Dynamin-Related Protein 1–Mediated Mitochondrial Mitotic Fission Permits Hyperproliferation of Vascular Smooth Muscle Cells and Offers a Novel Therapeutic Target in Pulmonary Hypertension

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Rationale: Pulmonary arterial hypertension (PAH) is a lethal syndrome characterized by pulmonary vascular obstruction caused, in part, by pulmonary artery smooth muscle cell (PASMC) hyperproliferation. Mitochondrial fragmentation and normoxic activation of hypoxia-inducible factor-1α (HIF-1α) have been observed in PAH PASMCs; however, their relationship and relevance to the development of PAH are unknown. Dynamin-related protein-1 (DRP1) is a GTPase that, when activated by kinases that phosphorylate serine 616, causes mitochondrial fission. It is, however, unknown whether mitochondrial fission is a prerequisite for proliferation.

Objective: We hypothesize that DRP1 activation is responsible for increased mitochondrial fission in PAH PASMCs and that DRP1 inhibition may slow proliferation and have therapeutic potential.

Methods and Results: Experiments were conducted using human control and PAH lungs (n=5) and PASMCs in culture. Parallel experiments were performed in rat lung sections and PASMCs and in rodent PAH models induced by the HIF-1α activator, cobalt, chronic hypoxia, and monocrotaline. HIF-1α activation in human PAH leads to mitochondrial fission by cyclin B1/CDK1–dependent phosphorylation of DRP1 at serine 616. In normal PASMCs, HIF-1α activation by CoCl2 or desferrioxamine causes DRP1-mediated fission. HIF-1α inhibition reduces DRP1 activation, prevents fission, and reduces PASMC proliferation. Both the DRP1 inhibitor Mdivi-1 and siDRP1 prevent mitotic fission and arrest PAH PASMCs at the G2/M interphase. Mdivi-1 is antiproliferative in human PAH PASMCs and in rodent models. Mdivi-1 improves exercise capacity, right ventricular function, and hemodynamics in experimental PAH.

Conclusions: DRP1–mediated mitotic fission is a cell-cycle checkpoint that can be therapeutically targeted in hyperproliferative disorders such as PAH. (Circ Res. 2012;110:1484-1497.)

Key Words: hypoxia-inducible factor-1 ■ mitochondrial division inhibitor-1 ■ mitochondrial fission ■ mitotic checkpoint ■ cyclin B1/cyclin-dependent kinase 1

Pulmonary arterial hypertension (PAH) is a syndrome in which obstructed, constricted, and inflamed small pulmonary arteries increase pulmonary vascular resistance, leading to right ventricular hypertrophy and, ultimately, failure. The cause of PAH remains unclear, despite important advances in understanding the genetic and epigenetic basis of this syndrome. The discovery of mutations in bone morphogenetic protein receptor-2 in familial PAH,1,2 the recognition of somatic chromosomal abnormalities in sporadic PAH,3 and the discovery of epigenetic silencing of mitochondrial superoxide dismutase-2 and the resulting activation of hypoxia-inducible factor-1α (HIF-1α)4 have yet to alter treatment. Moreover, despite effective vasodilator therapies, mortality rates remain high (15% at 1 year),5 suggesting a new treatment paradigm is needed. Excessive pulmonary artery smooth muscle cell (PASMC) proliferation plays an important role in PAH,6 suggesting that antiproliferative therapies are needed. We recently discovered that the hyperprolifera-
tive phenotype in PAH PASMCs (human and rodent) is associated with normoxic activation of HIF-1α and fragmentation of the mitochondrial network. Normoxic activation of HIF-1α in human PAH occurs in PASMCs and endothelial cells. HIF-1α activation can result from the redox changes initiated by epigenetic silencing of superoxide dismutase-2 in PAH, consistent with previous descriptions of redox regulation of HIF-1α. Activated HIF-1α suppresses mitochondrial oxidative metabolism (by increasing the expression of pyruvate dehydrogenase kinases, thereby blocking pyruvate uptake into the Krebs cycle) while simultaneously upregulating enzymes and transporters that favor glycolysis (ie, hexokinase-2 and the glucose transporter-1). Targeting these mitochondrial metabolic abnormalities in PAH; using the pyruvate dehydrogenase kinase inhibitor dichloroacetate reduces glycolysis, restores oxidative metabolism, and regresses PAH in several experimental models. This demonstrates the susceptibility of PAH to mitochondrial metabolic therapies. HIF-1α is activated early in experimental models of PAH and leads to a pulmonary glycolytic pattern that can be detected with a 18F-fluoro-deoxyglucose positron emission tomography scan. This glycolytic switch is reversible with therapies including pyruvate dehydrogenase kinase inhibitors and Gleevec. However, the link between HIF-1α, mitochondrial fragmentation, and cell proliferation is unknown.

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Mitochondria exist in a dynamic network comprising individual organelles that continuously join (fusion) and fragment (fission). The GTPases mitofusin-1 (MFN1), mitofusin-2 (MFN2), and optic atrophy-1 regulate fusion. Conversely, fission-1 and dynamin-related protein-1 (DRP1) mediate mitochondrial fission. The activity of DRP1 results from phosphorylation by regulatory kinases, including the mitosis regulator, cyclin B1/cyclin-dependent kinase 1 (CDK1), which causes mitotic phosphorylation of serine 616 and activation of DRP1. On activation, DRP1 moves from the cytosol to the mitochondria, where it assembles in multimers that constrict and divide the mitochondria. Conversely, phosphorylation of DRP1 at serine 637, by protein kinase A, inhibits fission.

Here, we determine the molecular basis for mitochondrial fragmentation in PAH and its relationship to HIF-1α, and assess whether mitochondrial fission offers a novel antiproliferative therapeutic target in PAH. Studies are conducted in human PASMCs and lung sections from PAH and control patients and in three rodent models of PAH, including a model induced by chemical activation of HIF-1α. We demonstrate that HIF-1α activation is a proliferative stimulus that causes DRP1-mediated mitochondrial fission. In vivo HIF-1α activation (by cobalt) is sufficient to produce PAH in rats and Mdivi-1, a small-molecule DRP1 inhibitor, restores mitochondrial fission and reduces the hemodynamic and histological severity of PAH in this model. Increased fission in PAH PASMC reflects DRP1 activation by cyclin B1/CDK1 activity and accompanies cell-cycle progression from G2 to mitosis. We show that fission is required for normal cell division and that Mdivi-1 slows proliferation by locking mitochondria in fusion and inhibiting cell-cycle progression, causing G2/M arrest, as we recently also observed in lung cancer cells. Mdivi-1 also has antiproliferative therapeutic benefit in monocrotaline-induced and hypoxia-induced pulmonary hypertension. We conclude that normoxic activation of HIF-1α creates a fissogenic proliferative milieu in PAH. Cyclin B1/CDK1, which is upregulated in PAH, coordinates mitosis and mitochondrial fission, permitting rapid proliferation; conversely, inhibition of mitotic fission causes cell-cycle arrest and regresses PAH. Thus, mitotic fission is a checkpoint that can be therapeutically targeted by inhibiting DRP1 in hyperproliferative syndromes.

Methods

Human Lung Samples

Detailed Methods are provided in the Online Supplement. Paraffin-embedded tissue sections and fresh tissue for PASMC isolation were obtained from autopsied patients with idiopathic PAH or control patients without PAH (Online Table I).

