles that are essential for normal physiology and health. The respiratory chain (RC) is crucial to mitochondrial function and generates approximately 90% of cellular adenosine triphosphate (ATP) via oxidative phosphorylation (7). In the oxidative step, four large protein complexes transfer electrons from NADH (the reduced form of nicotinamide adenine dinucleotide) or FADH (the reduced form of flavin adenine dinucleotide) to oxygen while generating a proton gradient. Approximately 90% of the oxygen we breathe is used as a substrate for the RC (7). In the phosphorylation step, the proton gradient is dissipated by a fifth and final complex to generate ATP. Numerous additional chemical reactions and transport processes are intimately coupled to the redox and proton-pumping activities of the RC.

A spectrum of human diseases result from a faulty RC (2–4). Virtually all age-related disorders, including type 2 diabetes, neurodegeneration, and sarcopenia, are accompanied by a quantitative decline in the activity of the mitochondrial RC. The aging process itself is associated with a gradual decrease of oxidative phosphorylation in multiple tissues. Monogenic disorders of the mitochondrial RC represent the largest class of inborn errors of metabolism. To date, lesions in over 150 genes, encoded by the nuclear or mitochondrial (mtDNA) genomes, have been identified as disease-causing. Mutations in these genes lead to a biochemical deficiency of one or more of the RC complexes, resulting in either tissue-specific or multisystem disease with devastating effects on human health. Patients with RC disorders can present with blindness, deafness, gray- or white-matter brain disease, cardiomyopathy, skeletal muscle myopathy, gastrointestinal dysmotility, anemia, ataxia, liver disease, and kidney disease.

Management of these disorders remains challenging (5, 6). Although individual mutations are rare, the overall disease burden of mitochondrial disease is significant, with an estimated prevalence of 1 out of 4300 live births (7). Therefore, a general and effective therapeutic is needed. The current mainstream of managing mitochondrial disease involves the use of vitamin cofactors (such as coenzyme Q, α-lipoic acid, riboflavin, or L-carnitine) (8). Other proposed strategies include the use of small-molecule bypass of defective RC components, using electron carriers such as idebenone, and antioxidants. None of these approaches have demonstrated efficacy in randomized controlled clinical trials.

Several lines of evidence point to the existence of endogenous coping mechanisms for dealing with mitochondrial dysfunction. Mitochondrial disorders can be highly tissue-specific and episodic (2, 9). These disorders are often triggered by drugs, alcohol, or viral illnesses, implying that a genetic lesion is not always sufficient to cause cellular dysfunction, but rather that the lesion may need to be compounded with an environmental insult. Such observations suggest the existence of cellular pathways or environments that buffer against mitochondrial lesions.

A genome-wide screen to spotlight suppressors of mitochondrial disease

We modeled mitochondrial disease in the human leukemia suspension cell line K562 and performed a Cas9-mediated knockout (KO) screen (10, 11). We used the natural product antimycin as a complex III inhibitor of the RC. In the presence of antimycin, the RC is unable to oxidize high-energy reducing equivalents to power ATP production; however, cytoplasmic lactate dehydrogenase maintains NAD⁺ redox balance. Removal of pyruvate exacerbates reductive stress, further preventing cell proliferation (12). We modeled mitochondrial disease with the addition of antimycin alone (moderate disease) or antimycin in combination with the removal of pyruvate (severe disease), using cell growth as a proxy for disease magnitude (Fig. 1A). We infected K562 cells with a ~65,000 single-guide RNA (sgRNA) library, targeting ~18,000 genes (10). After 1 week of genome editing, we transferred the pool of KO cells to experimental conditions of untreated, moderate disease, and severe disease states (Fig. 1B). We collected samples for an enrichment screen by allowing the KO pool to grow in selection conditions for 3 weeks. The relative growth between untreated and moderate disease conditions was 300-fold and between untreated and severe disease conditions was 7000-fold (Fig. 1C).

