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Novel Peptide for Attenuation of Hypoxia-Induced Pulmonary Hypertension via Modulation of Nitric Oxide Release and Phosphodiesterase -5 Activity

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Abstract

Pulmonary vascular endothelial nitric oxide (NO) synthase (eNOS)-derived NO is the major stimulant of cyclic guanosine 5' monophosphate (cGMP) production and NO/cGMP-dependent vasorelaxation in the pulmonary circulation. We recently synthesized multiple peptides and reported that an eleven amino acid (SSWRRKRKES) peptide (P1) but not scrambled P1 stimulated the catalytic activity but not expression of eNOS and causes NO/cGMP-dependent sustained vasorelaxation in isolated pulmonary artery (PA) segments and in lung perfusion models. Since cGMP levels can also be elevated by inhibition of phosphodiesterase type 5 (PDE-5), this study was designed to test the hypothesis that P1-mediated vasorelaxation is due to its unique dual action as NO-releasing PDE-5 inhibitor in the pulmonary circulation. Treatment of porcine PA endothelial cells (PAEC) with P1 caused time-dependent increase in intracellular NO release and inhibition of the catalytic activity of cGMP-specific PDE-5 but not PDE-5 protein expression leading to increased levels of cGMP. Acute hypoxia-induced PA vasoconstriction ex-vivo and continuous telemetry monitoring of hypoxia (10% oxygen)-induced elevated PA pressure in freely moving rats were significantly restored by administration of P1. Chronic hypoxia (10% oxygen for 4 weeks)-induced alterations in PA perfusion pressure, right ventricular hypertrophy, and vascular remodeling were attenuated by P1 treatment. These results demonstrate the potential therapeutic effects of P1 to prevent and/or arrest the progression of hypoxia-induced PAH via NO/cGMP-dependent modulation of hemodynamic and vascular remodeling in the pulmonary circulation.

Keywords

Pulmonary hypertension; Hypoxia; Nitric oxide; Phosphodiesterase-5; Synthetic peptide; cyclic GMP; Telemetry; Pulmonary artery pressure

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1. Introduction

Pulmonary arterial hypertension (PAH) is a life-threatening condition characterized by elevated pulmonary artery pressure (PAP) with abnormal vascular remodeling of the lung, which ultimately leads to right-heart failure [4]. Despite therapeutic advances in the past 30 years, survival rate of PAH is modestly improved. Thus, PAH remains a devastating disease with an emerging need to consider development of mechanism-based novel targets and therapies. Increased exposure to multiple risk factors including hypoxia is considered a critical factor in the pathogenesis of PAH [2,10]. Hypoxia-induced endothelium dysfunction leads to imbalance between vasoconstriction and vasodilator mechanisms in the pulmonary vasculature. As such, vasodilator therapy has been the mainstay for treatment of PAH.

Vascular endothelium plays a critical role in the regulation of pulmonary vascular tone through multiple mechanisms, including release of potent vasoactive mediators such as nitric oxide (NO) synthesized by endothelial NO synthase (eNOS) and elevation of cyclic guanosine 5'-monophosphate (cGMP) production via NO-mediated activation of soluble guanylate cyclase (sGC) [3, 14]. To achieve similar or additive responses, pulmonary vascular cGMP levels can also be elevated by inhibition of the cGMP-dependent phosphodiesterase type 5 (PDE-5) responsible for cGMP hydrolysis in the lung [17, 23]. In fact, current therapeutic approaches for the treatment of PAH involve the combination of experimental cGMP-specific PDE-5 inhibitors such as sildenafil and conventional drugs that help reduce pulmonary vascular resistance in patients with PAH [20]. As such, development of a therapeutic approach involving sustained elevation of cGMP via increased NO release associated with decreased cGMP hydrolysis may be critical for the treatment of PAH.

