Mammalian Target of Rapamycin Complex 2 (mTORC2) Coordinates Pulmonary Artery Smooth Muscle Cell Metabolism, Proliferation, and Survival in Pulmonary Arterial Hypertension

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Background—Enhanced proliferation, resistance to apoptosis, and metabolic shift to glycolysis of pulmonary artery vascular smooth muscle cells (PAVSMCs) are key pathophysiological components of pulmonary vascular remodeling in idiopathic pulmonary arterial hypertension (PAH). The role of the distinct mammalian target of rapamycin (mTOR) complexes mTORC1 (mTOR-Raptor) and mTORC2 (mTOR-Rictor) in PAVSMC proliferation and survival in PAH and their therapeutic relevance are unknown.

Methods and Results—Immunohistochemical and immunoblot analyses revealed that mTORC1 and mTORC2 pathways are markedly upregulated in small remodeled pulmonary arteries and isolated distal PAVSMCs from subjects with idiopathic PAH that have increased ATP levels, proliferation, and survival that depend on glycolytic metabolism. Small interfering RNA– and pharmacology-based analysis showed that although both mTORC1 and mTORC2 contribute to proliferation, only mTORC2 is required for ATP generation and survival of idiopathic PAH PAVSMCs. mTORC2 downregulated the energy sensor AMP-activated protein kinase, which led to activation of mTORC1-S6 and increased proliferation, as well as a deficiency of the proapoptotic protein Bim and idiopathic PAH PAVSMC survival. NADPH oxidase 4 (Nox4) protein levels were increased in idiopathic PAH PAVSMCs, which was necessary for mTORC2 activation, proliferation, and survival. Nox4 levels and mTORC2 signaling were significantly upregulated in small pulmonary arteries from hypoxia-exposed rats at days 2 to 28 of hypoxia. Treatment with the mTOR kinase inhibitor PP242 at days 15 to 28 suppressed mTORC2 but not Nox4, induced smooth muscle–specific apoptosis in small pulmonary arteries, and reversed hypoxia-induced pulmonary vascular remodeling in rats.

Conclusions—These data provide a novel mechanistic link of Nox4-dependent activation of mTORC2 via the energy sensor AMP-activated protein kinase to increased proliferation and survival of PAVSMCs in PAH, which suggests a new potential pathway for therapeutic interventions. (Circulation. 2014;129:864-874.)

Key Words: AMP-activated protein kinase ■ energy metabolism ■ idiopathic pulmonary arterial hypertension ■ muscle, smooth, vascular ■ mTORC2 ■ remodeling ■ signal transduction

Pulmonary arterial hypertension (PAH) is a multifactorial disease with a poor prognosis that may be idiopathic, heritable, or associated with other diseases. All types of PAH share similar pathological manifestations, such as remodeling of the small muscular pulmonary arteries (PAs), which leads to increased right ventricular afterload and ultimately right-sided heart failure and death. Increased cell proliferation and survival in the intima and media of small muscular PAs are key cellular events that lead to the pathological components of pulmonary vascular remodeling. We propose that the underlying mechanisms involve an interplay of metabolic adaptation with growth-promoting cellular signals.

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PA vascular smooth muscle cells (PAVSMCs) in idiopathic PAH (IPAH) have increased expression of hypoxia-inducible
factor 1α (HIF1α) and a metabolic glycolytic shift similar to the Warburg effect seen in human tumors, which supports cancer cell growth and survival.1,2 Several of these characteristics are also present in hypoxic pulmonary hypertension (PH) and cellular responses caused by hypoxia. The majority of nontransformed cells, including human airway smooth muscle cells, respond to chronic hypoxia by upregulating AMP-activated protein kinase (AMPK), which suppresses cell proliferation via inhibition of mammalian target of rapamycin complex 1 (mTORC1), a key positive regulator of protein, nucleotide, and lipid synthesis.6 Cancer cells overcome this translational block by mutational upregulation of phos- phatidylinositol 3 kinase (PI3K)-Akt and Raf-extracellular signal-regulated kinases 1/2 (ERK1/2) pathways that acti- vate mTORC1 via inhibition or counterbalancing of AMPK, increase HIF1α expression or transcriptional activity, stimulate glycolysis, and protect cells from apoptosis.7 PA VSMCs respond to chronic hypoxia by Akt and mTORC1 activation, which is required for increased proliferation and vascular smooth muscle remodeling,8–11 without causing changes in PI3K and ERK1/2 activities,8 by mechanisms that are currently unknown.

In addition to rapamycin-sensitive mTORC1, mTOR acts via the functionally distinct mammalian target of rapamycin complex 2 (mTORC2), which is rapamycin resistant in most cell types, including human vascular smooth muscle cells,8,12 and phosphorylates S473 Akt.6,7 The only known activator of mTORC2 is growth factor– and insulin-induced PI3K signaling. Alternative mechanisms of mTORC2 activation and its function in pulmonary vasculature have not been studied. Several lines of evidence suggest that mTORC2 may act as a coordinator of the metabolic shift with proliferation and survival of PA VSMCs in human PAH. First, chronic hypoxia induces PI3K-independent mTORC2 activation, which is required for PA VSMC proliferation.8 Second, mTORC2 stimulates glycolysis and upregulates expression of HIF1α in certain cell types.13,14 Third, NADPH oxidase 4 (Nox4), an important regulator of pulmonary vascular remodeling in PAH,15 increases phosphorylated (P) S473-Akt (P-S473-Akt) in human PA VSMCs under chronic hypoxia16 and contributes to HIF1α expression in heart.17 Finally, mTORC2 is required for cancer cell survival,18,19 which suggests its possible role in the regulation of PA VSMC glycolytic metabolism, proliferation, and survival in PAH.

In the present study, we aimed to dissect the role of mTOR signaling in PA VSMC proliferation and survival in IPAH. Our data show a novel role for mTORC2 as a coordinator of PA VSMC energy metabolism, proliferation, and survival in PAH and provide a novel mechanistic link of Nox4-dependent activation of mTORC2 via AMPK to the increased proliferation and survival of PA VSMCs in PAH, which suggests a new potential pathway for therapeutic interventions.

