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PDGF induces SphK1 expression via Egr-1 to promote pulmonary artery smooth muscle cell proliferation

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Sysol JR, Natarajan V, Machado RF. PDGF induces SphK1 expression via Egr-1 to promote pulmonary artery smooth muscle cell proliferation. Am J Physiol Cell Physiol, 310: C983–C992, 2016. First published April 20, 2016; doi:10.1152/ajpcell.00059.2016.—Pulmonary arterial hypertension (PAH) is a progressive, life-threatening disease for which there is currently no curative treatment available. Pathologic changes in this disease involve remodeling of the pulmonary vasculature, including marked proliferation of pulmonary artery smooth muscle cells (PASMCs). Recently, the bioactive lipid sphingosine-1-phosphate (SIP) and its activating kinase, sphingosine kinase 1 (SphK1), have been shown to be upregulated in PAH and promote PASMC proliferation. The mechanisms regulating the transcriptional upregulation of SphK1 in PASMCs are unknown. In this study, we investigated the role of platelet-derived growth factor (PDGF), a PAH-relevant stimuli associated with enhanced PASMC proliferation, on SphK1 expression regulation. In human PASMCs (hPASMCs), PDGF significantly increased SphK1 mRNA and protein expression and induced cell proliferation. Selective inhibition of SphK1 attenuated PDGF-induced hPASMC proliferation. In silico promoter analysis for SphK1 identified several binding sites for early growth response protein 1 (Egr-1), a PDGF-associated transcription factor. Luciferase assays demonstrated that PDGF activates the SphK1 promoter in hPASMCs, and truncation of the 5′-promoter reduced PDGF-induced SphK1 expression. Stimulation of hPASMCs with PDGF induced Egr-1 protein expression, and direct binding of Egr-1 to the SphK1 promoter was confirmed by chromatin immunoprecipitation analysis. Inhibition of ERK signaling prevented induction of Egr-1 by PDGF. Silencing of Egr-1 attenuated PDGF-induced SphK1 expression and hPASMC proliferation. These studies demonstrate that SphK1 is regulated by PDGF in hPASMCs via the transcription factor Egr-1, promoting cell proliferation. This novel mechanism of SphK1 regulation may be a therapeutic target in pulmonary vascular remodeling in PAH.

gene expression; PDGF; PASMC; Egr1; SphK1; proliferation

PULMONARY arterial hypertension (PAH) is a severe disease of multiple etiologies characterized by increases in pulmonary vascular resistance (PVR) primarily due to uncontrolled vascular remodeling, sustained vasoconstriction, or thrombosis in situ (12, 24). The precise mechanisms of these disease processes are poorly understood and few treatments are available (30). Remodeling of small pulmonary arteries is a hallmark of PAH, with human pulmonary artery smooth muscle cell (hPASMC) hyperproliferation and apoptosis resistance contributing to vessel obstruction and impaired blood flow (54). Understanding the mechanistic regulation of hPASMC proliferation in PAH is critical for developing novel therapeutics. With the discovery that some familial and sporadic cases of PAH arise from mutations in members of the transforming growth factor beta (TGF-beta) cell-signaling superfamily, including the bone morphogenetic protein type II receptor (BMPR2) gene (25), the role of different growth factors in disease pathogenesis has been explored. One of these growth factors, platelet-derived growth factor (PDGF), has been demonstrated to contribute to pulmonary vascular remodeling in human PAH and experimental models and is a potential therapeutic target (42, 47).