We recently reported that an 11-amino acid (SSWRRKRKES) synthetic peptide (P1) but not scrambled P1 derived from the eNOS sequence enhances the catalytic activity of eNOS and cGMP levels in pulmonary artery endothelial cells/smooth muscle cell (PAEC/PASM) co-culture system leading to NO/cGMP-dependent sustained vasorelaxation in isolated porcine PA segments and mice lung perfusion models [9]. The present study is focused on examining whether P1-mediated NO/cGMP-dependent sustained vasorelaxation is associated with its dual action as NO releasing PDE-5 inhibitor function using endothelial cell culture and hypoxia-induced rat model of PAH.

2. Materials and methods

2.1 Synthetic peptide and reagents

Eleven amino acid (SSWRRKRKES) peptide (P1) or scrambled P1 with arbitrarily shuffled amino acid sequence with >95% purity, as determined by HPLC and mass-spectrometry was synthesized by EZBiolab (Carmel, IN). Primary antibody for western blotting was commercially obtained as rabbit polyclonal anti-PDE-5 (Cell Signaling, Danvers, MA). Secondary antibody (peroxidase-conjugated donkey anti-rabbit IgG) was obtained from Jackson Immuno Research Laboratories (West Grove, PA). [³H]cGMP and [³H]cAMP were obtained from ICN (Irvine, CA) and cGMP assay kit was obtained from Amersham (Arlington Heights, IL). NG-nitro-L-arginine methyl ester (L-NAME), acetylcholine, zaprinast, and all other reagents/chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Orlando, FL).

2.2 Animals

Male Sprague Dawley rats (250-300g) (Harlan Laboratories) were housed in specific pathogen free, 12/12 light/dark cycle conditions with free access to standard food and water. Experimental protocol was approved by the Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals.

2.3 Tissue culture and treatments

PAECs were obtained from the main PA of 6-to7-month pigs and propagated in monolayer cultures as previously described [9]. Confluent monolayers of porcine PAECs were treated in RPMI 1640 containing P1 (0.5 -10 μ M), zaprinast (5 μ M) or RPMI 1640 alone (control) for up to 1 hr at 37°C. After incubation, cells were used to determine intracellular NO release, PDE-5 expression and activity, cGMP levels, PDE-5 immunoprecipitation, and cGMP-PDE-5 binding.

2.4. NO Measurement

The time-dependent effects of P1 stimulation on NO release in PAEC was measured by monitoring a fluorescence of NO-bound 4, 5-diaminofluorescein diacetate (DAF-FM) using a confocal microscopy as we previously reported [27].

2.5 PDE-5 activity and expression

The potential effects of P1 treatment on cGMP- and cAMP-specific PDE activity in PAEC as well as the effect of P1 on PDE-5 expression in PAEC were examined. PAEC in culture were treated with P1 (0.5- 5.0 μ M) or zaprinast (5 μ M) in RPMI 1640 or RPMI 1640 only (control) for 1 hr at 37° C. After incubation, cells were homogenized in buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.1 mM benzamidine, 1 mM EDTA, 5 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, 2 μ M each of leupeptin and pepstatin, 1 % Triton X-100. The homogenates were centrifuged at 15,000 $\times$ g for 15 min at 4° C and supernatant and particulate fractions were collected. The supernatants, which consist of >98% of the total PDE activity, were used for PDE activity assay using 0.5 μ M each of [³H]cGMP or [³H]cAMP as previously described [16,22].

For PDE-5 expression, western blot analysis of lysates from P1 and control PAEC (30 μ g protein) were performed. Cell lysates were fractionated on 7.5 SDS PAGE and blots were probed with the polyclonal rabbit anti-PDE-5 antibody (1:500 dilution) as previously reported [13]. The blots were scanned to determine the optical density of PDE-5 protein content using a Fluor-S MultiImager system (Bio-Rad, Richmond, CA) and the data was normalized to GAPDH protein level in the same lysate samples.

2.6 cGMP assay

Intracellular cGMP levels in P1-stimulated and control cells or in rat lung tissue were measured using an Amersham cGMP enzyme immunoassay system kit as we previously described [9].