**Methods**

Methods are expanded in the online-only Data Supplement.

**Human Tissues and Cell Cultures**

Lung tissues from 4 nondiseased (control) and 4 IPAH lungs from females were provided by the Pulmonary Hypertension Breakthrough Initiative and the National Disease Research Interchange under protocols approved by the Pulmonary Hypertension Breakthrough Initiative, the National Disease Research Interchange, and the University of Pennsylvania and University of Colorado institutional review boards (Table I in the online-only Data Supplement). Distal (type III) PA VSMCs (Table I in the online-only Data Supplement) were isolated and characterized as described (Figures I and II in the online-only Data Supplement). Each experiment was repeated using primary (passages 3–8) PA VSMCs of the same passage from a minimum of 3 control and 3 IPAH subjects. Lonza media with 0.1% BSA (Lonza Group, Basel, Switzerland) was used for serum deprivation.

Immunohistochemical analysis was performed on lung tissue sections snap-frozen in optimal cutting temperature embedding compound (Tissue-Tek, Tokyo, Japan) as described previously.4 Transfection and immunoblot analysis were performed as described previously.5,19 Small interfering RNAs (siRNAs), pCMV6-Myc-DDK-Nox4 and pCMV6-Bim, were purchased from Dharmacon/Thermo Scientific (Lafayette, CO) and OriGene (Rockville, MD). Apoptosis analysis was performed with an in situ cell death detection kit (Roche, Nutley, NJ) as described previously.19 DNA synthesis analysis was performed with a bromodeoxyuridine (BrdU) incorporation assay as described previously.19

**Cell Growth and Viability Assay**

Cells plated on 6-well cultured plates (180,000 cells/well) were placed in Lonza media supplemented with 0.1% BSA (day 0). Cells were harvested at days 0, 5, and 10, and cell counts or viability was measured with a Countess automated cell counter (Invitrogen, Grand Island, NY).

**ATP Analysis**

Cell extracts were prepared as described previously.20 An ATP colorimetric/fluorimetric assay (Abcam, Cambridge, United Kingdom) was performed according to the manufacturer’s protocol.

**Animals**

All animal procedures were conducted in accordance with University of Pennsylvania Animal Care and Use Committee guidelines. Male Sprague-Dawley rats 6 to 8 weeks of age were randomly assigned to control and experimental groups (n=6 per group). Experimental groups were exposed to hypoxia (10% O2) for 2, 14, or 28 days or treated with PP242 (20 mg/kg i.p. 5 d/wk) or vehicle at days 15 to 28. Controls included normoxia-maintained animals.21 Animals were euthanized with pentobarbital overdose; the lungs were subjected to immunohistochemical or apoptosis analysis or stained with hema- toxylin and eosin. Images were taken with a Nikon TE2000 microscope; blinded morphometric analysis of PA medial wall thickness was performed as described previously.21 The lumen area at the level of the basement membrane and the total vascular area at the adventitial border in muscular PA (25–150 μm outer diameter) per lung section were outlined, and area sizes were measured with Image-Pro Plus 7 (Media Cybernetics, Rockville, MD). Medial wall thickness was calculated as (total vascular area−lumen area)/total vascular area×100. Analysis of fluorescent intensity in smooth muscle actin–positive areas of small muscular PAs was performed with Image-Pro 7. For visualization of the pulmonary vascular tree, the lung vascular- ture of rats randomly selected from experimental groups was rinsed with PBS and inflated with AltaBlu reagent (Numira Biosciences, Salt Lake City, UT), and micro-computed tomography analysis was performed by Numira Biosciences.

**Data Analysis**

Data are expressed as mean±SE by use of StatView software (SAS Institute, Cary, NC). Statistical comparisons between 2 groups were performed with the unpaired Student t test. Comparisons among ≥3 groups were performed with 1-, 2-, or 3-way ANOVA without repeated measures as appropriate. Comparisons among ≥2 groups were performed with 1-way ANOVA followed by the Dunnett post
hoc test. Comparisons among ≥3 groups were performed with 2- or 3-way ANOVA followed by a stratified independent \( t \) test with Bonferroni correction for multiple comparisons. Statistical significance was defined as \( P≤0.05 \).

**Results**

**mTORC1 and mTORC2 Pathways Are Activated in PAVSMCs in PAH**

Immunohistochemical analysis of lung tissues from 4 IPAH and 4 nondiseased (control) subjects revealed a marked increase in P-S2481-mTOR, a marker for mTOR catalytic activity,\(^22\) mTORC1-specific P-S235/236-S6,\(^6,8\) and mTORC2-specific P-S473-Akt\(^7,19\) in smooth muscle actin-positive areas in small muscular remodeled PAs (50–250 \( \mu \)m outer diameter) in IPAH lungs (Figure 1A through 1D). Distal PAVSMCs from IPAH patients demonstrated significant elevation of P-S2481-mTOR, P-S6, and P-S473-Akt in serum-replete conditions, which persisted after 48 hours of serum deprivation (Figure 2A and 2B) and was associated with increased DNA synthesis, growth, and viability (Figure 2C through 2E). These data show that PAVSMCs from IPAH patients have activated mTORC1 and mTORC2 pathways and in vitro elevated proliferation and survival without mitogenic stimuli.

**Increased ATP Generation, Proliferation, and Survival of IPAH PAVSMCs Depend on Glycolytic Metabolism**

Because glycolytic shift is proposed to play a role in pulmonary vascular cell proliferation in PAH,\(^4\) we evaluated the relative contributions of glycolytic versus mitochondrial metabolism to IPAH PAVSMC ATP generation, proliferation, and survival. IPAH PAVSMCs had ≥2-fold higher cellular ATP content than controls in both serum-replete and -deplete conditions, which was markedly reduced by the glycolytic inhibitor 2-deoxy-d-glucose, whereas the mitochondrial respiratory chain inhibitor rotenone had a modest effect (Figure 3A). 2-Deoxy-d-glucose, but not rotenone, markedly decreased proliferation and promoted apoptosis in IPAH PAVSMCs (Figure 3B and 3C). Conversely, rotenone inhibited ATP levels and proliferation and survival of control PAVSMCs, whereas 2-deoxy-d-glucose had a lesser effect (Figure 3A through 3C). These data show that in contrast to nondiseased cells, the increased ATP generation, proliferation, and survival of IPAH PAVSMCs depend predominantly on glycolytic metabolism.

