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Original Article

Hypoxic upregulation of preproendothelin-1 gene expression is associated with protein tyrosine kinase-PI3K signaling in cultured lung vascular endothelial cells

Jianliang Zhang[1], Vikram M. Narayan[1], Noah Juedes[1], Jawaharlal M. Patel[1,2]

[1]Department of Medicine, University of Florida College of Medicine; and [2]Research Service, Malcom Randall Department of Veterans Affairs Medical Center, Gainesville, FL 32608-1197, USA

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Abstract: Hypoxia-increased endothelin-1 (ET-1) expression contributes to vasoconstriction and vessel wall thickening, often seen in the progression of pulmonary hypertension. We sought to investigate whether hypoxic modulation of preproET-1 transcription is associated with protein tyrosine kinase and phosphatidylinositol-3-kinase (PI3K). ET-1 is predominantly produced in and secreted from the vascular endothelium. Cultured human pulmonary artery endothelial cells (PAEC) in basic medium EBM-2 were exposed to hypoxia (1% oxygen, 5% CO_2, 37°C) or normoxia (room air containing 5% CO_2) for 0-48 hr. RNA was extracted from the treated cells and subjected to quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Hypoxia increases the relative levels of steady-state preproET-1 mRNA. The results of actinomycin D chase studies suggest that hypoxia-increased levels of preproET-1 mRNA are unlikely to be caused by increased RNA stability. A modified nuclear run-on method coupled with the sensitive qRT-PCR technique was used to assess preproET-1 gene transcription. The synthesis rate of preproET-1 mRNA in the cells exposed to hypoxia is higher than that in normoxic cells. The inhibitors of protein tyrosine kinases and PI3K, genistein and PI3Kγ inhibitor II, were used to elucidate the role of protein tyrosine kinase and PI3K in hypoxic modulation of preproET-1 expression. Pre-incubation of human PAEC with genistein or PI3Kγ inhibitor II abolishes hypoxia-increased levels of preproET-1 mRNA. Our observations support the notion that hypoxia increases the level of preproET-1 mRNA through upregulation of RNA synthesis, which is associated with protein tyrosine kinase- and PI3K-mediated signal transduction pathways. This implies that therapeutic interventions targeting protein tyrosine kinases and/or PI3K might be used to treat hypoxic pulmonary hypertension.

Key words: Preproendothelin-1 gene (PreproET-1), protein tyrosine kinase (PI3K), lung vascular endothelial cells, hypoxia, signal transduction

Introduction

Overproduction of endothelin-1 (ET-1) contributes to the pathophysiology of hypoxic pulmonary hypertension (PH). Exposure of humans and animals to reduced oxygen concentrations leads to increased ET-1 levels. For instance, hypoxia is associated with increased levels of human plasma ET-1 [1]. Chronic alveolar hypoxia is present in patients with chronic obstructive pulmonary diseases (COPD). A subset of COPD patients develop PH, which is correlated with elevated levels of plasma ET-1 [2]. Exposure of rats to hypoxia results in ET-1 elevation in the lungs [3].

ET-1, a 21-residue peptide, is the most potent endogenous vasoconstrictor. ET-1 induces contraction of pulmonary arteries derived from hypoxic rats [4]. In addition, ET-1 has been shown to modulate multiple cell functions including proliferation in the development of PH [5,6]. ET-1 modulates vascular tone and cell proliferation via interaction with endothelin receptors type A and type B (ETA and ETB). Endothelin receptor antagonists have been developed to treat PH patients [7].

Hypoxic upregulation of preproET-1 transcription can contribute to elevation of plasma ET-1

peptides. Hypoxic exposure increases the levels of preproET-1 mRNA in the lungs of rats and sheep [3,8]. Immunohistochemical examination suggested a hypoxia-induced increase in the levels of preproET-1 mRNA in the endothelium of pulmonary arteries [9]. The vascular endothelium is a primary site for the synthesis and secretion of ET-1, while lung endothelial cells make up the major portion of total vascular endothelium. Hypoxia has been reported to increase ET-1 expression in bovine coronary artery and human microvascular endothelial cells [10,11], but decrease its expression in rat lung endothelial cells [12]. The reported observations imply that endothelial cells derived from different species and/or vascular beds may respond to low oxygen tensions in a different manner. Determining the effects of hypoxia on ET-1 expression in human pulmonary artery endothelial cells (PAEC) will help us better understand the role of the endothelium in hypoxic vasoconstriction and remodeling of large vessels.