PDGF is a mitogenic and promigratory stimulus for hPASMCs with both autocrine and paracrine functions (6, 42, 53). Many cell types in the lung can synthesize PDGF, including vascular smooth muscle cells and endothelial cells, and it has been shown to be induced in alveolar hypoxia, causing vascular remodeling in lung parenchyma (5). Five ligand isoforms of PDGF are known (PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD) along with two distinct receptor tyrosine kinase isotypes (PDGFR-α and PDGFR-β). The PDGF-BB isoform, which can bind to both PDGFR-α and PDGFR-β, is released at sites of vascular injury by endothelial cells and platelets and is a potent stimulus for hPASMC proliferation and migration (31, 42). Activation of PDGFRs leads to receptor dimerization, autophosphorylation, and subsequent signal transduction mainly via the mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) cascade, resulting in targeted gene transcription that promotes cell proliferation, migration, and differentiation, and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, promoting cell survival (35). Recent studies have implicated PDGF and its receptors in both lung development and disease, including lung cancer, lung fibrosis, and PAH (3, 10, 23, 35). The circulating concentration of PDGF has been shown to be significantly elevated in patients with PAH (48), and concentrations within the pulmonary vasculature environment are likely to be much greater since small remodeled pulmonary arteries of PAH patients have increased PDGF and PDGFRs expression in the hPASMCs and pulmonary artery endothelial cells (PAECs) (42). In addition, inhibition of PDGFR-β using
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PDGF increases SpbhK1 expression in hPASMCs. Given the upregulation of both SpbhK1 and PDGf in PAH and their association with pulmonary vascular remodeling, we first tested whether PDGF can induce SpbhK1 expression in hPASMCs. Following stimulation of hPASMCs with PDGF-BB (20–100 ng/ml, 0–6 h), SpbhK1 protein was increased by ~1.5-fold at 6 h measured by Western blotting and normalized to β-actin expression (Fig. 1, A and B). PDGF did not increase SpbhK2 expression in hPASMCs (data not shown). Next, quantitative real-time RT-PCR was used to

the specific tyrosine kinase inhibitor, imatinib (STI-571), has been shown to reverse vascular remodeling in severe experimental pulmonary hypertension (PH) (47). The therapeutic use of imatinib in refractory PAH has also been reported in several clinical case reports, leading to improvements in exercise capacity, hemodynamics, and functional class (17, 41). The precise mechanisms by which PDGF-BB stimulates hPASMC proliferation and vascular remodeling are not fully understood. CROSSTALK BETWEEN PDGF/PDGFR SIGNALING AND SPHINGOLIPID SIGNALING HAS BEEN DEMONSTRATED IN NUMEROUS CELL TYPES, INCLUDING AIRWAY SMOOTH MUSCLE CELLS, WHERE PDGFRs can be trans-activated by the bioactive lipid, sphingosine-1-phosphate (SIP), to elicit pro-proliferative and pro-migratory signaling (6a, 34a, 52a). Interestingly, PDGF has also been shown to stimulate activity of sphingosine kinase 1 (SpbhK1), the lipid kinase that generates SIP, and increase intracellular SIP 1 in fibroblasts, while competitive inhibition of SpbhK1 prevents PDGF-induced cell proliferation (36, 43). PDGF has also been shown to increase expression of SpbhK1 in coronary artery smooth muscle cells (14). We recently reported that levels of SIP and SpbhK1 are elevated in patients with PAH and promote hPASMC proliferation (9). In addition, genetic deficiency or pharmacologic inhibition of SpbhK1 protects from the development of experimental PH in several rodent models (9). The mechanisms of SpbhK1/SIP upregulation and their role in pulmonary vascular remodeling in PAH are still largely unknown.

In this study, we investigated the mechanisms controlling SpbhK1 expression in PASMCs which may contribute to cell proliferation. We found that PDGF-induced SpbhK1 expression and PASMC proliferation is mediated in part by activation of the Egfr-1 transcription factor.

MATERIALS AND METHODS

Materials. Recombinant human PDGF-BB was purchased from Sigma Aldrich (St. Louis, MO). The primary antibodies for SpbhK1, Egfr1, Lamin B1, Erk1/2, phospho-Erk1/2, and HRP-conjugated β-Actin, and secondary anti-rabbit IgG HRP-linked antibody were purchased from Cell Signaling Technology (Danvers, MA). U0126 was purchased from Cell Signaling Technology. Pf-543 was purchased from Cayman Chemical Company (Ann Arbor, MI).