**mTORC2 Is Required for Elevated ATP Generation, Proliferation, and Survival of IPAH PAVSMCs**

To determine the specific roles of mTORC1 and mTORC2 in PAVSMC remodeling, we selectively disrupted the complexes using siRNA-induced knockdown of their specific regulatory proteins, Raptor and Rictor.\(^6\) Raptor siRNA reduced mTORC1-specific P-S6 and IPAH PAVSMC proliferation without effects on mTORC2-specific P-S473-Akt, HIF1\(\alpha\) protein levels, ATP content, or cell survival (Figure 4A through 4D, 4G, and 4H). Rictor siRNA suppressed both mTORC2-specific P-S473-Akt and mTORC1-specific P-S6, reduced cellular ATP and HIF1\(\alpha\) levels, and decreased proliferation and induced apoptosis in IPAH PAVSMCs (Figures 4A through 4E and 5C and Figure III in the online-only Data Supplement). These data demonstrate that mTORC2 is required for increased cellular ATP levels, mTORC1-S6 activity, and proliferation and survival of IPAH PAVSMCs, whereas mTORC1 contributes predominantly to increased proliferation. Comparison of the mTOR kinase inhibitor PP242 (which inhibits both mTORC1 and mTORC2) and the allosteric mTORC1 inhibitor rapamycin\(^8\) revealed that

![Figure 1](https://example.com/figure1.png)
both reduced proliferation, but only PP242 induced apoptosis in IPAH PAVSMCs (Figure IV in the online-only Data Supplement). Importantly, mTORC2 inhibition did not significantly affect ATP or the proliferation and apoptosis rates of control PAVSMCs (Figure 4B through 4D and Figure IV in the online-only Data Supplement), which indicates the specificity of mTORC2 upregulation for the diseased PAVSMC phenotype.

**mTORC2 Downregulates AMPK, Enabling mTORC1 Activation and Proliferation of IPAH PAVSMCs**

Because mTORC2 positively regulates mTORC1-S6, we next determined whether mTORC2 controls mTORC1 activation and cell proliferation via the energy sensor, AMPK. IPAH PAVSMCs showed a significant reduction of P-T172-AMPK and AMPK-specific P-acetyl-CoA carboxylase (Figure 5A and 5B), which demonstrates that AMPK signaling is downregulated in IPAH PAVSMCs. mTORC2 inhibition with Rictor siRNA markedly increased P-acetyl-CoA carboxylase and P-AMPK while suppressing P-S6 (Figure 5C and 5D). siRNA-induced AMPK knockdown in IPAH PAVSMCs rescued Rictor siRNA–dependent inhibition of mTORC1-specific P-S6K1, P-S6, and proliferation (Figure 5E through 5G). In line with the role of AMPK, its activator AICAR decreased P-S6K1 and P-S6 without affecting mTORC2-specific P-S473-Akt (Figure V in the online-only Data Supplement). In aggregate, these data indicate that mTORC2 inhibition of AMPK leads to mTORC1 activation, which allows for proliferation of IPAH PAVSMCs.

**mTORC2 Regulates IPAH PAVSMC Survival via AMPK and Bim**

To determine the mechanisms by which mTORC2 stimulates IPAH PAVSMC survival, we tested whether mTORC2 regulates protein levels of Bim and Bcl2, its known downstream effectors in cancer cells. IPAH PAVSMCs showed deficiency of proapoptotic Bim and elevated levels of antiapoptotic Bcl2 (Figure 6A and 6B). mTORC2 inhibition with Rictor siRNA, rather than mTORC1 suppression with Raptor siRNA, markedly increased Bim levels without a significant effect on Bcl2 (Figure 6C through 6F). Because AMPK is shown to induce apoptosis via Bim, we evaluated whether mTORC2 suppresses Bim levels and enhances IPAH PAVSMC survival via inhibition of AMPK. AMPK siRNA prevented the Rictor siRNA–induced increase in Bim protein levels. Either AMPK siRNA or Bim siRNA rescued Rictor siRNA–dependent apoptosis (Figure 6G through 6J and Figures VI and VII in the online-only Data Supplement). Bim overexpression induced apoptosis in IPAH PAVSMCs (Figure 6K). These data demonstrate that mTORC2-dependent downregulation of AMPK promotes Bim deficiency, which is required for IPAH PAVSMC survival.

**Activation of mTORC2 Signaling, Proliferation, and Survival of IPAH PAVSMC Depend on Nox4**

Nox4 contributes to PAH pathogenesis and to chronic hypoxia–induced S473-Akt phosphorylation in human PAVSMCs. The link between Nox4 and mTORC2, however, has not been established. We found a marked increase of Nox4 protein levels in IPAH compared with control PAVSMCs (Figure 7A). Nox4 siRNA decreased P-S2481-mTOR, mTORC2-specific P-S473-Akt, and mTORC1-specific P-S6;

