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Inhibition of Phosphatidylinositol 3-kinase/Akt Signaling Attenuates Hypoxia-induced Pulmonary Artery Remodeling and Suppresses CREB Depletion in Arterial Smooth Muscle Cells

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Abstract

Hypoxia-induced pulmonary hypertension is characterized by progressive remodeling of the pulmonary artery (PA) system and loss of the transcription factor, cAMP response element binding protein (CREB) in PA smooth muscle cells (SMCs). Previous in vitro studies suggested that platelet-derived growth factor, a mitogen produced in the hypoxic arterial wall, elicits loss of CREB in medial SMCs via the PI3K/Akt pathway. These events trigger switching of SMCs from a quiescent, contractile phenotype to a proliferative, migratory, dedifferentiated, and synthetic phenotype, which contributes to PA thickening. Here, we investigated whether inhibition of PI3K or Akt could attenuate arterial remodeling in the lung and prevent CREB loss in PA medial SMCs in rats subjected to chronic hypoxia. Inhibition of either enzyme-blunted hypoxia-induced PA remodeling and SMC CREB depletion and diminished SMC proliferation and collagen deposition. Inhibition of Akt, but not PI3K, suppressed muscularization of distal arterioles and blunted right ventricular hypertrophy. Interestingly, mean PA pressure was elevated equally by hypoxia in untreated and inhibitor-treated groups but was normalized acutely by the Rho kinase inhibitor, Fasudil. We conclude that PI3K and Akt inhibitors can attenuate hypoxia-induced PA remodeling and SMC CREB depletion but fail to block the development of pulmonary hypertension because of their inability to repress Rho kinase-mediated vasoconstriction.

Keywords

pulmonary arterial hypertension; CREB; hypoxia; phosphatidylinositol 3-kinase; Akt; smooth muscle cell

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Introduction

Pulmonary arterial hypertension (PAH) is characterized by thickening or remodeling of the pulmonary artery (PA) wall and persistent vasoconstriction of the pulmonary vasculature.¹ In small animals like rats, the thickening of the PA wall does not seem to reduce lumen volume and therefore does not contribute to the increased workload placed on the right ventricle (RV) in PAH.^{2–4} However, in larger animals and humans, remodeling of the PA wall is progressive, and reduction in lumen volume and occlusive lesions are found in both primary and secondary forms of human PAH. Although most current therapies for PAH target persistent vasoconstriction, few if any have been designed to address remodeling of the PA wall.¹

Thickening of the PA wall is the result of medial smooth muscle cells (SMCs) proliferation, hypertrophy and extracellular matrix (ECM) production, and collagen and elastin deposition in the adventitia. In addition, SMCs appear around previously thin-walled nonmuscularized distal arterioles, transforming them into “resistance vessels” with decreased distensibility. Hypoxia triggers these events by eliciting the expression of growth factors including platelet-derived growth factor (PDGF)-BB, vascular endothelial growth factor, transforming growth factor- β , endothelin-1, and thrombospondin-1 that are potent SMC mitogens.^{5–7} These growth factors stimulate several intracellular signaling systems including MAP kinases,^{8,9} protein kinase C,^{10,11} Rho/RhoA kinase,^{12,13} and the PI3-kinase/Akt pathway.^{14–20}

The PI3K/Akt signaling pathway plays especially important roles in vascular development and normal vascular function. Angiogenesis and vascular development involve the activation of PI3K/Akt signaling in response to extracellular stimuli like vascular endothelial growth factor^{21,22} and various fibroblast growth factors.^{23,24} During vascular development, PI3K/Akt signaling pathways promote the proliferation, differentiation, and survival of resident cells in the vessel wall and the recruitment of cells from other regions of the vasculature. The importance of PI3K/Akt signaling in normal vascular development is exemplified by gene knockout studies such as the Akt-1 null mouse, which exhibits delayed vessel maturation and increased vascular permeability.²⁵ Another mouse model having an endothelial-specific knockdown of phosphatase and tensin homolog (PTEN), an inhibitor of PI3K/Akt, exhibits increased angiogenesis.²⁶