Cell culture and treatments. Primary human pulmonary artery smooth muscle cells (hPASMCs) were purchased from Lonza (Allendale, NJ) and used for all cell studies. Cells were cultured in a humidified atmosphere with 5% CO2 at 37°C in Medium-199 supplemented with 10% FBS and penicillin-streptomycin antibiotics. All studies were conducted from passages 4–8. For treatment studies, subconfluent hPASMCs plated in multi-well plates were subjected to serum deprivation and stimulation with PDGF-BB (20–100 ng/ml) before collection and analysis. The dose of PDGF-BB used was based on prior publications (20, 47). In some studies, pretreatment with the chemical inhibitor U0126 (10 μM) or Pf-543 (100 nM) for 1 h was used before PDGF-BB stimulation. Further details of treatment timing are provided in the respective figure legends. The NE-PER kit from Thermo Fisher Scientific was used for nuclear and cytoplasmic extractions.

Cell proliferation assays. Cell proliferation was determined using a 5-bromo-2′-deoxyuridine (BrdU) incorporation assay from Calbiochem (San Diego, CA) per manufacturer’s instructions in a 96-well format. Starting cell densities of 4,000 cells/well were used.

Chromatin immunoprecipitation (ChIP) assays. ChIP studies were done using the SimpleChIP Plus Magnetic Bead ChIP kit purchased from Cell Signaling Technology (Danvers, MA) per manufacturer’s instructions. In brief, cross-linking was completed after cell stimulation, followed by nuclei preparation and chromatin digestion. DNA gel electrophoresis was used to confirm adequate digestion. ChIP was then performed using the Egfr1, positive control Histone H3, and negative control normal rabbit IgG antibodies. Elution of chromatin from antibody/beads and reversal of crosslinks was performed. DNA was purified and analyzed by both standard PCR and quantitative real-time PCR. Primers used to amplify the Egfr1-B binding site were forward 5′-GGCCTGTGGCCTGCTAC-3′ and reverse 5′-CCAGCTTCCCTCTTCTTC-3′.

Promoter analysis. Putative Egfr1 binding sites were identified within the proximal ~2 kb promoter of SpbhK1 using Genomatix software (similarity threshold > 0.95). The identified binding sites were confirmed in the public ENCODE ChIP-seq database (44).

Transfection and luciferase assays. For promoter studies using the Gaussia Luciferase (Gluc) and Secreted Alkaline Phosphatase (SEAP) system, a human SpbhK1 promoter reporter clone was purchased from GeneCopoeia (Rockville, MD). Activities of Gluc/SEAP were analyzed using the Secretome-Pair Dual Luminescence Assay Kit (GeneCopoeia) per manufacturer’s guidelines, using a GloMax luminometer (Promega). For promoter deletion studies, fragments of the SpbhK1 promoter were amplified by PCR, purified, and ligated into pGL4.10[luc2] promoterless luciferase reporter vectors purchased from Promega (Madison, WI). The hRluc Renilla luciferase reporter vector pGL4.74[hRluc/TK] (Promega) was used as a transfection normalization control in these studies. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega). FuGENE HD transfection reagent (Promega) was used for all vector transfections in hPASMCs per manufacturer’s guidelines. For siRNA experiments, ON-TARGETplus siRNAs specific for Egfr1 and non-targeting control were purchased from GE Dharmacon (Lafayette, CO). Transfection of siRNAs (50 nM) was completed using Lipofectamine RNAiMAX reagent per manufacturer’s instructions (Thermo Fisher Scientific). Results are representative of at least three independent experiments.

Western blotting analysis. Solubilized protein lysates isolated from hPASMCs after stimulations were used for Western blotting. Cells were lysed using RIPA buffer (Sigma) supplemented with protease and phosphatase inhibitor cocktails (Calbiochem) and protein quantification and Western blot analysis were performed according to standard procedures.

RNA extraction and quantitative real-time PCR analysis. Total RNA was isolated from hPASMCs using the RNeasy Mini kit from Qiagen (Valencia, CA) and reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Expression of SpbhK1 mRNA was determined using a TaqMan primer assay with GAPDH used as an internal control (Thermo Fisher Scientific). Relative changes in mRNA expression were calculated using the comparative Ct method.

Statistical analysis. Results are shown as means ± SE from at least three experiments and statistical significance was calculated with Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001 vs. controls) using GraphPad Prism software.