As expected, 3 weeks of genome editing in untreated cells led to a significant depletion of sgRNAs corresponding to essential genes, including those related to transcription, translation, and splicing (fig. S1). Nearly 20% of the most essential genes were mitochondrial proteins, especially mitochondrial ribosomal proteins and electron transport chain subunits (table S1). Because mitochondrial proteins make up approximately 5% of the proteome (13), this enrichment highlights the dramatic effects of mitochondrial dysfunction on viability.

Of the ~18,000 genes tested, the KO screen identified inhibition of the Von Hippel-Lindau (VHL) factor as the most effective genetic suppressor of mitochondrial disease, in both the moderate and severe disease conditions (Fig. 1D). RIGER analysis ranked VHL KO cells as the most enriched over time in both infection replicates corresponding to severe and moderate disease (table S2). The five sgRNAs spanning all three exons of VHL ranked 1, 2, 3, 12, and 14 out of ~65,000 total guides for enrichment in disease conditions relative to pretreatment conditions (Fig. 1, D to F, and figs. S2 and S3). Furthermore, the most significant VHL sgRNAs were enriched approximately 20-fold in disease states (fig. S4). VHL KO cells were also enriched in untreated conditions over time, reflecting an overall effect on cell growth. However, this enrichment was significantly less than in disease conditions.
VHL activity is a key regulator of the hypoxic response pathway (14, 15). Organisms have evolved elaborate mechanisms to adapt to fluctuating oxygen tensions and extreme environments. In normoxic conditions, the hypoxia-inducible transcription factors (HIFs) are constitutively made and hydroxylated by the prolyl-hydroxylase (PHD) enzymes (Fig. 2A) (16–18). The hydroxylated form is recognized by the ubiquitin ligase, VHL, and targeted for degradation. In response to environmental hypoxia, the PHD reaction does not take place, allowing HIF stabilization and activation of the hypoxia transcriptional program (Fig. 2B). VHL KO cells show HIF stabilization, even during normoxic conditions, thereby bypassing cellular oxygen-sensing mechanisms (18–20). Our screen suggested that harnessing innate responses to hypoxia may be protective in the setting of inherited mitochondrial disease.

**Genetic and small-molecule proof of concept in cellular models**

We validated and characterized the hypoxic response as a therapeutic target by testing the ability of VHL KO cells to withstand respiratory-chain dysfunction. VHL KO cells showed increased cell proliferation in the presence of antimycin relative to nontargeting (dummy) sgRNA-modified cells (Fig. 2C). Furthermore, there was perfect correspondence between the degree of VHL sgRNA enrichment in the CRISPR (clustered regularly interspaced short palindromic repeats) screen and the rescue-effect size of individual sgRNAs (Fig. S5). VHL KO cells were also more resistant to complex I inhibition by piericidin and complex V (ATP synthase) inhibition by oligomycin, demonstrating the potentially broad utility of our therapeutic approach (Fig. 2C).

We next explored small molecules as tools for triggering the hypoxia response. Although a VHL inhibitor has been reported (21), it is not cell-permeant. PHD inhibitors have been developed as investigational drugs for anemia and ischemic disorders (22). FG-4592 is currently in phase III clinical trials for the treatment of anemia of chronic kidney disease and acts by upregulating the canonical marker of the hypoxia response, erythropoietin (Epo). We reasoned that FG-4592 treatment would mimic VHL KO, thus triggering a broader hypoxia transcriptional program. Normal growth rates were minimally increased by FG-4592. Complex I, III, or V inhibition stunts cell growth but does not increase cell death (fig. S6) in most cell lines, including HT-29s, HEK 293Ts, and K562s. Administering ~50 μM FG-4592 in advance and during RC dysfunction nearly or completely rescued this growth defect, in a dose-dependent manner (Fig. 2, D to F, and fig. S7). The nearly full rescue of the disease state across different cell lines and across chemical lesions highlights the general utility of our approach.

