Activation of phosphatidylinositol 3-kinase, Akt, and mammalian target of rapamycin is necessary for hypoxia-induced pulmonary artery adventitial fibroblast proliferation

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Gerasimovskaya, Evgenia V., Doug A. Tucker, and Kurt R. Stenmark. Activation of phosphatidylinositol 3-kinase, Akt, and mammalian target of rapamycin is necessary for hypoxia-induced pulmonary artery adventitial fibroblast proliferation. J Appl Physiol 98: 722–731, 2005. First published October 22, 2004; doi:10.1152/japplphysiol.00715.2004.—In contrast to cell types in which exposure to hypoxia causes a general reduction of metabolic activity, a remarkable feature of pulmonary artery adventitial fibroblasts is their ability to proliferate in response to hypoxia. Previous studies have suggested that ERK1/2, phosphatidylinositol 3-kinase (PI3K), Akt, and mammalian target of rapamycin (mTOR) are activated by hypoxia and play a role in a variety of cell responses. However, the pathways involved in mediating hypoxia-induced proliferation are largely unknown. Using pharmacological inhibitors, we established that PI3K-Akt, mTOR-p70 ribosomal protein S6 kinase (p70S6K), and EKR1/2 signaling pathways play a critical role in hypoxia-induced adventitial fibroblast proliferation. We found that exposure of serum-starved fibroblasts to 3% O2 resulted in a time-dependent activation of PI3K and transient phosphorylation of Akt. However, activation of PI3K was not required for activation of ERK1/2, implying a parallel involvement of these pathways in the proliferative response of fibroblasts to hypoxia. We found that hypoxia induced significant increases in mTOR, p70S6K, 4E-BP1, and S6 ribosomal protein phosphorylation, as well as dramatic increases in p70S6K activity. The activation of p70S6K/S6 pathway was sensitive to inhibition by rapamycin and LY294002, indicating that mTOR and PI3K/Akt are upstream signaling regulators. However, the magnitude of hypoxia-induced p70S6K activity and phosphorylation suggests involvement of additional signaling pathways. Thus our data demonstrate that hypoxia-induced adventitial fibroblast proliferation requires activation and interaction of PI3K, Akt, mTOR, p70S6K, and ERK1/2 and provide evidence for hypoxic regulation of protein translational pathways in cells exhibiting the capability to proliferate under hypoxic conditions.

Keywords: pulmonary hypertension; protein synthesis; environmental stress; oxygen sensing; vascular remodeling

Chronic exposure of most mammalian species to reduced oxygen concentrations, either persistent or intermittent, results in the development of pulmonary hypertension. Experiments in several models demonstrate that, in response to moderate levels of global hypoxia (10–13% oxygen), proliferative responses of cells in the pulmonary artery adventitial compartment consistently precede and greatly exceed proliferation of smooth muscle cells (SMC) in the medial compartment (55). In culture, pulmonary artery adventitial fibroblasts have been shown to retain their proliferation ability in response to hypoxia in the absence of any exogenous mitogens (13, 44). The signaling pathways, which ultimately act to cause proliferation in this specific cell population under conditions of moderate hypoxia, remain unclear, although signaling through G proteins, MAPK, and PKC have been shown to be necessary (13, 21, 55). Improved insight into the mechanisms contributing to hypoxia-induced fibroblast proliferation could thus have a significant impact on our understanding of hypoxia-induced pulmonary vascular remodeling.

The phosphatidylinositol 3-kinase (PI3K) pathway has been shown to be important in growth, survival, and proliferation of a wide range of cell types (33, 60). Recent studies have demonstrated that PI3K signaling is involved in vascular SMC and endothelial cell mitogenesis in response to specific growth stimuli (7, 24, 30, 31, 53). Shigematsu et al. (53) have shown that activation of PI3K is essential for the initiation of medial vascular SMC replication after carotid balloon injury in rats. A direct role of PI3K in angiotensin II-induced DNA synthesis and proliferation has been described in cultured porcine carotid artery SMC (49). Importantly, there is indirect evidence that PI3K is involved in hypoxia-induced signaling. The PI3K pathway has been implicated in hypoxic induction of hypoxia-inducible factor-1α in certain transformed cell lines (29, 39, 62). However, in rat vascular SMC and endothelial cells, hypoxia-induced augmentation of growth factor-induced proliferation occurs independent of PI3K (27). Currently, little or no information exists regarding the role of PI3K in regulating hypoxia-induced proliferation of pulmonary artery adventitial fibroblasts, cells capable of serum-independent proliferation under hypoxic conditions (13, 21). Indirect assessment of PI3K activation through Akt phosphorylation is usually performed in vascular cells exposed to hypoxia. However, to our knowledge, there is no direct evidence for PI3K activity in response to hypoxia in these cells.

