PI3K is required for proliferation and migration of human pulmonary vascular smooth muscle cells

ELENA A. GONCHAROVA,1 ALAINA J. AMMIT,1 CARLA IRANI,1 RICHARD G. CARROLL,2 ANDREW J. ESZTERHAS,1 REYNOLD A. PANETTIERI,1 AND VERA P. KRYMSKAYA1

1Pulmonary, Allergy, and Critical Care Division, Department of Medicine; and 2Abramson Family Cancer Research Institute, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104-6160

Received 10 January 2002; accepted in final form 3 March 2002

Goncharova, Elena A., Alaina J. Ammit, Carla Irani, Richard G. Carroll, Andrew J. Eszterhas, Reynold A. Panettieri, and Vera P. Krymskaya. PI3K is required for proliferation and migration of human pulmonary vascular smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 283: L354–L363, 2002. First published March 8, 2002; 10.1152/ajplung.00010.2002.—Human vascular smooth muscle cells proliferation and migration contribute to vascular remodeling in pulmonary hypertension and atherosclerosis. The precise mechanisms that regulate structural remodeling of the vessel wall remain unknown. This study tests the hypothesis that phosphatidylinositol 3-kinase (PI3K) activation is both necessary and sufficient to mediate human pulmonary vascular smooth muscle (PVSM) cell proliferation and migration. Microinjection of human PVSM cells with a dominant-negative class IA PI3K inhibited platelet-derived growth factor (PDGF)-induced DNA synthesis by 65% (P < 0.001; χ² analysis) compared with cells microinjected with control plasmid, whereas microinjection of cells with a constitutively active class IA PI3K (p110*-CA) was sufficient to induce DNA synthesis (mitotic index of p110*-CA-microinjected cells was 15% vs. 3% in control cells; P < 0.01). Transfection of PVSM cells with p110*-CA was also sufficient to promote human PVSM cell migration. In parallel experiments, stimulation of human PVSM cells with PDGF induced PI3K-dependent activation of Akt, p70 S6 kinase, and ribosomal protein S6 but not mitogen-activated protein kinase. PDGF-induced proliferation and migration was inhibited by LY-294002. These results demonstrate that PI3K signaling is both necessary and sufficient to mediate human PVSM cell proliferation and migration and suggest that the activation of PI3K may play an important role in vascular remodeling.

phosphatidylinositol 3-kinase; hypertension; Akt; S6K1; ribosomal protein S6

VASCULAR REMODELING, CHARACTERIZED BY smooth muscle cell proliferation and migration, contributes to the pathology of pulmonary hypertension and atherosclerosis (9, 32, 36, 40). In part, altered smooth muscle cell function, which is modulated by growth factors, contractile agonists, inflammatory mediators, and the extracellular matrix, is a compensatory response to changes in the vascular microenvironment such as stress or injury. The precise molecular mechanisms that regulate vascular remodeling, however, remain unknown.

Phosphatidylinositol 3-kinase (PI3K), a family of enzymes with complex multifunctional roles, is activated by a variety of upstream signals and controls the activity of multiple downstream effectors (21). PI3K is activated by receptor and nonreceptor protein tyrosine kinases and by G protein-coupled receptors, and PI3K coordinates downstream cellular events by generating 3-phosphoinositide lipids (42). Current evidence suggests that PI3K activation modulates vascular smooth muscle cell function in cell- and species-specific manners. PI3K activation appears important for medial but not intimal vascular smooth muscle cell growth (39). Migration of human coronary (27) and canine pulmonary (46) arterial vascular smooth muscle cells appears to be PI3K dependent, whereas chemotaxis of rat (13) and rabbit (6) aortic smooth muscle cells is PI3K independent. No information exists regarding the role of PI3K activation in regulating human pulmonary arterial vascular smooth muscle (PVSM) cells. In this study, using molecular approaches, we examined the role of PI3K in human PVSM cell proliferation and migration.

In some cell types, several downstream targets of PI3K have been identified, such as serine/threonine kinase Akt/protein kinase B (PKB), which controls insulin signaling and apoptosis, and p70 S6 kinase (S6K1), a critical enzyme that exerts translational control of protein synthesis by phosphorylation of ribosomal protein S6 (42). PI3K-dependent activation of Akt/PKB has been shown in porcine (7) and rat (8) pulmonary arterial, rat (20) and human (37) aortic, and human coronary (37) vascular smooth muscle cells. S6K1 was also found to be activated in response to balloon injury of rat carotid artery (3). Studies demonstrate a role for S6K1 in angiotensin II-mediated protein synthesis in rat pulmonary artery (8) and aorta (11). No studies were performed to examine downstream effectors of PI3K in human PVSM cells.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Our recent studies demonstrated a role for the PI3K signaling pathway in regulating airway smooth muscle (ASM) cell growth, which is a common pathological feature of chronic severe asthma. Expression of constitutively active class IA PI3K was sufficient to induce human ASM cell proliferation (23). PI3K-mediated ASM cell proliferation is also dependent on activation of S6K1 but was not dependent on p42/p44 mitogen-activated protein kinase (MAPK) activation (26). PI3K signaling also appears to be critical for ASM cell migration. Our studies show that expression of the constitutively active class IA PI3K promotes smooth muscle cell migration (17). Collectively, this evidence suggests that PI3K activation plays a pivotal role in regulating smooth muscle cell proliferation and migration.

