

Alterations of cellular bioenergetics in pulmonary artery endothelial cells

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Idiopathic pulmonary arterial hypertension (IPAH) is pathogenetically related to low levels of the vasodilator nitric oxide (NO). Because NO regulates cellular respiration and mitochondrial biogenesis, we hypothesized that abnormalities of bioenergetics may be present in IPAH. Evaluation of pulmonary artery endothelial cells from IPAH and control lungs *in vitro* revealed that oxygen consumption of IPAH cells was decreased, especially in state 3 respiration with substrates glutamate-malate or succinate, and this decrease paralleled reduction in Complex IV activity and IPAH cellular NO synthesis. IPAH pulmonary artery endothelial cells had decreased mitochondrial dehydrogenase activity and lowered mitochondrial numbers per cell and mitochondrial DNA content, all of which increased after exposure to NO donors. Although IPAH/pulmonary artery endothelial cells' ATP content was similar to control under normoxia, cellular ATP did not change significantly in IPAH cells under hypoxia, whereas ATP decreased 35% in control cells, identifying a greater dependence on cellular respiration for energy in control cells. Evidence that glucose metabolism was subserving the primary role for energy requirements of IPAH cells was provided by the \approx 3-fold greater glycolytic rate of IPAH cells. Positron emission tomography scan with [¹⁸F]fluoro-deoxy-D-glucose performed on IPAH patients and healthy controls revealed significantly higher uptake in IPAH lungs as compared with controls, confirming that the glycolytic rate was increased *in vivo*. Thus, there are substantial changes in bioenergetics of IPAH endothelial cells, which may have consequences for pulmonary hypertensive responses and potentially in development of novel imaging modalities for diagnosis and evaluation of treatment.

cellular respiration | nitric oxide | oxygen consumption | pulmonary hypertension | mitochondrion

Idiopathic pulmonary arterial hypertension (IPAH) is a fatal disease of unknown etiology characterized by a progressive increase in pulmonary artery pressure and vascular growth (1, 2). Secondary forms of pulmonary arterial hypertension (PAH) are associated with known diseases, such as collagen vascular diseases or portal hypertension but in the absence of an identifiable etiology are classified as IPAH. Abnormalities in vasodilators, specifically nitric oxide (NO), have been implicated in the pathogenesis of pulmonary hypertension (1–5). NO is produced in the lung by NO synthases (NOS; EC 1.14.13.39) (6–8). There is conclusive evidence from animal models of pulmonary hypertension, mice genetically deficient in endothelial NOS (eNOS), and complementation studies with gene transfer of NOSs for the concept that NO is a critical determinant of pulmonary vascular tone (6, 7, 9). Furthermore, pulmonary and total body NO are lower in IPAH patients as compared with healthy controls (3, 10–12), and the decrease of NO has been linked to increased arginase II and decreased eNOS expression in IPAH pulmonary endothelial cells *in vivo* (10, 13).

In addition to effects on vascular tone, NO regulates cellular bioenergetics through effects on glycolysis, oxygen consumption by mitochondrion, and mitochondrial biogenesis (14–17). For exam-

ple, eNOS-deficient mice, which have mild pulmonary hypertension under normoxia and an exaggerated pulmonary vasoconstrictive response to hypoxia (18), have reduced mitochondria content in a wide range of tissues in association with significantly lower oxygen consumption and ATP content (14–17). Mitochondria are essential to cellular energy production in all higher organisms adapted to an oxygen-containing environment, i.e., ATP produced through oxidative phosphorylation. The electrochemical gradient used by mitochondrial F₀F₁ ATP synthase to synthesize ATP from ADP is generated by the proton pump action performed by Complexes I, III, and IV of the respiratory chain. The proton pumping is accompanied by electron shuttling, whereby Complexes I and II, along with the flavoprotein-ubiquinone oxidoreductase, transfer electrons from different sources to ubiquinone (coenzyme Q). The electrons are then transferred sequentially to Complex III, cytochrome *c*, Complex IV, and finally to molecular oxygen, the terminal electron acceptor. All multisubunit complexes of the respiratory chain (I–IV) are located in the mitochondrial inner membrane. Thus, mitochondria are the primary oxygen demand in the body, accounting for \approx 90% of cellular oxygen consumption. Conversely, under limiting oxygen conditions, cells turn to glycolysis to generate energy. In endothelial cells, ATP is generated nearly equivalently by glycolysis and cellular respiration (19), accounting for a relative tolerance to hypoxia because of low oxygen demand and relatively high glycolytic activity (19, 20).