Hypoxia-increased stability and/or synthesis of preproET-1 mRNA may contribute to elevated steady-state levels of preproET-1 mRNA. For instance, hypoxia stimulates promoter activity of the *preproET-1* gene in cultured microvascular endothelial cells [13]. Signaling events that transmit hypoxic stimulation to modulation of ET-1 expression in human PAEC are unclear. However, tyrosine kinase-PI3K cascades may contribute to hypoxic modulation of ET-1 expression. For instance, tyrosine kinase transactivation is essential for Hypoxia-Inducible Factor (HIF)-1α translation through a PI3K/p70S6 kinase pathway in vascular smooth muscle cells [14]. A tyrosine kinase inhibitor, genistein, prevented hypoxia-stimulated production of plasminogen activator inhibitor-1 in bovine aortic endothelial cells [15]. Hypoxia stimulates the transcription of the endothelial receptor tyrosine kinase tie-1 in endothelial cells [16].

The aims of the present studies are to investigate 1) whether hypoxia modulates preproET-1 transcription in human PAEC, and 2) whether hypoxic upregulation of preproET-1 expression is associated with protein tyrosine kinase-PI3K signaling.

Materials and methods

Chemicals

Genistein and PI3Kγ inhibitor II were purchased from Calbiochem (San Diego, CA). All other chemicals were obtained from Fisher Scientific (Orlando, FL).

Cell culture

Human PAEC (Cat# cc-2530, Lonza) were used as a cell model to determine hypoxic modulation of preproET-1 expression. PAEC were cultured in EGM-2 medium containing growth factors, fetal bovine serum, and antibiotics.

Exposure of PAEC to hypoxia

The cells on dishes containing fresh EBM-2 medium were placed in a modular incubator chamber (Billups-Rothenberg). The exposure chamber was connected to a gas cylinder containing 1% oxygen, 5% CO_2, and balanced nitrogen. The cells were exposed to the 1% oxygen gas mixture at 37°C for 6-48 hr. Control cells were exposed to room air containing 5% CO_2 in an incubator for 6-48 hr. The cells were incubated in serum-free medium to induce the quiescent status. This could reduce the effects of proliferation on preproET-1 expression due to loss of cell contact inhibition caused by subconfluence and/or cell detachment under hypoxic conditions. After hypoxic exposure, morphology of the monolayers was assessed by phase contrast microscopy as described earlier [17] to monitor hypoxia-induced cell injury or death. Some floating cells were observed 24-48 hr post exposure. The dishes were rinsed to discard the floating cells, and only attached cells were harvested and analyzed.

Assessment of the relative levels of preproET-1 mRNA using quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the Pure-link RNA purification system by following the manufacturer's instructions. Briefly, after hypoxic exposure, the monolayers of human PAEC were rinsed with cold phosphate-buffered saline, and 0.3 ml of the RNA lysis buffer was immediately added to the dish. The lysate was passed seven times through a syringe with a 21-gauge needle. The lysate was mixed with 0.3 ml of 70% ethanol and vortexed for 1 min. The mixture was loaded on a RNA binding column, washed three times, and eluted in 29 μl of RNase-free water. To assess the rates of RNA degradation,

actinomycin D (Act-D) was used to arrest RNA synthesis. After 48-hr normoxia or hypoxia exposure, PAEC were immediately incubated in the medium containing 10 μg/ml Act-D for 0-30 min. Then, total RNA was extracted from the Act-D-treated cells.

RNA was converted into cDNA using the High Capacity cDNA Reverse Transcription kit following the manufacturer's instructions (Applied Biosystems). Briefly, 29 μl of RNA were mixed with 28 μl of a cDNA synthesis master mix containing RT buffer, dNTP, random primers, and a reverse transcriptase. The mixture was incubated at room temperature for 10 min, 37°C for 2 hr, and then at 85°C for 10 seconds.

The relative steady-state levels of preproET-1 mRNA were assessed using qRT-PCR. Two μl of cDNA were mixed with 1 μl of preproET-1 (ACA CTC CCG AGC ACG TTG TTC CGTA, Hs00174961_m1, TaqMan Gene Expression Assay, Applied Biosystems) or α-tubulin (Hs00428633_m1, TaqMan Gene Expression Assay, Applied Biosystems) primers, 7 μl of distilled water, and 10 μl of TaqMan master mix (Applied Biosystems). α-tubulin was used as an internal standard. Delta delta Ct was used to calculate the relative quantities of preproET-1 mRNA in the normoxic and hypoxic cells in the presence or absence of Act-D, protein tyrosine kinase, or PI3K inhibitors.