RESULTS

PDGF increases SpbhK1 expression in hPASMCs. Given the upregulation of both SpbhK1 and PDGF in PAH and their association with pulmonary vascular remodeling, we first tested whether PDGF can induce SpbhK1 expression in hPASMCs. Following stimulation of hPASMCs with PDGF-BB (20–100 ng/ml, 0–6 h), SpbhK1 protein was increased by ~1.5-fold at 6 h as measured by Western blotting and normalized to β-actin expression (Fig. 1, A and B). PDGF did not increase SpbhK2 expression in hPASMCs (data not shown). Next, quantitative real-time RT-PCR was used to

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measure SphK1 mRNA following stimulation of hPASMCs with PDGF-BB (20 ng/ml, 0–24 h). SphK1 mRNA normalized to GAPDH expression was significantly increased at 1 and 3 h, and expression returned to baseline levels by 24 h (Fig. 1C). These findings demonstrate the role of PDGF-BB in SphK1 upregulation in hPASMCs.

**PDGF activates the SphK1 promoter in hPASMCs.** To investigate the mechanism(s) by which PDGF could induce activation of the SphK1 promoter using several approaches. First, a commercially available dual reporter construct was used to express and secrete Gaussia Luciferase (GLuc) under control of the ~1.3-kb upstream human SphK1 promoter sequence, with Secreted Alkaline Phosphatase (SEAP) used as a control. PDGF-BB (20–100 ng/ml) stimulated SphK1 promoter activity at 1.5 and 24 h, as measured by quantification of relative luminescence of GLuc/SEAP in the culture media (Fig. 2A). To confirm these findings, the ~2.1-kb human SphK1 promoter was isolated via PCR and cloned into the pGL4 luciferase reporter vector. Stimulation of hPASMCs transfected with this vector with PDGF-BB (20 ng/ml, 1.5 h) resulted in a 2.5-fold increase in transcriptional activity over basal levels (Fig. 2B). The empty control vector was not activated under basal or PDGF-BB stimulated conditions.

Next, in silico analysis of the SphK1 promoter was conducted to identify transcription factor binding sites that may mediate PDGF-induced SphK1 expression. Three highly predicted binding sites for early growth response protein 1 (Egr-1), a transcription factor by which PDGF is known to signal intracellularly (45), were identified within the SphK1 promoter (labeled EGR1-"A", "B", and "C") (Fig. 2C). These three binding sites were also observed in the public ENCODE ChIP-seq database (44). To determine whether the promoter regions containing these Egr-1 binding sites were functionally important in PDGF-mediated SphK1 activation, truncated promoter fragments containing the sites were amplified by PCR and cloned into the pGL4 luciferase reporter vector, as depicted in Fig. 2C. hPASMCs were transfected with these vector constructs and stimulated with PDGF (20 ng/ml, 1.5 h). Truncations that excluded binding sites for EGR1-"B" and EGR1-"C" demonstrated a significant loss in SphK1 promoter activity, with exclusion of the EGR1-"B" site fragment showing the largest decrease in activity (Fig. 2D). These results suggest that Egr-1 binding to the SphK1 promoter may be important for PDGF-induced transcriptional activation.

**PDGF increases nuclear expression of Egr-1 in hPASMCs.** Since Egr-1 transcription factor binding sites may play a role in PDGF-induced SphK1 activation, we tested whether PDGF-BB could increase Egr-1 expression in hPASMCs. Stimulation with PDGF-BB (20–100 ng/ml, 1–3 h) resulted in a dramatic increase in Egr-1 protein expression by 1 h that subsided by 3 h (Fig. 3A). PDGF-BB also significantly increased Egr-1 mRNA levels, with maximum expression observed at 1 h (Fig. 3B). The basal expression of Egr-1 protein expression was also measured in three hPASMC lines derived from PAH patients, and no basal differences were observed compared with control PASMCs (data not shown). Although higher circulating PDGF concentrations within the pulmonary vascular microenvironment of PAH patients have been demonstrated (48) which may lead to elevations in Egr-1 expression, this elevation may not be retained following isolation of PASMCs and culturing in vitro.