Interestingly, studies have identified site-specific defects in the electron transport chain in avian spontaneous idiopathic pulmonary hypertension; the lower respiratory chain coupling and inefficient use of oxygen have been linked to the development of pulmonary hypertension in avian species (21–23). Similarly, Fawn Hooded rats, a spontaneously pulmonary hypertensive rodent strain, have dysmorphic mitochondria with reduced expression of electron transport chain components. Dichloroacetate, a mitochondrial pyruvate dehydrogenase kinase inhibitor, prevents and reverses the pulmonary hypertension of Fawn Hooded rats, indicating a pathogenetic relation between mitochondria function and pulmonary hypertension (24, 25). Given

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Abbreviations: IPAH, idiopathic pulmonary arterial hypertension; PAH, pulmonary arterial hypertension related to risk factors or associated conditions; NOS, NO synthases; eNOS, endothelial NOS; MTT, 3-(4,5-dimethylthiazolyl-2)-5-diphenyltetrazolium bromide; PET, positron-emission tomography; CT, computed tomography; FDG, [¹⁸F]fluoro-deoxy-D-glucose; SUV, standardized uptake values; LTF, lung tissue fraction; SNAP, S-nitroso-N-acetyl-D,L-penicillamine.

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Table 1. Oxygen consumption of pulmonary artery endothelial cells

| Substrate | Cell type | Oxygen consumption* | | Mitochondrial function |
|------------------|--------------|--------------------------|--------------------------|------------------------|
| | | State 4 | State 3 | RCI |
| Glutamate-malate | Control PAEC | 2.04 ± 0.10 | 5.44 ± 0.43 | 2.74 ± 0.20 |
| Glutamate-malate | IPAH PAEC | 1.28 ± 0.12 [†] | 3.36 ± 0.35 [†] | 2.65 ± 0.16 |
| Succinate | Control PAEC | 3.51 ± 0.25 | 9.40 ± 0.17 | 2.71 ± 0.23 |
| Succinate | IPAH PAEC | 1.83 ± 0.10 [†] | 4.76 ± 0.68 [†] | 2.60 ± 0.38 |

RCI, respiratory control index.

*Nanomoles of O₂ per minute per 10⁶ cells; †, $P < 0.01$, $n = 4$ (IPAH), $n = 3$ (control), and $n = 8$ replicate experiments for substrates glutamate-malate, and $n = 5$ replicate experiments for substrate succinate.

these data and the low NO state of IPAH, we hypothesized that abnormalities of cellular metabolic energy pathways would be present in IPAH patients. To test this, oxygen consumption, ATP content, NO production, mitochondrial function, morphology and amount, and mitochondrial complexes activities were evaluated in pulmonary artery endothelial cells (PAEC) from IPAH and healthy control lungs. Subsequently, cellular glycolytic rate was determined in cells *in vitro*, and lung glycolytic rate was evaluated in patients with IPAH in comparison to healthy controls *in vivo*.

Results

Clinical Characteristics. Altogether, six controls, nine IPAH, and four PAH subjects were studied. PAEC were derived from donor lungs not used in transplantation and five IPAH and four PAH explanted lungs obtained at transplantation. Positron-emission tomography (PET)-computed tomography (CT) scans were conducted in three healthy female controls and four IPAH female patients. Clinical characteristics among subjects were similar [age (years): IPAH 45 ± 3, PAH 40 ± 4, control 35 ± 6; sex (female/male): IPAH 8/1, PAH 2/2, control 5/1; race (Caucasian/African American/Hispanic): IPAH 9/0/0, PAH 3/1/0, control 6/0/0]. Pulmonary hypertension was diagnosed by right heart catheterization performed for clinical care [pulmonary artery pressures (mmHg, 1 mmHg = 133 Pa): IPAH, systolic 94 ± 6, diastolic 41 ± 4, mean 59 ± 2; PAH, systolic 97 ± 12, diastolic 44 ± 14, mean 50 ± 6]. PAH was secondary to congenital heart diseases, except for one case of sarcoidosis. IPAH individuals were receiving vasodilators, anticoagulants, diuretics, digitalis, and/or oxygen by nasal cannula.