Several downstream targets of PI3K have been identified, including phosphoinositide-dependent kinase-1, serine/threonine kinase Akt, mammalian target of rapamycin (mTOR), and the p70 ribosomal protein S6 kinase (p70S6K) (15, 22, 49). All may be either dependent on or independent of upstream activation by PI3K in the context of cell proliferation. For exam-
ple, a recent study showed that mTOR may be activated by hypoxia independent of PI3K and may be critical in mediating the augmented proliferation of SMC and endothelial cells under hypoxic conditions in the presence of growth factors (27). One downstream target of mTOR, p70S6K, increases activation of the ribosomal machinery and induces G1 phase-promoting genes such as cyclin-D. mTOR can also transmit hypoxia-induced signals via 4E-BP1 and other downstream targets. However, the role of mTOR, p70S6K, and 4E-BP1 in cell proliferation may be cell type and/or stimulus specific. For example, angiotensin II-induced proliferation in rat aortic SMC was shown to be dependent on PI3K and Akt signaling pathways, yet independent of mTOR. This suggests that, under certain conditions, mTOR and p70S6K are not downstream targets of PI3K in proliferating cells (18). The role of PI3K, mTOR, and p70S6K activation in mediating hypoxia-induced fibroblast proliferation has not been elucidated.

The purpose of this study was, therefore, to evaluate the role of PI3K and some of its potential downstream targets in hypoxia-induced adventitial fibroblast proliferation. Our approach was to utilize an established model cell system, in which cultured bovine pulmonary artery adventitial fibroblasts were serum starved for a period of 72–84 h and then exposed to hypoxia (3% oxygen) in the absence of exogenous mitogens. In this cell system, the adventitial fibroblasts consistently proliferate to hypoxia (3% oxygen) in the absence of exogenous mitogens. In certain conditions, mTOR and p70S6K are not downstream targets of PI3K in the proliferative response. In addition, kinase assays were performed to evaluate the direct effects of hypoxia on PI3 and p70S6 kinases.

MATERIALS AND METHODS

Cell culture. Pulmonary artery adventitial fibroblasts were isolated from tissue explants of 120- to 180-day gestational bovine fetuses and cultured as previously described (21). Cells were grown in DMEM, supplemented with 20 mM L-glutamine (Cellgro), nonessential amino acids (1:100, vol/vol), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, St. Louis, MO), and 10% FBS (Gemini Bio-Products, Woodland, CA) and used between passages 2 and 6. For all assays, cells were plated at a density of 5 × 10^4 cells/cm^2, cultured to 80% of confluence, growth arrested in serum-free DMEM without serum for 72–84 h, and then used for the experiments.

DNA synthesis. DNA synthesis was determined by [methyl-^3H]thymidine incorporation. Cells were plated in 24-well plates at a density of 12 × 10^3 cells/well in DMEM, supplemented with 10% FBS. On the next day, cells were rinsed with PBS and incubated in serum-free DMEM for 72 h. Cells were preincubated either with or without LY-294002 (20 µM, 60 min), wortmannin (100 nM, 2 h), rapamycin (5 nM, 20 µM, 60 min) (Cell Signaling, Beverly, MA), or NL-71-101 (5 µM, 2 h) (Calbiochem, La Jolla, CA), and labeled with 1.0 µCi of [methyl-^3H]thymidine (NEF Life Science Products, Boston, MA). Cells were then placed into a Bellco chamber filled with a 3% oxygen-5% CO2-92% N2 gas mixture for 24 h. At the end of incubation, fibroblasts were washed with PBS, incubated with 0.5 µl of 0.2 M perchloric acid, and lysed by 1% SDS/0.1 M NaOH.