Animal Studies

The University of Chicago Animal Care Committee approved all protocols. Male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). To induce pulmonary hypertension in rats, we injected 2 mg CoCl2 intraperitoneally per day for 4 weeks (Sigma Aldrich). This dose was well-tolerated and did not induce mortality. Higher doses caused death or severe lethargy. Control rats received saline injections. Other animal models for PAH were chronic hypoxia (10% oxygen) and monocrotaline (60 mg/kg intraperitoneal). The DRP1 inhibitor Mdivi-1 (Enzo Life Sciences) was dissolved in DMSO and injected at a dose based on the literature (50 mg/kg) for prevention studies (weekly injections for CoCl2 and biweekly injections for chronic hypoxia), whereas five daily injections were administered 3 weeks after monocrotaline injection for regression studies. Control animals received intraperitoneal DMSO injections.

Immunoblotting and Immunofluorescence

Immunoblotting was performed on 25 μg of protein from PASMCs. The primary antibodies used were anti-cyclin B1 (Santa Cruz Biotechnology), anti-cyclin E (Cell Signaling Technology), anti-DRP1 (Novus Biologicals), anti-phospho Ser16 DRP1 (Cell Signaling Technology), anti-phospho Ser637 DRP1 (Cell Signaling Technology), anti-HIF-1α (Novus Biologicals), anti-smooth muscle

Non-standard Abbreviations and Acronyms

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<th>Abbreviation</th>
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<tr>
<td>CDK1</td>
<td>cyclin-dependent kinase 1</td>
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<tr>
<td>DRP1</td>
<td>dynamin-related protein-1</td>
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<tr>
<td>HIF-1α</td>
<td>hypoxia-inducible factor-1α</td>
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<td>Kv</td>
<td>voltage-gated K+ channel</td>
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<td>Mdivi-1</td>
<td>mitochondrial division inhibitor-1</td>
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<td>MFC</td>
<td>mitochondrial fragmentation count</td>
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<td>MFN1</td>
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<td>Mito-GFP</td>
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<td>Mito-PA-GFP</td>
<td>mitochondrial matrix-targeted photoactivatable GFP</td>
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<td>MNF</td>
<td>mitochondrial networking factor</td>
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<td>PA</td>
<td>pulmonary artery</td>
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<td>PAH</td>
<td>pulmonary arterial hypertension</td>
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<td>PASMC</td>
<td>pulmonary artery smooth muscle cell</td>
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cell actin (MP Biomedicals), and anti-von Willebrand factor (Abcam). To confirm equal protein loading, blots were stripped in Restore Western blot stripping buffer (Thermofischer) for 15 minutes and then reprobed with anti-actin (Millipore). Antigen retrieval, secondary antibodies, signal amplification, and imaging parameters are described in detail in the Online Supplement.

Small Interfering RNA Treatment of PASMCs
For small interfering RNA (siRNA) treatment, PASMCs were grown to approximately 80% confluence and then transfected using oligo-fection (Invitrogen) and 25 mmol/L validated Silencer Select siRNAs (Applied Biosystems). After 4 hours, normal culture medium was added and gene knockdown was assessed after 48 hours using quantitative reverse transcription polymerase chain reaction and immunocytochemistry. For each experiment there was a scrambled siRNA control (Applied Biosystems).

Live Cell Imaging to Assess Mitochondrial Networks
Mitochondria of live PASMCs were imaged with a Zeiss 510META confocal laser scanning microscope equipped with an environmental chamber to maintain humidity, 37°C, and 5% CO₂. We used coexpression of mitochondrial matrix-targeted photoactivatable green fluorescent protein (GFP) (a kind gift of Dr. Richard Youle, National Institutes of Health)22 and a mitochondrial matrix-targeted DsRed Protein (mito-DsRed, a kind gift of Dr Michael Frohman, Stony Brook University)23 to assess the degree of mitochondrial connectivity in live cells. PASMCs were transfected using Fugene HD transfection reagent (Roche). After acquiring control images, mitochondrial-targeted photoactivatable green fluorescent protein was photoactivated with 10 passes of the two-photon laser (set to 700 nm) in a confined 200×60-pixel area. Photoactivated GFP was measured at 488 nm excitation and 500 to 550 nm emission. For assessing mitochondrial morphology in the whole cell, we used a mitochondrial matrix-targeted GFP protein (a kind gift of Dr Michael Frohman, Stony Brook University).23

Image Analysis of Mitochondrial Morphology
Two recently validated techniques were used for assessing mitochondrial morphology in the whole cell: measurement of the mitochondrial fragmentation count and the mitochondrial networking factor.20 Briefly, images of mitochondrial matrix-targeted green fluorescent protein–transfected PASMCs were obtained, thresholded, and binarized to identify mitochondrial segments using ImageJ (National Institutes of Health). The mitochondrial fragmentation count was calculated by normalizing the number of individual mitochondria to the entire mitochondrial pixel count.20 To quantify functional networking of the mitochondria, PASMCs were transfected with mitochondrial-targeted photoactivatable green fluorescent protein and mitochondrial-targeted DsRed protein. The extent of mitochondrial networking is reflected in the diffusion of the photoactivated mitochondrial GFP away from the site of photoactivation, as measured after 17.6 seconds using ImageJ (National Institutes of Health). A highly interconnected network allows the photoactivated GFP to distribute in a larger area, resulting in a higher mitochondrial networking factor.20

Replication-Deficient Adenoviruses
The HIF-1α dominant-negative adenovirus was constructed using a plasmid from Dr Jian Chen (Hokkaido University, Japan).24 Viral methodology is described in the Online Supplement.

Proliferation Assay
Proliferation was quantified using the Click-It EdU kit according to the manufacturer’s instructions (Invitrogen).

Transmission Electron Microscopy
Freshly isolated pulmonary arteries (third-order and fourth-order) were fixed and processed for electron microscopy (Online Supple-

Measurement of Cyclin B1/CDK1 Activity by Förster Resonance Energy Transfer
A biosensor for cyclin B1/CDK1 activity was recently developed. It consists of a plasmid encoding two fluorescent proteins (mCerulean and YPet) linked by a cyclin B1/CDK1 phosphorylation site and a phosphobinding domain.25 Phosphorylation induces a conformational change that facilitates energy transfer from mCerulean to YPet. PASMCs were transfected with this Förster resonance energy transfer (FRET) biosensor plasmid and mCerulean was exited with a 458-nm argon laser. Emission was recorded at 465 nm to 510 nm for mCerulean and at 520 nm to 555 nm for YPet. Increased YPet/mCerulean signal indicates cyclin B1/CDK1 activation.25

Statistics
Values are stated as mean±SEM. Intergroup differences were assessed by an unpaired Student t test and simple or repeated-measures ANOVA with post hoc analysis using Tukey test, as appropriate. When applicable, normality was confirmed with a Kolmogorov-Smirnov test. For comparing the number of cyclin E–positive cells after Mdivi-1 treatment, a Fisher exact test was used. P<0.05 was considered statistically significant.

Supplemental Methods
Human and rat PASMC culture, quantitative reverse transcription polymerase chain reaction, O₂ consumption and metabolic measurements, electrophysiology, cytosolic calcium measurements, hemodynamic measurements, and histology were performed as previously described,4,26 and details are in the Online Supplement.