The present study examines whether PI3K activity is both necessary and sufficient to mediate human PVSM cell proliferation and migration and defines the signaling molecules that act downstream of PI3K. The regulation of human PVSM cell proliferation and migration is likely important in a variety of disease processes. By determining the signaling mechanisms that are responsible for mediating human PVSM cell proliferation and migration, we can address new therapeutic approaches to treat pulmonary vascular diseases.

METHODS

Cell culture. Human PVSM cells were dissociated from human pulmonary arteries, which were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. A segment of pulmonary artery just proximal to the lung entry was removed under aseptic conditions, cleaned from connective and fat tissues, and digested as follows: the media of pulmonary artery was digested from the adventitia and intima and subjected to an enzymatic digestion in 10 ml of buffer containing 0.2 mM CaCl₂, 640 U/ml collagenase, 1 mg/ml soybean trypsin inhibitor, and 10 U/ml elastase for 60 min in a shaking water bath at 37°C. The cell suspension was filtered through a 105-µm Nytex mesh, and the filtrate was washed with equal volumes of cold Ham’s F-12 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT). Aliquots of the cell suspension were plated at a density of 1.0 x 10⁴ cells/cm² on tissue-culture plates covered with Vitrogen (Cohesion Technologies, Palo Alto, CA). The cells were cultured in Ham’s F-12 medium supplemented with 10% FBS, 30 µg/ml endothelial cell-growth supplement (Becton Dickinson, Bedford, MA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin, and medium was replaced every 72 h.

To provide adequate cell numbers for our biochemical and molecular studies, we transfected PVSM cells with the vector LXSN16E6E7 (American Type Culture Collection, Rockville, MD), which is a retroviral vector encoding the E6 and E7 transforming proteins of human papillomavirus type 16 (HPV-16) and the neomycin phosphotransferase gene (5, 12). Briefly, 24 h after plating, retroviral transfection was performed using passage 10 primary human PVSM cells. Cells that were transfected with the LXSN16E6E7 retrovirus or that were mock-transfected were selected by using geneticin (G418, Life Technologies) at a concentration of 300 µg/ml. On day 6 after transduction in the presence of G418, death of all mock-transfected cells was observed. G418-resistant colonies that successfully incorporated the LXSN16E6E7 retrovirus were harvested and replated as pooled populations of PVSM/HPV-16 cells. The plates were then grown to confluency and propagated via a 1:6 split.

The E6 and E7 transforming proteins specifically target p53 and Rb protein, thereby promoting cell-cycle progression (31). A comparative study of human PVSM/HPV-16 cells and primary human PVSM cells by morphological, immunofluorescent, and biochemical analyses demonstrated that PVSM/HPV-16 cells retain much of the primary PVSM cell phenotype including the expression of the smooth muscle marker, smooth muscle α-actin, and the appropriate growth responses to platelet-derived growth factor (PDGF), which was consistent with previously published observations (5). Furthermore, immortalization of PVSM cells did not change the functional properties of PI3K. As seen in Fig. 1, basal and transforming growth factor-α (TGF-α)-induced PI3K activities in primary and immortalized human PVSM cells were comparable. Similar data were obtained for PDGF- and thrombin-induced PI3K activities (data not shown).

Primary human PVSM cells in subculture during cell passages 2–10 and PVSM/HPV-16 cells during passages 11–24 were used. All experiments were performed using a minimum of three different cell lines. Each human PVSM cell line was established using pulmonary arterial tissue from a single human donor.

[³H]thymidine incorporation assay. DNA synthesis was measured using a [³H]thymidine incorporation assay. Near-confluent cells were growth-arrested by incubating the cultures at day 8 in serum-free Ham’s F-12 medium supplemented with 0.1% BSA (Sigma, St. Louis, MO). After 48 h in serum-free media, the cells were stimulated with 10 ng/ml PDGF-BB, 10 ng/ml TGF-α, or 1 U/ml thrombin. The concentrations of mitogens were chosen because these concentra-
tions have been reported to induce cell proliferation in other cell types (26, 44). After 16–18 h of stimulation, cells were labeled with 3 μCi/ml of [3H]thymidine (40–60 Ci/mmol; Amersham/Pharmacia, Arlington Heights, IL) for 24 h. The cells were then lysed and the protein/DNA was precipitated with 10% TCA. The precipitant was aspirated onto glass filters, extensively washed, dried, and counted (33).