Samples were mixed with liquid scintillation cocktail (Ecoscint H, National Diagnostics, Atlanta, GA) and counted in a scintillation β-counter (Beckman LS 6500).

Cell extracts and Western blot analysis. Adventitial fibroblasts were cultured to near confluence and growth arrested in serum-free DMEM for 72–84 h. For the experiments with PI3K, Akt and mTOR inhibitor fibroblasts were preincubated with LY-294002, wortmannin, U0126, NL-71-101, or rapamycin as described in the figure legends. After stimulation with hypoxia (3% oxygen) for various periods of time, cells were washed twice with ice-cold PBS and lysed with Tris-HCl buffer (40 mM, pH 7.5, 4°C) containing 0.1% Triton X-100, 0.25 M sucrose, 3 mM EGTA, 3 mM EDTA, 50 µM β-mercaptoethanol, 1 mM PMSF, and complete protease inhibitors cocktail (Calbiochem). Samples containing 10–25 µg of total cellular protein were subjected to 10% SDS-PAGE, transferred to PVDF membrane, and probed with rabbit polyclonal antibodies against phospho-p70S6K (Thr421/Ser424), phospho-S6 (Ser235/236), phospho-Akt (Ser473), phospho-ERK1/2 (Tyros202/Thr204), and phospho-4E-BP1 (Thr37/40) under conditions recommended by the manufacturer (Cell Signaling Technology, Beverly, MA). After washing with TBS-Tween, membranes were incubated with mouse anti-rabbit peroxidase-conjugated IgG (Amersham; 1:10,000 dilution for 1 h at room temperature). Immunoreactive bands were detected by enhanced chemiluminescence kit (Renaissance, NEN Life Science Product) followed by exposure to Hyperfilm and quantitatively analyzed by Image J densitometry system.

Assay of in vitro PI3K activity. Adventitial fibroblasts were grown to 80% of confluence in 100-mm^2 dishes, growth arrested, and stimulated with hypoxia (3% oxygen). After stimulation, cells were washed twice with ice-cold PBS, containing 0.2 mM activated orthovandate, and incubated in 750 µl of lysis buffer containing 0.1% Triton X-100, 137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM CaCl2, 10% glycerol, 1% Nonidet P-40, 1 mM sodium orthovandate, 1 mM PMSF, and complete protease inhibitor cocktail (Calbiochem) and incubated for 20 min. After a 20-min incubation at 4°C, cell lysates were centrifuged at 10,000 g for 10 min to sediment insoluble material. Supernatants were normalized for protein content (Bio-Rad assay kit) and were incubated with anti-phospho antibodies or anti-phospho-tyrosine antibodies (clone 4G10) linked to protein A-agarose (1 µg/0.5 mg of total cell protein) (Upstate Biotechnology, Lake Placid, NY). After rocking at 4°C for 1–2 h, agarose beads containing antibody–enzyme complexes were centrifuged at 10,000 g for 20 s and washed three times with buffer A: 137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM CaCl2, 1% Nonidet P-40, 0.1 mM sodium orthovandate; three times with buffer B: 0.1 mM Tris-HCl, pH 7.5, 5 mM LiCl, 0.1 mM sodium orthovandate; and three times with buffer C: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM sodium orthovandate.

The phosphorylation reaction (final volume of 80 µl) was started by addition of 10 µl of mixture, containing 15 µCi [γ-^32P]ATP (10 mM, 3,000 Ci/mmol) and 0.88 µl of 10 mM ATP. The samples were incubated for 15 min at 37°C, and the reaction was stopped by the addition of 20 µl of 6 N HCl. Radiolabeled lipids were extracted with 160 µl of chloroform methanol (1:1, vol/vol). Organic phase was separated by centrifugation for 10 min at 12,000 g at 4°C, and lipids were separated on oxalate-coated silica TLC plates (Silica Gel 60, Sigma) by chromatography in CH3:MeOH:H2O:NH4OH (60:40:11.5:2, vol/vol) solvent system in parallel with nonradioactive standard. Radioactive spots were revealed by autoradiography and quantitatively analyzed by Image J densitometry system.