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*Figure 2.* Pulmonary artery vascular smooth muscle cells (PAVSMCs) from lungs with idiopathic pulmonary arterial hypertension (IPAH) have activated mammalian target of rapamycin complex 1 (mTORC1) and 2 (mTORC2) signaling and increased proliferation and survival. A and B, Distal PAVSMCs from 4 nondiseased (control [Contr]) and 4 IPAH subjects cultured in complete media (+FBS) or serum deprived for 48 hours (-FBS) were subjected to immunoblot analysis to detect indicated proteins. Data represent fold changes in phosphorylated (P)/total protein ratios; P/total ratio for controls taken as 1 fold. Four subjects per group; *P<0.05 by unpaired Student *t* test. C, DNA synthesis analysis of serum-deprived (for 48 hours) PAVSMCs (bromodeoxyuridine incorporation) from 4 control and 4 IPAH subjects; 3 measurements per subject; data from each subject presented as a separate bar. Data represent percentage of bromodeoxyuridine-positive cells per total number of cells. **P<0.001 vs controls by unpaired Student *t* test. D and E, Cell counts and viability of PAVSMCs from 4 IPAH (squares) and 4 control subjects (circles) maintained in serum-free conditions. Data represent quantity of cells per well (D) and percentage of dead cells per total number of cells (E). Four subjects per group; *P<0.05 by unpaired Student *t* test.
upregulated P-AMPK, AMPK-specific P-acetyl-CoA carboxylase, and Bim levels without affecting Bcl2 (Figure 7B through 7D); and reduced proliferation and increased apoptosis of IPAH PAVSMCs (Figures 7E and Figure VIII in the online-only Data Supplement). Nox4 overexpression in non-diseased PAVSMCs increased P-S473-Akt and P-S6, reduced P-acetyl-CoA carboxylase and Bim levels, and elevated cell proliferation (Figure 7F through 7H). These data show that Nox4 acts as an upstream positive regulator of mTORC2 signaling, proliferation, and survival of IPAH PAVSMCs.

**mTOR Is Required for PAVSMC Survival and Pulmonary Vascular Remodeling In Vivo**

To evaluate the role of mTORC2 and Nox4 in the development of pulmonary vascular remodeling, we performed immunohistochemical analysis of lung tissues from rats with chronic hypoxia-induced pulmonary vascular remodeling. We found significant upregulation of P-S4281-mTOR, P-S473-Akt, and Nox4 in smooth muscle actin–positive areas of small muscular PAs (25–150 µm outer diameter) at day 2 of hypoxia exposure, with a further increase at day 14 (Figure 8A, 8B, 8D, and 8E). Morphometric analysis under the same conditions showed significant smooth muscle cell remodeling at day 14 of hypoxia, with no changes in PA medial wall thickness at
day 2 (Figure 8C). Thus, upregulation of mTORC2 and Nox4 in distal PAVSMCs occurs at the early stages of hypoxia preceding pulmonary vascular remodeling. Treatment with the mTOR kinase inhibitor PP242 at days 15 to 28 of hypoxia exposure markedly reduced P-S2481-mTOR and P-S473-Akt (Figure 8F and 8G and Figure IX in the online-only Data Supplement), which supports our observations that Nox4 acts upstream of mTORC2. PP242 induced apoptosis in smooth muscle actin–positive cells in small muscular PAs, which was associated with an increase in Bim protein levels (Figure 8H and Figure X in the online-only Data Supplement), and decreased PA medial wall thickness to levels comparable to normoxia-exposed controls (Figure 8I). Micro-computed tomography analysis showed improved pulmonary vascular density in PP242-treated rats compared with vehicle-treated animals (Figure 8J and 8K and Movies I through VI in the online-only Data Supplement). These data demonstrate that PP242 inhibits mTORC2, which induces smooth muscle actin–specific apoptosis in small muscular PAs and reverses existing pulmonary vascular remodeling in vivo in a model relevant to PH in humans.

**Discussion**

Increased proliferation and survival of PAVSMCs in small PAs coupled with deregulated expression of HIF1α and glycolysis are critical components of the pathophysiology of pulmonary vascular remodeling in PAH. The present study identifies mTORC2 as an important positive regulator of glycolysis-dependent proliferation and survival of PAVSMCs in IPAH. We report the novel mechanistic link from mTORC2 via AMPK to the activation of mTORC1 signaling and increased proliferation, as well as Bim deficiency and survival of IPAH PAVSMCs. We also show that Nox4 acts proximally to mTORC2-mediated effects to positively regulate IPAH PAVSMC proliferation and survival. Lastly, we demonstrate benefits of dual mTORC1/mTORC2 inhibition to reduce proliferation and promote apoptosis in IPAH PAVSMCs and reverse hypoxia-induced pulmonary vascular remodeling in rats (Figure XI in the online-only Data Supplement), which suggests the attractiveness of mTORC2 as a potential target to treat deregulated proliferation and survival in human PAH.

The metabolic shift to glycolysis, similar to the Warburg effect in cancer, contributes to increased PAVSMC proliferation and pulmonary vascular remodeling in PAH. The present data provide direct evidence that the elevated ATP generation and proliferation and survival of PAVSMCs from subjects with IPAH depend on glycolytic metabolism and can occur without the need for mitogenic stimuli, which indicates the critical role of the glycolytic shift in PAVSMC proliferation and survival in IPAH.

Currently, the mechanisms that coordinate the metabolic shift with increased vascular cell proliferation and survival in IPAH are not well understood. Recent studies in cancer indicate that the maintenance of a glycolytically active proliferative cell phenotype requires mutational activation of major proliferative prosurvival pathways, including PI3K-Akt and