Decreased Oxygen Consumption in IPAH PAEC. To initially evaluate cellular respiration (21, 22), oxygen consumption in IPAH and control PAEC was determined. Compared with control cells ($n = 3$), IPAH PAEC ($n = 4$) had less oxygen consumption for state 3 and state 4 respirations with glutamate-malate or succinate as substrate (Table 1). However, the coupling between oxygen consumption and ATP production, the respiratory control index, was similar among IPAH and controls.

Activity and Expression of Mitochondrial Complexes. To determine causes of lower oxygen consumption, the activities of Complexes III and IV and the ability of mitochondria to reduce 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were determined (Fig. 1 *A* and *B*). Activity of Complex IV, the terminal enzyme complex of the respiratory chain that catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen, was significantly lower in IPAH PAEC ($n = 2$) than in control ($n = 2$), whereas Complex III activity was similar among the controls and IPAH cells (nanomoles of cytochrome *c* per minute per 10⁶ cells: Complex IV, IPAH 9.83 ± 0.47, control 12.33 ± 0.73, $P < 0.05$, $n = 9$ replicate experiments, cells derived from each of two lungs from IPAH and controls in these series of experiments; Complex III, IPAH 5.65 ± 0.19, control

5.97 ± 0.22, $P > 0.05$, $n = 4$ replicate experiments) (Fig. 1*A*). MTT reduction by IPAH PAEC was strikingly lower than control, indicating less mitochondrial dehydrogenase activity (absorbance at 570 nm per 60,000 cells: IPAH 0.57 ± 0.03, control 0.75 ± 0.02, $P < 0.05$, $n = 9$ replicate experiments) (Fig. 1*B*).

Quantitation of mitochondria in electron microscopy images of cells (Fig. 1*C*) and Southern blot analysis of mtDNA (Fig. 1*D* and *E*) revealed decreased mitochondrial numbers in IPAH PAEC compared with control (mitochondrial number per cell, IPAH 69 ± 7, control 191 ± 23, $P < 0.01$; mtDNA relative units per microgram of total DNA, IPAH 1 ± 0.16, control 2.29 ± 0.64, $P < 0.05$) but no discernible difference in mitochondrial morphology (Fig. 1*F*).

NO Production in IPAH PAEC. IPAH patients have low levels of exhaled NO (3, 11), and IPAH PAEC produce lower total amounts of NO products than control cells *in vitro* (10). Here, NO production by IPAH PAEC ($n = 3$) detected by measuring nitrite (NO₂⁻) in the culture supernatants after ionomycin stimulation was also significantly lower than controls ($n = 3$, IPAH 0.30 ± 0.27, controls 3.91 ± 1.38 pmol/min per 10⁶ cells, $P < 0.05$). Ionomycin-stimulated nitrite (NO₂⁻) concentrations of IPAH and control cells were correlated to oxygen consumption for state 3 and state 4 respirations with glutamate-malate substrates (all $P < 0.01$) (Fig. 1*G*).

To test whether NO affects mitochondrial numbers and functions in IPAH cells, mitochondria numbers in electron microscopy images (Fig. 1*C*), mtDNA by Southern blot analysis (Fig. 1*D* and *E*), and mitochondrial dehydrogenase activity were analyzed in IPAH cells ($n = 3$) after exposure to NO donors in culture. Mitochondrial numbers increased in IPAH PAEC after treatment with NO donors, DETA NONOate (detaNO), or *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) for 6 days compared with untreated cells (mitochondrial number/cell, untreated 69 ± 7, detaNO 93 ± 9, SNAP 90 ± 6, $P < 0.01$; mtDNA relative units per microgram of DNA, untreated 1 ± 0.16, detaNO 1.52 ± 0.47, SNAP 1.52 ± 0.40; $P = 0.06$ untreated vs. detaNO; $P < 0.05$ untreated vs. SNAP; $n = 3$) (Fig. 1 *C–F*). Mitochondrial dehydrogenase activity as measured by MTT also increased after NO donors (absorbance at 570 nm per 60,000 cells: untreated 0.42 ± 0.01, SNAP 0.49 ± 0.01, $P < 0.01$, $n = 3$), and Complex III-1 and cytochrome *c* protein levels were ≈2-fold greater after NO donors treatment (data not shown). Altogether, these data indicate that levels of NO in IPAH cells affect mitochondrial numbers and hence cellular bioenergetics in IPAH cells.