Results
DRP1-Mediated Mitochondrial Fragmentation in Human PAH PASMCs
Mitochondria are more fragmented in PASMCs from idiopathic PAH patients compared with control PASMCs (Figure 1A). Quantitative reverse transcription polymerase chain reaction for the genes that regulate mitochondrial fission and fusion16 shows a profile that favors fission (increased DRP1 and fission-1 and decreased MFN2: Figure 1B). Optic atrophy-1 and MFN1 expression are unchanged (Online Figure IA). We focused on the role of DRP1 because preliminary data indicated that Mdivi-1, a small-molecule inhibitor of DRP1, substantially rescues the mitochondrial network and slows proliferation. Expression of total and activated DRP1 (phosphorylated at Ser616) are upregulated in PAH PASMC (Figure 1C–E). Direct inhibition of CDK1, using 10 μmol/L of the specific inhibitor RO-3306,27 significantly reduces DRP1 Ser616 phosphorylation, implicating CDK1 as the key kinase driving DRP1 activation in PAH. In contrast, inhibition of other putative mediators of DRP1 phosphorylation, such as calmodulin, with 5 μmol/L W7 or calcium/calmodulin-dependent protein kinase II, with 20 μmol/L KN93, has no effect on DRP1 Ser616 phosphorylation (Figure 1E). Serum withdrawal (like CDK1 inhibition) reduces both the percentage of PASMCs with DRP1 Ser616 phosphorylation and the expression of the G2/mitotic-specific cyclin B1 in PAH PASMCs (Figure 1E–F), indicating that external growth factors/mitogens promote DRP1 activation. Using a FRET biosensor for cyclin B1/CDK1, we confirmed that increased
levels of cyclin B1 in PAH PASMCs translate into higher cyclin B1/CDK1 activity (Figure 1G).

Mitochondrial networking, measured using the mitochondrial networking factor, is decreased in human PAH PASMC, and this is reversed by 25 μmol/L Mdivi-1 (Figure 1H). Online Movies I to III show representative 8-minute time-lapse movies of control, PAH, and PAH plus Mdivi-1-treated PASMCs immediately before and after photoactivation, documenting the more rapid spread of the signal in cells treated with Mdivi-1.

Molecular or Pharmacological DRP1 Inhibition Blocks Cell-Cycle Progression

Compared to control human PASMCs, PAH PASMCs have increased proliferation rates at baseline, confirming the persistence of their hyperproliferative phenotype in vitro. DRP1 inhibition by Mdivi-1 causes a dose-dependent reduction in proliferation rates (Figure 2A). The antiproliferative effects of Mdivi-1 are attributable to induction of cell-cycle arrest in the G2/M phase (Figure 2B, Online Figure IIA), suggesting that cell-cycle progression is dependent on fission of mitochondria. To exclude nonspecific effects of Mdivi-1, we used siRNA directed against DRP1 and found a similar reduction in proliferation (Figure 2C) attributable to accumulation of PASMCs at the G2/M phase of the cell cycle (Figure 2D). To more exactly establish at which point the cell cycle is interrupted, we used immunoblotting to assess whether CDK1 activation is altered by Mdivi-1. CDK1 activation

Figure 1. Dynamin-related protein-1 (DRP1) activation causes excessive fission in human pulmonary arterial hypertension (PAH) pulmonary artery smooth muscle cells (PASMCs). A, Mitochondria are more fragmented in PAH vs control PASMCs. Quantification (mitochondrial fragmentation count) reveals a doubling of the number of individual mitochondria in PAH vs control PASMCs. Scale bar=20 μm. B, Quantitative reverse transcription polymerase chain reaction reveals increased expression of the profission proteins DRP1 and fission-1 and decreased expression of the profusion protein mitofusin-2 (MFN2) in human PAH PASMCs. C, Immunoblotting confirms the upregulation of DRP1 at the protein level (n=4 independent control cell lines and n=5 independent PAH cell lines). Expression was normalized to actin for each sample. D, More PAH PASMCs are positive for DRP1 Ser616 phosphorylation than control PASMCs. E, PASMCs with DRP1 phosphorylated at Ser616. Scale bar=100 μm. F, High levels of DRP1 Ser616 phosphorylation in PAH PASMCs vs control PASMCs. Only direct inhibition of CDK1 with 10 μmol/L RO-3306 and 16 hours of serum withdrawal (indicated by “–FBS” on the graph) decrease Ser616 phosphorylation levels. Inhibition of calmodulin with 5 μmol/L W7 or calcium/calmodulin kinase II with 20 μmol/L KN93 have no effect on DRP1 Ser616 phosphorylation. Approximately 100 PASMCs were counted for each group. F, Cyclin B1 expression was measured with flow cytometry and was significantly higher in PAH PASMCs compared with control PASMCs. Serum withdrawal (“–FBS”) reduces cyclin B1 levels in PAH PASMCs. G, Activity of the cyclin B1/CDK1 complex was measured using a biosensor Förster resonance energy transfer (FRET) probe. A higher yellow (YPet) over cyan (mCerulean) emission ratio indicates increased cyclin B1/CDK1 activity. PAH PASMCs have a higher activity compared with control PASMCs (n=10–11 cells/group). H, Phototactivation experiments confirm the decreased mitochondrial networking in PAH PASMCs. The mitochondrial networking factor (MNF) is lower in human PAH PASMCs and Mdivi-1 (25 μmol/L), an inhibitor of DRP1, reverses the fragmented phenotype and increases the MNF, demonstrating that increased DRP1 activity is the major determinant of the fragmented mitochondrial morphology in PAH. Scale bar=10 μm.
during mitosis depends on dephosphorylation of Thr14 and Tyr15. This dephosphorylation is not impaired by Mdivi-1 (Figure 2E), suggesting that cell-cycle arrest occurs specifically in prometaphase (between late G2 and metaphase), because this is the last phase in which cells have dephosphorylated CDK1. Subsequently, CDK1 becomes dephosphorylated at Thr14 and Tyr15. Mdivi-1 treatment (25 μmol/L) results in a clear activation of CDK1, suggesting that cells are either in late G2 phase or in prometaphase of the cell cycle. Protein expression levels are expressed as arbitrary units (AU) vs untreated PASMCs after normalizing for actin expression. F, Prevention of microtubule assembly with 50 ng/mL nocodazole synchronizes PAH PASMCs in the G2 phase of the cell cycle (increased percentage of cyclin B1–positive cells that have an increased DNA content). Removal of nocodazole allows cells to rapidly enter mitosis and after 4 hours the percentage of cyclin B1+ cells is normalized. However, if 25 μmol/L Mdivi-1 is added, then cells are unable to progress through mitosis.