![Figure 5. Mammalian target of rapamycin complex 2 (mTORC2) regulates mammalian target of rapamycin complex 1 (mTORC1) signaling and proliferation of pulmonary artery vascular smooth muscle cells (PAVSMCs) from lungs with idiopathic pulmonary arterial hypertension (IPAH) via AMP-activated protein kinase (AMPK). A and B, Immunoblot analysis of serum-deprived (for 48 hours) PAVSMCs from 4 IPAH and 4 control (Contr) subjects performed to detect indicated proteins. Data represent phosphorylated (P)/total protein ratios; mean ratio for control PAVSMCs was taken as 1 fold. Four subjects per group; *P<0.05 by unpaired Student t test. C–G, Serum-deprived PAVSMCs from 3 IPAH subjects were transfected for 48 hours with 100 nmol/L Rictor small interfering RNA (siRictor) or control scrambled (−) small interfering RNAs (siRNAs; C, D) or cotransfected with 50 nmol/L siRictor, AMPK siRNA (siAMPK), or control scrambled siRNA (--; E–G) followed by immunoblot (C–F) and DNA synthesis (bromodeoxyuridine incorporation; G) analyses. Data represent fold changes in phosphorylated (P)/total protein ratios; ratio for control siRNA-transfected cells taken as 1 fold (D, F) and percentage of bromodeoxyuridine-positive cells from total number of cells (G). D, Three subjects per group; **P<0.001 by unpaired Student t test. F and G, Four (F) and 3 (G) subjects per group; *P<0.05, **P<0.001 by 1-way ANOVA with post hoc Dunnett test. ACC indicates acetyl-CoA carboxylase.
Our previous studies, however, have shown that suppression of mTORC2 reduces cellular ATP levels, decreases P-S473-Akt and protein levels of HIF1α (2 confirmed stimulators of glycolysis in other cell types35), and inhibits proliferation and promotes apoptosis in IPAH PAVSMCs, which strongly suggests a role for mTORC2 as an upstream positive regulator of glycolytic metabolism and PAVSMC growth in human IPAH.

The lack of understanding of the role of mTORC2 in normal and diseased PAVSMCs is likely attributable to the exclusive use of the allosteric mTORC1 inhibitor rapamycin or its analogs (rapalogs) in prior studies. Rapalogs in the doses used for clinical applications have therapeutically proven cytostatic function with no appreciable proapoptotic effect in vascular smooth muscle cells, including human PAVSMCs.6,8,27 Indeed, we found that rapamycin inhibits proliferation but does not induce IPAH PAVSMC apoptosis. Rapamycin in clinically relevant doses attenuates development of pulmonary vascular remodeling in experimental PH but had a cytostatic effect on smooth muscle–like cells in clinical trials of pulmonary lymphangioleiomyomatosis and tuberous sclerosis, prevented apoptosis in a rat carotid model of vascular injury, and did not reverse existing monocrotaline-induced PH in rats.3,10,19,28,29 High doses of rapamycin, however, attenuated pulmonary vascular remodeling and downregulated both mTORC1-specific S6 and mTORC2-specific P-S473-Akt in the same experimental model.30 Although not pharmacologically applicable to humans because of the nonphysiological doses of rapamycin, the present study suggests a potential link between mTORC2 and remodeling in experimental PH.

The present findings show that the mTORC1 pathway is activated in small PAs and in PAVSMCs from IPAH lungs and is critical for IPAH PAVSMC proliferation. An siRNA-based approach to selectively inhibit mTORC1 showed that mTORC1 inhibition does not affect cellular ATP levels and survival of IPAH PAVSMCs. These data support the critical role of mTORC1 for cell proliferation but strongly suggest that mTORC1 acts downstream of signaling pathways regulating IPAH PAVSMC energy metabolism.

mTORC1 is a homeostatic energy-triggered molecular relay and is activated by increased ATP levels.20 Hypoxic stress suppresses mTORC1 and cell proliferation via the energy sensor AMPK, which has been identified recently as a key regulator of cardiovascular homeostasis, the dysfunction of which underlies several cardiovascular pathologies.31 In addition to its growth-inhibitory effects, AMPK may act as a proapoptotic or antiapoptotic molecule in an isoform-specific manner32 by upregulating p53 signaling or downregulating the proapoptotic or antiapoptotic molecule in an isoform-specific manner.32 Our previous studies, however, have shown that hypoxia-induced PAVSMC proliferation was not associated with changes in PI3K activity or ERK1/2 signaling but required activation of mTORC1 and mTORC2 pathways.8 That led us to hypothesize about the critical role of mTORC2 in mediating glycolytic metabolism and the increased proliferation and survival of PAVSMC in PAH. Here, we have demonstrated that mTORC2 signaling is upregulated in small remodeled PAs and in proliferative apoptosis-resistant distal IPAH PAVSMCs without the need for exogenous mitogenic stimuli. Using both molecular and pharmacological interventions, we subsequently showed that suppression of mTORC2 reduces cellular ATP levels, decreases P-S473-Akt and protein levels of HIF1α (2 confirmed stimulators of glycolysis in other cell types35), and inhibits proliferation and promotes apoptosis in IPAH PAVSMCs, which strongly suggests a role for mTORC2 as an upstream positive regulator of glycolytic metabolism and PAVSMC growth in human IPAH.
Hypoxia-induced Nox4 overexpression

PAs, and we recently reported an association of genetic variation in Nox4 with the risk of PAH in patients with portal hypertension.37–41 Hypoxia-induced Nox4 overexpression contributes to S473-Akt phosphorylation and increased proliferation of PAVSMCs and pulmonary vascular remodeling in hypoxic PH.16,38–41 The present data indicate that Nox4 acts as an upstream positive regulator of mTORC2 signaling, proliferation, and survival in IPAH PAVSMCs, providing a potential mechanism of mitogen-independent mTORC2 activation, increased cell proliferation, and survival in PAH.

Recognizing that human PAH is a multifactorial disease, we anticipate that other factors such as dysregulation of BMPRII (bone morphogenetic protein receptor type 2), PPAR-γ (peroxisome proliferator-activated receptor-γ) signaling, and mitogen exposure might have a further impact on mTORC2-dependent regulation of the proliferative apoptosis-resistant PAVSMC phenotype. Although there is no direct evidence linking BMPRII deficiency with mTORC2 activation, the BMPRII downstream effector PPAR-γ inhibits mTORC1 signaling in cancer cells,42,43 which provides a link between BMPRII and PPAR-γ deficiency and mTOR activation. Note, a recent report from Green et al44 showed that Nox4 modulates chronic hypoxia–induced expression of PPAR-γ and transforming growth factor-β1, which suggests potential cross talk between Nox4, growth factors, BMPRII, PPAR-γ, and mTORC2 signaling in human PAH that requires further investigation.