Assessment of preproET-1 transcription rates using a modified nuclear run-on protocol coupled with qRT-PCR

A sensitive nuclear run-on method was used to determine the transcription rate of preproET-1 mRNA. Briefly, normoxic and hypoxic (1% oxygen, 48 hr) human PAEC were lysed in an ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40. The nuclei were isolated using a Dounce homogenizer and resuspended in a buffer containing 20 mM Tris-HCl (pH 8.1), 75 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, and 50% glycerol. The nuclear run-on mixture contains 1-5 million nuclei derived from one 100-mm dish, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 100 mM KCl, 0.25 mg/ml BSA, 50 μM EDTA, 1 mM S-adenosylmethionine, 1 mM dithiothreitol, 250 units/ml RNasin, and 0.5 mM each of CTP, ATP, UTP, and GTP. The mixture was incubated at 28°C for 60 min. Total RNA was isolated from the reaction mixture, converted to cDNA, and subjected to qRT-PCR using the primers of preproET-1 (Hs00174961_m1) or α-tubulin (Hs00428633_m1, TaqMan Gene Expression Assay, Applied Biosystems).

Western blot analysis

Human PAEC were exposed to normoxia or hypoxia for 18 hrs. Lysates were collected by adding Laemmli sample buffer directly to the dishes and scraping the lysates into 1.5 ml-tubes. Supernatants (20 μg) were separated on a 10 % SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. After the detection of phosphor-tyrosine proteins using an anti-phosphor-tyrosine antibody (SC-7020, Santa Cruz Biotechnology), the membrane was stripped and then reprobed using an anti-α-tubulin antibody (SC-51503, Santa Cruz Biotechnology) to confirm the equal loading.

Inhibition of protein tyrosine kinases and PI3K activity

Human PAEC were exposed to normoxia (room air plus 5% CO_2) or hypoxia (1% oxygen, 5% CO_2, and balanced nitrogen) in the presence or absence of the protein tyrosine kinase inhibitor genistein or PI3Kγ inhibitor II. Pre-experiments indicated that prolonged (>24 hr) exposure of human PAEC to hypoxia in the presence of genistein (50 μM) or PI3Kγ inhibitor II (40 μM) resulted in elevated cell death since protein tyrosine kinases and PI3K are related to cell survival. In order to achieve minimal cell death and maximal inhibition of protein tyrosine kinases and PI3K, human PAEC were exposed to hypoxia for 18 hr in the inhibition studies.

Statistical analysis

Significance for the effects of hypoxia on the levels of preproET-1 mRNA, degradation, and transcription was determined by t-Test and ANOVA using the data analysis tools of Microsoft Excel [18].

Results

Exposure of human PAEC to hypoxia leads to increased levels of preproET-1 mRNA

Since the vascular endothelium is the primary site for ET-1 production, an endothelial cell model was used to assess the effects of

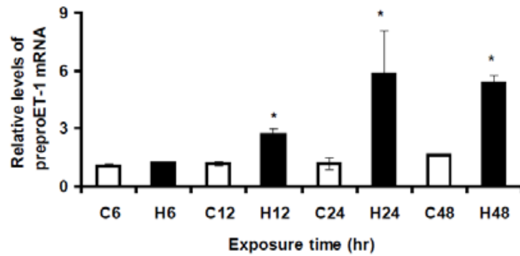

Figure 1. Hypoxia-increased levels of preproET-1 mRNA. Cultures of human PAEC in EBM-2 medium were exposed to room air containing 5% CO_2 (C6-C48, normoxia) or a gas mixture containing 1% oxygen, 5% CO_2, and balanced N_2 (H6-H48, hypoxia) at 37°C for 6-48 hr. RNA was extracted using PureLink™ Micro-to-Midi™ Total RNA Purification System (Invitrogen). RNA was converted to cDNA using the High-capacity cDNA Archive kit and assessed for the preproET-1 mRNA levels using the Taqman technique-based qRT-PCR (Applied Biosystems). *: P<0.01 vs controls (C6-C48), n=3.

hypoxia on ET-1 expression. To determine whether hypoxia increases the steady-state levels of preproET-1 mRNA in human PAEC, cell monolayers cultured on 35-mm dishes were exposed to hypoxia (1% oxygen, 5% CO_2, and balanced nitrogen) or normoxia for 6-48 hr. Exposure of human PAEC to 1% oxygen increases the relative levels of preproET-1 mRNA as early as 12 hr. For instance, exposure of human PAEC to hypoxia for 48 hr increases the level of the preproET-1 mRNA over two times, compared to normoxic cells (**Figure 1**). The relative levels of preproET-1 mRNA were normalized to the levels of α-tubulin mRNA of the same sample.