Assay of in vitro p70S6K activity. The p70S6K immunocomplex kinase assay was performed according to the manufacturer’s protocol with small modifications (Upstate Biotechnology). Quiescent fibroblasts in 100-mm dishes were growth arrested as indicated above and stimulated with hypoxia (3% oxygen) for the indicated periods of time, washed twice with ice-cold PBS, and solubilized in 700 µl of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 120 mM NaCl, 50 mM NaF, 5 mM sodium pyrophosphate, 0.1% β-mercaptoethanol, 1% Triton X-100, 0.1 mM PMSF, complete protease inhibitors cocktail). Lysates were incubated for 20 min on ice and centrifuged at 12,000 g for 15 min to sediment insoluble material and were precleared with 40 µl of protein A/G Agarose Plus (Santa Cruz, CA). Equivalent amounts (350 µg) of total cell protein were

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incubated with 2.5 μg of sheep polyclonal anti-p70S6K antibodies (Upstate Biotechnology), rocking at 4°C for 2 h, and further with 40 μl of protein A-Sepharose for 4°C for 1–2 h. Beads containing antibody-enzyme complexes were centrifuged at 10,000 g for 20 s and washed twice with lysis buffer containing 0.5 M NaCl, twice with lysis buffer alone, and twice with assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT). Kinase reaction was carried out for 10–30 min at 37°C with S6 ribosomal protein peptide (AKRRRLSSLRA) as a substrate. p70S6K activity was determined as picomoles of phosphate incorporated into substrate per minute.

Data analysis. Density of bands from Western and Northern blot film images was determined by using National Institutes of Health Image J program. Data are expressed as means ± SE; n equals the number of replicates in one experiment or a number of observations in independent experiments. To evaluate significance of the obtained data, Student-Newman-Keuls test followed by one-way ANOVA was performed.

RESULTS

Hypoxia-induced fibroblast proliferation requires PI3K, ERK1/2, and mTOR activation. Consistent with our previously reported data, exposure of serum-starved fibroblasts (72 h, serum-free DMEM) to hypoxic conditions (3% oxygen, 24 h) caused significant increases in DNA synthesis (13, 21). To assess the importance of the PI3K-Akt pathway in this response, [H3]thymidine incorporation was measured in the presence of pharmacological inhibitors of PI3K (LY-294002 and wortmannin). Both agents inhibited hypoxia-induced DNA synthesis (Fig. 1). LY-294002 was also noted to cause a significant reduction in basal [H3]thymidine incorporation under normoxic conditions. To investigate whether stimulation of Akt is necessary for hypoxia-induced proliferative responses, we examined the effect of the newly developed Akt inhibitor NL-71-101. This compound has been shown to be an ATP competitive inhibitor, which prevents phosphorylation of the Akt substrate GSK3 (46). Our data showed that hypoxia-induced increase in DNA synthesis in adventitial fibroblasts was completely blocked in the presence of NL-71-101.

Because in certain cell types PI3K activation leads to mitogen-activated protein kinase kinase 1/2 and ERK1/2 phosphorylation, we evaluated the effect of U0126, a specific mitogen-activated protein kinase kinase 1/2 inhibitor, on hypoxia-induced fibroblast proliferation. We found that U0126 was as effective at blocking hypoxia-induced DNA synthesis in adventitial fibroblasts as the PI3K inhibitors (LY-294002 and wortmannin). This is consistent with our laboratory’s previous observations demonstrating that ERK1/2 activation is necessary for hypoxia-induced adventitial fibroblast proliferation (13, 21).

To determine whether mTOR was involved in hypoxia-induced proliferation of pulmonary artery adventitial fibroblasts, we evaluated the effect of rapamycin, a potent pharmacological inhibitor of mTOR. We found that rapamycin completely abolished hypoxia-induced DNA synthesis. It should be noted that the inhibitors used in this study were found not to affect cell viability, as assessed by trypan blue exclusion or lactate dehydrogenase release during the 24-h incubation.

Hypoxia increases PI3K activity and Akt phosphorylation in adventitial fibroblasts. Although many inhibitor studies have suggested that activation of PI3K is involved in hypoxia-mediated responses, there is little direct evidence for increases in PI3K activity under hypoxic conditions in vascular wall cells. We therefore investigated whether moderate (3% oxygen) hypoxic exposure caused activation of PI3K in adventitial fibroblasts. We found that within 10–30 min of hypoxic exposure, PI3K activity associated with p85 regulatory subunit and with phosphotyrosine immunoprecipitates was increased severalfold (Fig. 2). These data, along with the pharmacological data, support a role for PI3K in hypoxia-induced adventitial fibroblast proliferation.