Egr-1 has a bipartite nuclear localization domain allowing for nuclear expression that is pivotal for its functional role as a transcription factor (16, 39), although Egr-1 expression in the cytoplasm has also been reported in some cancer cell lines (32). We next tested whether PDGF-induced Egr-1 expression was localized in the nucleus. Stimulation with PDGF-BB (20 ng/ml, 0.5–3 h) resulted in abundant and transient nuclear Egr-1 protein expression that was maximal at 1 h (Fig. 3C), with Lamin B1 used as a nuclear loading control. These results show upregulation of Egr-1 by PDGF in hPASMCs.
PDGF induces Egr-1 binding to the SphK1 promoter in hPASMC. Regions of the SphK1 promoter containing both EGR1-“B” and EGR1-“C” transcription factor binding sites are important for PDGF-induced SphK1 expression, with loss of the EGR1-“B” resulting in the greatest reduction in expression (Fig. 2D). Given these results, we sought to determine whether PDGF could induce direct binding of Egr-1 to the EGR1-“B” predicted site within the SphK1 promoter. Following stimulation of hPASMCs with PDGF-BB (20–100 ng/ml, 1 h), chromatin immunoprecipitation (ChiP) studies coupled with quantitative real-time PCR demonstrated significantly increased binding of Egr-1 protein to the EGR1-“B” DNA binding site within the SphK1 promoter (Fig. 4A). Optimal chromatin shearing was achieved for these studies as demonstrated in Fig. 4B. To confirm these findings, standard PCR was used to amplify the EGR1-“B” binding site following immunoprecipitation with Egr-1 in hPASMCs treated with PDGF-BB (20–100 ng/ml, 1 h). DNA gel electrophoresis for these studies demonstrated increased Egr-1 protein binding to the EGR1-“B” site within the SphK1 promoter relative to IgG controls, (Fig. 2A).
PDGF promotes SphK1 expression and hPASMC proliferation via EGR1. PDGF-BB (20–100 ng/ml, 48 h) increases proliferation of hPASMCs (Fig. 5A). Since PDGF also enhances Egr-1 transcription factor expression, SphK1 expression, and binding of Egr-1 to the Sphk1 promoter, we aimed to determine whether silencing of Egr-1 could prevent PDGF-induced SphK1 expression. SiRNA-mediated silencing of Egr-1 in hPASMCs resulted in 80% reduction in basal Egr-1 protein expression after 48 h (Fig. 5, B and C). Egr-1 silencing also prevented induction of Egr-1 expression by PDGF-BB (20 ng/ml, 1 h) (Fig. 5, B and C).

Uncontrolled proliferation of PASMCs contributes to pulmonary vascular remodeling in PAH, but the mechanisms regulating the proliferative phenotype of these cells are poorly understood. Therefore, we next tested whether silencing of Egr-1 could alter proliferation of hPASMCs. Compared with scrambled siRNA controls, silencing of Egr-1 decreased basal and PDGF-induced (20 ng/ml, 48 h) proliferation of hPASMCs as measured by relative BrdU incorporation (Fig. 5D). In addition, silencing of Egr-1 attenuated PDGF-induced (20 ng/ml, 6 h) expression of SphK1 in hPASMCs (Fig. 5, E and F). Next, we used the highly specific SphK1 inhibitor, PF-543, to assess the importance of SphK1 in mediating PDGF-induced proliferation. Treatment of hPASMCs with PF-543 (100 nM, 1 h pretreatment) did not alter basal hPASMC proliferation, but significantly reduced PDGF-induced (20 ng/ml, 48 h) proliferation (Fig. 5G). These results demonstrate the critical importance of the Egr-1/SphK1 signaling axis in the induction of hPASMC proliferation by PDGF.