ATP Content in IPAH PAEC Under Normoxia (21% O₂) and Hypoxia (2% O₂). On the basis of the lower oxygen uptake and decreased mitochondrial function, we reasoned that the IPAH cells might have lower levels of energy production (20, 26, 27). To test this hypothesis, ATP content in IPAH ($n = 3$) and control cells ($n = 4$) was quantitated (Fig. 2*A*). Under 21% O₂, ATP content in IPAH cells was similar to controls ($P > 0.05$). Under hypoxia of 2% O₂,

tional Review Board, and written informed consent was obtained from individuals.

Cell Culture. Human PAEC were dissociated and cultured as described in *SI Text*. A549 cells, an epithelial cell line derived from lung adenocarcinoma, were cultured in MEM (Invitrogen Corporation, Carlsbad, CA) with 10% heat-inactivated FCS. Cells were subjected to hypoxia in a sealed chamber with 2% O₂, 5% CO₂, and 93% N₂ or placed directly in a 5% CO₂ and 95% air incubator (20% O₂) and cultured at 37°C. For NO donor treatment, 125 μM detaNO or 100 μM SNAP was added once a day to cell culture from day 1 to day 6 (15).

Detection of Endothelium-Derived NO. To evaluate NO synthesis, nitrite (NO₂⁻) concentrations in the culture supernatants were measured in ionomycin-stimulated cell supernatant from subconfluent PAEC from IPAH lung or control lung as described (10). Nitrite concentrations were determined by using the ISO-NOP Nitric Oxide Sensor (World Precision Instruments, Sarasota, FL), an amperometric sensor specific for NO.

Mitochondrial Respiration. Oxygen consumption of cells vs. healthy controls was measured at 37°C in circulator chambers by using an 5300A biological oxygen monitor (YSI, Yellow Springs, OH) and Clark-type polarographic oxygen electrodes as described with slight modifications (47). Briefly, reactions were conducted using 0.01% digitonin (wt/vol) (Sigma-Aldrich, St. Louis, MO) permeabilized PAEC (7.5 × 10⁶ cells in 3-ml reaction volume) in a reaction medium [250 mM sucrose, 20 mM D-glucose, 40 mM KCl, 5 mM KH₂PO₄, 3 mM MgCl₂, 0.5 mM EDTA, 30 mM Tris·HCl, pH 7.5, and protease inhibitors aprotinin (5 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), and Pefabloc SC (24 μg/ml)]. Oxygen consumption measurements were made under state 3 and state 4 respiration using either 5 mM glutamate and 2.5 mM malate or 5 mM succinate as substrate. State 3 respiration was stimulated by the addition of 1 mM ADP. The respiratory control index was calculated by dividing oxygen consumption rate at state 3 by state 4.

Respiratory Chain Complex Assays. The activities of Complex III and IV of the mitochondrial respiratory chain were measured spectrophotometrically as described (48). Briefly, cells (20 × 10⁶ per ml) were incubated with 0.01% digitonin (Sigma-Aldrich) in PBS containing protease inhibitors [5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 24 μg/ml Pefabloc SC (Sigma-Aldrich)] (pH 7.4) for 5 min on ice. After centrifugation at 16,000 × *g* and 4°C for 1 min, the supernatant, containing cytosolic proteins, was removed, and the organelle pellet was resuspended in an equal amount of assay buffer. For Complex III and Complex IV activity, an organelle volume correlating to 40,000 and 10,000 cells was used per reaction, respectively. Complex III activity was measured at 550 nm and 30°C with 15 μM cytochrome *c* (III), 0.6 mM *n*-dodecyl-β-D-maltoside (EMD Biosciences, Inc., La Jolla, CA), 2 μg/ml rotenone, and 35 μM ubiquinol in assay medium [25 mM potassium phosphate, 5 mM MgCl₂, 2 mM KCl, 2.5 mg/ml BSA (fraction V), pH 7.2]. Ubiquinol was prepared by dissolving ubiquinone (10 μmol) in 1 ml of ethanol acidified to pH 2 with 6 M HCl. The quinone was reduced with excess solid sodium borohydride. Ubiquinol was extracted into diethylether/cyclohexane (2:1, vol/vol), evaporated to dryness under nitrogen gas, and redissolved in 1 ml of ethanol acidified to pH 2 with 6 M HCl. For Complex IV activity, cytochrome *c* was reduced with 1 M ascorbate in 0.1 M Mes (pH 7.0) for 20 min and desalted afterward by using a D-Salt Excellulose GF-5 column (Pierce Biotechnology, Inc., Rockford, IL). Complex IV activity was measured at 550 nm and 25°C with 15 μM cytochrome *c* (II), 0.45 mM *n*-dodecyl-β-D-maltoside in 20 mM potassium phosphate (pH 7.0).