**Microinjection and measurement of DNA synthesis.** Near-confluent human PVSM cells were growth-arrested and microinjected with either control (pCG), constitutively active (pCG-p110*-CA), or dominant-negative (pCG-p110-DN) plasmids (15) as described previously (1). To identify single cells that underwent DNA synthesis after microinjection, incorporation of 5-bromo-2′-deoxyuridine (BrduU), an analog of thymidine, was measured. Approximately 2 h after microinjection, human PVSM cells were treated with 10 ng/ml PDGF-BB, 1 μM thrombin, 10 ng/ml TGF-α, or diluent. Fifteen minutes later, 10 μM of BrdU were added to all wells. Twenty-four hours after the addition of BrdU, the cell monolayers were fixed with 3.7% paraformaldehyde and then permeabilized with 0.1% Triton X-100. After denaturation of DNA with 4 N HCl (for 3 min at room temperature), the monolayers were incubated for 1 h at 37°C with 2 μg/ml murine anti-BrdU antibody (Becton Dickinson, San Jose, CA) and then for 1 h at 37°C with 10 μg/ml Texas Red conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) to detect BrdU-positive cells. The cells were examined using a fluorescent microscope (Aristoplan, Leica, Wetzlar, Germany) under ×200 magnification with the appropriate fluorescent filters. Results are presented as a mitotic index, which is defined as the percentage of BrdU-positive nuclei per number of cells microinjected.

**Transfection of human PVSM cells.** Cultures of human PVSM cells were transfected using the Calcium Phosphate Transfection System (Life Technologies) according to the manufacturer’s protocol. Plasmid DNA used in these studies was purified with the Qiagen EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA) and was endotoxin free. Cells were plated in 20 mM Tris (pH 7.5) with 150 mM NaCl (TBS) plus 1 mM EDTA (pH 7.5). All solutions also contained 0.2 mM vanadate (pH 7.5). The lysates were centrifuged at 14,000 rpm for 10 min. The supernatant was aspirated and the pellets were resuspended with 1% NP-40; two times in 0.1 M Tris-HCl (pH 7.5) and 0.5 M LiCl; and two times in 10 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA (pH 7.5). All solutions also contained 0.2 mM vanadate.

**PI3K activity assays.** PI3K activity assays were performed as previously described (26). Briefly, sonicated phosphatidylinositol in Tris·HCl/EGTA (0.2 mg/ml final concentration) was added to the immunoprecipitates; the phosphorylation reactions were started by the addition of MgCl2, ATP, and [γ-32P]ATP (30 μCi/sample) to achieve final concentrations of 4 mM MgCl2 and 50 mM ATP for 10 min at room temperature. Reactions were stopped by the addition of 100 μl of 1 N HCl and extracted with 160 μl of a 1:1 chloroform-methanol mixture. Lipids were separated on oxalate-coated thin-layer chromatography (TLC) plates (Silica Gel 60, Merck KGaA, Darmstadt, Germany) using a solvent system that contained acetone/methanol/2% 3 M aqueous ammonium hydroxide mixture. The lipids were then detected by autoradiography. The position of [32P]phosphatidylinositol monophosphate (PIP) was determined by the position of a PIP standard subsequently separated on TLC in parallel and developed in iodine vapor.

**Identification of proteins by immunoblot assay.** Whole cell lysates were prepared from growth-arrested human PVSM cells treated with agonists or LY-294002 at 37°C for the times indicated. Cells were then washed with ice-cold PBS, and whole cell lysates were prepared. Protein contents were measured using a Bio-Rad Protein Assay Reagent Kit. Equal amounts of lysate, adjusted to protein content, were subjected to SDS-PAGE and immunoblot analysis as described previously (24). The blots were exposed to anti-phospho-Akt (Thr308), anti-Akt, anti-phospho-S6K1 (Thr389), anti-phospho-S6K1 (Thr421/Thr424), anti-S6 ribosomal protein antibodies (Cell Signaling Technology, Beverly, MA), anti-phospho-ribosomal protein S6 antibody (Upstate Biotechnology), anti-S6K1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-phospho p42/p44 MAPK (Thr202/Thr204; New England Biolabs, Beverly, MA); all antibodies were in 20 mM Tris (pH 7.5) with 150 mM NaCl (TBS) plus 0.5% Tween 20 (TBST), and all incubations were for overnight at 4°C. After three washes in TBST, the nitrocellulose filters were exposed to horseradish peroxidase-conjugated secondary antibody (Boehringer-Mannheim, Indianapolis, IN). Filters were washed five times in TBST and were visualized using enhanced chemiluminescence (ECL, Amersham/Pharmacia).

**Data analysis.** Data points from individual assays represent the mean values of triplicate measurements. Statistically significant differences among groups were assessed with one-way ANOVA (Bonferroni-Dunn test) or by χ2-analysis with values of P < 0.05 sufficient to reject the null hypothesis for all analyses.