Because Akt is a major downstream target of PI3K involved in proliferative responses to mitogenic agonists, we examined the effects of hypoxia on Akt phosphorylation. We found that hypoxia induced a transient phosphorylation of Akt at Ser473 with a maximal effect observed at 30 min (Fig. 3).
PI3K and ERK1/2 pathways act independently in hypoxia-induced fibroblast proliferation. Because inhibitors of PI3K and ERK1/2 (LY-294002, wortmannin, and U0126, respectively) reduced hypoxia-stimulated cell responses to nearly the same extent, we examined whether PI3K was required for ERK1/2 activation under hypoxic conditions. We found that hypoxia consistently induced phosphorylation of ERK1/2 by severalfold (Fig. 4). The PI3K inhibitor wortmannin had no significant effect on hypoxia-induced ERK1/2 phosphorylation, indicating that hypoxia initiates independent activation of ERK1/2 and PI3K. We also found that inhibitors of ERK pathway, PD-98059 and U0126, were not effective in blocking hypoxia-induced PI3K activation (not shown). These data are consistent with the findings described for thrombin-, ATP-, and angiotensin II-induced proliferative responses, in which PI3K and ERK1/2 are shown to act as independent signaling pathways, both necessary for cell proliferation (18, 24, 58).

Hypoxia induces phosphorylation of mTOR, p70S6K, and 4E-BP1 in adventitial fibroblasts. The data shown in Fig. 1 demonstrate that rapamycin inhibits hypoxia-induced cell pro-
liferation and thus strongly suggest that mTOR is an important regulator of hypoxia-induced cell proliferation. The sensitivity of mTOR to hypoxia was further confirmed by Western blot analyses with phospho-specific mTOR antibodies that recognize the activated state of the protein (Fig. 5A). We found that exposure of adventitial fibroblasts to hypoxia resulted in elevated mTOR phosphorylation at both Ser2448 and Ser2481 in a time-dependent manner, with the maximal responses at 10 and 30 min, respectively.

Two downstream targets of mTOR believed to be important for cell growth are p70S6K and 4E-BP1. We therefore evaluated the effects of hypoxia on p70S6K and 4E-BP1 phosphorylation and found that hypoxia caused a significant increase in phosphorylation of p70S6K at Thr421/Ser424. This effect was

Fig. 5. Hypoxia activates mTOR, p70S6K, and 4E-BP1 in adventitial fibroblasts. Adventitial fibroblasts were growth arrested as described above and exposed to hypoxia (3% O2) for the indicated time. Forty micrograms of total cell protein were analyzed by Western blotting with anti-phospho-mTOR (Ser2481) and anti-phospho-mTOR (Ser2448) (A), anti-phospho-p70S6K (Thr421/Ser424) (B) or with anti-phospho-4E-BP1 (Thr37/40) antibodies (C). Phospho-mTOR, phospho-p70S6K, and phospho-4E-BP1 were normalized to total mTOR, p70S6K, and 4E-BP1, respectively. Quantitative data (bar graphs) are expressed as percent of basal level. Data are means ± SE from 3–6 independent experiments. D: growth-arrested adventitial fibroblasts were preincubated either with rapamycin (5 nM, 60 min) or vehicle (methanol, 1:1,000) and stimulated with hypoxia (3% O2) for indicated times. After stimulation, total cells lysates were prepared, and 350 μg of protein were immunoprecipitated with anti-p70S6K antibodies for kinase assays as described in MATERIALS AND METHODS; p70S6K activity is expressed in relative units; basal activity corresponds to 80.02 pmol Pi incorporated into substrate/min. Data are means ± SE from 5 independent experiments performed on 3 separate cell populations. *P < 0.05 compared with nonstimulated control.
evident at 30 min and was sustained for at least 120 min (Fig. 5B). Similarly, hypoxia caused an increase in 4E-BP1 phosphorylation at Thr37/40, with a maximal effect observed at 30 min (Fig. 5C).