MTT Assay. Mitochondrial dehydrogenase activity in cells was determined by the ability of mitochondria to reduce MTT according to the protocol from American Type Culture Collection (Manassas, VA). Absorbance at 570 nm was measured.

ATP Content in IPAH PAEC. ATP in cells was measured under normoxia (21% O₂) or hypoxia (2% O₂) for 4 h by using a luciferase-based luminescence assay kit (PerkinElmer, Boston, MA).

Glycolytic Rate. Glycolytic rate of cells was measured by monitoring the conversion of 5-³H-glucose to ³H₂O (49, 50). Briefly, 2 × 10⁵ PAEC from IPAH or healthy controls were suspended in 0.2 ml of endothelial cell growth medium (EGM-2 containing 1,000 mg/L glucose; Cambrex, Walkersville, MD), and 10 μl of 5-³H-glucose (PerkinElmer Life Sciences Inc., Boston, MA) was added to each well. Samples were incubated for 1 h at 37°C in a humidified incubator under 5% CO₂. Reactions were terminated with 0.5 ml of 0.2 N HCl, and 100 μl of the cell/HCl mixture was added to open tubes, which were then placed upright in 4-ml scintillation vials containing 0.5 ml of H₂O. The vials were capped, sealed with parafilm, and incubated for 2 days at room temperature. During the incubation, ³H₂O generated by glycolysis diffused from the tube to the H₂O in the scintillation vial through evaporation and condensation. The contents of the tube were transferred to a new scintillation vial with 0.5 ml of H₂O, the tube was discarded, and scintillation fluid was added to both the original (diffused counts) and second (undiffused counts) vials. The amounts of diffused and undiffused ³H were determined by scintillation counting. Appropriate ³H-glucose-only and ³H₂O-only controls were included, enabling the calculation of ³H₂O in each sample and thus the rate of glycolysis as described (49). Lactate concentration in supernatants overlying culture cells was determined spectrophotometrically (Trinity Biotech, St. Louis, MO). Lactate was converted to pyruvate and hydrogen peroxide (H₂O₂) by lactate oxidase. H₂O₂ in the presence of a peroxidase subsequently catalyzes oxidative condensation of a precursor, producing a chromogen with an absorption maximum at 540 nm.

Southern Blot Analysis. Total DNA was extracted from cultured cells (Qiagen Inc., Valencia, CA). Primers (5'-TGATCAGAG-GATTGAGTAAACGG-3' and 5'-GGTACCCTAACCGTGCA-AGGTA-3') were used to amplify a 1,090-bp DNA fragment (2,574–3,663 bp; GenBank accession no. AY339547) from 16,569-bp human mitochondrial genomic DNA (51). The PCR products were purified, sequenced, and used as a probe for Southern blot analysis. For Southern blot analysis, the amount of total DNA from cultured cells was digested with restriction enzyme PvuII, electrophoresed through a 0.8% agarose gel, and transferred to Duralon-UV membranes (Stratagene, Cedar Creek, TX). The filters were hybridized with the ³²P-labeled PCR-generated mitochondrial DNA (mtDNA) probe and visualized with autoradiography.

Ultrastructural Analyses. Cells were fixed at 4°C for >1 h in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde and 4% formaldehyde. After washing three times in the same buffer, cells were postfixated with 1% aqueous osmium tetroxide for 1 h at 4°C, washed in sodium cacodylate buffer, followed by maleate buffer (pH 5.1), and then dehydrated with ascending grades of ethanol and propylene oxide. Samples were embedded in Eponate12 kit, polymerized at 70°C for 48 h, trimmed, sectioned at 70–90 nm, poststained in saturated uranyl acetate and lead citrate, and examined with a transmission electron microscope (Philips CM12; Philips, Eindhoven, The Netherlands).