**RESULTS**

**PI3K regulates PDGF-induced human PVSM cell proliferation and migration.** Because PI3K activation is required for mitogen-induced cell proliferation and migration in some cell types, we investigated whether PI3K mediates human PVSM cell mitogenesis and motility. As seen in Fig. 2A, pretreatment of cells with LY-294002 inhibited PDGF-, TGF-α-, and thrombin-induced DNA synthesis in a concentration-dependent manner (IC50 values of ~1, 0.2, and 0.5 μM, respectively). LY-294002 also inhibited PDGF-induced mi-
Migration in a concentration-dependent manner (IC$_{50}$ value of $\sim$3 $\mu$M). Interestingly, 30 $\mu$M LY-294002 abrogated mitogen-induced PVSM cell migration, whereas it did not completely inhibit PDGF-induced cell migration (Fig. 2). The effect of 30 $\mu$M LY-294002 on the basal level of $[^{3}H]$thymidine incorporation and migration was not different from that of diluent alone. Over the duration of this assay, PVSM cell viability was also unaffected by LY-294002 as determined by trypan blue staining and CaspaTag fluorescein caspase activity assay (data not shown). These data suggest that PI3K modulates signaling events involved in agonist-induced PVSM cell growth and migration.

PI3K is sufficient for human PVSM cell proliferation and migration. We next addressed the role of PI3K in modulating DNA synthesis by microinjecting human PVSM cells with pCG-p110*-CA, pCG-p110-DN, and control plasmids (Fig. 3B). Constitutively active p110*-CA alone markedly induced DNA synthesis by 2.2-fold compared with cells microinjected with control plasmid (mitotic index of p110*-CA-microinjected cells was 6.5% vs. 2.9% in control cells, $P < 0.01$; $\chi^2$-analysis). Interestingly, stimulation of p110*-CA-microinjected cells with PDGF did not increase DNA synthesis compared with PDGF-stimulated control cells. The microinjection of cells with p110-DN had little effect on the basal level of DNA synthesis, whereas expression of p110-DN markedly attenuated PDGF-induced DNA synthesis (Fig. 3B). As shown in Fig. 3B and Table 1, microinjection of PVSM

![Fig. 2. PI3K modulates human PVSM cell proliferation and migration. A: confluent, growth-arrested PVSM cells were pretreated with 0.1, 1, 10, and 30 $\mu$M LY-294002 for 30 min and stimulated with either 10 ng/ml platelet-derived growth factor (PDGF), 10 ng/ml TGF-\(\alpha\), 1 U/ml thrombin, or diluent. $[^{3}H]$thymidine incorporation was subsequently assessed. Data are means $\pm$ SE from 6 experiments, each containing 6 replicates for each condition; $^*P < 0.01$ for PDGF-BB plus LY294002 vs. PDGF; $^{**}P < 0.01$ for TGF-\(\alpha\) plus LY294002 vs. TGF-\(\alpha\); $^{***}P < 0.01$ for thrombin plus LY294002 vs. thrombin by one-way ANOVA (Bonferroni-Dunn test). B: growth-arrested PVSM cells were pretreated with diluent or 1, 10, or 30 $\mu$M LY-294002 for 30 min, then cells were allowed to migrate for 4 h in the presence of PDGF (10 ng/ml). Data are means $\pm$ SE of 6–12 replicates in 3 separate experiments; $^*P < 0.001$ for 10 $\mu$M LY-294002 plus PDGF vs. 10 ng/ml PDGF; $^{**}P < 0.001$ for 30 $\mu$M LY-294002 plus PDGF vs. PDGF by ANOVA (Bonferroni-Dunn test).]

![Fig. 3. PI3K is sufficient for human PVSM cell proliferation and migration. A: transient expression of constitutively active (p110*-CA) or dominant-negative (p110-DN) PI3K in human PVSM cells. Cells were transfected with constitutively active pCG-p110*-CA, dominant-negative pCG-p110-DN, or control pCG vectors and then PI3K activity was measured in anti-myc-tag immunoprecipitate. B: effects of pCG-p110-DN and pCG-p110*-CA on the mitotic index of human PVSM cells. Cells, microinjected with pCG-p110*-CA, pCG-p110-DN, or control vector pCG, were stimulated with 10 ng/ml PDGF. Mitotic index was measured as the percentage of 5-bromo-2'-deoxyuridine (BrdU)-positive cells compared to the total number of cells. Data are representative of 3 separate experiments; $^*P < 0.01$ for control plus pCG-p110*-CA vs. control plus pCG ($\chi^2$-analysis); $^{**}P < 0.01$ for PDGF plus pCG-p110-DN vs. PDGF plus pCG ($\chi^2$-analysis). C: PVSM cells were transfected with pCG-p110*-CA plasmid or control pCG plasmid and growth arrested for 48 h. Migration assay was performed in the presence of PDGF-BB (10 ng/ml) or diluent. Data represent means $\pm$ SE from 6 replicates in 2 separate experiments; $P < 0.001$ for p110*-CA vs. pCG by ANOVA (Bonferroni-Dunn test).]