DRP1 Activation in Human PAH Pulmonary Arteries

We obtained human lung tissue from five autopsied idiopathic PAH patients and five age-matched controls (patient characteristics in Online Table I) and confirmed the presence of plexiform lesions and increased pulmonary artery muscular...
larization (Figure 3A). Plexiform lesions and muscularized pulmonary arteries from idiopathic PAH patients have 10%±7% and 12%±3% increases in DRP1 expression, respectively, compared with arteries from normal subjects (Online Figure IIIA). DRP1 Ser616 is clearly enriched in pulmonary arteries (versus lung parenchyma) in PAH and is greater in PAH versus control lungs (Figure 3B), consistent with the PASMC data (Figure 1D-E). Moreover, the G2/mitotic-specific cyclin B1 is not only upregulated in PAH PASMCs but also activated, evident from its nuclear translocation (Figure 3C), which is in line with the increased cyclin B1/CDK1 activity measured in PAH PASMCs using a FRET biosensor (Figure 1G). Increased cyclin B1/CDK1 activity leads to DRP1 Ser616 phosphorylation.31 Whereas endothelial cells stain strongly for the inhibitory Ser637 form of DRP1, we did not detect this form of DRP1 in the media of control or PAH lungs (Online Figure IIIB) and therefore focused on DRP1 Ser616 in subsequent experiments.

**HIF-1α Activation Increases Proliferation and Glycolysis in Human PAH PASMCs**

We confirmed that HIF-1α activation occurs in PASMCs in lungs from PAH patients but not in control patients (Figure 4A). Even when PAH PASMCs are cultured in vitro at oxygen concentrations of approximately 140 mmHg, most have HIF-1α activation, evident from its nuclear translocation, greatly exceeding the rate in control PASMCs (Figure 4B). Metabolically, HIF-1α activation increased glycolysis, as measured with a Seahorse Bioscience XF24 analyzer (Online Supplement). Compared with control PASMCs, we observed a more than three-fold higher lactate production rate and a two-fold lower ratio of oxygen consumption/lactate production in PAH versus control human PASMCs (Online Figure IVA). The upregulated glycolysis is also evident in increased gene expression of glucose transporter-1, pyruvate dehydrogenase kinase-2, and pyruvate dehydrogenase kinase-4 (Online Figure IVB).
We next examined the consequences of HIF-1α activation on proliferation and metabolic function in human PASMCs. The importance of HIF-1α activation for proliferation was studied using chetomin, a small molecule that inhibits the interaction of HIF-1α with its p300 coactivator, preventing hypoxia-inducible gene transcription.32 Chetomin caused minimal cell death (Figure 4C), consistent with the low doses (5–50 nmol/L) we used, relative to those reported in the literature.33,34 Chetomin dose-dependently inhibited proliferation (Figure 4D) and DRP1 Ser616 phosphorylation (Figure 4E).

HIF-1α Activation in Normal PASMCs Recapitulates the PAH Phenotype and Induces Mitochondrial Fragmentation

We used two chemically discrete HIF-1α activators (CoCl2 and desferrioxamine)35 to establish a direct relationship between normoxic HIF-1α activation and mitochondrial fragmentation in normal rat PASMCs. This approach avoids confounders, such as genetic abnormalities and upregulation of growth factor pathways that might exist in human PAH cells. CoCl2 activated HIF-1α in most PASMCs (evident from nuclear accumulation of HIF-1α; Online Figure VA). Whole-cell patch-clamp traces show a reduction in voltage-gated K+ currents (Kv) with CoCl2, which is rescued with a HIF-1α dominant-negative virus (Online Figure VIA-B). The reduced Kv currents lead to depolarization of the PASMC (lower EM; Online Figure VIC) and increased cytosolic calcium levels (Online Figure VID). Both abnormalities can be prevented by overexpression of HIF-1α dominant-negative virus construct, confirming the role of HIF-1α in the current inhibition caused by ex vivo CoCl2 treatment. These HIF-1α-induced electrophysiological changes recapitulate those seen in PAH PASMCs.7,36–39

HIF-1α Dependence of Cobalt-Induced Mitochondrial Fission

CoCl2 (500 μmol/L) rapidly induces mitochondrial fission, again mimicking PAH (Figure 4F). To exclude nonspecific effects of the chemical HIF-1α activators, we first showed that 50 μg/mL cycloheximide, an inhibitor of protein translation, prevents cobalt or desferrioxamine-induced mitochondrial fragmentation. This indicates that protein synthesis is required for HIF-1α-induced fission (Figure 5A, B). Next, we showed that HIF-1α siRNA largely prevents cobalt-
induced fission, proving the fragmentation is attributable to HIF-1α activation (Figure 5C). Efficacy of HIF-1α/siRNA is demonstrated in Online Figure VB and VC. Finally, we showed that cobalt increases the phosphorylation of DRP1 Ser616 (Figure 5D). Thus, cobalt-induced HIF-1α activation mimics the PAH phenotype described in Figure 1D.

**Mdivi-1 Reverses HIF-1α–Induced Mitochondrial Fragmentation**

We used Mdivi-1 to confirm that the mitochondrial fragmentation observed after CoCl2-induced HIF-1α activation is dependent on DRP1. Mdivi-1 treatment (25 μmol/L) not only normalizes mitochondrial morphology after CoCl2 treatment (Figure 5E-F) but also enhances networking, as demonstrated by the more rapid and broader spread of photoactivated, mitochondrial matrix-targeted DsRed and photoactivatable green fluorescent protein (GFP). Mdivi-1 (25 μmol/L) restores the mitochondrial network (lowers the mitochondrial fragmentation count; F) and increases the mitochondrial networking factor (G). Scale bar=10 μm.

**Chronic Treatment With CoCl2 Causes Experimental PAH**

Because cobalt recapitulates many of the features of PAH PASMCs in culture, we determined whether activation of HIF-1α using cobalt would cause PAH in vivo. As expected, chronic cobalt treatment increased hematocrit, confirming that the selected dose of CoCl2 was biologically active (Figure 6A). Moreover, cobalt decreased maximal walking distance (Figure 6B). Echocardiography confirmed that the pulmonary artery acceleration time, a measure of vascular compliance that is inversely related to pulmonary artery pressures, decreases significantly after cobalt treatment (Figure 6C, Online Figure VIIA), whereas right and left heart catheterization confirmed that cobalt increased pulmonary vascular resistance (Figure 6D–F, Online Figure VIIB, C). Cobalt-induced PAH is not caused by left ventricular dysfunction because the left ventricular end diastolic pressures did not change between groups (Figure 6F). Postmortem analysis revealed increased right ventricular hypertrophy and percentage medial thickness of small pulmonary arteries (Figure 6G–I).

**DRP1 Inhibition Attenuates Pulmonary Hypertension In Vivo**

To determine whether inhibition of mitochondrial fission in vivo would attenuate cobalt-induced PAH, we coinjected 50 mg/kg Mdivi-1 (or the DMSO vehicle) on a weekly basis in
rats receiving chronic CoCl2 injections. Exercise capacity, which is reduced in cobalt-treated rats and Mdivi-1 treatment normalizes exercise capacity (B). Cobalt decreases pulmonary artery acceleration time (PAAT), indicative of increased pulmonary arterial pressures. Mdivi-1 partially normalizes the PAAT (C). CoCl2 doubles the pulmonary vascular resistance (defined as mean pulmonary artery pressure divided by the cardiac output) and Mdivi-1 significantly reduces pulmonary vascular resistance (D). Right ventricular systolic pressure is increased by CoCl2 treatment (E), but this is not induced by left ventricular dysfunction because the left ventricular end diastolic pressure (LVEDP) is not changed by any treatment (F). Postmortem determination of right ventricular fractional weight (right ventricular weight divided by left ventricular plus septum weight [RV/LV+S]) showed that CoCl2 increased right ventricular hypertrophy, which is normalized by Mdivi-1 treatment (G). Immunohistochemistry for von Willebrand factor (green) and smooth muscle cell actin (red) reveals that small precapillary resistance vessels in the lungs of CoCl2-treated animals have a thicker media compared with control lungs, and Mdivi-1 partially reverses this (H, I). Scale bar=50 μm. J, Electron microscopy imaging of pulmonary arteries derived from control animals and rats treated with CoCl2 and CoCl2 plus Mdivi-1. Pulmonary artery smooth muscle cells (PASMCs) were identified at a low magnification (5900x) by their characteristic cytoplasmic phenotype, their close proximity to collagen fibers, and the absence of Weibel Palade bodies (upper panels, scale bar=1 μm). Higher-resolution images (25 000x, lower panels, scale bar=200 nm) were then acquired to measure mitochondrial size. Note the smaller mitochondrial size in the CoCl2 rats and the normalization in the CoCl2 plus Mdivi-1 rats.

