Phosphodiesterase Type 5 as a Target for the Treatment of Hypoxia-Induced Pulmonary Hypertension

A. Sebkhi, PhD; Julian W. Strange, MB; Steven C. Phillips, PhD; John Wharton, PhD; Martin R. Wilkins

Background—Phosphodiesterase type 5 (PDE5) is a novel therapeutic target for the treatment of pulmonary hypertension. This study examined the distribution of PDE5 in normal and hypoxic lung and the effect of chronic PDE5 inhibition with sildenafil, initiated before and during exposure to hypoxia, on pulmonary artery pressure (PAP) and structure.

Methods and Results—Sprague-Dawley rats were exposed to hypoxia (10% O\textsubscript{2}) for up to 42 days. PAP, measured continuously by telemetry, increased gradually by 20 to 40 mm Hg, reaching a plateau between 10 and 14 days, and declined to normal levels on return to normoxia. PDE5 immunoreactivity was localized to smooth muscle cells in the medial layer of pulmonary arteries and veins in the normal lung and in distal muscularized arteries (<25 μm diameter) after hypoxia-induced pulmonary hypertension. Sildenafil (25 or 75 mg·kg\textsuperscript{-1}·d\textsuperscript{-1}) given before hypoxia produced marked dose-dependent inhibition in the rise of PAP (60% to 90% reduction; \(P<0.0001\)) and vascular muscularization (28.4±5.0% reduction; \(P<0.001\)). When begun after 14 days of hypoxia, sildenafil significantly reduced PAP (30% reduction; \(P<0.0001\)) and partially reversed pulmonary artery muscularization (39.9±4.9% reduction; \(P<0.001\)).

Conclusions—PDE5 is found throughout the muscularized pulmonary vascular tree, including in newly muscularized distal pulmonary arteries exposed to hypoxia. PDE5 inhibition attenuates the rise in PAP and vascular remodeling when given before chronic exposure to hypoxia and when administered as a treatment during ongoing hypoxia-induced pulmonary hypertension. (Circulation. 2003;107:3230-3235.)

Key Words: inhibitors ■ hypertension, pulmonary ■ hypoxia ■ telemetry

Pulmonary arterial resistance vessels constrict in response to hypoxia. Prolonged exposure to a low oxygen environment leads to structural remodeling of these vessels, comprising increased thickness of the adventitial and medial layers and, perhaps more importantly, the muscularization of precapillary vessels that are normally either poorly muscularized or devoid of muscle altogether.\(^1\) The combination of vasoconstriction and vascular remodeling, coupled with an increase in hematocrit, results in pulmonary hypertension and subsequently right ventricular hypertrophy.

Hypoxia-induced pulmonary hypertension is found among lowlanders traveling to or living at altitude (above 2500 m) and also complicates chronic obstructive pulmonary disease.\(^2,3\) Hypoxia is also used frequently to induce pulmonary hypertension in animal models to examine novel treatments for pulmonary arterial hypertension from different causes.\(^1,4,5\)

It is well recognized that the current treatments for pulmonary arterial hypertension are unsatisfactory. The most obvious treatment for hypoxia-induced pulmonary arterial hypertension is continuous oxygen administration. Although this reduces the mortality rate in chronic obstructive pulmonary disease, it has little effect on pulmonary artery pressure (PAP), it is cumbersome to administer, and it is inappropriate for habitation to rugged environments.

Recently, there has been interest in targeting cGMP-dependent phosphodiesterases (PDEs) in pulmonary hypertension, particularly PDE type 5 (PDE5). There is an abundance of this enzyme in the lung, and it hydrolyzes cGMP, the mediator of nitric oxide and natriuretic peptide activity.\(^6\) Orally active PDE5 inhibitors are available, and studies in animal models and humans suggest that these drugs markedly attenuate hypoxia-induced pulmonary hypertension when given before exposure to hypoxia.\(^7\) However, little is known about the distribution of PDE5 in the lung, the effect of hypoxia on PDE5 expression, and the effect of inhibition of the enzyme on PAP when hypoxia-induced pulmonary hypertension is established. Accordingly, we have addressed this in a rat model using immunohistochemistry to evaluate PDE5 expression and telemetry to measure changes in PAP during chronic PDE5 inhibition.
Hypoxia and Sildenafil Treatment

Pulmonary hypertension was induced by exposure to hypoxia (10% inspired O2 fraction) in a normobaric chamber, as described previously. Pulmonary hypertension was induced by exposure to hypoxia (10% O2) for 14 days in a normal oxygen environment.

Methods

Animals

Male Sprague-Dawley rats weighing 260 to 300 g were obtained from Charles River Laboratories (Margate, UK) and were studied at least 7 days after arrival. Rats were housed under controlled temperature (22 °C) and lighting (12/12-hour light/dark cycle), with access to food (ERD; Special Diets Services) and water.