To further investigate the effect of hypoxia on the protein translation pathway, we sought to determine whether an increase in p70S6K phosphorylation was associated with an increase in p70S6K activity. To answer this question, we performed an in vitro kinase assay using the peptide corresponding to S6 ribosomal protein as a substrate. Exposure of adventitial fibroblasts to hypoxia resulted in a time-dependent increase in p70S6K activity (Fig. 5D). The maximal effect of hypoxia on p70S6K activation was observed at 30 min, a finding consistent with the Western blot data demonstrating a time-dependent p70S6K phosphorylation.

**Hypoxia induces phosphorylation of p70S6K through mTOR-, PI3K-, and ERK1/2-dependent pathways.** To determine the relationship between PI3K, mTOR, and ERK1/2 pathways in mediating hypoxia-induced activation of p70S6K, we evaluated the effects of the PI3K inhibitor LY-294002, the mTOR inhibitor rapamycin, and the mitogen-activated protein kinase kinase 1/2 inhibitor U0126 on p70S6K phosphorylation. We found that each of these compounds markedly attenuated the hypoxia-induced p70S6K phosphorylation (Fig. 6A). In addition, treatment of cells with the specific Akt inhibitor NL-71-101 resulted in a dramatic decrease in hypoxia-induced p70S6K phosphorylation (Fig. 6B). We also found that LY-294002 and rapamycin both significantly reduced phosphorylation of S6 ribosomal protein (Fig. 7), suggesting that hypoxia-induced activation of the protein translation pathway in adventitial fibroblasts is mediated through mTOR and PI3K.

**DISCUSSION**

It is generally assumed that living organisms respond to reduced oxygen concentrations in a cell type- and tissue-
specific manner. Pulmonary artery adventitial fibroblasts possess the rather distinct capability, at least for nontransformed cells, to proliferate in response to hypoxia (3% oxygen) independent of exogenous growth factors (44, 51, 55). This capability distinguishes adventitial fibroblasts from many other cell types that often undergo metabolic suppression and inhibition of protein synthesis (hibernation) in response to hypoxia. The goal of this study was to understand the molecular mechanisms that contribute to the autonomous proliferative phenotype of adventitial fibroblasts under hypoxic conditions. Specifically, we were interested in determining the sensitivity of PI3K, Akt, and mTOR to hypoxic stimulation and in understanding the role of these pathways in hypoxia-induced proliferative responses. We provide definitive evidence that hypoxia (3% oxygen) directly activates PI3K signaling in these cells, which ultimately leads to mTOR activation. Activation of the mTOR-p70S6 pathway in fact appears to be a necessary step and a key point of convergence in ERK1/2 and PI3K-Akt pathways that regulate cell cycle progression under hypoxic conditions. These data, when taken in context with our laboratory’s previous work demonstrating the importance of Gαi in hypoxia-induced proliferation (13, 21), support a model of hypoxia-induced proliferation of adventitial fibroblasts as shown in Fig. 8.

The involvement of PI3K-Akt and mTOR pathways in mitogenic responses has been shown in various cell types, including vascular endothelial cells and SMC (32, 33, 49, 54, 61). In many tissues, activation of PI3K, Akt, and mTOR is associated with hypoxia-inducible factor-1α-dependent VEGF expression, implicating involvement of these kinases in hypoxia-induced angiogenesis (30, 39, 62). However, to our knowledge, no evidence has been presented that demonstrates a role of PI3K in hypoxia-induced cell proliferation. Our studies revealed that hypoxia-induced increases in DNA synthesis in adventitial fibroblasts are highly sensitive to PI3K inhibitors, indicating that PI3K is a critical signaling component in this response (Fig. 1). It is known that several structurally distinct PI3K isoforms are selectively activated in response to various stimuli (33, 60). Class I PI3Kα is activated by receptor tyrosine kinases, whereas PI3Kβ is synergistically activated by receptor tyrosine kinases and G-protein βγ subunits (38, 43). We previously found that p110α, p110β, and p85 PI3K subunits are expressed in adventitial fibroblasts (data not shown). The current studies demonstrate that hypoxia-induced PI3K activity can be measured in an in vitro kinase assay after immunoprecipitation with anti-p85 regulatory subunits (38, 43). We previously found that p110α, p110β, and p85 PI3K subunits are expressed in adventitial fibroblasts (data not shown). The current studies demonstrate that hypoxia-induced PI3K activity can be measured in an in vitro kinase assay after immunoprecipitation with anti-p85 regulatory subunits and phospho-tyrosine antibodies (Fig. 2), suggesting that
PI3Kα, as well as PI3Kβ, are involved in hypoxia-initiated responses in adventitial fibroblasts.