In addition to participating in normal vascular development and function, the PI3K/Akt pathway participates in vascular remodeling and the development of various vascular pathologies. In particular, growth factors that are elevated in the vessel wall during remodeling like angiotensin II,^{14,15} insulinlike growth factor 1,^{16,17} PDGF-BB,^{18,19} and endothelin-1²⁰ stimulate PI3K/Akt signaling in medial SMCs. Activation of PI3K/Akt by these factors promotes SMC proliferation, hypertrophy, survival, and neointimal growth; increases SMC contractility, polyploidization, and migration; and downregulates expression of SMC markers. Recently, Furgeson et al²⁷ demonstrated that targeted knockdown of PTEN in SMCs promotes vascular remodeling and that highly proliferative SMCs from

injured aortas exhibit constitutive Akt signaling. Thus, PI3K/Akt signaling seems to be an important pathway governing SMC phenotype and arterial remodeling.

We have previously shown that levels of the transcription factor, cAMP response element binding protein (CREB) are reduced in medial SMCs in remodeled PAs from hypertensive calves and rats exposed to chronic hypoxia.²⁸ In vitro studies with PA SMCs in culture demonstrate that forced depletion of CREB with small interfering RNA promotes SMC proliferation and migration, leads to decreased expression of SMC markers like SM-myosin and calponin, and stimulates collagen and elastin production. These changes are consistent with those observed in the PA wall as it remodels, wherein SMC dedifferentiation, proliferation, migration, and ECM synthesis are upregulated and contribute to vessel thickening. PDGF, which is produced in the hypoxic PA wall during remodeling, stimulates CREB depletion in primary PA SMCs. This occurs through the PI3K/Akt pathway, which promotes nuclear export, ubiquitination, and proteasomal degradation of CREB.²⁹ The data suggest that PDGF activation of the PI3K/Akt pathway in response to hypoxia leads to CREB depletion in SMCs, which in turn promotes switching of SMCs from a quiescent contractile phenotype to a proliferative, migratory, dedifferentiated, and synthetic phenotype, which contribute to PA thickening.

With these factors in mind, we investigated whether inhibition of PI3K with LY294002 or Akt with triciribine in vivo could block PA remodeling and inhibit CREB loss in medial PA SMCs in a chronic hypoxic rat model of PAH. Inhibition of either PI3K or Akt reduced medial PA thickening and blockade of Akt reduced neomuscularization of distal arterioles. No reduction in mean PA pressure was observed with either compound. The inhibitors also prevented loss of CREB in medial PA SMCs in rats exposed to hypoxia. Repression of hypoxia-induced remodeling seemed to be due in part to decreased medial SMC proliferation and ECM deposition. We conclude that targeting of the PI3K/Akt signaling pathway may be an effective strategy to address the PA wall thickening in human PAH.

Methods

Materials

Male Wistar-Kyoto rats were purchased from Harlan (Indianapolis, IN). Triciribine was obtained from EMD Biosciences (San Diego, CA) and LY294002 was purchased from Cayman Chemical (Ann Arbor, MI). For chronic triciribine and LY294002 administration, osmotic minipumps (model 2ML4) were from Alzet-Durect (Cupertino, CA). Polyclonal antibodies to phospho-glycogen synthase kinase-3 (GSK-3) (a + b) were purchased from Abcam (Cambridge, MA) and to proliferating cell nuclear antigen (PCNA) were obtained from Dako Cytomation (Carpenteria, CA). Polyclonal antibodies to α -smooth muscle actin, Cardiogreen polymethine dye, and Fasudil (HA-1077) were purchased from Sigma (St Louis, MO). Rabbit polyclonal antibody to CREB was from Cell Signaling Technology, Inc (Beverly, MA). Picrosirius red stain was obtained from Electron Microscopy Sciences (Hatfield, PA). Alexa Fluor 594 and Alexa Fluor 488-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). VectaShield mounting medium with 4,6-diamidino-2-phenylindole was purchased from Vector Laboratories (Burlingame, CA).