PDGF increases Egr-1 and SphK1 in hPASMCs via ERK. Previous studies have identified the importance of the MAPK/ERK cascade in cell proliferation (9), Egr-1 activation (26), and PDGF-induced cell signaling (53). Here we explored whether MAPK/ERK signaling could be an upstream mediator of PDGF-induced EGR1 activation in hPASMCs. Treatment of hPASMCs with PDGF-BB (20 ng/ml) resulted in enhanced ERK1/2 phosphorylation at 15 min (Fig. 6, A and B). Inhibition of ERK phosphorylation with U0126 (10 μM, 1 h pretreatment), a highly selective inhibitor of MAPK/ERK kinases MEK1 and MEK2, resulted in reduced basal and PDGF-induced (20 ng/ml, 48 h) hPASMC proliferation (Fig. 6C) and significant attenuation of PDGF-induced (20 ng/ml, 1 h) Egr-1 protein expression in hPASMCs (Fig. 6, D and E). U0126 (10 μM) also inhibited induction of downstream SphK1 expression by PDGF (20 ng/ml, 6 h) (Fig. 6, F and G). These results demonstrate the critical importance of MAPK/ERK signaling.
in PDGF-induced proliferation and Egr-1 and SphK1 expression in hPASMCs.

**DISCUSSION**

The mechanistic processes contributing to PASMC proliferation and the development of pulmonary vascular remodeling in PAH are poorly understood. Given the important role of both PDGF and SphK1/S1P in the pathobiology of PAH (9, 42), we investigated whether PDGF-induced proliferation of hPASMC involves induction of SphK1 expression and potential mechanisms by which this may occur. In this study, we demonstrate that 1) PDGF induces SphK1 and Egr-1 transcription factor expression in hPASMCs, 2) PDGF-mediated SphK1 transcriptional activity is mediated through binding of Egr-1 to the proximal SphK1 promoter, 3) Egr-1 and SphK1 are important for PDGF-induced hPASMC proliferation, 4) downregulation of Egr-1 attenuates PDGF-induced expression of SphK1 in hPASMC, and 5) MAPK/ERK phosphorylation is critical for induction of Egr-1 and SphK1 by PDGF. Our data describe a novel mechanism contributing to PASMC proliferation in PAH and identify the PDGF/EGR1/SphK1 pathway as a potential therapeutic target.

PDGF-BB is released by endothelial cells and platelets during vascular injury and can stimulate hPASMC proliferation and migration (31, 42). The role of PDGF and its receptors in the pathobiology of PAH is well established, and the utility of pharmacologically targeting this pathway in PAH has been proposed (3, 42). One recent study found that in a rodent model of chronic hypoxia-mediated PH, mice genetically engineered with constitutively active PDGFR-β expression developed more severe pulmonary vascular remodeling than with chronic hypoxia alone (10). PDGF receptor antagonism using imatinib has also been shown to reverse advanced pulmonary vascular disease in several animal models of PH, including reversal of...
vascular remodeling and cor pulmonale (47). Clinically, the use of imatinib in several cases of refractory PAH has led to improvements in exercise capacity, hemodynamics, and functional class (17, 41). Here, we demonstrate that PDGF can stimulate SphK1 expression in hPASMCs and that regions of the proximal SphK1 promoter are important for this induction. In other cell types, PDGF-induced SphK1 activity has been shown to increase intracellular S1P levels and is important in mediating proliferation, due in part to the ability of S1P to mobilize calcium and enhance levels of mitogenic phosphatidic acid (36, 43, 55). In addition, silencing of SphK1 in mouse embryonic fibroblast cells reduces PDGF-induced migration (18). We recently reported that levels of both SphK1 and S1P are elevated in patients with PAH and in rodent models of experimental PH, and that SphK1/S1P promote hPASMC proliferation (9). Genetic deletion or pharmacologic inhibition of SphK1 also protects from the development of experimental PH in several rodent models (9). The present data indicate the importance of PDGF signaling in activating SphK1 in hPASMCs, mechanistically linking these important pathways in PAH which contribute to vascular remodeling.