Hypoxia does not alter the degradation rates of preproET-1 mRNA

Act-D was used to arrest new RNA synthesis, thus allowing quantification of the degradation rate of mRNA. The levels of preproET-1 mRNA in hypoxic cells are higher than that in normoxic cells after 0-30-min Act-D treatment (**Figure 2A**). In order to compare the degradation rates of preproET-1 mRNA in hypoxic cells to that in normoxic cells, the levels of preproET-1 mRNA in the 15-min and 30-min Act-D-treated hypoxic cells were normalized to 0-min hypoxic cells and expressed as percentages. The percentages of remaining preproET-1 mRNA levels in the presence of Act-D under normoxic and hypoxic conditions are comparable (**Figure 2B**). This indicates that hypoxia-increased levels of preproET-1 mRNA are unlikely to be caused by increased stability of preproET-1 mRNA.

Hypoxia increases the synthesis of preproET-1 mRNA

A modified nuclear run-on technique coupled with qRT-PCR was used to assess the rates of *preproET-1* gene transcription. Human PAEC in

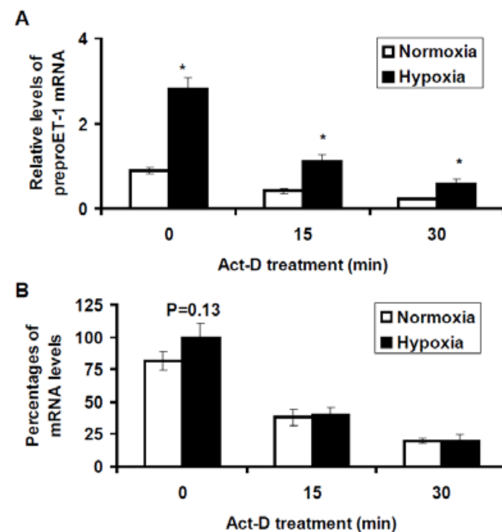

Figure 2. Hypoxia does not alter the stability of preproET-1 mRNA. Human PAEC in EBM-2 medium were exposed to room air containing 5% CO_2 (normoxia) or a gas mixture containing 1% oxygen, 5% CO_2, and balanced N_2 (hypoxia) for 48 hr to stimulate *preproET-1* expression. The normoxic and hypoxic cells were incubated with Act-D (10 μg/ml) for 0, 15, or 30 min. RNA was isolated from the Act-D-treated normoxic and hypoxic cells, converted to cDNA, and quantified using qRT-PCR as described in Materials and Methods. The values of relative quantities (RQ) were normalized to the values of RQ for the 0-min Act-D-treated normoxic cells (Fig 2A). In order to compare the relative rates of RNA degradation, the values of RQ for 15-min and 30-min Act-D-treated hypoxic cells were normalized to the 0-min Act-D-treated hypoxic cells and shown as percentages (Fig 2B). Three sets of samples were analyzed in the same time to minimize the differences caused by changes in experimental conditions. In panel A, in order to assess the relative levels of preproET-1 mRNA, the values of all samples were normalized to C1 (0-min Act-D-treated normoxic cells). The values of triplicate samples C1, C2 and C3 were 1; 0.872; and 0.833. The average of C1, C2, and C3 was 0.9, but not 1. *: P<0.01 vs normoxic cells (0, 15, or 30 min Act-D-treated normoxic cells), n=3.

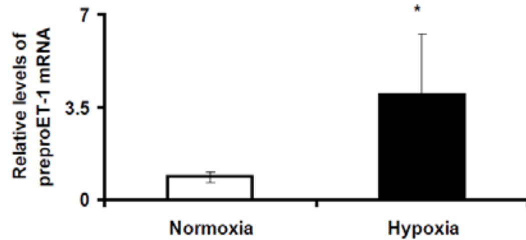

Figure 3. Hypoxia increases the synthesis rate of preproET-1 mRNA. Human PAEC in EBM-2 medium were exposed to normoxia or hypoxia for 48 hr to stimulate *preproET-1* expression. A sensitive nuclear run-on method was used to determine the transcription rate of preproET-1 mRNA as described in Materials and Methods. Nuclei derived from normoxic and hypoxic human PAEC were incubated in the nuclear run-on mixture at 28°C for 60 min. RNA was isolated from the reaction mixture, converted to cDNA, and subjected to qRT-PCR. Normoxia: nuclei isolated from normoxic cells, and hypoxia: nuclei isolated from hypoxia-stimulated cells. *: P<0.05 vs normoxic cells, n=6.