Mitochondrial Fragmentation In Vivo in Response to CoCl2
To prove that the antiproliferative effects of Mdivi-1 in vivo reflected inhibition of fission, we performed transmission electron microscopy studies on pulmonary arteries isolated from control rats and rats receiving CoCl2 or CoCl2 plus Mdivi-1. These images demonstrate that mitochondrial fragmentation is present in vivo in the cobalt PAH model and that these morphological changes are reversed with Mdivi-1 (Figure 6J).

Mdivi-1 Attenuates the Development of Chronic Hypoxia-Induced Pulmonary Hypertension
We also studied whether similar benefits of Mdivi-1 therapy could be observed in additional experimental models of PAH. As shown in Figure 7A, Mdivi-1 prevents the decrease in exercise capacity caused by chronic hypoxia-induced pulmonary hypertension. Echocardiography confirms that Mdivi-1
improved pulmonary artery acceleration time (consistent with a reduction in pulmonary artery pressure) and tricuspid annular plane systolic excursion (indicating improved right ventricular function) while reducing right ventricular hypertrophy (Figure 7A). Mdivi-1 also has antiproliferative effects in the chronic hypoxia model. In line with our previous findings, we observed decreased muscularization and fewer proliferating PASMCs in rats treated with Mdivi-1 (Figure 7B–D).

Mdivi-1 Reduces Proliferation in the Monocrotaline Model of PAH
Having shown that Mdivi-1 can attenuate the development of CoCl₂ and chronic hypoxia-induced PAH, we tested Mdivi-1 as regression therapy in the monocrotaline model. More specifically, we wanted to test whether inhibiting fission reduces PASMC proliferation. Daily Mdivi-1 treatment was started 3 weeks after monocrotaline injection, when PAH is already established (confirmed by echocardiography), and continued for 5 days. This relative short treatment significantly improved tricuspid annular plane systolic excursion (indicating improved right ventricular function) while reducing right ventricular hypertrophy (Figure 7A). Mdivi-1 also has antiproliferative effects in the chronic hypoxia model. In line with our previous findings, we observed decreased muscularization and fewer proliferating PASMCs in rats treated with Mdivi-1 (Figure 7B–D).

Discussion
We demonstrate that the hyperproliferative diathesis of human PAH PASMCs reflects increased DRP1 expression and activation (Figures 1, 2). The molecular mechanism of DRP1 activation in human PAH patients is increased DRP1 Ser616 phosphorylation (Figure 1D, E, Figure 3B), reflecting increased activation of the regulatory kinase of DRP1 (cyclin B1/CDK1). DRP1 Ser616 phosphorylation and fission can be induced in normal PASMCs by chemically activating HIF-1α. The relevance of HIF-1α-induced DRP1 activation to PAH is evident in the discovery that cobalt-induced HIF-1α activation elicits PAH in normal rats and that PAH is attenuated by Mdivi-1. Notably, the hemodynamic benefits were associated with restoration of mitochondrial fusion in vivo (Figure 6J). The antiproliferative effects of blocking mitotic fission also are evident in two well-established models of pulmonary hypertension. By locking the mitochondria in fusion, Mdivi-1 and siDRP1 prevent mitotic fission and cause cell-cycle arrest in the G2/M phase (Figure 2A–D), which is consistent with the recent demonstration that inhibition of DRP1 regresses lung cancer cell xenografts.20 Thus, excessive fission, which occurs in human PAH PASMCs in vitro and in vivo, contributes to the disease by driving proliferation and can be therapeutically targeted.
Mitochondrial morphology reflects the balance of fission and fusion. Fusion merges the contents of adjoined mitochondria, creating a homogenous network of elongated mitochondria, allowing the redistribution of mitochondrial DNA and proteins. Conversely, fission is necessary during cell division to evenly distribute mitochondria between daughter cells. Fission occurs when DRP1 is recruited from the cytoplasm to the mitochondrial surface, where it forms oligomeric complexes that hydrolyze GTP and mechanically constrict the mitochondria causing fragmentation.

Taguchi et al showed that the interconnected mitochondrial network structures in interphase HeLa cells become fragmented in the early mitotic phase via cyclin B1/CDK1-mediated DRP1 phosphorylation. In the current study, we show that in human PAH there is both an upregulation of total and Ser616 phosphorylated DRP1. The profission phenotype is seen in lungs from patients with PAH and persists in human PAH PASMCs in culture (Figure 1D, E, Figure 3B). The elevation of cyclin B1 and CDK1, which coordinate mitosis and mitotic fission, is a major stimulus for Ser616 phosphorylation in PAH (Figure 1F, G, Figure 3C). Cell proliferation is driven by many factors in PAH, including elevated levels of mitogens and growth factors and mutations of the bone morphogenetic protein receptor (in familial PAH).

In carefully controlled experiments, using molecular tools including siHIF-1α and a HIF-1α dominant-negative virus, we showed that HIF-1α activation is sufficient to confer a PAH phenotype on previously normal PASMCs (Online Figure VI) and to induce mitochondrial fission (Figure 5). The increased HIF-1α in human PAH lungs (Figure 4A) persists in culture, despite PASMCs being cultured at ambient oxygen concentrations (approximately 140 mmHg). This is consistent with the recently described epigenetic suppression of mitochondrial superoxide dismutase, which is an upstream heritable cause of the HIF-1α activation and proliferation in human and fawn-haired rat PAH that persist in cells in culture. In addition, normoxic HIF-1α activation can be induced in aortic smooth muscle cells by growth factors such as serotonin and platelet-derived growth factor-BB.

HIF-1α activation causes a glycolytic shift that confers a proliferative advantage in PASMCs. Similar HIF-1α–dependent glycolytic metabolic changes also occur in the endothelial cells of PAH patients. Proof that HIF-1α underlies the proliferation advantage comes from experiments in which HIF-1α inhibition by chetomin reduces PASMC proliferation (Figure 4D). Activation of HIF-1α results in glycolytic metabolic changes that can be detected using lung 18F-fluorodeoxy-glucose positron emission tomography scans. These glycolytic changes occur simultaneously with the development of pulmonary hypertension after monocrotaline administration and appear to be related to increased proliferation. Reversing the glycolytic phenotype with dichloroacetate also regresses experimental PAH. Our findings are consistent with the observations that HIF-1α haplo-insufficient mice are relatively protected from chronic hypoxic pulmonary hypertension, whereas HIF-1α activation increases proliferation of human PASMCs in response to platelet-derived growth factor. These data provide compelling evidence that HIF-1α activation contributes to disease progression in human PAH.