Collectively, the present study data demonstrate that mTORC2 coordinates preferential energy generation by glycolysis and the increased proliferation and survival of PAVSMCs in IPAH. The attractiveness of mTORC2 as a potential therapeutic target is further supported by our observations that inhibition of mTORC2 signaling by Rictor siRNA and the mTOR kinase inhibitor PP242 targets predominantly IPAH PAVSMCs with no significant effects on nondiseased cells. Similar selectivity of PP242 has already been demonstrated in a mouse leukemia model in which PP242 showed improved therapeutic response compared with rapamycin but only mildly affected normal lymphocytes.45 Although the present study focused on the IPAH PAVSMC and not other pulmonary vascular cell types, endothelial cells and adventitial fibroblasts in human IPAH also have a glycolytic phenotype,5,46 and
hypoxia-induced endothelial cell proliferation requires constitutive mTORC2 activation.\textsuperscript{47}

We recognize that the present study has the limitations associated with small human sample size that arise from the nature of studied disease. IPAH is a rare disease, which limits the availability of human lung tissue specimens and primary human cell cultures of early passage for mechanistic research of this type. However, given our present findings on the pro-apoptotic effects of PP242 on pulmonary vascular smooth muscle cells from both IPAH subjects and rats exposed to chronic hypoxia, as well as recent advances in the pharmacological use of PP242 in animal models of cancer,\textsuperscript{46-50} preclinical testing of dual mTORC1/mTORC2 inhibitors on other human PAH cells and experimental PH models is worthy of further investigation.

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**Disclosures**

None.

**References**


**CLINICAL PERSPECTIVE**

Pulmonary arterial hypertension is characterized by remodeling of the small muscular pulmonary arteries, which leads to increased right ventricular afterload, right-sided heart failure, and death. Enhanced proliferation, impaired apoptosis, and a metabolic shift to glycolysis of pulmonary arterial vascular smooth muscle cells (PAVMCs) are important pathophysiological components of pulmonary vascular remodeling, the understanding of which is critical for identification of novel molecular targets. mTOR (mammalian target of rapamycin) is a key regulator of cell growth and metabolism that acts through 2 distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 1 (mTORC2). Here, we show that both mTORC1 and mTORC2 pathways are upregulated in small remodeled pulmonary arteries and primary distal PAVSMCs from subjects with idiopathic pulmonary arterial hypertension (IPAH) and contribute to increased proliferation. Only mTORC2 regulates ATP levels and IPAH PAVSMC survival. Using molecular and pharmacology-based analyses, we demonstrate a novel mechanistic link from NADPH oxidase Nox4-dependent activation of mTORC2 via energy sensor AMP-activated protein kinase (AMPK) to the activation of mTORC1 and increased proliferation, as well as deficiency of proapoptotic Bim and IPAH PAVSMC survival. We also provide evidence that in contrast to the mTORC1 inhibitor rapamycin, the dual mTORC1/mTORC2 inhibitor PP242 not only inhibits proliferation but also induces apoptosis in PAVSMCs from IPAH patients without a significant effect on control cells. Treatment with PP242 induces apoptosis in small pulmonary arteries and reverses existing pulmonary vascular remodeling in the rat chronic hypoxia model of pulmonary hypertension. These data suggest a novel role for mTORC2 in pulmonary vascular remodeling and provide a new potential target pathway for therapeutic interventions for this incurable disease.
Mammalian Target of Rapamycin Complex 2 (mTORC2) Coordinates Pulmonary Artery Smooth Muscle Cell Metabolism, Proliferation, and Survival in Pulmonary Arterial Hypertension
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SUPPLEMENTAL MATERIAL
Supplemental Methods

Human tissues and cell cultures. Lung tissues from four non-diseased (control) and four IPAH female lungs were provided by the Pulmonary Hypertension Breakthrough Initiative (PHBI) tissue core and National Disease Research Interchange (NDRI) under protocols approved by PHBI, NDRI, and the University of Pennsylvania and University of Colorado institutional review boards (Table S1). PAVSMC from non-diseased (control) and IPAH lungs (Table S1) were isolated from distal segments of arteries from the left lower lobe in the following manner. Pre-capillary pulmonary arteries were dissected out from the left lower lobe and adherent lung parenchymal tissue was removed using a microscissor and scalpel. The arteries were then minced to 1 mm² blocks that were plated on tissue culture plates with a small drop of LONZA culture medium supplemented with SmGm-2 media kit (LONZA, Walkersville, MD). On the following day, a full volume of culture medium was added to the plates and left undisturbed for 3-5 days. The medium was then subsequently changed every other day until the cells reached confluence. Isolated PAVSMC were characterized by immunocytochemical analysis of smooth muscle cell-specific proteins SMA, SM22alpha, myosin heavy chains (Cell Signaling Technology, Danvers, MA) and cell morphology (Figure S1) and by FACS analysis with anti-SMA antibody (Sigma Aldrich, St. Louis, MO) while the endothelial cell marker CD31 antibody (BD Biosciences, San Jose, CA) was used for negative control (Figure S2). Primary (3-8 passage) PAVSMC of the same passage from a minimum of three control and three IPAH subjects were used in each experiment. For serum-deprivation, cells were maintained for 48 h in LONZA media supplemented with 0.1% BSA.

Immunohistochemical analysis was performed using sections of lung tissues which were snap-frozen in OCT embedding compound (Tissue-Tek, Tokyo, Japan) as described previously. Briefly, tissue sections were co-immunostained with anti-P-S2481 mTOR, anti-P-S6, anti-P-S473, anti-Bim (Cell Signaling Technology, Beverly, MA), anti-Nox4 (Abcam, Cambridge, MA), and anti-SMA antibodies (Sigma, St. Louis, MO). Staining with 4′,6-diamidino-
2-phenylindole (DAPI) was performed to detect cell nuclei. Tissues from four subjects with IPAH and four non-diseased donor lungs and from three chronic hypoxia-exposed and three normoxia-maintained rats were analyzed. Staining was visualized using a Nikon Eclipse TE2000-E microscope with appropriate filters and then images were analyzed using Image Pro-Plus 7 software (Media Cybernetics Inc., Rockville, MD). After subtraction of background noise, fluorescence intensity (mean optical density) within SMA-positive areas of small PAs was calculated in arbitrary units (AU), and analysis of variance (ANOVA) was performed to assess statistically significant differences.