Animal Care and Teatment

All animal procedures were performed with approval and in accordance with the guidelines of the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee. Male Wistar-Kyoto rats (6 weeks old) were acclimatized to Denver altitude for 2 weeks before experimentation. Twenty-four hours before initiation of hypoxic exposure, rats were anesthetized with ketamine/xylazine (75 and 6 mg/kg, respectively), the skin was shaved and cleaned with iodine soap, and miniosmotic pumps (Alzet 2ML4) containing either 50% of dimethyl sulfoxide (DMSO) or LY294002 or triciribine dissolved in 50% of DMSO were inserted into a subcutaneous pocket through a small incision made into the abdominal cavity. These pumps delivered a volume of 2.5 mL/h, which was equivalent to 1 mg·kg⁻¹·d⁻¹ of LY294002 or to 0.5 mg·kg⁻¹·d⁻¹ of triciribine. These concentrations were selected based on the known ID50s for kinase inhibition, the LD50s for rodents, and levels used by other investigators.^{30–33} There were no complications from insertion of the pumps, including bleeding, infection, or deaths in vehicle or inhibitor-treated animals.

Twenty-four hours after pump implantation, rats were maintained under isobaric normoxia (1600 m, 630 mm Hg) or hypobaric hypoxia (5500 m, 410 mm Hg) for 3 weeks. Fresh food, water, and clean cages with fresh bedding were provided every other day. Light was maintained on a 12-hour cycle and humidity was 40%–45% with a temperature of 25–27°C. The animals were monitored daily and weight was checked once a week.

Hemodynamic Measurements and Tissue Procurement

Rats received a combination of ketamine (75 mg/kg) and xylazine (6 mg/kg) intraperitoneally as anesthetic. The ventral neck area and the area dorsally between the scapulae were shaved and the areas scrubbed with betadine solution. A 2-cm incision was made over the right ventral neck area to expose the internal jugular vein. A polyvinyl (PV-1) catheter was threaded into the PA through the right jugular vein. A catheter pressure tracing was transduced and monitored with an oscilloscope, and the location of the tip was identified by the characteristic shapes of the PA pressure waveforms. The catheter was then securely sutured into place. An additional catheter was inserted into the jugular vein for drug infusion and venous return. A polyethylene (PE-50) catheter was placed into the right carotid artery to obtain cardiac output values. Cardiac output was measured by infusion of Cardiogreen polymethine dye and a specially designed densitometer and software (Deterministic-systems, Boulder, CO) to detect and calculate cardiac output from the dye dilution.³⁴ Total pulmonary resistance was calculated by dividing the mean PA by cardiac output.³⁵ All catheters were routed, exteriorized, and sutured in place. Rats were placed in a 3-inch wide, 3-inch high, and 7-inch long Plexiglas box with gas delivery ports for the delivery of air. Sampling of the gas inside the box over time showed that complete gas exchange within the box occurred within 2 minutes. The implanted catheters were attached to pressure transducers for hemodynamic measurements. All measurements were made while the animals were anesthetized.

After hemodynamic measurements, the anesthetized animals were treated with the RhoA/Rho kinase inhibitor Fasudil (10 mg/kg intravenously) to dilate the vasculature before fixation. The rats were then euthanized by exsanguination. Lungs were fixed with 4% of

paraformaldehyde in phosphate-buffered saline (PBS) containing 5 mM of ethylenediaminetetraacetic acid (EDTA) by airway inflation at 30 cm H₂O pressure. PAs were perfused with 4% of paraformaldehyde in PBS containing 5 mM of EDTA (to maintain vasodilation) at a pressure similar to that measured in vivo. The heart and lungs were then removed in bloc. Wet RV weights were measured and used to calculate the ratio of RV weight to total body weight. Lung tissue was fixed overnight in 4% of paraformaldehyde and embedded in paraffin.

Immunostaining Protocol

Five-micrometer sections of paraformaldehyde-fixed, paraffin-embedded lung tissue were deparaffinized with Hemo-D and rehydrated in a graded ethanol-water series. Sections were subjected to antigen retrieval in citrate buffer in a microwaveable pressure cooker for 20 minutes. Sections were blocked with PBS containing 5% of horse serum for 30 minutes at room temperature. The sections were incubated overnight in PBS containing 5% of fetal bovine serum at 4°C with the primary antibodies as indicated. The sections were then washed and incubated with the indicated Alexa Fluor-conjugated secondary antibodies for 1 hour at room temperature. Picrosirius red staining was performed according to the supplier's instructions.