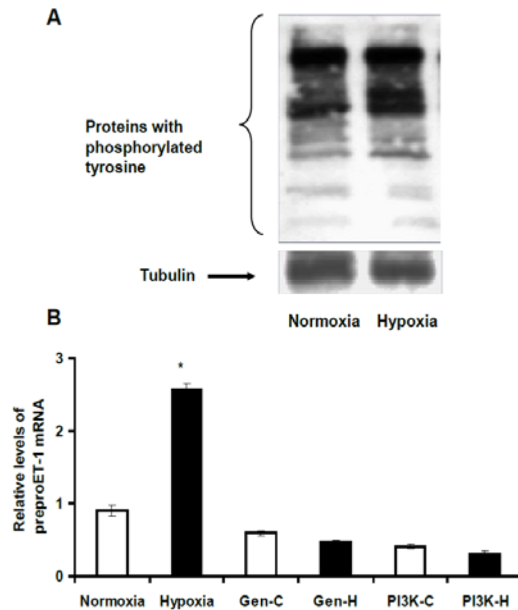

Figure 4. Inhibition of protein tyrosine kinase or PI3K activity leads to attenuation of hypoxia-increased levels of preproET-1 mRNA. Human PAEC in EBM-2 medium were exposed to room air containing 5% CO_2 (normoxia) or a gas mixture containing 1% oxygen, 5% CO_2, and balanced N_2 (hypoxia) for 18 hr to stimulate *preproET-1* expression in the presence or absence of the protein tyrosine inhibitor, genistein (50 μM), or PI3Kγ inhibitor II (40 μM). Cell lysates were subjected to Western blot analysis using an anti-phosphorylated tyrosine antibody. After being stripped, the membrane was reprobed using an anti-tubulin antibody to verify equal loading. A representative image of the exposed X-ray film is shown (Fig 4A). Total RNA was extracted, converted to cDNA, and assessed for the preproET-1 mRNA levels using qRT-PCR (Fig 4B). Normoxia: normoxic cells; Hypoxia: hypoxic cells; Gen-C: normoxic cells in medium containing genistein; Gen-H: hypoxic cells in medium containing genistein; PI3K-C: normoxic cells in medium containing PI3Kγ inhibitor II; and PI3K-H: hypoxic cells in medium containing PI3Kγ inhibitor II. *: P<0.01 vs normoxia, n=3.

tyrosine proteins in hypoxic cells are higher than those in normoxic cells (Figure 4A). To determine whether protein tyrosine kinases and one of their downstream effecters, PI3K, are associated with hypoxia-increased levels of preproET-1 mRNA, human PAEC were exposed to hypoxia in the presence or absence of the protein tyrosine kinase inhibitor, genistein, and PI3Kγ inhibitor II. The levels of preproET-1 mRNA in the treated cells were assessed. Genistein and PI3Kγ inhibitor II attenuated hypoxia-increased levels of preproET-1 mRNA (Figure 4B). This suggests that protein tyrosine kinase and PI3K cascades contribute to hypoxia upregulation of preproET-1 expression. The levels of preproET-1 mRNA in genistein and PI3Kγ inhibitor II-treated normoxic PAEC are lower than that in control normoxic cells, indicating that protein tyrosine kinases and PI3Kγ are associated with basal *preproET-1* expression.