**Apoptosis analysis** was performed using In Situ Cell Death Detection Kit (Roche, Nutley, NJ) based on terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) technology. Briefly, serum-deprived for 48 h cells incubated with 100 mM 2-DG, 10 µM rotenone, 0.1, 1, or 10 µM PP242, 2, 20 or 200 nM rapamycin, or transfected with siRNA rictor, siRNA raptor, siRNA AMPK, siRNA Bim, siRNA Nox4 (Dharmacon, Lafayette, CO), and control scrambled siRNA (Santa Cruz, Dallas, TX) or pCMV6-Bim or pCMV6-Myc-DDK-Nox4 transfection vectors (OriGene, Rockville, MD), and then apoptosis analysis was performed according to the manufacturer's protocol. The cells were visualized on the Nikon Eclipse E400 microscope using the appropriate filters, and automatic counts of TUNEL-positive and total number of cells were performed using Image-Pro Plus 7 software. Data are represented as the percentage of apoptotic cells per total number of cells taken as 100%. A total of 200 cells were counted for each condition in each experiment.

**DNA synthesis analysis.** Cells were treated as described above and then DNA synthesis analysis was performed using BrdU incorporation assay as we previously described. The cells were examined using the Nikon Eclipse TE2000-E microscope with the appropriate filters, and counts of BrdU-positive and DAPI-positive cells were performed using Image-Pro Plus 5.1 software. The mitotic index was defined as the percentage of BrdU-positive cells per field.
divided by the total number of cells per field detected by DAPI. A total of 200 cells were counted for each condition.

**Animals.** All animal procedures were performed accordingly to the protocols approved by the University of Pennsylvania Animal Care and Use Committee. 6-8-week-old male Sprague-Dawley rats (n≥6 per each experimental group) were placed into plexiglas chambers and exposed to hypoxia (10% O2) for up to 28 days as described; treatment with PP242 (20 mg/kg, IP 5 days/week) or vehicle (IP, 5 days/week) was performed at days 15-28 of hypoxia exposure. The oxygen concentration was maintained using ProOx Oxygen Controller (BioSpherix); the forced circulation and instant homogenization of gases was provided by fan (BioSpherix). All animals had access to standard rat chow and water ad libitum under both normoxic and hypoxic conditions. Chambers opened 5 days a week to replenish food and water. Animal health, weight and overall behavior were monitored through experiment. Negative controls included age- and gender-matched animals maintained under normoxia. At days 0, 2, 14, and 28 animals were euthanized with pentobarbital overdose; the lungs were perfused via PA, filled with 80% OCT/20% saline solution at 25 cm H2O, snap-frozen in OCT embedded compound, and sectioned. The slides (5 µm thickness) were subjected to immunohistochemical analysis, apoptosis analysis using In Situ Cell Death Detection Kit (Roche, Nutley, NJ), or stained with hematoxylin and eosin. Images were taken using Nikon TE2000 microscope; blinded morphometric analysis of PA medial wall thickness was performed as described. Briefly, the lumen area at the level of the basement membrane and total vascular area at the adventitial border in muscular PA (25-150 µm outer diameter) per lung section were outlined, area sizes were measured using Image Pro-Plus 7. The medial wall thickness was calculated as [(total vascular area - lumen area)/total vascular area]x100. Analysis of fluorescent intensity in SMA-positive areas of small muscular PA (25-150 µm outer diameter) was performed using Image-Pro 7 software. Fluorescence intensity (mean optical density) within SMA-positive areas of small PAs was measured in arbitrary units (AU), subtraction of background noise was performed.
Data represented as means±SE from 3 rats, minimum of 12 separate measurements per rat per experimental condition; statistically significant differences assessed by ANOVA. For visualization of pulmonary vascular tree at day 28 of experiment, animals were randomly selected from each experimental group; lung vasculature was rinsed with PBS, inflated with AltaBlue reagent, fixed with 10% paraformaldehyde, and subjected to microCT analysis by Numira Biosciences. Briefly, lungs were scanned on a high-resolution, volumetric microCT scanner (µCT40, ScanCo Medical, Zurich, CH). The image data was acquired with the following parameters: 10 µm isotropic voxel resolution at 300 ms exposure time, 2000 views, and 3 frames per view. The microCT-generated DICOM files were converted to 3D images using SCIRun (Scientific Computing and Imaging Institute, University of Utah) and then a label map generated using Numira Biosciences’ VHLab software. SCIRun (SCI Institute) was used to generate the frames for the rotating 3D movies. The movie frames were then converted into QuickTime (Apple, Inc) movies for viewing. After the segmentation process, the voxel count associated with each label map was calculated using VHLab. The voxel count for each region of interest was then multiplied by the cubic voxel resolution to obtain volume measurements. The centerline of the vessels was calculated by topologically thinning the mask until just a skeleton of voxels remains. The skeleton was then converted into a points-and-edges representation, which was smoothed using a volume-preserving mesh fairing algorithm. The vessel radius was calculated as the average distance of the closest points on the surface of the vessel label mask to each point on the centerline. The centerline was also used to calculate vessel distance (shortest linear distance from the centerline to the nearest tumor boundary), vessel tortuosity (vessel path length divided by chord length), and vessel branching (number of times the vessel branches per millimeter that it travels). Average values for each sample’s vasculature are reported.
Table S1. Subjects characteristics.