Microscopy and Lung Morphometric Analysis

Five-micrometer sections of fixed lung or heart tissue were subjected to hematoxylin and eosin staining by the University of Colorado Denver Histology Core Laboratory. Picrosirius red staining of lung sections was performed as directed by the supplier's instructions.

Distal pulmonary vessels (outside diameter 10–50 µm) were assessed by a blinded observer for degrees of circumferential α -smooth muscle actin-positive staining indicative of muscularization. Vessels smaller than that of 10 µm were considered capillaries and excluded from further considerations. Proximal vessels (outside diameter 50–250 µm) were analyzed for medial wall thickness at 4 points around the vessel circumference and for lumen diameter along 2 axes.

Microscopy was performed on a Nikon TE2000-U inverted epifluorescent microscope. Brightfield, phase-contrast, and fluorescent digital deconvolution images were captured to a personal computer with either a Spot RT/KE monochrome camera or Spot Insight color camera (Diagnostic Imaging, Sterling Heights, MI). Images were analyzed and processed with MetaMorph 6.1 software (Molecular Devices, Sunnyvale, CA). The nuclear fluorescence intensity of nuclear-localized CREB, total medial (between inner and outer elastic lamellae) phospho-GSK-3 (P-GSK-3) staining was determined by inclusive thresholding in each micrograph followed by computer-assisted fluorescence signal integration. The cellularity of the vascular media in each treatment group was evaluated by counting the number of nuclei (4,6-diamidino-2-phenylindole stained) between the internal and the external elastic lamellae. This number was divided by the lumen radius of the vessel to account for artery size.

Statistics

Statistical analysis was performed using the Super ANOVA software program (Abacus Concepts, Berkeley, CA). Comparisons were performed using 2-way analysis of variance followed by the Scheffé multiple comparison test for individual comparisons within and between groups of data points. Data were considered statistically significant with a *P* value of <0.05.

Results

Inhibition of PI3-kinase or Akt Blocks Hypoxia-stimulated Phosphorylation of GSK-3 In Vivo

There was no statistical difference between the body weights of animals in each treatment group at the end of the treatment period. The animals subjected to chronic hypoxia for 3 weeks demonstrated the expected polycythemia; however, there was no statistical difference between the hematocrit of hypoxic animals receiving vehicle and those treated with LY294002 or triciribine (normoxic/50% of DMSO, 44.25 ± 1.71 ; hypoxic/50% of DMSO, 69.00 ± 3.37 ; normoxic/LY294002, 45.00 ± 1.60 ; hypoxic/LY294002, 73.00 ± 2.83 ; normoxic/triciribine, 43.00 ± 2.94 ; and hypoxic/triciribine, 61.00 ± 5.51).

The goal of our studies here was to determine whether inhibition of PI3-kinase/Akt signaling in vivo was sufficient to prevent the development of hypoxia-induced PAH and CREB loss in arterial SMCs. As a first step, we examined whether LY294002 or triciribine effectively suppressed PI3-kinase/Akt signaling in vivo by assessing their impact on a well-characterized Akt substrate, GSK-3.³⁶ Lung sections from normoxic, hypoxic, hypoxic + LY294002, or hypoxic + triciribine-treated rats were subjected to immunohistochemical staining for phosphorylated GSK-3 (Fig. 1A). Modest P-GSK-3 signal was seen throughout the lung parenchyma and PA wall in normoxic animals. In hypoxic animals, P-GSK-3 levels were elevated in the SMC-rich layer of the PA wall between the inner and the outer elastic lamellae, but no increase was seen in the parenchyma. However, in rats treated with either LY294002 or triciribine, virtually no P-GSK-3 immunoreactive material was detected in the PA wall, but again, little change was noted in the parenchyma compared with normoxic controls. P-GSK-3 immunoreactive fluorescence between the elastic lamellae was 3- to 4-fold higher in hypoxic/50% DMSO animals than in normoxic/50% DMSO rats (Fig. 1B), but no increase was measured in hypoxic animals treated with either LY294002 or triciribine. The data indicate that in vivo delivery of PI3 kinase or Akt inhibitors effectively suppress activation of the PI3kinase/Akt signaling pathway by chronic hypoxia.