AJP-Lung Cell Mol Physiol • VOL 283 • AUGUST 2002 • www.ajplung.org
cells with p110-DN inhibited PDGF-induced DNA synthesis by 65% (mitotic index of PDGF-stimulated cells was 21.3% compared to 7.4% after microinjection of p110-DN). It is interesting that the expression of p110-DN did not completely inhibit PDGF-induced DNA synthesis, which suggests that other signaling pathways that are PI3K independent may be involved in regulating mitogen-induced human PVSM cell proliferation.

In parallel experiments, we examined whether transient expression of p110*-CA induced PVSM cell migration and whether p110*-CA modulates PDGF-induced PVSM cell migration. As seen in Fig. 3A, human PVSM cells transfected with pcG-p110*-CA showed a robust intrinsic PI3K activity of expressed p110*-CA protein, whereas p110-DN showed as little activity as control pcG plasmid. As shown in Fig. 3C, expression of p110*-CA was sufficient to promote cell migration in the absence of PDGF stimulation. Migration of p110*-CA-transfected cells was 66.0 ± 1.9 cells/field compared with 42.5 ± 8.2 cells/field in control cells (P < 0.01 by Bonferroni/Dunn test). Interestingly, no significant differences were observed in PDGF-stimulated migration of cells transfected with control and p110*-CA plasmids (PDGF-stimulated migration of p110*-CA-transfected cells was 90 ± 10.6 cells/field compared with 80 ± 4.6 cells/field in PDGF-stimulated control cells). We also performed transient transfection of cells with the pcG-p110-DN. Unfortunately, transfection of cells with the dominant-negative PI3K induced cell detachment from the culture plates. We speculate that the prolonged exposure of cells to the dominant-negative PI3K may alter cell-cell or cell-matrix attachment.

**Mitogens activate PI3K in PVSM cells.** Because LY-294002 inhibited PDGF-, TGF-α-, and thrombin-induced human PVSM-cell proliferation and PDGF-induced migration, studies were performed to show that mitogens can directly activate PI3K in human PVSM cells. PDGF, TGF-α, and thrombin significantly induced PI3K activation in a time-dependent manner (Fig. 4). PDGF and TGF-α maximally increased PI3K activity at 10 and 1 min, respectively (Fig. 4, A and B); maximal PI3K activation by thrombin was observed at 20 min (Fig. 4C). Interestingly, PDGF-induced PI3K activation was substantially greater than TGF-α- and thrombin-induced PI3K activation, and this is consistent with the observation that PDGF is a more effective mitogen for human PVSM cells. These data demonstrate that PDGF, TGF-α, and thrombin activate PI3K activity in human PVSM cells.

**Mitogen-induced activation of Akt/PKB in PVSM cells is mediated by stimulation of PI3K.** To dissect the PI3K-dependent molecular signaling processes that regulate PVSM cell proliferation, we examined whether PDGF, TGF-α, and thrombin induce activation of the serine/threonine protein kinase Akt/PKB, a downstream effector of PI3K, and whether Akt/PKB activation is PI3K dependent. Activation of Akt was assessed by immunoblot analysis of cell lysates using an antibody that recognizes the phosphorylated Ser473 residue, which is a critical site required for full activation of Akt. As shown in Fig. 5, PDGF, TGF-α, and thrombin activated Akt/PKB, and pretreatment of cells with LY-294002 inhibited PDGF-α-, TGF-α-, and thrombin-induced phosphorylation of Akt/PKB in a concentration-dependent manner. These studies demonstrate that LY-294002 inhibits Akt activity and suggest that PDGF-α-, TGF-α-, and thrombin-induced activation of Akt/PKB is PI3K dependent. Activation of S6K1 and ribosomal protein S6 in PVSM cells is mediated by stimulation of PI3K. To further assess the mechanisms by which mitogens induce PVSM cell proliferation, we investigated the relationship between PI3K and S6K1 activation in modulating PVSM cell mitogenesis. Additionally, we examined whether the inhibition of S6K1 activity by rapamycin, a specific S6K1 inhibitor, modulates PVSM cell proliferation. Rapamycin inhibited PDGF-α-, TGF-α-, and thrombin-induced DNA synthesis in a concentration-dependent manner with IC_{50} values of ~0.2, ~2, and ~2, respectively, and 200 nM of rapamycin blocked PDGF-α, TGF-α, and thrombin-induced DNA synthesis by 80.3, 69.5, and 73.5%, respectively (Fig. 6). These data demonstrate that S6K1 modulates PDGF-α, TGF-α, and thrombin-induced DNA synthesis in human PVSM cells.