<table>
<thead>
<tr>
<th>Group</th>
<th>Gender</th>
<th>Age, years</th>
<th>Mean age ± SE, years</th>
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<td>43.0 ± 7.3</td>
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<td>Non-diseased</td>
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Figure S1. Distal PAVSMC from three non-diseased (control) and three IPAH lungs of 5 passage were subjected to immunocytochemical analysis to detect smooth muscle alpha-actin (SMA, green), SM22 (red), and smooth muscle myosin heavy chain (SM-MHC, red). Normal rabbit IgG were used as a negative control. Representative images were taken using Nikon TE2000 microscope. Bar equals 100 µM.
Figure S2. Distal PAVSMC from three non-diseased (control) and three IPAH lungs of 5 passage were stained with FITC-conjugated anti-SMA (FITC-SMA) and PE-conjugated anti-CD31 antibodies (PE-CD31) followed by FACS analysis to detect percentage of SMA- and CD31-positive cells per total number of cells. PE- and FITC-conjugated normal mouse IgG were used as a negative control. Data represent % of PE (upper left) and FITC (lower right) gated cells.
Figure S3. siRNA rictor promotes apoptosis in IPAH PAVSMC. Cells transfected with siRNA rictor or control siRNA were serum-deprived for 48 h followed by apoptosis analysis using In Situ Cell Death Detection Kit (TUNEL, green). DAPI staining (blue) was performed to detect nuclei. Bar equals 100 µM.
Figure S4. Effects of PP242 and rapamycin on proliferation and apoptosis rates of IPAH and control (non-diseased) PAVSMC. Serum-deprived for 48 h PAVSMC from three non-diseased (control) and three IPAH lungs were treated for 18 h with indicated concentrations of PP242, rapamycin or diluent and then subjected to DNA synthesis (BrdU incorporation assay) (A) and apoptosis analysis (B). Data represent percentage of BrdU- (A) or TUNEL-positive cells (B) per total number of cells taken as 100%; three separate measurements were performed per each cell culture for each experimental condition. Data are means ± SE. A: *p < 0.05 for diluent- vs. agonist-treated IPAH PAVSMC. B: *p < 0.05 for diluent- vs. PP242-treated IPAH PAVSMC by two-way ANOVA (stratified independent t-test with corrections for multiple comparisons).
**Figure S5.** AICAR inhibits mTORC1-specific P-S6, but not mTORC2-specific P-S473-Akt in IPAH PAVSMC. Serum-deprived for 48 h PAVSMC from two subjects with IPAH were treated with 100 mM AICAR or diluent for 18 h followed by immunoblot analysis to detect indicated proteins.
Figure S6. siRNA AMPK rescues siRNA rictor-induced apoptosis in IPAH PAVSMC. Apoptosis analysis using *In Situ* Cell Death Detection Kit (TUNEL, green) performed on serum-deprived for 48 h IPAH PAVSMC transfected with 50 nM siRNA rictor, siRNA AMPK or control scrambled siRNA separately or in combination. DAPI staining (blue) was performed to detect nuclei. Representative images were taken using a Nikon Eclipse 2000 microscope. Bar equals 50 µM.
Figure S7. siRNA Bim prevents siRNA rictor-induced apoptosis in IPAH PAVSMC. Apoptosis analysis using *In Situ* Cell Death Detection Kit (TUNEL, green) and DAPI staining (blue) performed on serum-deprived for 48 h PAVSMC transfected with 50 nM siRNA rictor, siRNA Bim, or control scrambled siRNA separately or in combination. **A:** Representative images were taken using a Nikon Eclipse 2000 microscope. Bar equals 50 µM. **B:** siRNA-induced Rictor and Bim down-regulation was confirmed by immunoblot analysis.
Figure S8. siRNA-induced Nox4 knock-down promotes apoptosis in IPAH PAVSMC. Cells transfected with siRNA Nox4 or control siRNA were subjected to apoptosis analysis using *In Situ* Cell Death Detection Kit (TUNEL, green) DAPI staining (blue) was performed to detect nuclei. Bar equals 100 µM
Figure S9. 6-8-week-old male Sprague-Dawley rats were maintained under chronic hypoxia (10% O₂) for 28 days; treatment with PP242 or vehicle was performed at days 15-28 of hypoxia exposure. Negative control included age-and gender-matched animals maintained under normoxia for 28 days. At day 28, lung tissue sections were subjected to immunohistochemical analysis with anti-Nox4 (red) and anti-SMA antibodies (green). DAPI staining (blue) was performed to detect nuclei. A: Representative images were taken using Nikon TE 2000 microscope. Bar equals 50 µm. B: Comparative analysis of Nox4 levels in SMA-positive areas of small PAs (25-150 µm outer diameter) was performed using Image-Pro 7 software. Data represent arbitrary units (AU). Data are mean±SE from 3 rats, minimum of 40 separate measurements for each experimental condition. *p<0.001 by ANOVA (Dunnett’s).
Figure S10. Immunohistochemical analysis with anti-Bim antibody (red) performed on the lung of 6-8-week-old male Sprague-Dawley rats exposed to normoxia or chronic hypoxia (10% O₂) for 28 days and treated with PP242 or vehicle at days 15-28 of hypoxia exposure. Staining with anti-SMA antibody (green) and DAPI (blue) were performed to detect SMA-positive cells and nuclei. Representative images were taken using Nikon TE 2000 microscope. Bar equals 50 µm.
Figure S11. Schematic representation of proposed function of mTOR signaling in PAVSMC in PAH.
Supplemental References


Legends for the Supplemental Movie Files:

**Movie S1.** MicroCT analysis of the lung vasculature from rat maintained for 28 days under normoxia. Bar equals 5 mm.

**Movie S2.** MicroCT analysis of the lung vasculature from rat exposed to chronic hypoxia for 28 days. Bar equals 5 mm.

**Movie S3.** MicroCT analysis of the lung vasculature from rat exposed to chronic hypoxia for 28 days and treated with PP242 at days 15-28 of hypoxia exposure. Bar equals 5 mm.

**Movie S4.** Vessel-radius hit map of the lung vasculature from rat maintained for 28 days under normoxia. Bar equals 5 mm.

**Movie S5.** Vessel-radius hit map of the lung vasculature from rat exposed to chronic hypoxia for 28 days. Bar equals 5 mm.

**Movie S6.** Vessel-radius hit map of the lung vasculature from rat exposed to chronic hypoxia for 28 days and treated with PP242 at days 15-28 of hypoxia exposure. Bar equals 5 mm.