2.7 cGMP-PDE-5 binding assay

Because P1-stimulation inhibits PDE-5 activity and the cGMP binding to the catalytic site of PDE-5 is critical for its activity, we examined the effects of P1 on [³H]cGMP binding to PDE-5. Cell lysates from control or P1 (10 μ M)-stimulated PAEC were immunoprecipitated with anti-PDE-5 antibody and an equal volume of immunoprecipitates (5 μ l each sample) or buffer (control) were incubated in a reaction mixture consisting of 50 mM Tris-HCl buffer, pH 7.5 , 5 mM MgCl₂, 0,2 mM EDTA, 15 mg/ml bovine serum albumin, and varying amounts (10 to 75 nM) of [³H]cGMP for 15 min at 30° C. After incubation, reactions were terminated by adding cold 10 mM potassium phosphate, pH 6.8, and samples were filtered using 0.45 μ m HAWP filters, and radioactivity was counted. Blanks without immunoprecipitates were run under identical conditions [16]. Non-specific activity was determined by using excess (1 mM) unlabeled cGMP.

2.8 Vasorelaxation of rat PA segments

To determine the potential effects of P1-mediated vasorelaxation, ex-vivo models of hypoxia-induced vasoconstriction of isolated rat PA segments were used. PA segments were isolated from the left lung of 3 month old Sprague Dawley rats and suspended in individual organ chambers with 5 ml of Krebs buffer and oxygenated with 95% O₂ and 5% CO₂ at 37°C as we previously described [9]. After equilibration of resting force of 0.5 g, smooth muscle/endothelium integrity was confirmed by monitoring 0.5 μ M U-46619 (a thromboxane A₂ mimetic)-mediated PA contraction and acetylcholine (5 μ M)-mediated vasodilation before further experimentation. Smooth muscle/endothelium-responsive segments were washed with Krebs buffer and the contraction was initiated with 0.5 μ M phenylephrine (PE, an α -adrenoreceptor agonist) for 5 min and then with the exposure to 0% O₂, 5% CO₂, and 95% N₂ for 30 min when a stable contraction was induced, the segments were then incubated with or without P1 or scrambled P1 (10 μ M each) for 30 min. In some experiments, PA segments were pre-incubated with P1 prior to hypoxic exposure. We also examined the effects of eNOS inhibitor L-NAME (100 μ M) to determine the contribution of reduced NO generation on P1-mediated vasodilatation.

2.9 Chronic hypoxia-induced modulations of pulmonary vasculature

To examine in vivo physiologic relevance of P1 treatment for PAH, a chronic hypoxia-induced rat model of PAH was used with or without telemetry transmitter implantation for constant pulmonary artery pressure (PAP) monitoring. The model was also used for the evaluation of the P1's effects on systemic artery pressure (SAP), pulmonary artery perfusion pressure, changes in right ventricular (RV) hypertrophy, and vascular remodeling.

Chronic exposure to hypoxia was carried out using a Coy Laboratory (Grass Lake, MI) chamber equipped with auto purge, airlock, and automated live animal filtration and dehumidification systems. 12 groups Sprague-Dawley male rats exposed to hypoxia (10% oxygen) or normoxia (21% oxygen) received intraperitoneal administration of P1 (60 mg/kg/day in saline) or equal amounts of saline (control) starting day 21 until end of exposure on day 34. All animals were maintained in similar humidity and temperature. Hypoxia exposure was 24 hr/day, except when the chamber was opened for 10-15 min for cleaning the cages, replenishing food, water and administering P1 or saline.