Because activation of S6K1 requires the phosphorylation of specific amino acid residues, the effects of LY-294002 and rapamycin on the level of S6K1 phosphorylation were next examined. As shown in Fig. 7, PDGF, TGF-α-, and thrombin increased Thr421/Ser424 S6K1 (Fig. 7A) and Thr389 (Fig. 7B) phosphorylation. LY-294002 and rapamycin pretreatment inhibited mitogen-induced Thr389 and Thr421/Ser424 S6K1 phosphorylation, which suggests that the inhibitory effects of LY-294002 and rapamycin on PVSM cell proliferation are modulated via the inhibition of S6K1 activity.

Because S6K1 is activated by phosphorylation that subsequently leads to the phosphorylation of ribosomal protein S6, we next investigated whether PI3K mediates ribosomal protein S6 phosphorylation. As shown in Fig. 8, PDGF, TGF-α, and thrombin increased phos-

### Table 1. Effect of microinjection of p110*-CA and p110-DN on BrdU incorporation

<table>
<thead>
<tr>
<th>Plasmid and Treatment</th>
<th>Number of Microinjected Cells</th>
<th>Number of BrdU-Positive Cells</th>
<th>Mitotic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcG Diluent</td>
<td>173</td>
<td>5</td>
<td>2.89</td>
</tr>
<tr>
<td>PDGF</td>
<td>209</td>
<td>48</td>
<td>23.0</td>
</tr>
<tr>
<td>Thrombin</td>
<td>146</td>
<td>12</td>
<td>8.22</td>
</tr>
<tr>
<td>TGF-α</td>
<td>204</td>
<td>22</td>
<td>10.9</td>
</tr>
<tr>
<td>p110*-CA Diluent</td>
<td>138</td>
<td>9</td>
<td>6.52</td>
</tr>
<tr>
<td>PDGF</td>
<td>178</td>
<td>38</td>
<td>21.3</td>
</tr>
<tr>
<td>Thrombin</td>
<td>151</td>
<td>17</td>
<td>11.3</td>
</tr>
<tr>
<td>TGF-α</td>
<td>162</td>
<td>25</td>
<td>15.4</td>
</tr>
<tr>
<td>p110-DN Diluent</td>
<td>135</td>
<td>3</td>
<td>2.22</td>
</tr>
<tr>
<td>PDGF</td>
<td>175</td>
<td>13</td>
<td>7.43</td>
</tr>
<tr>
<td>Thrombin</td>
<td>155</td>
<td>11</td>
<td>7.10</td>
</tr>
<tr>
<td>TGF-α</td>
<td>184</td>
<td>12</td>
<td>6.52</td>
</tr>
</tbody>
</table>

BrdU, 5-bromo-2′-deoxyuridine; PDGF, platelet-derived growth factor; TGF-α, tumor-growth factor-α.
phorylation of ribosomal protein S6, and pretreatment of cells with LY-294002 and rapamycin inhibited mitogen-induced S6 phosphorylation.

Together these data suggest that PDGF, TGF-α, and thrombin induce activation of S6K1 and ribosomal protein S6 in PVSM cells and that mitogen-induced activation of S6K1 and ribosomal protein S6 is PI3K dependent.

LY-294002 has little effect on p42/p44 MAPK activation. Evidence suggests that in some cell types there is cross talk between mitogen-induced PI3K and p42/p44 MAPK signaling pathways. We therefore examined whether LY-294002 modulates PDGF-, TGF-α-, and thrombin-induced p42/p44 MAPK activation. As shown in Fig. 9, LY-294002 pretreatment has little effect on mitogen-stimulated p42/p44 MAPK phosphorylation. These data suggest that the effects of PI3K and LY-294002 on mitogenesis are primarily mediated through pathways distinct from those dependent on p42/p44 MAPK activation.

DISCUSSION

Understanding the molecular mechanisms that regulate human PVSM cell proliferation and migration may lead to insights into defining the role of PVSM cells in vascular remodeling that occurs in pulmonary hypertension. Our study addresses the central hypothesis that PI3K activity is both necessary and sufficient to mediate mitogen-induced human PVSM cell proliferation and migration. Expression of the constitutively active p110*-CA class IA PI3K promoted human PVSM cell proliferation and migration. Conversely, expression of the dominant-negative p110-DN class IA PI3K inhibited PDGF-induced DNA synthesis. Collectively, these data represent the first description that class IA PI3K is sufficient to promote human PVSM cell proliferation and migration.