We characterized the rescue mechanism of FG-4592 by studying its effect on the hypoxia response and energy metabolism. Although HIFα is undetectable during normoxic exposure, treatment with FG-4592 stabilized the transcription factor even during normoxia. It has previously been noted that a paradox exists between severe mitochondrial dysfunction and cellular sensing of hypoxia (23). In cell culture, full inhibition of the RC prevents HIF stabilization,
even under low oxygen conditions that would otherwise trigger the hypoxia response (23, 24). FG-4592 treatment bypassed this paradox and enabled HIF1α stabilization in the face of mitochondrial dysfunction, during states of normoxia or hypoxia (Fig. 3A). Further work is needed to determine whether the paradox contributes significantly to disease pathology or whether it is simply a feature of severe RC blockade in cultured cells.

The HIF transcriptional response is believed to be protective during states of hypoxia, at least in part by shifting the cell’s reliance away from mitochondrial oxidative energy metabolism. The HIF1α response can preserve energy supply at low oxygen tensions in a redox-neutral manner. Indeed, treatment with FG-4592 for 24 hours up-regulated the transcription of glycolytic enzymes (Fig. 3B and fig. S8) such as glucose transporter 1 (GLUT1), hexokinase 2 (HK2), and lactate dehydrogenase A (LDHA). HIF1α activation is also known to shunt the carbon supply away from the TCA cycle and toward the LDHA reaction (25–28), as evidenced by the significant up-regulation of pyruvate dehydrogenase kinase (PDK1) (Fig. 3B). Although lesions to the RC and hypoxia can in principle limit RC activity, cells do not mount the hypoxia response upon RC inhibition, because the signal is lacking. However, FG-4592 treatment artificially triggers the hypoxia transcriptional program, even during normoxic conditions (Fig. 3B and fig. S8).

To corroborate the transcriptional changes, we also measured lactic acid production and oxygen consumption as proxies of glycolysis and oxidative phosphorylation. Glycolysis was somewhat increased by RC inhibition, probably as a result of allosteric mechanisms (Fig. 3C). Treatment with FG-4592 increased glycolysis by nearly 25% in HEK293T cells under basal conditions and beyond allosteric mechanisms in response to RC inhibition. Furthermore, basal oxygen consumption was decreased by approximately twofold with FG-4592 treatment (Fig. 3D). This may be protective in the setting of mitochondrial dysfunction, because it may limit the amount of reactive oxygen species (ROS) produced by impaired electron transport.

**Genetic and small-molecule proof of concept in zebrafish models**

To further establish proof of concept, we asked whether genetic or small-molecule activation of the hypoxia response would be protective against RC poisoning in zebrafish embryos. *vhl*-null zebrafish continuously up-regulate the hypoxia response throughout early development (29). Just as *vhl* KO cells are protected against mitochondrial dysfunction, we asked whether *vhl*-null zebrafish might be more resilient to RC poisoning. Zebrafish
embryos exhibit sensitivity to multiple specific RC inhibitors, including antimycin (30–32). We demonstrated a significant improvement in the survival of vhl-null embryos exposed to 2.5 nM antimycin as compared to heterozygous and wild-type (WT) controls (Fig. 4A).

We then extended our small-molecule approach to the zebrafish model of RC dysfunction. A previously generated zebrafish reporter strain, Tg(phd3::EGFP), expresses green fluorescent protein (GFP) under the control of a HIF-responsive promoter, thereby enabling in vivo assessment of activating the hypoxia transcriptional response (33, 34). FG-4592 treatment of Tg(phd3::EGFP) embryos at 96 hours post fertilization (hpf) resulted in a time-dependent increase in the fluorescence of individual reporter fish (Fig. 4B). Furthermore, in situ hybridization for the glycolytic HIF targets glut1 and ldha1 demonstrated significant up-regulation upon FG-4592 treatment (Fig. 4C), confirming that FG-4592 engages the zebrafish PHDs to trigger the hypoxia transcriptional program. We then demonstrated that cotreatment of embryos with FG-4592 rescued antimycin-induced death by nearly twofold (Fig. 4D). The genetic and small-molecule experiments in zebrafish provide proof of concept that activation of the hypoxia program can protect against insults to the mitochondrial RC.