2.10 Real time PAP measurement by telemetry

Male Sprague-Dawley rats weighing 250-270 g were surgically implanted with telemetry probes into the pulmonary artery as previously described [8]. The system consists of a fluid-filled sensing catheter (10 cm long, diameter 0.7 mm, -20 to 300 mm Hg pressure range; model PA-C40 (DSI, St Paul, MN) connected to a transmitter that signals to a remote receiver (model RPC-1) and a data exchange matrix connected to a computer. The radiotelemetry implants were calibrated as per supplier's instructions. Animals were allowed to recover for 9 days after implantation. Starting day 10 four groups (normoxia, normoxia + P1, hypoxia, and hypoxia + P1) of fully recovered animals were exposed to hypoxia or normoxia up to day 34. Starting day 21, P1 (60 mg/kg in saline i.p.) or equal volume of saline were administered to hypoxia or normoxia exposed animals per day for 14 days, respectively. The real time changes in PAP (mm Hg) were recorded constantly by radiotelemetry throughout the 34 day experiment period.

2.11 Measurement of PAP, SAP, RV hypertrophy, and vascular remodeling

To verify whether P1 administration specifically prevents or attenuates PAH, separate groups of animals were used to measure lung perfusion PAP and SAP. Male Sprague Dawley rats (270-300g) were exposure to hypoxia or normoxia for 4 weeks with or without

intraperitoneal administration of P1 (60 mg/kg/day) and used for one time direct in vivo measurements of PAP or SAP. After 4-week hypoxia exposure, one time direct RV pressure (RVP) in vivo was measured using the Seldinger technique, a 7Fr catheter was inserted through the RV and connected to the pressure transducer for monitor the pressure trace as PAP. To confirm the effect of P1 on the PA resistance, in situ lung perfusion PAP was measured with a constant flow of RPMI 1640 containing 4% Ficoll at 10 ml/min for each of lung after exsanguinate as we described [9]. Separate groups of animals were used for measurement of SAP. Briefly, rats were anesthetized, the left carotid artery isolated, and 17-gauge intravenous needle used to tunnel the catheter to the carotid artery. The needle was retracted, thus leaving the catheter in place. A pressure transducer was then connected to the catheter and the blood pressure in carotid artery was measured and calculated by using Gould Instrument System as we used for PAP measurement [9]. To determine the RV hypertrophy and vascular modeling, the hearts and lungs from each group were isolated. After isolation of the heart, the major vessels were removed from the ventricles. The RV was dissected from the left ventricle (LV) and septum, and each was weighed. The degree of RV hypertrophy was expressed as the ratio of RV weight to left ventricular plus septum weight (RV/LV+S). Lungs were fixed with 10% formaldehyde and stained with hematoxylin and eosin-stained for histological examination to determine degree of vascular remodeling.

2.12 Statistical analysis

Significance of the effects of hypoxia, P1, and enzyme inhibitors on PDE-5 activity, NO and cGMP generation, pulmonary hemodynamic, vasorelaxation in PAEC, PA segments, ex-vivo, and in-vivo lung vasorelaxation were determined with ANOVA and a two-tailed Student's t test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. P1 stimulates NO release in cultured lung endothelial cells

Incubation of porcine PAEC with P1 (10 μ M) for 10 min significantly increased intracellular DAF-FM fluorescence (Fig. 1A) indicating increased NO production in PAEC. Quantitative analysis of the intensity of DAF-FM fluorescence in PAECs demonstrated that P1-stimulation resulted in about 4-fold increase of NO production at 5 and 10 min as compare to controls (Fig. 1B).

3.2. P1 inhibits PDE-5 activity and elevates cGMP levels in PAEC

We examined the effect of varying concentrations of P1 (0.5- 5 μ M) on PDE-5 activity and/or PDE-5 protein expression in PAEC. We also compare the effects of P1 (5 μ M) and zaprinast (5 μ M), a known inhibitor of PDE-5, on the catalytic activity of PDE-5. Since P1-mediated increased NO generation and/or reduced cGMP hydrolysis due to inhibition of PDE-5 can enhance cGMP levels, we also determine whether P1- or zaprinast-stimulation increases cGMP levels in PAEC. As shown in Fig. 2, P1-mediated inhibition of PDE-5 was dose-dependent (panel A). Western blot analysis demonstrated that P1 (μ M) stimulation did not change PDE-5 expression (panel B). The inhibitory effects of P1- and zaprinast on the catalytic activity of cGMP-specific PDE-5 were comparable (panel C). The elevated levels of P1-mediated intracellular cGMP were higher than in zaprinast-treated cells (panel D).