Akt is a downstream target of PI3K known to play a central role in cell growth and survival (14, 31, 54). Consistent with the stimulatory effect of hypoxia on PI3K activity, Akt phosphorylation is also increased under exposure of adventitial fibroblasts to hypoxia. The time course of hypoxia-induced Akt phosphorylation is similar to the early Akt activation observed in response to G protein-coupled receptor stimulation (Refs. 23, 28, 45, and our unpublished data), but it is different from the hypoxia-induced responses observed in rat pheochromocytoma-12 cells or in U373 cells, which showed prolonged and sustained Akt phosphorylation, implicating PI3K-Akt pathways in cell survival (1, 5, 63). The underlying mechanisms through which Akt may be involved in cell proliferation include Akt-dependent phosphorylation, inactivation, and subcellular redistribution of p21Cip1 and p27kip1 proteins, which are known as inhibitors of cell cycle progression, as well as accumulation of cyclin D1 mediated by GSK-3β (17, 37, 47).

In addition to existing data demonstrating serum- and growth factor-induced Akt activation, one recent report clearly demonstrates a role of Akt in angiotensin II-induced proliferation (13). A remarkable feature of this study is the demonstration that PI3K inhibitors decrease the basal rate of DNA synthesis (Fig. 1), are compatible with the idea that hypoxia-proliferative adventitial fibroblasts exhibit a basal or “constitutive” activation of PI3K and Akt and that maintenance of this pathway might be a unique aspect of specific populations of cells capable of exhibiting hypoxia-induced proliferative responses.

mTOR has been shown to play a central role in regulating cell growth, homeostasis, and proliferation (15, 20, 49). Our study demonstrates that mTOR is a critical target of hypoxia, important in mediating hypoxia-induced proliferative responses in adventitial fibroblasts (Fig. 1). We found that hypoxia significantly increases phosphorylation of mTOR as well as its downstream targets p70S6K and 4E-BP1. Moreover, using an in vitro kinase assay (Fig. 5B), we found that hypoxia dramatically induces (up to 9-fold) p70S6K activity. It is remarkable that the magnitude of this response is comparable to the response to 10% serum (up to 11-fold over the basal level, used in our study as a control for maximal stimulation). Although the effect of hypoxia on mTOR phosphorylation is not as dramatic, the sensitivity of hypoxia-induced activation of p70S6K to rapamycin clearly indicates that mTOR is an upstream regulator of p70S6K under hypoxic conditions in these cells. We also found that exposure of adventitial fibroblasts to hypoxia leads to phosphorylation of S6 ribosomal protein, again indicating the importance of protein translation pathways in hypoxia-induced proliferative responses. The sensitivity of mTOR to hypoxic stimulation reported in this study is consistent with the data of Humar et al. (27), demonstrating mTOR-dependent sprout formation and enhanced endothelial cell proliferation in response to hypoxia, as well as with data of Hudson et al. (26), demonstrating a key role of mTOR in hypoxia- and CoCl2-induced hypoxia-inducible factor-1α expression in pheochromocytoma-3 cells.

The results of the present study show that hypoxia-induced phosphorylation of p70S6K and its downstream target S6 ribosomal protein is significantly decreased by PI3K, ERK1/2, and mTOR inhibitors, indicating the requirement of all these pathways for the response (Figs. 6–8). In addition, the observation that specific Akt inhibitor NL-71-101 completely attenuated hypoxia-induced p70S6K phosphorylation suggests an intermediary role of Akt in PI3K-dependent regulation of 70S6K. A role for Akt in the cross talk between PI3K and mTOR-p70S6K pathways has been shown in several studies in other cell types (8, 41, 52, 59). In addition, there is evidence that mTOR and PI3K pathways may operate in parallel and converge on common downstream targets to support cell growth and proliferation (20). Current knowledge in the field suggests that mTOR may operate as a nutrient and energy sensor to permit mitogens to stimulate cell proliferation. However, specific and coordinated signaling cross talk between mTOR and PI3K pathways is generally believed to occur in a cell type- and stimulus-specific manner, and thus further studies are required for better understanding the pathways specifically involved in linking hypoxia to protein translation in pulmonary artery adventitial fibroblasts. In this regard, it should also be noted that, in our cells, hypoxia induces PI3K-independent activation of ERK1/2 (Fig. 4), a signaling pathway that appears to converge at p70S6K to support the proliferative response. Similar interactions between ERK1/2, PI3K, and p70S6K pathways have been reported in vascular cells and hepatocytes in response to other mitogenic stimuli (12, 35, 58).