Whereas CoCl2 clearly induces HIF-1α in PASMCs (Online Figure VA), it is possible that nongenetic effects of cobalt contribute to our observations. Gene expression arrays in fibroblasts and carcinoma cells have shown that CoCl2 and HIF-1α induce a common set of genes, but some genes are specifically induced by cobalt. Yet, desferrioxamine, another HIF-1α activator, similarly leads to mitochondrial fragmentation (Figure 5A) and, conversely, siRNA-mediated knockdown of HIF-1α prevents CoCl2-induced mitochondrial fragmentation (Figure 5C), indicating that mitochondrial fragmentation depends on HIF-1α activation. Moreover, changes in K⁺ currents, membrane potential, and cytoplasmic calcium concentrations induced by CoCl2 are almost completely negated by adenosine overexpression of a HIF-1α dominant-negative construct (Online Figure VI), again suggesting that in PASMCs most effects of CoCl2 are HIF-1α–mediated. In addition, in vivo, CoCl2 will induce HIF-1α in other tissues, as exemplified by increased hematocrit, which is dependent on renal erthropoietin production. Despite possible nongenetic effects, the net result of chronic CoCl2 administration is pulmonary vascular remodeling (Figure 6).

We acknowledge that although Mdivi-1 clearly improves mitochondrial networking both in human (Figure 1H) and rat PASMCs (Figure 5E), and in vivo (Figure 6J) it potentially could have off-target effects. However, knockdown of DRP1 expression by using a specific siRNA prevents viralPasmc, Mdivi-1 knockdown of DRP1 prevents proliferation in human PAH PASMCs (Figure 2A, C). The notion that mitochondrial fragmentation is essential for cell-cycle progression past the G2/M checkpoint is an important and possibly controversial discovery. In support of our findings, gene therapy with Mitofusin-2, which causes mitochondrial fusion, also slow smooth muscle cell proliferation in systemic arterial injury. Moreover, DRP1 activation recently has been shown to be crucial to the hyperproliferative phenotype in lung cancer, and Mdivi-1 is therapeutic in regressing tumors in a xenotransplantation model. More study is required to determine whether mitotic division of organelles (not just mitochondria) is relevant to proliferative disorders. Supporting this notion, blocking Golgi fragmentation prevents progression through mitosis. Interestingly, CDK1 is involved in controlling Golgi fragmentation as well.

Electron microscopy confirms that CoCl2 induces mitochondrial fragmentation in vivo (Figure 6J), and chronic cobalt administration leads to pulmonary hypertension in line with previous publications that describe its ability to increase hematocrit, pulmonary hypertension, and right ventricular
hypertrophy in rats. Importantly, Mdivi-1 already has been shown to protect against ischemia-induced kidney damage and cardiac ischemia-reperfusion injury. For the first time to our knowledge, we used Mdivi-1 for long-term treatment (during the 4 weeks of CoCl2 administration) and observed that it is well-tolerated and significantly improves exercise capacity and reduces pulmonary vascular resistance and right ventricular hypertrophy. Moreover, muscularization of the pulmonary small blood vessels is attenuated by Mdivi-1 treatment, consistent with its antiproliferative effects on PAH PASMCs. We only looked at the effect of Mdivi-1 on PASMC proliferation, and it is possible that Mdivi-1 could influence other cell types as well. However, we did observe increased hematocrit in CoCl2 rats treated with Mdivi-1, indicating that hematopoietic stem cell proliferation is not or is only minimally impaired. Moreover, impaired intestinal stem cell proliferation would result in impaired nutrient absorption and weight loss, which we did not observe. After intraperitoneal injection, Mdivi-1 is taken-up in the mesenteric vein and transported to the right ventricle and subsequently to the pulmonary circulation. Therefore, Mdivi-1 concentrations are expected to be higher in the pulmonary vascular bed compared with other vascular beds. A similar mechanism is responsible for the relative pulmonary specificity of monocrotaline-induced pulmonary hypertension. Future pharmacological studies are necessary to evaluate tissue distribution and retention times of Mdivi-1. Mdivi-1 reduces PASMC proliferation in both monocrotaline and chronic hypoxic pulmonary hypertension. Therefore, it is reasonable to anticipate that Mdivi-1 is more effective in preventing than in reversing PAH. Thus, inhibition of mitochondrial fission may have broad therapeutic benefit in preventing disease progression in various forms of PAH. Future research is necessary to elucidate the role of fission-1 and MFN-2 in the hyperproliferative phenotype of PAH.

In conclusion, HIF-1α-stimulated DRP1-mediated mitochondrial fission leads to increased proliferation in human PAH PASMCs. This is the first direct evidence that an acquired abnormality of mitochondrial fission contributes to the maintenance of human PAH and demonstrates the feasibility of targeting mitochondrial dynamics as a therapeutic option for PAH. Taken together, our data indicate that mitotic fission is required for rapid proliferation and, conversely, that locking the mitochondria in a networked state prevents mitotic fission, causing cell-cycle arrest. A schematic representation of our conclusions is provided in Figure 8. Our findings have great therapeutic potential in PAH, evidenced by the effective and well-tolerated response to Mdivi-1 in the human PAH PASMC and several experimental models of pulmonary hypertension. This work also demonstrates an important interaction between the mitochondrial cycle and the cell cycle, which perhaps can be exploited in other proliferative disorders, such as cancer.

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Disclosures
None.

References


Mortality in PAH remains high despite current vasodilator therapies. Excessive proliferation of PASMCs contributes to PAH by obstructing the vasculature, suggesting that antiproliferative therapies may be therapeutic. This study explores two previous observations associated with hyperproliferation of PAH PASMCs: normoxic activation of HIF-1α and mitochondrial fragmentation. PASMC mitochondria exist in dynamic networks regulated by the balance between mitochondrial fusion and fission. Mitochondria divide (fission) during cell division, allowing equitable distribution of these organelles to daughter cells. Fission and mitosis are coordinated by the cell-cycle regulator cyclin B1–CDK1.