3.3. P1 inhibits cGMP binding to PDE-5

Since cGMP binding to the catalytic site in PDE-5 is critical for its activity, we examined the effect of P1-stimulation on cGMP binding to PDE-5. As shown in Fig. 3, P1-stimulation

significantly reduces [^3H]cGMP binding to PDE-5 at 100-500 nM concentrations of cGMP as compared to binding in control PAEC.

3.4 P1 treatment increased lung cGMP levels and vasorelaxation of hypoxia-contracted rat PA segments

Since NO/cGMP-mediated vasorelaxation plays a critical role in PAH, we examine the effect of P1 on lung cGMP levels and vasorelaxation response in hypoxia-contracted PA segments. As shown in Fig 4A, P1 treatment significantly increased lung cGMP levels under both normoxic and hypoxic conditions. P1- but not scrambled P1-stimulation significantly induced vasorelaxation of persistently-pre-contracted PA with hypoxic. This P1-mediated response on vasorelaxation was diminished by eNOS inhibitor L-NAME (Fig. 4B).

3.5 P1-treatment attenuated hypoxia-elevated PAP in real time

To examine the potential therapeutic benefits of P1 treatment for PAH, we used a physiologically relevant rat model of hypoxia-induced PAH to determine whether hypoxia causes progressive increase in PAP. If so, what are the effects of P1 treatment on hypoxia-induced real time changes in PAP monitored by radiotelemetry. As shown in Table 1 and Fig. 5, mean PAP (mPAP) in animals under normoxic condition remained stable for 1-34 days. Hypoxia exposure progressively increased mPAP from days 11 to 34. P1 treatment from day 21 to 34 resulted in attenuation mPAP in hypoxia expose group. However, mPAP continue to increase in saline administered rats during the same period. As shown in Table 1, PAP in normoxia and normoxia + P1 treated animals remained stable for up to 34 days (not shown in Fig. 5).

3.6 P1 attenuates hypoxia-induced PAP but not SAP

To determine whether P1 selectively attenuates hypoxia-induced PAP but not SAP, the end stage experiments were conducted using normoxia and hypoxia exposed animals with or without P1 administration for 4 weeks. As shown in Fig. 6, hypoxia-induced PAP was significantly attenuated by P1 treatment. However, under normoxic condition, the PAP remained comparable with or without P1 treatment (panel A). The change in SAP under normoxic or hypoxic conditions with or without P1 treatment remained unchanged (panel B).

3.7 P1 attenuates hypoxia-induced RV hypertrophy and vascular remodeling

To determine the potential therapeutic benefits of P1 treatment for PAH in context with changes in hemodynamic and vascular remodeling, we examined the effects of P1 on RV hypertrophy and histopathology of lung vasculature. As shown in Fig 6, 4 weeks of hypoxia-induced hemodynamic changes measured as RV hypertrophy was significantly attenuated by P1 treatment (panel C). P1 treatment reduced hypoxia-induced muscularization of pulmonary artery (lower panel) which appears to be progressing to the level of normoxic lung (panel D).

4. Discussion

We report here for the first time that an 11 amino acid peptide derived from eNOS protein sequence promotes vasorelaxation and significantly attenuates hypoxia-induced PAH through sustained elevation of cGMP levels via its dual action as NO releasing PDE-5 inhibitor in the pulmonary circulation. Our results using cell culture, isolated PA segments, and intact animal models demonstrate that P1 enhances NO release and selectively inhibits cGMP-specific PDE-5 activity in PAEC. P1-mediated inhibition of the catalytic activity of cGMP-specific PDE-5 is associated with reduced cGMP binding affinity to PDE-5 in PAEC.