Inhibition of PI3-kinase or Akt In Vivo Attenuates Chronic Hypoxia-induced Pulmonary Arterial Remodeling in Medial SMCs

Examination of hematoxylin and eosin-stained lung tissue sections revealed a profound increase in PA wall thickness in animals exposed to hypoxia (with or without vehicle) (Fig. 2A). However, in animals treated with either LY294002 or triciribine, remodeling was attenuated, although not normalized. Morphometric analysis (vessel wall thickness/lumen radius) confirmed a marked ~37% decrease in wall thickness with the PI3K inhibitor and ~62% decrease with triciribine (Fig. 2B). To ensure that these differences were not due to

variations in vascular tone, we treated the animals with the vasorelaxant Fasudil (RhoA/Rho kinase inhibitor) immediately before lung removal and perfused the lungs with buffers containing 5 mM of EDTA.

Examination of sections also revealed a marked increase in actin-positive arterioles within the distal lung parenchyma in hypoxic animals (Figs. 3A, B). Interestingly, triciribine diminished hypoxia-induced muscularization of distal arterioles, whereas LY294002 had no measurable effect.

Inhibition of PI3-kinase or Akt In Vivo Attenuates RV Hypertrophy in Response to Hypoxia But Fails to Block the Development of Pulmonary Hypertension

PA catheterization demonstrated an increase in mean PA pressure (Fig. 4A) and total pulmonary resistance (Fig. 4B) in response to chronic hypoxia. However, neither inhibitor was capable of blocking the increase in PA pressure or pulmonary resistance. When animals were subsequently treated with RhoA/Rho kinase inhibitor Fasudil (10 mg/kg intravenously),^{37,38} mean PA pressure decreased significantly but did not reach normal levels. Cardiac output was not significantly different between normoxic and hypoxic groups or between treatments (Fig. 4C). Although low cardiac output values are consistent with those we previously reported for anesthetized, male Wistar-Kyoto rats at Denver, CO, altitude.³⁴

As anticipated, RV hypertrophy (RV weight divided by left ventricle + septum weight) was observed in vehicle-treated rats exposed to hypoxia. However, ventricular thickening was significantly reduced in the hypoxic + triciribine group compared with untreated hypoxic controls (Fig. 4D). Cardiac hypertrophy was not attenuated in the hypoxic + LY294002 group.

Inhibition of PI3-kinase or Akt Regulates SMC Proliferation and ECM Metabolism in the Hypoxic PA Wall

To delineate the mechanisms by which LY294002 or triciribine attenuate PA remodeling in response to hypoxia, we examined the impact of these inhibitors on SMC proliferation in PAs of lung tissue sections. Using immunostaining for PCNA as a marker for proliferating cells, we detected numerous proliferating cells in the thickened PA walls of hypoxic animals (Fig. 5). These proliferating cells were detected primarily in the medial SMC layer and adventitia. However, PCNA-positive cells were virtually absent in PA walls from hypoxic animals treated with LY294002 or triciribine indicating that these agents block proliferation of cells in the PA wall. Another potential mechanism by which PI3-kinase or Akt inhibitors might reduce the PA wall remodeling is by regulating the synthesis and deposition of ECM. To test this hypothesis, we measured collagen deposition on the PA wall by picrosirius red staining. We noted substantial amounts of collagen (red material in picrosirius red-stained sections) in the media and adventitia of vessels from hypoxic animals (Fig. 5). However, collagen staining in hypoxic animals treated with either LY294002 or triciribine was reduced to levels comparable with those observed in normoxic animals.