Hypoxic breathing alleviates disease and extends life span in a mouse model of Leigh syndrome

The cellular and zebrafish models provided proof of the concept that individual components of the cellular response to hypoxia are protective.
against mitochondrial toxins. Although small molecules are capable of activating specific branches of the hypoxia program, we reasoned that they may not have as broad and potent an effect as the naturally evolved, whole-body physiological response to hypoxia itself. Moreover, small-molecule drugs for activating the hypoxia response are currently in clinical trials for kidney disease and anemia. However, a large fraction of mitochondrial disease originates in the central nervous system, and our preliminary pharmacokinetic studies suggested limited blood-brain barrier penetration of these drugs in mice. Higher doses resulted in whole-body toxicity. Because mammals have evolved a complex homeostatic program to adapt to low oxygen tensions in their environment, we reasoned that a similarly broad hypoxic stress response might protect animal models of mitochondrial disease.

Leigh syndrome is the most common pediatric form of mitochondrial disease. Though relatively healthy at birth, patients develop irreversible neurodegeneration by 2 years of age (6). They suffer bilaterally symmetric lesions in the brain stem and basal ganglia, with marked gliosis. Most patients die between the ages of 3 and 16 months. To date, over 75 different genes have been implicated in this syndrome, with complex I deficiency being the most frequent biochemical cause of disease. One of the more severe recessive forms of Leigh syndrome is caused by inactivation of the NDUF54 gene, which codes for NADH:ubiquinone oxidoreductase subunit S4. Recently, a mouse model that recapitulates many features of Leigh syndrome was generated by disruption of the murine Ndufs4 gene (35). Ndufs4 KO mice breathing ambient air (21% O2) display retarded growth rates, impaired visual acuity, and a delayed startle response. Their body temperature falls progressively until reaching 32°C, shortly before death at 50 to 60 days of age. Diseased mice also display locomotor deficits and failure to thrive by 50 days of age. Their neuropathology closely resembles clinical findings, with a substantial inflammatory response in the brainstem and cerebellum.

We first performed experiments to confirm that Ndufs4 KO mice can tolerate a brief exposure to environmental hypoxia and that they activate a hypoxic response in a manner similar to WT mice. We exposed four WT mice and four KO mice to 8.5% O2 at sea-level pressure for 6 hours. Acute exposure of WT mice to hypoxia triggers HIF stabilization, resulting in Epo transcription and translation. After a 6-hour exposure, we measured Epo protein levels in plasma and showed that both WT and KO mice up-regulated Epo production by approximately 40-fold (fig. S9). These results...
indicate that the KO mice mount a hypoxic transcriptional response and that the RC inhibition–HIF stabilization paradox does not extend to this disease setting.

We next tested whether chronic exposure to moderate environmental hypoxia (breathing 11% O2, a level known to be tolerated by humans (equivalent to 4500 m altitude)) could alleviate the disease phenotype in the Ndufs4 KO mice. Environmental hypoxia was generated by adjusting the relative concentration of nitrogen and oxygen in the input gas mixture. This created environmental oxygen tensions similar to those found in the high mountain communities of Nepal and Peru (36); Continuous gas flow and CO2 absorption by Ca(OH)2 within the 11% O2 hypoxic chamber maintained CO2 levels below 0.4% with continuous monitoring. A control ambient environment for breathing 21% O2 was created with continuous monitoring.

Hypoxia-related vascular responses (constriction of pulmonary circulation and dilation of the ductus arteriosus) occur in early postnatal development, we initiated chronic hypoxic exposure treatments after the mice were 30 days old.

Remarkably, chronic hypoxia prevented the development of many disease symptoms in the Ndufs4 KO mice and significantly extended their survival. All normoxia-exposed Ndufs4 KO mice either fulfilled criteria for humane euthanasia or died at a median age of ~60 days, with none surviving past 75 days (Fig. 5A). In contrast, there were no deaths in Ndufs4 KO mice that were chronically breathing 11% O2. Several mice showed a mild clumping phenotype at ages greater than 120 days. At the time of submission of this manuscript, the oldest KO mice breathing 11% O2 were 170 days old.