P1 treatment increases cGMP levels in PAEC and in the lung leading to attenuation of hypoxia-induced vasoconstriction in isolated PA segments and in conscious moving rats. P1 treatment improves changes in RV hypertrophy and vascular remodeling with selective attenuation of hypoxia-induced elevation of PAP but not affect SAP.

PAH is characterized by vascular obstruction via vasoconstriction and PA remodeling, leading to narrowed vascular lumen and increased pulmonary vascular resistance. Although multiple therapeutic approaches are currently available to help prolong survival and improve quality of patient's life, none of these approaches cures this devastating condition due to the fact that the exact mechanism causing PAH is not clear(4,10,18,19). One of the critical pathogenic mechanisms of PAH involves diminished synthesis of vascular endothelium-derived NO, a potent pulmonary vasodilator that activates soluble guanylate cyclase and increased intracellular cGMP production, critical to maintain sustained vesorelaxation in the pulmonary circulation. In fact, decrease level of endothelium-dependent NO production has been observed in the pulmonary vascular tissue of patients with PAH (6). To achieve similar or additive responses, pulmonary vascular cGMP levels can also be elevated by inhibition of the cGMP-dependent PDE-5 responsible for cGMP hydrolysis in the lung (20,21,25,26). Our results indicate that P1 enhances endothelium-derived NO production. We recently reported that P1-mediated activation of eNOS is associated with caveolae internalization of P1 and compartmentalization of eNOS that increases its catalytic activity without change in eNOS protein expression in PAEC (11). Several studies have shown that in caveolae, eNOS:Caveolin-1 association or dissociation is linked with inhibition or stimulation of the enzyme activity, respectively (5,6). Our more recent report demonstrated that P1-stimulation enhances phosphorylation of caveolar PKC- α and increases intracellular calcium release, both of these events are known to be involved in eNOS/caveolin-1 dissociation and increased eNOS activity in vascular endothelial cells (6,12). Thus, P1-stimulated increased NO production and NO/cGMP-dependent regulation of vascular function is critical for treatment of PAH.

The experimental evidence suggests that cGMP binds to the allosteric site(s) of PDE-5 leading to activation of cGMP-dependent protein kinase which in turn phosphorylates Ser-92 or Ser-102 in regulatory domain of PDE-5 resulting in enhanced activity. Thus, cGMP binding at the catalytic site of PDE-5 and activation of PKG are critical events for the regulation of PDE-5 activity. In fact, classical inhibitors of PDE-5 are designed to compete with cGMP binding at the catalytic site of PDE-5, which then prevents activation of PKG and consequently phosphorylation of PDE-5. Based on these mechanisms, the order of potency of some known PDE-5 inhibitors is sildenafil > zaprinast > dipyridamole > 3-isobutyl-1-methylxanthine (IBMX) > cilostamide > theophylline > rolapram [1]. Our data show that P1-mediated response appears at the zaprinast order in context with inhibition of PDE-5. An important question that remains elusive is that the precise mechanism by which P1 inhibits the catalytic activity of PDE-5 in vitro or in vivo. Nonetheless, the unique dual action of P1 as NO releasing PDE-5 inhibitor offers mechanism-based potential therapeutic agent for treatment of PAH.