Inhibition of PI3-kinase or Akt In Vivo Prevents Hypoxia-induced CREB Depletion in PA SMCs

Finally, we examined whether LY294002 or triciribine would prevent the loss of CREB in medial SMCs in rats exposed to chronic hypoxia. Lung sections from normoxic, hypoxic, and hypoxic LY294002- or triciribine-treated rats were subjected to immunohistochemical staining for CREB. CREB was easily detected in nuclei throughout the lungs of normoxic animals (Fig. 6A). In hypoxic animals, CREB was detected in nuclei in the lung parenchyma, PA endothelial cells, and PA adventitia. However, no CREB signal was observed in nuclei of medial SMCs cells (located between the autofluorescent elastic lamellae). CREB was detected throughout the lung and PA media of hypoxic + LY294002 or hypoxic + triciribine animals. Quantitation of nuclear CREB fluorescence in the media (Fig. 6B) confirmed significant loss of CREB in hypoxic rats treated with vehicle alone, whereas LY294002 and triciribine completely prevented CREB depletion. In fact, CREB levels were higher in the hypoxic/triciribine group than in normoxic/50% of DMSO animals. Thus, inhibition of PI3-kinase/Akt signaling in the lungs of hypoxia-treated rats inhibited CREB depletion in medial PA SMCs.

Discussion

These studies were based on 3 previous observations. First, CREB is reduced in medial PA SMCs in bovine and rodent models of chronic hypoxia-induced pulmonary hypertension (PH).^{28,39} Second, loss of CREB in PA SMCs can be recapitulated in vitro by treating cells with PDGF-BB, which activates the PI3-kinase/Akt pathway resulting in CREB polyubiquitination and proteasomal degradation.²⁹ Third, small interfering RNA-induced loss of CREB in SMCs in vitro stimulates their proliferation and alters their expression of contractile proteins and ECM components in a manner consistent with changes in SMC phenotype observed in the hypertensive PA wall. These observations suggested that chronic activation of PI3K/Akt signaling in response to hypoxia/PDGF might promote PA remodeling and the development of PH through downregulation of CREB.

Here, we show that inhibition of PI3K/Akt signaling attenuates PA thickening and CREB loss in medial PA SMCs in rats exposed to 3 weeks of hypobaric hypoxia. Both inhibitors also suppressed SMC proliferation and collagen deposition in the PA wall. Inhibition of Akt also attenuated muscularization of distal arterioles and blunted RV hypertrophy. These results support a model in which chronic hypoxia stimulates sustained PI3K/Akt signaling, most likely through growth factors like PDGF that are upregulated in the hypoxic PA wall. The PI3K/Akt pathway subsequently regulates several aspects of PA remodeling including SMC proliferation and migration and increased deposition of ECM components by medial and adventitial cell populations. We propose that many of the effects of PI3K/Akt signaling on SMC phenotype are mediated by the loss of CREB; a concept we are currently testing in SMC-targeted CREB knockout mice.

The present findings are consistent with the results of Gary-Bobo et al⁴⁰ who demonstrated that monocrotaline-induced PH was associated with marked activation of Akt signaling in the lungs and the proximal PAs. Our observations are also consistent with numerous reports demonstrating a role for PI3K/Akt in systemic vascular function. This pathway participates

in multiple cellular activities including proliferation, adhesion, migration, metabolism, and survival. Previous studies from our laboratory have demonstrated that activation of the PI3K/Akt signaling pathway in cultured PA SMCs promotes phosphorylation of CREB by casein kinase 2, which targets CREB for polyubiquitination and proteosomal degradation.²⁵ A recent review focused on the role of PI3K/Akt in angiogenesis through loss of PTEN and/or increased expression of growth factor receptors such as epidermal growth factor receptor.⁴¹ Furgeson et al²⁷ further suggested that PTEN negatively regulates SMC phenotypic modulation with loss of PTEN in SMC associated with multiple downstream events regulating neointima formation, including promotion of an activated SMC phenotype. Recently, CREB was identified as a novel substrate of PTEN in the nucleus.⁴²

Although blockade of the PI3K/Akt pathway significantly curtailed PA remodeling and RV hypertrophy in response to chronic hypoxia, it had little impact on elevated PA pressure. We previously observed this decoupling of PA pressure from structural changes in the PA wall in experiments testing the efficacy of thiazolidinediones in the treatment of hypoxia-induced PH in rats,³⁴ and other groups have reported this phenomenon in a variety of models.^{2,4,43} The persistent elevation of PA pressure was due primarily to RhoA/Rho kinase-mediated vasoconstriction and could be normalized with the Rho kinase inhibitor, Fasudil. Although blockade of PA thickening and RV hypertrophy may seem to be a beneficial outcome, failure of these tissues to remodel appropriately could lead to failure of the PA and/or RV wall in the face of sustained elevated PA pressure. Therefore, inhibition of PI3K/Akt signaling may be an important therapeutic strategy for treating PH only in conjunction with vasodilatory agents.