Discussion

Exposure of cultured human PAEC to 1% oxygen leads to elevation of preproET-1 mRNA. Our observation is in agreement with many recent reports indicating that hypoxia increases levels of preproET-1 mRNA in cultured bovine coronary artery endothelial cells [10], rat aortic endothelium [19], and human microvascular endothelial cells [11]. Hypoxic elevation of preproET-1 mRNA contributes to increased levels of circulating ET-1 peptides *in vivo* [1,2]. However, some early reports suggested that hypoxia decreased preproET-1 mRNA levels in rat lung endothelial cells [12] and bovine PAEC [20]. The reason for the different observations related to hypoxic modulation of ET-1

expression is unclear. Different experimental conditions used in these studies may be responsible for the conflicting observations. For instance, exposure of human PAEC to a constant flow of a gas mixture containing 1% oxygen, 5% CO_2, and balanced nitrogen leads to elevation of preproET-1 under our experimental conditions, whereas exposure of bovine PAEC to nearly 0% oxygen resulted in hypoxic downregulation of ET-1 expression [20]. This implies that different concentrations of oxygen may cause different modulation of preproET-1 expression in cultured PAEC. In addition, the endothelium derived from different species (rats, sheep, or human) and vascular beds (systemic or pulmonary vasculatures) may respond to hypoxia in a different manner. Hypoxic upregulation of ET-1 expression in human PAEC suggests that the endothelium of large pulmonary vessels can play a role in hypoxic vasoconstriction and remodeling.

Hypoxia-increased preproET-1 mRNA is associated with upregulation of preproET-1 transcription. Our results indicate that hypoxia has minimal effects on the stability of preproET-1 mRNA with a short half-life (10-20 min) in human PAEC. This implies that hypoxic exposure may not alter “AUUUA” motif-mediated destabilization of the preproET-1 mRNA [21]. However, we have shown that hypoxia increases the synthesis rates of preproET-1 mRNA in human PAEC as determined by a modified nuclear run-on analysis. The *preproET-1* promoter contains an inverted HIF-1 binding sites that is essential for the promoter response to hypoxia [13]. Exposure of transgenic mice bearing a *preproET-1* promoter-*luciferase* gene to hypoxia leads to the strongest transgene expression in the lung vasculature [22]. These observations support the notion that hypoxia-stimulated *preproET-1* expression is mediated through upregulation of the *preproET-1* transcription in human PAEC.

Hypoxic upregulation of *preproET-1* expression is associated with protein tyrosine kinase signaling in human PAEC. Inhibition of protein tyrosine kinases using a selective inhibitor, genistein, attenuates hypoxia upregulation of *preproET-1* expression. Receptor tyrosine kinase cascades have been suggested to play a role in hypoxia-induced gene expression in vascular remodeling of the pulmonary artery [23]. The transcriptional responses of the *preproET-1* promoter are inhibited by genistein in human microvascular endothelial cells [11]. Genistein therapy decreases plasma ET-1 levels in postmenopausal women [24]. These results demonstrate a vital role of protein tyrosine kinase signaling in hypoxic modulation of ET-1 expression.

Hypoxia modulates preproET-1 expression of human PAEC in a PI3K-dependent manner. PI3K signaling is linked to hypoxia-induced phosphorylation of endothelial nitric oxide synthase in porcine coronary artery endothelial cells [25]. Some cancer cells live in a hypoxic microenvironment. The upregulation of HIF correlates with increased progression of the cancer, and activation of PI3K signaling contributes to increased HIF-1 [26]. The exact molecular mechanisms underlying hypoxic modulation of preproET-1 expression via protein tyrosine kinase-PI3K are unclear. However, hypoxia may directly or indirectly stimulate protein tyrosine kinases, leading to PI3K activation. This notion is supported by the observations that genistein and PI3Kγ inhibitor II attenuate hypoxia-increased levels of preproET-1 mRNA.

Our present investigation demonstrates that hypoxic upregulation of preproET-1 transcription is associated with protein tyrosine kinase-PI3K signaling in cultured PAEC. The protein tyrosine kinase-PI3K cascades in modulation of preproET-1 expression can add to current knowledge of signal transduction pathways that are involved in pulmonary vascular tone and remodeling through ET-1-mediated vascular homoeostasis in hypoxic PH. ET-1 receptor antagonists such as bosentan and sitaxsentan are used in PH patients. The clinical outcomes of blocking ET-1 receptors are beneficial but limited. New therapeutic targets aimed at the upstream signaling in modulation of ET-1 expression to reduce ET-1-dependent vasoconstriction and remodeling may be required. Elucidating tyrosine kinase-PI3K-ET-1 signaling can provide additional upstream targets to be tested.

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Address correspondence to: Jianliang Zhang, Ph.D. Pulmonary Division, MSB Rm M452, Department of Medicine, University of Florida College of Medicine, 1600 S. W. Archer Road, Gainesville, FL 32610-0225, USA. Tel: 353-376-1611, Ext. 6265, Fax: 352-374-6170, E-mail: Zhangjl@medicine.ufl.edu

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