The combined use of available drugs with compatible mechanisms of action to maximize the clinical benefit is an emerging strategy for the treatment of PAH. Two interrelated approaches involving NO/cGMP-dependent responses have shown promising results for treatment of PAH: i) inhalation therapy with authentic NO gas, which attenuates vasoconstriction in patients with PAH or in animal models of hypoxia-induced PAH (21,25).and ii) combination therapy involving sildenafil, a potent and specific inhibitor of cGMP-specific PDE5, which increases intracellular cGMP levels (24,25). However, both approaches have limitations with regard to their efficacy and long-term safety for treatment of patients with PAH. For example, administration of NO over the long term is difficult and

risks cytotoxic potential. Similarly, the short- and long-term safety and efficacy of sildenafil alone or co-administered with other conventional drugs remains to be established. The results of our study using in situ and in vivo models of hypoxia-induced PAH demonstrate that P1 administration improved pulmonary hemodynamic and vascular remodeling with selective attenuation of hypoxia-induced elevation of PAP. Although change in mean PAP has been used to classify the severity of PAH, the most common mechanism of high mortality in patients with PAH is progressive RV failure. As such, the use of a single therapeutic agent such as P1 over combination therapy of two or more drugs offers distinct advantage for treatment of PAH. However, more extensive studies will be needed to determine the effects of dose, time course, route of administration, and bioavailability and toxicologic potential to evaluate P1's pharmacodynamic relevance as a therapeutic agent.

5. Conclusion

Our study demonstrates that P1 exerts pulmonary protective effect against hypoxia-induced pathophysiologic changes observed in PAH. Since prominent feature of PAH pathology is associated with remodeling of the lung vasculature with endothelium dysfunction, the modulation of eNOS activity to maintain sustained levels of NO may be crucial for NO/cGMP-mediated vasorelaxation in the pulmonary circulation. An alternative strategy to enhance vasodilatory effect in the pulmonary vasculature involves increasing the level of intracellular cGMP by reduction in the rate of cGMP hydrolysis. This can be achieved by inhibition of cGMP-specific PDE-5, a major PDE isoform present in the pulmonary vasculature. In fact, the potential action of P1 as a NO-releasing PDE5 inhibitor offers an excellent opportunity to develop a new class of single agent multi-faceted therapy for the treatment of pulmonary vascular diseases. The overall implications of understanding P1-mediated dual action via activation of the eNOS/NO/cGMP pathway, and maintaining elevated levels of cGMP via inhibition of PDE-5 can be of considerable value with an enormous impact for treatment of patients with PAH.

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Abbreviations

eNOS	endothelial nitric oxide synthase
NO	nitric oxide
cGMP	cyclic guanosine-3',5'-monophosphate
cAMP	cyclic adenosine monophosphate
PDE-5	phosphodiesterase type 5
PAH	pulmonary artery hypertension
DAF-FM	4,5-diaminofluorescein diacetate
PA	pulmonary artery
PAP	pulmonary artery pressure

SAP	systemic artery pressure
RV	right ventricular
PAEC	pulmonary artery endothelial cells
L-NAME	NG-nitro-L-arginine methyl ester

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Highlights

Peptide therapy attenuates hypoxia-induced pulmonary artery hypertension and vascular remodeling.

The effect was found to be mediated by increased NO-release and inhibition of cGMP-specific PDE-5.

Dual action of the peptide that enhance NO release and diminished PDE-5 activity resulted in sustained elevation of cGMP.

This novel peptide has therapeutic potential for NO/cGMP-dependent modulation of hemodynamic and structural changes in the pulmonary circulation.

Fig.1.

P1-stimulates intracellular NO release in PAEC. P1 (10 μ M)-stimulated NO release was monitored and measured as fluorescence change for NO-bound 4,5-diaminofluorescein diacetate (DAF-2). Panel A= representative confocal images of control (a, 0 min; c, 10min) and P1 (b, 0 min; d, 10 min) of three independent experiments. Panel B = P1-induced time-dependent NO release in PAEC (n=3 for each time point). *p<0.05 or **p <0.01 vs control.

Fig. 2.