We also noted that triciribine blocked all manifestations of PH in vivo except elevated PA pressure, whereas LY294002 alone suppressed only a subset of endpoints. Insufficient LY294002 dosage may explain these disparate results. However, both LY294002 and triciribine repressed GSK-3 phosphorylation, medial SMC proliferation, and adventitial collagen deposition in hypoxic subjects indicating that inhibition of PI3K/Akt signaling was likely achieved by both agents. A related possibility is that different concentrations of LY294002 may be required for complete inhibition of distinct PI3K isoforms. Failure to fully inhibit PI3K signaling with the levels of LY294002 we used may explain why not all endpoints were affected. This argument also raises the possibility that distinct PI3K isoforms are involved in the regulation of specific aspects of SMC phenotype. Unfortunately, higher concentrations of LY294002 exhibited toxicity in our animal trials, and we were unable to test this hypothesis further. Finally, there may be Akt-independent pathways regulated by PI3K or collateral signaling systems that circumvent PI3K inhibition. In this respect, Akt may be a more important therapeutic target than PI3K in treating or preventing PAH.

In closing, we conclude that inhibition of the PI3K/Akt signaling pathway may be an effective means of blocking the remodeling of the PA tree in response to hypoxia and other insults. Although vessel remodeling does not seem to contribute to the development of PH in rodent models, encroachment of the arterial wall into the vessel lumen in conjunction with vasoconstriction both contribute to PH in humans. Thus, therapies that target PI3-kinase and Akt in particular may be especially effective when used in combination with vasorelaxant agents.

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Figure 1.

LY294002 and triciribine block hypoxia-induced phosphorylation of GSK-3 in rat PA SMCs. Adult male Wistar-Kyoto rats were implanted with osmotic minipumps delivering vehicle (50% DMSO), LY294002 or triciribine, and subjected to isobaric normoxia or hypobaric hypoxia for 3 weeks. The animals were then euthanized, and their lungs were inflated and perfused with PBS containing 4% of paraformaldehyde. The sections were deparaffinized, rehydrated, and subjected to immunohisto-chemistry for P-GSK-3. A, The top row shows representative phase contrast micrographs of intralobar PAs adjacent to airways (AW). The middle row shows the corresponding fluorescence deconvolution images in which the signal for P-GSK-3 (red) has been combined with the autofluorescence of the internal and external elastic lamellae (green). The bottom row shows enlargements of the regions within the blue squares in the middle row. Bar = 75 μ m. B, Total medial P-GSK-3 levels were quantitated by measuring P-GSK-3– related fluorescence intensity between the

inner and the outer lamellae. Data were averaged for at least 5 vessels per animal from 6 animals. * $P < 0.05$ compared with normoxic, 50% of DMSO values.

Figure 2.

LY294002 and triciribine markedly decreased hypoxia-induced PA remodeling. Adult rats were implanted with osmotic minipumps delivering vehicle (50% DMSO), LY294004 or triciribine. Another cohort was left untreated. All groups were then maintained under normoxic or hypoxic conditions for 21 days. A, Representative brightfield photomicrographs of hematoxylin and eosin-stained lung sections show that LY294002 and triciribine inhibited hypoxia-induced PA remodeling. Bar, 100 μ m. B, Morphometric analysis confirms that the inhibitors attenuated hypoxia-induced (solid bars) PA wall thickening compared with controls. Open bars = normoxic and black bars = hypoxic. Data are average values obtained from at least 30 vessels with diameters between 50 and 250 μ m from 6 animals per group. * P 0.05 versus corresponding normoxia/DMSO-, LY294002-, and triciribine-treated groups. # P 0.05 versus hypoxia/DMSO-treated group.

Figure 3.