Hypoxia-treated mice showed an improvement in body weight gain, core temperature maintenance, and neurologic behavior. All Ndufs4 KO mice continued to gain weight between 30 and 37 days of age (Fig. 5, B and C). At this stage, untreated KO mice lose weight, become hypothermic, and die. In contrast, Ndufs4 KO mice breathing 11% O2 gained weight for several weeks, at which point body weight gain slowed, similar to the growth kinetics of WT mice. The growth rate of hypoxia-treated KO mice matched that of WT mice breathing 11% O2 upon treatment, suggesting that the primary cause of weight loss was alleviated by hypoxic exposure. At 30 days of age, untreated Ndufs4 KO mice had similar core body temperatures to control mice. However, by 50 days there was nearly a 4°C drop in temperature (Fig. 5D). KO mice chronically breathing 11% O2 showed no reduction of core body temperature. Thus, chronic hypoxic breathing improves the underlying metabolic phenotype that directly or indirectly results in alterations of energy and nutrient metabolism.

Ndufs4 KO mice, as well as patients suffering from Leigh syndrome, exhibit striking defects in locomotor activity. Ataxia and failure to thrive are hallmarks of mitochondrial dysfunction. Behavioral tests were performed at 10-day intervals in normoxia and hypoxia-treated WT and KO mice. The rotarod test (37) measures the ability of mice to maintain grip strength and balance and resist fatigue on an accelerating rotating rod. At 30 days of age, KO mice breathing air displayed a slight depression in the median time that they could stay on a rotarod (Fig. 5E). This ability declined by 40 days, and at 50 days, untreated KO mice were no longer able to stay on the rod for more than a few seconds, due to a combination of muscular weakness, inability to balance, and loss of visual acuity. Hypoxia-treated WT mice performed similarly to normoxia-treated control mice. KO mice breathing 11% O2 displayed a near-complete rescue of this locomotor phenotype. As a further neurological behavioral
test, spontaneous locomotor activity was measured as total distance traveled within an hour (Fig. 5F). Untreated KO mice showed drastically reduced spontaneous locomotor activity and jump counts. These defects were rescued in hypoxia-treated mice, although only to 50% of the values of normoxic WT mice (Fig. S10).

Modest hyperoxic breathing exacerbates disease in a mouse model of Leigh syndrome

The striking therapeutic effect of hypoxia led us to investigate whether oxygen itself was a key molecular parameter determining Leigh syndrome progression. Thus, we asked whether the converse environmental scenario of chronic mild hyperoxic exposure (55% O₂) would affect the disease. We exposed WT and Ndufs4 KO mice to 55% normobaric oxygen starting at 30 days of age. We found that breathing 55% oxygen had no effect on the survival of the WT mice. In contrast, all Ndufs4 KO mice breathing 55% oxygen died within 2 to 11 days of this exposure; this is significantly earlier than Ndufs4 KO mice breathing ambient air (21% O₂), which typically succumb within 3 to 4 weeks after experimental exposure begins (Fig. 5A). The reduced survival of the KO mice breathing 55% O₂ along with the markedly extended health span of the KO mice that were breathing 11% O₂, points to the essential and previously unappreciated role of arterial oxygen tension in determining the progression of mitochondrial disease. This suggests that patients with mitochondrial disease may be highly sensitive to oxygen toxicity.

Circulating biomarkers and histopathology in a hypoxia-treated mouse model of Leigh syndrome

We further characterized Ndufs4 KO mice after treatment with chronic hypoxia. As expected, the hematocrit in these mice was elevated from 40% during normoxia to ~60% during hypoxia, indicating EPO target engagement by hypoxic breathing (Fig. 6A). Given how effective hypoxia is in treating these mice, we asked whether they still retained a deficiency in complex I activity. Indeed, we found that although Ndufs4 KO mice appeared healthy after hypoxia treatment, brain complex I activity remained dramatically reduced to the same levels as in untreated Ndufs4 KO mice (Fig. 6B).

Normoxia-treated KO mice exhibited substantial neuronal degeneration. Lesions were accompanied by Iba-1+ microglial proliferation within the olfactory lobes, cerebellum, and brainstem, as documented elsewhere (38). In contrast, KO mice breathing 11% O₂ exhibited minimal to no lesions (Fig. 6C) and were virtually indistinguishable histologically from WT controls.