Here, we identify HIF-1α-related mechanism of mitochondrial fission in PAH. In human and experimental PAH, excessive activation of the GTPase DRP1 promotes mitochondrial fission. Conversely, inhibition of mitochondrial fragmentation arrests cells in the G2/M phase of the cell cycle and slows PASMC proliferation in experimental PAH, identifying mitochondrial fission as an important mitotic checkpoint. These findings shed light on the coordination between cell division and mitochondrial division. Pathological rates of mitochondrial fission in PAH may present an “Achilles heel” for these fast-growing cells. Inhibiting mitochondrial fission constitutes a novel therapeutic strategy in PAH.
Dynamin-Related Protein 1–Mediated Mitochondrial Mitotic Fission Permits Hyperproliferation of Vascular Smooth Muscle Cells and Offers a Novel Therapeutic Target in Pulmonary Hypertension

Glenn Marsboom, Peter T. Toth, John J. Ryan, Zhigang Hong, Xichen Wu, Yong-Hu Fang, Thenappan Thenappan, Lin Piao, Hannah J. Zhang, Jennifer Pogoriler, Yimei Chen, Erik Morrow, E. Kenneth Weir, Jalees Rehman and Stephen L. Archer

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Supplemental Material

Supplemental Methods

Human and rat PASMC culture. Cultures of PASMCs from normal subjects and humans with WHO category 1 idiopathic pulmonary arterial hypertension (PAH) were obtained from the University of Chicago and University of Minnesota (<passage 8>). Resistance human pulmonary arteries (500–1,500µm diameter) were dissected free and placed in Ca²⁺-free Hanks’ solution. The arteries were then transferred to Hanks’ solution containing 0.5 mg/ml papain (Worthington, Lakewood, NJ), 1 mg/ml albumin (Sigma Aldrich, St. Louis, MO), 0.5 mg/ml collagenase type I (Worthington, Lakewood, NJ) and 1 mg/ml dithiothreitol without EGTA and kept at 4°C for 30 min and then digested at 37°C for 40-70 min. Cells were maintained in medium 231 containing smooth muscle cell growth supplement (Invitrogen, Carlsbad, CA) in a humidified incubator at 37ºC under 5% CO². Additional human PAH cell lines were a gift from Dr. Patricia A. Thistlethwaite (University of California-San Diego). Rat pulmonary artery smooth muscle cells (PASMCs) were derived from small, intrapulmonary arteries of rats by isolating the pulmonary artery and plating explants on rat tail collagen (Millipore, Billerica, MA) in DMEM supplemented with 20% FBS and penicillin/streptomycin (Invitrogen, Carlsbad, CA). After reaching confluency, cells were passaged with 2.5mg/ml collagenase type II (Invitrogen, Carlsbad, CA) in 0.25% trypsin and maintained in culture in DMEM supplemented with 10% FBS. Cells were used up to passage 5 for experiments.

qRT-PCR. Total RNA was extracted using the PureLink Micro-to-midi total RNA isolation kit (Invitrogen, Carlsbad, CA). Reverse transcription was performed on 500ng of RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Quantification was performed on a 7900HT fast real-time PCR system with FAM-labeled probes (Applied Biosystems, Carlsbad, CA). Results are normalized to 18S and compared to control samples.

O₂-consumption and metabolic measurements. Simultaneous measurements of lactate production and oxygen consumption were performed on a XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA). PASMCs were plated on a XF24 cell culture microplate and used for measurements the day after equilibration in XF assay medium supplemented with 4.5g/L glucose.

Electrophysiology. Membrane potential (Em) and potassium currents were measured using an Axopatch 700B patch clamp amplifier (Axon Instruments, Foster City, CA) in whole-cell mode using voltage-clamp and current clamp configuration, respectively. The data were sampled using pClamp software (pClamp 10) and a Digidata 1322A analog to digital interface. Analysis was performed using pClamp software (ClampFit10). To generate current-voltage (I-V) relationships, 250msec depolarizing test pulses were applied from -70mV to +70mV, with 20mV increases, holding the potential at -70mV. All potassium currents were normalized to cell size (measured as capacitance in pF) and expressed as current density (pA/pF).

Measurement of cytosolic calcium concentrations ([Ca²⁺]cyt). PASMCs were plated on coverslips and loaded with Fura-2 AM (3 µmol/L for 30 minutes at 37⁰C, Invitrogen, Carlsbad, CA). The ratio of 510nm fluorescence signals elicited at 380 and 340nm was
calculated to measure cytosolic calcium as previously described. By using a ratiometric approach, the influence of possible quenching by cobalt is avoided.

**Hemodynamic measurements.** Echocardiography was performed using a Vevo 770 ultrasound system with a 37.5 MHz transducer (VisualSonics Inc., Toronto, ON, Canada). Rats were lightly anesthetized with isoflurane (1.6 – 2.0%). Pulmonary artery acceleration time (PAAT), a parameter which varies inversely with mean PAP, was recorded, as previously described. After echocardiography, open-chest invasive hemodynamic measurements were performed by placing a fluid-filled catheter (22 Gauge catheter) in the left ventricle via the apex and in the pulmonary artery via the right ventricular outlet. Cardiac output was measured using the thermodilution technique. Right ventricle hypertrophy was assessed postmortem as the weight ratio of the right ventricle to the left ventricle plus septum.

**Immunofluorescence and immunohistochemistry.** Immunofluorescence and immunohistochemistry were performed on paraffin-embedded sections after heat-induced antigen retrieval (30’ at 95°C in 0.01M sodium citrate, pH6). For immunofluorescence, fluorescent Alexa Fluor-488 and Alexa-568 (Invitrogen, Carlsbad, CA) labeled secondary antibodies were imaged on a Zeiss LSM 510 META confocal microscope at 488/490/505LP and 561/565/575-630 (excitation/dichroic/emissions). The mounting medium (Prolong Gold, Invitrogen, Carlsbad, CA) contained DAPI which was imaged with a 2P laser (770/KP-725/390-465). For immunohistochemistry, the signal was amplified using the Vectastain ABC system (Vector Labs, Burlingame, CA) and detected with diaminobenzidine (Invitrogen, Carlsbad, CA).

**Quantification of lung histology.** Lungs were perfused with saline and inflated with Z-fix (Anatech, Battle Creek, MI) at a pressure of 25cm H₂O. After overnight fixation, lungs were paraffin-embedded. Antibodies against α-Smooth Muscle Cell Actin (MP Biomedicals, Solon, OH) and vWF (Abcam, Cambridge, MA) were used. For each animal, at least 40 blood vessels measuring between 25 and 50μm diameter (precapillary resistance vessels) were randomly selected by their von Willebrand factor expression, without knowledge of their muscularization, as described previously. Images were obtained with a Zeiss Axio Observer Z1 inverted microscope using a LD Plan-Neofluar 40x objective. Morphometric analysis was performed with Zeiss Axiovision 4.6 software. Using the staining for α-Smooth Muscle Cell Actin, the outer and inner diameters of the muscular coat were measured and the percentage thickness was calculated as 100*(outer diameter-inner diameter)/outer diameter. When blood vessels had an ovoid shape, percentage thickness was determined for both the long and the short axis and then averaged.

**Replication deficient adenoviruses.** Dr. Jian Chen (Hokkaido University, Japan) kindly provided us the HIF-1α dominant-negative adenoviral construct. Viruses were amplified and purified by ViraQuest (North Liberty, IA). A virus dose in the range of 1-5 x10^10 plaque forming units (PFU) was used for *in vitro* experiments. For *in vitro* transfection, the virus, with designated multiplicity of infection (MOI) in serum-free media, was added to cells that were counted and plated the day before the transfection. After overnight incubation, the virus was washed out and the cells were placed in regular culture media. Cells were harvested 24-48 hours after the transfection for analyses by quantitative PCR or other cell growth and survival assays described below. An adenovirus containing LacZ cDNA was used as vector control.
Transmission Electron Microscopy. Freshly isolated pulmonary arteries (3rd-4th generation) were fixed overnight in 2.5% glutaraldehyde/4% paraformaldehyde in 0.1 M sodium cacodylate buffer. Samples were then incubated with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h, rinsed in sodium cacodylate buffer and maleate buffer (pH 5.1), and then stained with 1% uranyl acetate in maleate buffer for 1 h. After dehydration (25%–100% ethanol with a final 100% propylene oxide incubation), samples were infiltrated with 2:1 propylene oxide:Spurr’s resin (Sigma) for 30 min, then with 1:1 propylene oxide:Spurr’s resin overnight, and finally with 100% Spurr’s resin for 6 hours. Polymerization at 60ºC proceeded for 2 days. Ninety-nm sections were cut with a Reichert-Jung Ultracut E microtome and stained with uranyl acetate and lead citrate. Images were taken at 300 kV with an FEI Tecnai F30 electron microscope equipped with a high-performance Gatan CCD camera.
Supplemental Figures

Online Figure I. Expression levels of additional mitochondrial fusion mediators and Mdivi-1 does not inhibit phosphorylation of DRP1 Ser 616.