The SphK1 promoter deletion and ChIP analyses identified the importance of Egr-1 in mediating PDGF-induced SphK1 expression via nuclear translocation and binding to the proximal promoter. Egr-1 is a highly conserved, Cys2His2 type zinc-finger transcription factor known to be induced by a variety of stimuli such as oxidative stress, shear stress, and growth factors, including PDGF (8, 22, 29, 38). In patients with both congenital heart disease–associated PAH and idiopathic PAH, Egr-1 expression is abundant in plexiform lesions and in smooth muscle cells in vessels with severe concentric intimal fibrosis (51). Enhanced expression of Egr-1 has also been shown in the pulmonary vascular smooth muscle cell layer in human PAH and in a severe MCT-induced PAH rat model, which directly correlated with the degree of pulmonary vascular remodeling (11). Expression of Egr-1 has also been shown to be increased in the lung and pulmonary vascular cells in response to hypoxia, where it can activate several downstream targets involved in vascular remodeling in PAH (4, 11). Interestingly, in a rat model of flow-associated PAH in rats, downregulation of Egr-1 in vivo led to reduced expression of vascular PDGF-BB, less vascular proliferation, and increased apoptosis (11).

Here, we report that Egr-1 expression is rapidly induced in the nucleus of hPASMCs following PDGF stimulation, and that activation of Egr-1 is important for PDGF-induced SphK1

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Fig. 6. ERK phosphorylation is required for PDGF-induced EGR1 and SphK1 expression in hPASMC. A and B: representative Western blotting images and ERK-normalized quantification of protein levels demonstrate induction of ERK phosphorylation by PDGF (20 ng/ml, 15 min) in hPASMC. C: U0126-mediated inhibition of ERK phosphorylation reduces basal hPASMC proliferation and attenuates PDGF-induced (20 ng/ml, 48 h) proliferation. D and E: representative Western blotting images and β-actin-normalized quantification of protein levels demonstrate U0126-mediated inhibition of ERK phosphorylation attenuates EGR1 induction by PDGF (20 ng/ml, 1 h) in hPASMC; and attenuates SphK1 induction by PDGF (20 ng/ml, 6 h) in hPASMC (F and G). Results are shown as means ± SE from at least 3 experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. untreated control unless otherwise indicated.
expression and cell proliferation. Using a specific SphK1 inhibitor, PF-543, we also demonstrate that SphK1 is involved in mediating PDGF-induced hPASMC proliferation. PF-543 did not alter basal proliferation, confirming previous findings that this drug does not inhibit DNA synthesis in hPASMCs (7). Our studies are also consistent with reports in other cell types that induced expression of Egr-1 is important in the control of cell proliferation, survival, and angiogenesis (40, 52). In addition to the novel activation of SphK1 by Egr-1 in hPASMCs presented here, the role of Egr-1 in activating other genes involved in regulation of vascular proliferation, inflammation, and apoptosis, including PDGF, TGF-β, IL-6, and p53, have been reported (11). These findings collectively highlight the importance of Egr-1 in key components of vascular remodeling, and suggest that targeting this pathway may be therapeutically beneficial in PAH. Several putative Egr-1 binding sites were identified within the SphK1 promoter, and this study explored the role of the EGR1-“B” site in detail due to the importance of the promoter region containing this site in SphK1 transcriptional activation. Future studies investigating the other potential Egr-1 binding sites may be important in determining the mechanistic regulation of SphK1 in other cell types or in response to other stimuli that activate Egr-1.

To further explore the mechanisms of PDGF-induced Egr-1 and downstream SphK1 expression in hPASMCs, we investigated the influence of the MAPK/ERK signaling pathway. We demonstrate that ERK1/2 phosphorylation is important for basal and PDGF-induced hPASMC proliferation and is critical for activation of Egr-1 and SphK1 expression by PDGF. The U0126 inhibitor used in these studies acts directly upon MEK1/2, so is highly specific in blocking ERK1/2 phosphorylation. The other MAPKs JNK and p38 are phosphorylated by MEK4/7 and MEK3/6, respectively (27). Our results demonstrate a nearly complete loss of PDGF-induced Egr-1 expression with U0126, suggesting that JNK and p38 are less involved in this signaling pathway in hPASMCs. Importantly, several supportive lines of evidence from the literature have demonstrated that inhibition of ERK1/2 phosphorylation can reduce hPASMC proliferation induced by a variety of stimuli in vitro. For example, pro-proliferative effects of hypoxia and brain-derived neurotrophic factor (BDNF) in hPASMCs have been attenuated using U0126 (28, 49). Another recent study demonstrated that both U0126 and inhibition of PDGF receptor signaling blocked peroxynitrite-induced proliferation and ERK1/2 phosphorylation in hPASMC (1). These studies highlight the multifaceted mechanisms by which ERK1/2 may regulate hPASMC proliferation and support our findings that downstream targets of ERK1/2 are involved in this process. Experiments to more precisely characterize the role of other downstream targets of ERK1/2 in PDGF-induced PASMC proliferation may be relevant to PAH pathology and could be explored in future studies.