Recently, α-hydroxybutyrate (α-HB) was identified as a circulating plasma marker of Leigh syndrome (39). Consistent with this, we found that α-HB was elevated in the plasma of Ndufs4 KO mice breathing ambient air, but not in Ndufs4 KO mice treated with chronic hypoxia (Fig. 6D). Similarly, plasma lactate levels were increased in Ndufs4 KO mice breathing ambient air between 50 and 65 days of age, whereas this increase was partially prevented by 11% hypoxic exposure (Fig. 6E).

Collectively, these studies confirm that chronic hypoxic exposure to breathing 11% O₂ activates the endogenous hypoxic response. Hypoxia does not correct the proximal lesion within mitochondrial complex I, but rather prevents the onset of subsequent biochemical and histopathological defects.

Discussion

The preclinical studies described here suggest that hypoxic gas mixtures may be useful in treating or preventing mitochondrial disorders. This approach is seemingly counterintuitive, because oxygen is a key substrate for the RC. However, hypoxia activates an evolutionarily conserved adaptive program that allows mammals to cope with limited oxygen levels. This program decreases an organism’s reliance on mitochondrial oxidative metabolism. Such adaptive programs are not necessarily triggered by mitochondrial disease, because the hypoxic signal is absent in those cases. Moreover, hypoxia leads to a state in which oxygen delivery and consumption are simultaneously reduced, whereas in mitochondrial disease, oxygen delivery continues in the face of impaired oxygen utilization. Such a mismatch between delivery and utilization potentially contributes to oxygen toxicity. Hence, hypoxia may represent nature’s solution for overcoming mitochondrial disease pathology, both by triggering innate adaptive programs and by simultaneously limiting the substrate for oxygen toxicity.

Multiple cellular and systemic mechanisms are probably acting in concert to provide the therapeutic effect we observed in mice. First, hypoxia may be triggering the HIF-dependent transcriptional program that is known to activate key biochemical pathways, including glycolysis for redox-neutral ATP production and decreased flux through PDH to prevent mitochondrial ROS generation. Second, breathing 11% O₂ reduces the provision of oxygen to the cell—oxygen that would otherwise be available as a substrate for free radical production or aberrant signaling. Many enzymes are designed to operate under reducing conditions and are highly sensitive to ambient oxygen levels. Hypoxia may establish a new oxygen set point that is better suited to the cellular environment created by impaired RC activity, whereas hypoxia may create an environment that is less favorable. Third, it is likely that hypoxia is also operating at the level of organ systems physiology (such as O₂ delivery and CO₂ clearance by the cardiovascular system, endocrine function, and immune signaling), which are inherently non-cell-autonomous and previously reported to be altered in humans living at high altitudes.

Future studies will fully decipher the role of HIF and the cellular versus cell-autonomous mechanisms underlying the in vivo therapeutic benefit.

Our results may hold therapeutic potential for patients with mitochondrial disease. However, before the chronic hypoxia strategy described here can be evaluated in the clinical setting, additional preclinical studies are needed to establish its safety and efficacy. First, studies of additional mouse models will determine the generalizability of this approach to other rare mitochondrial disorders, common disorders of mitochondrial dysfunction, and disorders of oxidative stress.

Second, in the current study, we used a treatment regimen consisting of chronic 11% inspired oxygen. Healthy humans can acclimate to high altitudes such as those encountered in Mount Blanc, Peru, and Nepal, where ambient oxygen tensions are comparable to those used in our experiments, but it is possible that these conditions would not be well tolerated by patients. Future studies should thus evaluate whether intermediate oxygen levels between 11 and 20% are effective in mouse disease models. Finally, if intermittent hypoxia proves as effective as chronic hypoxia, it may allow for a nighttime therapy for which face masks and sleeping tents have already been devised by the sports industry.