Evidence suggests a possible role for PI3K activation in systemic vascular smooth muscle cell mitogenesis; however, there exist markedly different cell-specific responses. Although in vivo studies demonstrate that administration of wortmannin (a potent PI3K inhibitor) to rats after catheter injury of carotid arteries significantly reduced medial smooth muscle cell replication, there was no decrease in the replication of intimal smooth muscle (39). Interestingly, differences also exist in the cross talk between signaling pathways in vascular smooth muscle cells. Mitogen-induced human aortic smooth muscle cell growth is mediated in part by PI3K-dependent activation of Ras and MAPK (16). Insulin-induced activation of MAPK and mitogenesis in rat aortic vascular smooth muscle cells also appears to be PI3K dependent (2). In other studies using rat aortic vascular smooth muscle cells, MAPK activity appears to be PI3K independent (8). Furthermore, proliferation of porcine coronary artery...
and PVSM (7) cells occurs via PI3K- and MAPK-independent signaling pathways. In our study, PDGF-, TGF-α-, and thrombin-induced DNA synthesis was inhibited by LY-294002 that was MAPK independent. We also showed that the level of p110*-CA-induced DNA synthesis was significantly lower than that induced by PDGF, TGF-α, or thrombin; thus although PI3K activation was sufficient to induce DNA synthesis in PVSM cells, other signaling pathways may act in parallel to regulate mitogen-induced DNA synthesis in human PVSM cells. This notion is also supported by our experiments using a dominant-negative PI3K construct. PDGF-, TGF-α-, and thrombin-induced BrdU incorporation was markedly decreased in cells microinjected with the p110-DN but not completely inhibited. It is noteworthy that expression of p110-DN significantly decreased DNA synthesis in PDGF-stimulated PVSM cells but not in TGF-α- and thrombin-stimulated cells, which suggests that activation of class IA PI3K is critically important for PDGF-induced DNA synthesis but is less important in mediating mitogenesis induced by TGF-α and thrombin.

Although the expression of p110*-CA in human ASM cells significantly increased DNA synthesis in epidermal growth factor (EGF)-stimulated cells (23), DNA synthesis of PDGF-stimulated PVSM cells microinjected with p110*-CA was comparable with that of PDGF-stimulated cells microinjected with control plasmid. We speculate that although PDGF and EGF both activate specific receptor protein tyrosine kinases, there are differences in the mode of PI3K activation by the PDGF and EGF receptors: the activated PDGF receptor, which has two specific binding sites for the SH2 domain of class IA PI3K, recruits and activates simultaneously two molecules of PI3K and appears to be the most potent activator of the PI3K signaling pathway in many cell types (19, 35). In human PVSM cells, PDGF induces robust activation of PI3K compared with that induced by TGF-α and thrombin. Possibly, PDGF maximally activates PI3K, and the expression of p110*-CA is unable to significantly enhance DNA synthesis. The EGF receptor, which does not have binding sites for PI3K,
requires transactivation of ErbB2 to activate PI3K (4, 25). As a result, EGF induces a modest activation of PI3K, and overexpression of p110*-CA can significantly enhance EGF-induced DNA synthesis. These data suggest that activation of PI3K is growth-factor specific, and further studies are required to address the growth-factor specificity of PI3K activation.

The role of PI3K in the regulating cell motility also appears to be cell specific. Although inhibition of PI3K attenuated PDGF-induced migration in rat thoracic aorta vascular smooth muscle cells (34) and in canine pulmonary artery smooth muscle cells (46), expression of dominant-negative PI3K mutants had little effect on PDGF-induced rat aorta vascular smooth muscle cells and Chinese hamster ovary cell migration (13). PDGF-induced migration of rabbit aortic vascular smooth muscle cells also appears to be PI3K independent (6).

In our study, LY-294002 attenuated but did not completely inhibit PDGF-induced human PVSM cell migration. Furthermore, migration induced by the transient expression of constitutively active p110*-CA PI3K was less than that induced by growth-factor stimulation, which suggests that other parallel or upstream signaling pathways, in addition to PI3K activation, are important in regulating cell motility. Given our data showing that human PVSM cell motility in-

---

**Fig. 7.** LY-294002 and rapamycin inhibit PDGF-, TGF-α-, and thrombin-induced activation of p70 S6 kinase (S6K1) in PVSM cells. Confluent, growth-arrested cells were pretreated with 30 μM LY-294002 or 200 nM rapamycin for 30 min then stimulated with 10 ng/ml PDGF, 10 ng/ml TGF-α, or 1 U/ml thrombin for 20 min. Cells were lysed and cell lysates were subjected to SDS-PAGE and immunoblot analysis with phosphospecific Thr421/Ser424 (A), Thr389 (B), or S6K1 (A, B) antibodies. Examples are representative of 3 separate experiments that yielded similar results.

**Fig. 8.** LY-294002 and rapamycin inhibit PDGF-, TGF-α-, and thrombin-induced phosphorylation of ribosomal protein S6 in PVSM cells. Confluent, growth-arrested cells were preincubated with 30 μM LY-294002 or 200 nM rapamycin for 30 min before incubation with 10 ng/ml PDGF, 10 ng/ml TGF-α, or 1 U/ml thrombin for 20 min. Cell lysates were subjected to immunoblot analysis with phosphospecific S6 and S6 antibodies. Immunoblots and graphs are representative of 3 separate experiments that yielded similar results.