The role of MAPK/ERK-dependent activation of Egr-1 by numerous stimuli, including PDGF, has been described in other vascular smooth muscle cell types (21, 22, 29). In mouse macrophages, inhibition of in ERK1/2 phosphorylation and activation resulted in reduced Egr-1-induced tissue factor (TF) expression. Lysoosphatidic acid-induced Egr-1 expression has also been shown to be dependent on the MEK/ERK and JNK cascades (26). Importantly, increased levels of ERK phosphorylation have been demonstrated in mouse and rat lung tissues and in the medial layer of vascular lesions in several models of experimental PH (2, 33, 47). Administration of imatinib in a monocrotaline-induced PH rat model strongly inhibited phosphorylation/activation of both PDGFR and ERK1/2 and reversed PH development (47), demonstrating an important relationship between PDGF and ERK signaling.

Notably, Egr-1 is also known to enhance expression of PDGFR (11), and S1P can induce mRNA and protein expression of PDGF-A and -B in vascular smooth muscle cells and neointimal cells from injured arteries (50). We therefore hypothesize that signaling of PDGF/ERK/Egr-1 in hPASMCs may create a positive feedback loop to generate additional PDGF/PDGFR. This would then lead to increased expression of SphK1 and other Egr-1 target genes involved in pulmonary vascular remodeling. Interestingly, the role of SphK1/S1P signaling as a positive regulator of Egr-1 has also been reported in several other cell types (15, 19, 46). Since our studies indicate that Egr-1 can activate expression of SphK1, which produces S1P, a positive feedback loop may be formed by the generation of additional Egr-1 expression. However, this loop may not be evident in cultured PASMCs since a biphasic induction of Egr-1 expression following PDGF stimulation has not been observed at time points as late as 48 h in our studies (data not shown). Future mechanistic studies to explore additional directions of the SphK1 activation pathway in PASMCs are warranted.

The process of pulmonary vascular remodeling in PAH also involves dysfunction and activation of other cell types besides smooth muscle, including endothelial cells and fibroblasts (34).
During end-stage disease in a rat model of flow-associated PAH, Egr-1 expression was shown to be increased in both the medial smooth muscle and endothelial vessel layers (11). This study also demonstrated that downregulation of Egr-1 increased vascular cell apoptosis, predominantly in the endothelial layer (11). While the present study focused on the proliferative phenotype of PASMCs, the role of PDGF activation of SphK1 in these other cells types and their crosstalk with PASMCs warrants further investigation.

Vascular smooth muscle cells, including PASMCs, have high plasticity compared with terminally-differentiated skeletal or cardiac muscle cells (37). Various environmental stimuli, including growth factors and changes in oxygen tension, can cause PASMCs to undergo a transition from a quiescent to highly proliferative phenotype (37). These changes are apparent under pathological conditions in PAH, where PASMCs demonstrate an increased proliferation and migration rate due in part to increases in cytosolic calcium concentration, ultimately leading to vessel obstruction and enhanced pulmonary vascular resistance (13, 34). Here, we have demonstrated that PDGF-induced ERK/Egr-1 signaling is a novel pathway in hPASMCs that enhances SphK1 expression and cell proliferation, as outlined in Fig. 7. These studies advance our understanding of the ability of external stimuli in PAH to upregulate SphK1 expression and highlight the therapeutic potential of targeting this pathway in PAH to prevent or reverse pulmonary vascular remodeling.

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REFERENCES