An important principle in the management of mitochondrial disease is to avoid exposures, such as aminoglycoside or tetracycline antibiotics, that are known to be toxic to mitochondria. Patients with mitochondrial disease (like many other patients) are routinely treated with supplemental oxygen and breathe high levels of inspired oxygen during general anesthesia, recovery from surgery, and intensive care. Retrospective or prospective studies may help to extend our observations to humans, and if confirmed, would imply that caution should be exercised in O₂ exposure, and that administering supplemental oxygen should be limited to those instances in which it is clinically indicated.

At present, how lesions in the RC lead to such diverse pathology remains a mystery. Given the striking therapeutic efficacy of hypoxic breathing and the detrimental effect of moderate hyperoxia we saw in our studies, we propose that aberrant oxygen metabolism, signaling, or toxicity lies at the heart of mitochondrial pathogenesis. The identification of such a critical parameter suggests that a real understanding of mitochondrial pathogenesis is within reach.

Finally, it is notable that the aging process and virtually all age-related degenerative diseases are associated with secondary mitochondrial dysfunction and oxidative stress (40). Although antioxidants have been proposed as a strategy to alleviate these disorders by scavenging free radicals, our work suggests that simply limiting the substrate for oxygen toxicity may prove more effective. Moreover, hypoxia can trigger an adaptive program designed to decrease our body’s reliance on mitochondrial oxidative metabolism. It is conceivable that breathing hypoxic gas mixtures may prevent the onset of more common disorders.

REFERENCES AND NOTES

FLOW CHEMISTRY

On-demand continuous-flow production of pharmaceuticals in a compact, reconfigurable system

Andrea Adamo,† Rachel L. Beingsesser,‡ Mohsen Behnam,† Jie Chen,† Timothy F.Jamison,†‡ Klavs F. Jensen,†‡ Jean-Christophe M. Monbaliu,†‡ Allan S. Myerson,†‡ Eve M. Revalor,‡† David R. Sneed,‡† Torsten Stelzer,‡† Nopphon Weeranoppanant,† Shin Yee Wong,† Ping Zhang‡‡

Pharmaceutical manufacturing typically uses batch processing at multiple locations. Disadvantages of this approach include long production times and the potential for supply chain disruptions. As a preliminary demonstration of an alternative approach, we report here the continuous-flow synthesis and formulation of active pharmaceutical ingredients in a compact, reconfigurable manufacturing platform. Continuous end-to-end synthesis in the refrigerator-sized [1.0 meter (width) × 0.7 meter (length) × 1.8 meter (height)] system produces sufficient quantities per day to supply hundreds to thousands of oral or topical liquid doses of diphenhydramine hydrochloride, lidocaine hydrochloride, diazepam, and fluoxetine hydrochloride that meet U.S. Pharmacopeia standards. Underlying this flexible plug-and-play approach is substantial enabling advances in continuous-flow synthesis, complex multistep sequence telescoping, reaction engineering equipment, and real-time formulation.

whereas manufacturing of automobiles, electronics, petrochemicals, polymers, and food use an assembly-line and/or continuous, steady-state strategy, pharmaceutical synthesis remains one of the last industrial processes to apply a noncontinuous or “batch” approach. Moreover, pharmaceutical companies generally assemble the active pharmaceutical ingredient (API) using molecular fragment

Supplementary Materials

www.sciencemag.org/content/352/6281/S4/suppl/DC1 Materials and Methods Figs. S1 to S30 Tables S1 and S2 References (41–44) 14 August 2015; accepted 9 February 2016 Published online 25 February 2016 10.1126/science.a95642
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Editor's Summary

Thriving on a breath of low oxygen
Mitochondrial diseases are debilitating and largely untreatable. Most are caused by genetic mutations that impair the mitochondrial respiratory chain, which generates cellular energy. Because these diseases do not affect all tissues equally, it is thought that endogenous mechanisms exist that can help cells cope with mitochondrial defects. Jain et al. identified the hypoxia response, a mechanism that helps cells adapt when oxygen is limited, as a potent suppressor of mitochondrial dysfunction (see the Perspective by Shoubridge). Mouse models of the mitochondrial disease Leigh syndrome showed fewer symptoms and a dramatically extended life span when raised in a hypoxic environment.

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