LY294002 and triciribine inhibit muscularization of distal pulmonary arterioles. Adult male Wistar-Kyoto rats were implanted with osmotic minipumps delivering vehicle (50% of DMSO), LY294002 or triciribine, and subjected to isobaric normoxia or hypobaric hypoxia for 3 weeks. Representative brightfield photomicrographs of lung parenchyma immunostained with antibodies to α -smooth muscle actin show that the inhibitors prevent muscularization of distal arterioles. A, Arrows indicate scored actin-positive arterioles. B, Morphometric analysis of the number of actin-positive vessels per square millimeter in vehicle or inhibitor-treated male animals. Open bar, normoxic and black bar, hypoxic. Data confirm that triciribine blocks or reverses neomuscularization. Data show average values obtained from at least 25 fields from 6 animals per group. * P 0.05 versus corresponding normoxia/DMSO-, LY294002-, and triciribine-treated groups. # P 0.05 versus hypoxia/DMSO-treated group.

Figure 4.

Impact of LY294002 and triciribine on mean PA pressure and RV hypertrophy in rats exposed to chronic hypoxia. Adult male Wistar-Kyoto rats were implanted with osmotic minipumps delivering vehicle (50% of DMSO), LY294002 or triciribine, and subjected to isobaric normoxia or hypobaric hypoxia for 3 weeks. A, Average mean PA pressure was measured as described in Methods. Data show that neither agent lowers mean PA pressure. However, intravenous injection of Fasudil during hemodynamic evaluation (gray bars) normalized mean PA pressures in animals exposed to hypoxia. N = 6 for all groups. * $P < 0.05$ versus corresponding normoxic DMSO-, LY294002-, and triciribine-treated groups. † $P < 0.05$ versus hypoxic/DMSO-treated group. B, Total pulmonary resistance (TPR) was measured as described in Methods. * $P < 0.05$ versus corresponding normoxic DMSO-, LY294002-, and triciribine-treated groups. LY294002 and tri-ciribine failed to normalize TPRs in hypoxic animals. C, Cardiac output was measured by dye dilution as described in

Methods. No significant differences were noted between any of the groups. D, Hypoxia-induced RV hypertrophy (assessed as average RV weight divided left ventricle + septum weight) was attenuated by triciribine but not LY294002. N = 6 for all groups. * $P < 0.05$ versus corresponding normoxia/DMSO-, LY294002-, and triciribine-treated groups.

Figure 5.

LY294002 and triciribine inhibit proliferation and collagen production in the PA wall in response to chronic hypoxia. Adult male Wistar-Kyoto rats were implanted with osmotic minipumps delivering vehicle (50% DMSO), LY294002 or triciribine, and subjected to isobaric normoxia or hypobaric hypoxia for 3 weeks. Five-micrometer sections of fixed lung tissue were subjected to immunohistochemistry for PCNA or picrosirius red staining for collagen production as indicated. Representative brightfield photomicrographs show that the inhibitors blocked proliferation and collagen deposition in rats exposed to hypoxia.

Figure 6.

LY294002 and triciribine prevent SMC CREB loss in rats exposed to chronic hypoxia.

Adult male Wistar-Kyoto rats were implanted with osmotic minipumps delivering vehicle (50% of DMSO), LY294002 or triciribine, and subjected to isobaric normoxia or hypobaric hypoxia for 3 weeks. At 3 weeks, the animals were euthanized and their lungs were inflated and perfused with PBS containing 4% of paraformaldehyde. The sections were deparaffinized, rehydrated, and subjected to immunohisto-chemistry for CREB. A, The top row shows representative phase contrast micrographs of intralobar PAs adjacent to airways (AW). The middle row shows the corresponding fluorescence deconvolution images in which the signal for CREB (red) has been combined with the auto-fluorescence of the internal and external elastic lamellae (green). The bottom row shows enlargements of the regions within the blue squares in the middle row. Bar = 75 μ m. B, Nuclear CREB levels were quantitated by measuring fluorescence intensities of nuclei located between the inner

and the outer lamellae. Data were averaged for at least 5 vessels per animal from 6 animals.

* P = 0.05 versus normoxic/50% of DMSO values.