P1 selectively inhibits cGMP-specific PDE-5 activity and increases cGMP levels in PAEC. Cells were incubated with P1 (0.5 – 5 μM) or zaprinast (5 μM) for 1 hr at 37° C. After incubation, cell supernatants were used for PDE activity assay (panels A and C), whereas cell lysates were used for Western blot analysis of PDE-5 (panel B) and cGMP measurement of PDE5 (panel D). cGMP levels were measured using cGMP immunoassay kit and PDE5 expression using polyclonal rabbit anti-PDE5 antibody (1:500 dilution). Data in panels A, C, and D represent means $\pm$ SE; n= 4 for each data point. Panel B: Shows representative blot and densitometric analysis of blots from three separate experiments with identical results. **p < 0.01 or *p < 0.05 vs control in panels A, C and D, respectively. #p < 0.05 vs zaprinast in panel D.

Fig. 3.

P1 Inhibits cGMP Binding to PDE5 in PAEC. PDE5 immunoprecipitate from control and P1 (10 μ M)-stimulated PAEC were used to assess [³H]cGMP binding as described in Methods. Data represent mean $\pm$ SE, n=4 for each cGMP concentration. **p<0.01 vs control.

In rat lung tissue

Fig. 4.

P1 increases lung cGMP levels and PA vasorelaxation. Panel A, rats were exposed to hypoxia or normoxia for 4 weeks with or without administration of P1 (60 mg/kg/day i.p.). After exposure lung levels of cGMP were determined as described in Methods. Data represent mean $\pm$ SE; $n=4$ in each group. ** $p<0.001$ vs. normoxia, * $p<0.05$ vs. hypoxia.

Panel B, hypoxia-induced vasoconstriction of PA segments were determined with or without P1 (10 μ M) and in the presence of L-NAME (100 μ M) or using scrambled P1 (10 μ M) as described in Methods. Data represents mean $\pm$ SE; $n=3$ in each group. ** $p<0.01$ vs all other groups.

Fig. 5.

P1 attenuates hypoxia-elevated PAP. The real time changes in mean PAP (mPAP) were monitored for 34 days after telemetry implanted surgery. Hypoxia exposure was initiated on day 10 with continued monitoring of PAP changes. On day 21 one group were administered P1 in saline (60 mg/kg, once per day, i.p.) and other received equal volume of saline with continued monitoring of changes in mPAP until end of experiment as described in Methods. Data represents mean $\pm$ SE, n=4 in each group. *p< 0.05 vs saline/ respective day.

Fig. 6.

P1 attenuates hypoxia-induced PAP increase, RV hypertrophy, and vascular remodeling but not SAP. End stage measurements of mPAP (Panel A) and mean SAP (mSAP) (panel B) were monitored in rats exposure to hypoxia or normoxia for 4 weeks with or without administration of P1 (60 mg/kg/day, i.p.). PAP and SAP measurements were performed as described in Methods. All of rats were used for monitoring RV hypertrophy expressed as the ratio of right ventricle (RV) weight to left ventricular (LV) plus septum weight (RV/LV+S) (panel C) and vascular remodeling (panel D). Data in panels A and C represent mean $\pm$ SE, $n=8$ and in panel B $\pm$ SE, $n=4$ in each group. Panel D shows representative histograms of 4 rats in each group.

Table 1

Effects of P1 on hypoxia-induced PAP in real time

Exposure/Treatment	mPAP(mmHg)
Normoxia	
Day 1	17.4±0.8
Day 34	17.01±1.2
Hypoxia	
Day 11	17.7±1.1
Day 34	45.6±1.6 *
Normoxia+P1	
Day 21	17.2±0.9
Day 34	15.9±0.8
Hypoxia+P1	
Day 21	18.1±0.9
Day 34	24.7±0.7#

Rats with surgically implanted telemetry probes were exposed to normoxia for 1-34 days or to hypoxia (10% O₂) from 21-34 days. Starting day 21, P1 (60 mg/kg, i.p./day) in saline or equal volume of saline were administered to normoxia or hypoxia exposed animals for 14 days. The real time changes in mean PAP (mPAP) were calculated as described in Methods. Data represent mean ±SE, n=4 in each group.

* p<0.01 vs hypoxia day 11

p<0.05 vs hypoxia day 34.