**Fig. 9.** Effect of LY-294002 on p42/p44 MAPK phosphorylation in PVSM cells. Growth-arrested cells were pretreated for 30 min with 0.1, 1, 10, or 30 μM LY-294002 and then stimulated with 10 ng/ml PDGF-BB, 10 ng/ml TGF-α, or 1 U/ml thrombin for 20 min. Reactions were stopped, and cell lysates were immunoblotted with phosphospecific p42/p44 MAPK antibody and detected by enhanced chemiluminescence. Experiments were performed in triplicate with similar results.
duced by mitogens is PI3K dependent, it appears that the signal transduction pathways that regulate cell migration are species and cell specific.

Few studies have examined the role of PI3K isoforms in modulating cell function in physiologically relevant cells. Our data and current evidence demonstrate that the class IA p110-α PI3K mediates human PVSM mitogenic responses (23, 30, 35, 41), whereas migratory responses are not unique to a specific PI3K isoform (21, 43). Vanhaesebroeck et al. (41) showed that microinjection of antibodies to p110-β and p110-δ in macrophages results in reduced cell migration in response to colony stimulating factor 1 (CSF-1). Other studies show that neutrophils and peritoneal macrophages deficient in p110-γ have decreased chemotaxis to formyl-methionyl-leucyl-phenylalanine and reduced capacity for migration to the peritoneum in various mouse models of peritonitis (14, 29, 38).

In our studies, we utilized the constitutively active form of class IA p110-α PI3K to demonstrate that transient expression of this PI3K isoform is sufficient for human PVSM cell migration. Based on published data and our new data, it appears that PI3K-dependent cell migration can be modulated by a specific PI3K isoform. Because all PI3K isoforms increase levels of phosphatidylinositol-3, 4, 5-trisphosphate, this phospholipid seems to play a central role in promoting cell migration. Further studies will define the role of the particular growth factor(s) and PI3K-dependent and -independent pathways required for cell migration.

Our study also shows that PDGF, TGF-α, and thrombin stimulation of human PVSM cells occurs via the PI3K-dependent activation of serine/threonine kinase Akt, S6K1, and ribosomal protein S6. Interestingly, human PVSM cell proliferation demonstrates high sensitivity to rapamycin, the specific inhibitor of S6K1, which suggests that S6K1 plays a potentially important role in human PVSM cell mitogenesis. Other studies also demonstrate that S6K1 can play a role in vascular smooth muscle cell growth by controlling protein synthesis in rat aorta (11) and pulmonary artery (8, 10, 18). Activation of S6K1 has been reported in rat balloon-injured arteries (22) and pressure-overloaded hearts (28), which suggests the significance of S6K1 as a potential therapeutic target in cardiovascular diseases. Our experiments were not designed to definitely characterize the downstream pathways of PI3K that regulate mitogen-induced human PVSM cell proliferation and migration; this will be a focus of future studies.

Our study yields novel information on the regulation of human PVSM cell proliferation and migration. Several limitations regarding our study, however, need to be addressed. A major limitation concerns the variability of transfection efficiency and the difficulty in interpreting data obtained from a mixed population of transfected and untransfected cells. The physiological relevance of overexpressing target proteins can also be called into question. It was evident that prolonged overexpression of p110-DN PI3K induced the cells to detach from the culture plates. Fortunately, our microinjection experiments decreased cell exposure time to p110-DN and yielded valuable information. We attempted to overcome these obstacles by using complementary methods such as transient transfection and microinjection techniques.

Regulation of cell proliferation and motility is a critical step in vascular remodeling. Our results demonstrate that PI3K appears to simultaneously regulate the proliferation and migration of human PVSM cells, which suggests that PI3K plays a key role in the signaling network that operates in human PVSM cells. These studies are important in fostering an understanding of the role of PI3K in regulating cell functions such as proliferation and migration. Further studies that define the precise molecular mechanisms of PI3K-dependent human PVSM cell motility and proliferation may offer a potential target in blocking development of lesions in atherosclerosis, restenosis after angioplasty and bypass surgery, and hypertension.

This work was supported by the American Heart Association (V. P. Krymskaya); the National Institutes of Health (Grants HL-55301, HL-64063, and 1-P50-HL67663 to R. A. Panettieri); Glaxo Smith Kline (R. A. Panettieri); and the National Health and Medical Research Council of Australia (C. J. Martin Fellowship 977301 to A. J. Ammit). The authors thank Dr. A. Klippel for the generously provided pCG, pCG-p110β-CA, and pCG-p110-DN plasmids. Present address for A. J. Ammit: Respiratory Research Group, Faculty of Pharmacy, University of Sydney, NSW 2006 Australia.

REFERENCES