A) Mitofusin-1 (MFN1) and Optic atrophy-1 (OPA1) are proteins involved in mitochondrial fusion and are present in the outer and inner mitochondrial membrane, respectively. Their expression levels were not changed between control and PAH human PASMCs.

B-C) Mdivi-1 does not prevent cyclin B1/CDK1 mediated phosphorylation of DRP1 at Ser616. Immunocytochemistry (B) and immunoblotting (C) demonstrated increased Ser616 phosphorylation, in agreement with the increased CDK1 activation in response to Mdivi-1.
Online Figure II. Cell cycle analysis after Mdivi-1 treatment of PAH PASMCs and increased cyclin E expression after Mdivi-1 treatment.

A) Human PASMCs were incubated for 1 hour with the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) to assess proliferation and 7-Amino-Actinomycin D (7-AAD) was used to determine DNA content. The percentage of cells in the G2/M phase of the cell cycle (no EdU incorporation, more than diploid DNA content) increased in response to 25µM Mdivi-1.

B) Increased percentage of PASMCs are positive for nuclear cyclin E staining after Mdivi-1 treatment, asterisks point at PASMCs with nuclear cyclin E staining. The nuclear intensity in approximately 60 nuclei was analyzed in each group. Based on the no antibody control, nuclei with a mean relative fluorescence intensity >1200 were considered positive. Scale bar = 100µm.
Online Figure III. Immunostaining for DRP1 and DRP1 phosphorylated at Ser637 of human lungs.

A) Immunofluorescent staining for DRP1 in human lung sections. Double staining for DRP1 (green) and smooth muscle cell actin (red) allows identification of the smooth muscle cell layer and permitted quantification of DRP1 staining in the PASMCs. PASMCs in PAH lungs have a 12±3% increased DRP1 staining compared to PASMCs in control lungs. Approximately 50 blood vessels were analyzed per group. Scale bar = 100µm.

B) Immunohistochemistry for DRP1 phosphorylation at Ser637. When phosphorylated at this Serine amino acid, DRP1 is inactivated. While there is strong staining in endothelial cells and inflammatory cells, we could not detect Ser637 phosphorylation in small precapillary resistance PASMCs of control or PAH lungs. Scale bar = 50µm.
Online Figure IV. Glycolytic shift in PAH PASMCs.

A) PAH PASMCs have increased lactate production and a decreased oxygen consumption/lactate production ratio as measured using the Seahorse analyzer. This suggests that PAH PASMCs generate a larger proportion of their ATP from glycolysis compared to control PASMCs.

B) This glycolytic switch is confirmed by the upregulation of glucose transporter 1 (GLUT1), and pyruvate dehydrogenase kinases (PDK) 2 and 4.
Online Figure V. Confirmation of HIF-1α induction by CoCl₂ and of the effectiveness of siRNA mediated HIF-1α knockdown.

A) CoCl₂ leads to a strong induction and nuclear accumulation of HIF-1α in the majority of PASMCs.

B) siRNA mediated HIF-1α downregulation prevents HIF-1α accumulation in response to CoCl₂. Arrowheads point to examples of nuclei without HIF-1α, while asterisks point at nuclei with HIF-1α accumulation. There is a clear reduction in HIF-1α signal intensity when cells are pretreated with siHIF-1α. Scale bar = 100µm.

C) The glucose transporter 1 (GLUT1) is upregulated in response to HIF-1α and siRNA against HIF-1α prevent this increase, confirming the efficacy of this HIF-1α inhibition strategy.
Online Figure VI. HIF-1α recapitulates the features described for PAH PASMCs.

**A-B)** Representative patch clamp traces show a reduction in voltage-gated potassium (Kv) currents after chronic (24 hours) cobalt treatment of rat PASMCs. This is rescued with a HIF-1α dominant-negative virus (HIF-1α DN, n=4). The 4-aminopyridine (4-AP)-sensitive current is diminished in cobalt-treated cells, demonstrating decreased Kv currents. * P < 0.05 versus control.

**C-D)** Chronic (24 hours) cobalt treatment depolarizes cells (lower membrane potential, n=4) and increases cytosolic calcium concentrations ([Ca^{2+}]_{cyt}). Both effects are prevented by the HIF-1α DN virus (n=11).
Online Figure VII. Echocardiography and catheterization measurements in CoCl₂/Mdivi-1 treated animals.

A) Representative pulse wave Doppler tracings of the pulmonary artery. These traces were used to measure the pulmonary artery acceleration time (PAAT).

B-C) Representative catheterization traces of the pulmonary artery (B) and the right ventricle (C). The mean pulmonary artery pressure was used to calculate pulmonary vascular resistance. These traces were obtained using a 22 gauge fluid-filled catheter in anesthetized, open-chest rats.
Online Figure VIII. Therapeutic benefit of Mdivi-1 in the chronic hypoxia model.

A) Rats were injected with monocrotaline and 3 weeks later, when pulmonary hypertension is present, we started daily treatment for 5 days with Mdivi-1. We found that Mdivi-1 improves functional capacity measured on a treadmill. There is a trend for improved pulmonary artery acceleration time (PAAT) and decreased right ventricular fractional weight, while there is a significant improvement in tricuspid annular plane systolic excursion (TAPSE).

B) Rats injected with monocrotaline develop pulmonary hypertension characterized by excessive PASMC proliferation. Mdivi-1 both reduces the degree of muscularization of the pulmonary arteries and the number proliferating cell nuclear antigen (PCNA) positive PASMCs. Scale bar = 50µm.

C) We observed nuclear accumulation of HIF-1α in PASMCs of monocrotaline-treated rats. Scale bar = 50µm.
### Online Table I. Characteristics of patients of which lung tissue was studied by immunohistochemistry

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C: Caucasian; AA: African American; mPAP: Mean Pulmonary Artery Pressure; PCWP: Pulmonary Capillary Wedge Pressure; CO: Cardiac Output; PVR: Pulmonary Vascular Resistance; BNP: Brain Natriuretic Peptide; NA: not available.
Supplemental References


Supplemental Movie Legends

**Online Movie I:** Control PASMCs were cotransfected with mitochondrial matrix-targeted DsRed and mitochondrial matrix-targeted photoactivatable GFP. Upon photoactivation, GFP spreads throughout fused mitochondria. Images were obtained during 8 minutes following photoactivation.

**Online Movie II:** Photoactivation experiment in PAH PASMC.

**Online Movie III:** Photoactivation experiment in PAH PASMC treated with 25µM Mdivi-1.