In agreement with our studies showing a mitogenic effect of hypoxia (3% oxygen) in adventitial fibroblasts, data by Cogo et al. (10) and Cooper and Beasley (11) have showed that modest hypoxia (5% oxygen) was pro-proliferative for vascular and airway SMC, whereas very low oxygen tension (1% oxygen) resulted in a decrease of SMC proliferation (10). It should also be noted that, in contrast to adventitial fibroblasts, other cell types respond to hypoxia (1–2% oxygen) or anoxia (0% oxygen) by inhibiting protein synthesis and stimulating adaptive cellular pathways (6, 25). For example, in HEK 293 cells, severe hypoxia (1.5% oxygen) attenuated insulin-induced phosphorylation of mTOR and its downstream targets 4E-BP1, p70S6K, S6 ribosomal protein, and eIF4G (2). Although the effect of 1.5% oxygen on HEK 293 cell proliferation was not reported, it is likely that the growth response in these cells would not be increased. Therefore, the effects of hypoxia on HEK 293 cells are more compatible with an adaptive response, whereas adventitial fibroblasts, as shown in this study, exhibit a proliferative response under nearly the same oxygen concentrations (1–3% oxygen) and, not surprisingly, with a different activation profile of signaling intermediates. Basically, because an increase in protein synthesis is necessary for cell proliferation, a positive correlation between these two responses in hypoxia-proliferative cells is expected. The ability of the adventitial fibroblast to proliferate in response to hypoxia and to...
activate mTOR/p70S6K/S6 protein translational pathways supports this notion. Although not directly evaluated, adventitial fibroblasts might be expected to exhibit metabolic similarities to an oxygen-conforming skeletal muscle cell line (C2C12) in which hypoxic exposure does not result in a suppression of protein synthesis (3). Additional data obtained in animal and cell models suggest that hypoxic exposure is associated with enhanced synthesis of extracellular matrix proteins (19, 40, 55, 56) as well as stress-related proteins, including transcription factors and proteins of energy metabolism, all of which are known to be necessary to maintain cell homeostasis (36). Given the above data, it is important to note that, despite the diversity in the hypoxic regulation of the protein translational pathway (mTOR, p70S6K, and S6 protein), a remarkable feature is the capability of these kinases to respond to changes in oxygen concentration.

In conclusion, this study demonstrates that PI3K-Akt, mTOR-p70S6K, and ERK1/2 signaling pathways are key signaling components of hypoxia-induced proliferative responses in pulmonary artery adventitial fibroblasts (Fig. 8). However, intriguing questions remain with regard to exactly how hypoxia initiates activation of individual signaling proteins in cells capable of proliferating under conditions of moderate hypoxia. An essential role for reactive oxygen species as mediators of stimulation of PI3K, Akt, and p70S6K has been proposed in various cell types, including fibroblasts (4, 34, 50, 57). There exist data demonstrating redox- and reactive oxygen species-dependent activation of receptor tyrosine kinase and G protein-coupled signaling pathways (9, 16, 42). Because in adventitial fibroblasts both PI3K and p70S6K pathways can be regulated through G proteins, it will be of interest to test the possibility that G proteins can be activated in response to hypoxia in a ligand- and/or receptor-independent manner (Ref. 13 and our unpublished observations). Future understanding of the fundamental mechanisms underlying hypoxic activation of signal transducing proteins and their assembly into signaling networks should have significant implications for therapeutic strategies directed at specific targeting proteins critical for cell proliferation under hypoxic conditions.

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Hypoxic activation of PI3K, Akt, mTOR, and p70S6K