Measurement of PAP

PAP was measured with an implanted radiotelemetry system (Dataquest A.R.T. 2.1; Data Sciences Inc). The system comprises a fluid-filled sensing catheter (10 cm long, external diameter 0.7 mm, internal diameter 0.25 mm; model TA11PA) connected to a transmitter (9g) that signals to a remote receiver (model RPC-1) and a data-exchange matrix connected to a computer. The rat was ventilated via the trachea with a small rodent ventilator (Harvard Instrument) with a mixture of O2 and isoflurane anesthetic. The thoracic and peritoneal cavities were exposed via a small thoracotomy and midline abdominal incision, respectively. A 17-gauge intravenous needle (Viggo AB) was used to tunnel the catheter from the peritoneal cavity through the skin to the thoracic cavity. The transmitter was placed in the peritoneal cavity and sutured to the abdominal musculature. The tip of the sensing catheter was inserted into the right ventricle through a small hole and pushed slowly into the pulmonary artery. The waveform was displayed on the computer and used to ensure correct positioning of the catheter. When a typical PAP waveform was recorded, the catheter was secured in place at its entry into the right ventricle with a drop of tissue adhesive. The thoracic and abdominal incisions were sutured. Animals were allowed to regain consciousness and were housed individually in standard rat cages. Each cage was isolated inside a Faraday cage to standardize rat cages. Each cage was isolated inside a Faraday cage to standardize rat cages.

Data Analysis

For each animal, the 30-minute PAP measurements were averaged for each day. PAP measurements are presented as mean ±SD of daily averages for each group of rats. Statistical comparisons of PAP measurements were performed by 1-way ANOVA. Morphometry data were expressed as mean ±SEM, and changes in muscularization were assessed by 1-way ANOVA with post hoc Tukey’s test. P <0.05 was considered statistically significant.

Results

Radiotelemetry

Mean PAP in animals recovered from surgery (after 5 days) varied between 15 and 25 mm Hg when the animals were kept in a normal oxygen environment for up to 30 days (Figures 2A and B). Mean PAP increased 2- to 3-fold within 14 days after exposure to 10% O2 and remained elevated in this atmosphere (P <0.0001). PAP returned gradually to normal levels on return to a normal atmosphere (Figure 2B). Pretreatment with sildenafil exhibited a dose-dependent effect on hypoxia-induced pulmonary hypertension (Figure 2C). The increase in PAP was reduced by ~60% at 25 mg·kg⁻¹·d⁻¹ (P <0.0001) and ~90% at 75 mg·kg⁻¹·d⁻¹ (P <0.0001), the reduction in PAP being significantly greater at the higher dose of sildenafil (P <0.01). Sildenafil also
attenuated ongoing pulmonary hypertension when given 14 days after exposure to hypoxia (Figure 2D), reducing PAP by up to \( \approx 30\% \) (\( P < 0.0001 \)). The reductions in PAP produced by the 2 doses of sildenafil were similar in magnitude (\( P > 0.05 \); Figure 2D).

**Immunohistochemistry and Western Blotting**

PDE5 immunoreactivity was demonstrated in both vascular and nonvascular smooth muscle and exhibited a similar distribution to that of \( \alpha \)-SMA. Prominent PDE5 immunoreactivity was observed in elastic and muscular pulmonary arteries and veins, bronchial blood vessels, and airway smooth muscle, including that surrounding the alveolar ducts and openings of the alveoli (Figure 3). Immunoreactivity was not detected in the nonmuscular microvasculature, the endothelium, airway epithelium, and myocardium surrounding the pulmonary veins. After exposure to chronic hypoxia, PDE5 immunoreactivity was also localized to distal muscularized arteries associated with alveolar ducts and alveolar walls and to intimal cells in elastic arteries (Figure 4). The appearance of PDE5 and \( \alpha \)-SMA immunoreactivity in distal arteries during chronic hypoxia reflected the progressive muscularization of precapillary vessels; PDE5 effectively acted as a smooth muscle cell marker. A single \( \approx 100\)-kDa band was detected in extracts of rat lung (Figure 5A), which corresponded to that described for recombinant human PDE5A1,11 and the PDE5 immunostaining was abolished after preabsorption of the LIP-1 antiserum with 0.1 to 1 \( \mu \)g/mL of peptide antigen (Figure 5B).

**Figure 2.** Effects of hypoxia and sildenafil treatment on PAP measured by radiotelemetry. Each point represents mean±SD of daily averages (48 readings for each animal) for each group of animals. A, Response to chronic hypoxia (n=5) compared with normal oxygen environment (n=4); B, Effect of hypoxia and returning animals to normal atmosphere on PAP (n=4). C, Effects of sildenafil treatment (25 mg · kg\(^{-1} \) · d\(^{-1} \), n=4; 75 mg · kg\(^{-1} \) · d\(^{-1} \), n=4) commencing 4 days before exposure to hypoxia compared with vehicle treatment (n=3) on PAP response to hypoxia. D, Effect of sildenafil treatment (25 mg · kg\(^{-1} \) · d\(^{-1} \), n=4; 75 mg · kg\(^{-1} \) · d\(^{-1} \), n=4) starting 14 days after exposure to hypoxia compared with vehicle treatment (n=3) in ongoing pulmonary hypertension.

**Figure 3.** Immunohistochemical distribution of PDE5 immunoreactivity in normal rat lung. Representative photomicrographs of proximal pulmonary artery (A), vein (B, C), and bronchiole and associated artery (D) showing PDE5 (A, B, D) and \( \alpha \)-SMA immunostaining of smooth muscle cells (C). E and F, Partially muscularized pulmonary vein (E) and artery (F) displaying PDE5 immunoreactivity and distal nonmuscularized vessels (\( * \)) lacking immunostaining.
Morphometry
The number of distal muscularized PDE5-immunoreactive arteries (<25 μm external diameter), expressed as a proportion of vessels accompanying the alveolar ducts and alveolar walls, was increased significantly at 14 days (87.3±4.1%; \( P<0.001 \)) and 24 days (90.6±1.8%; \( P<0.001 \)) of chronic hypoxia compared with normoxic controls (13.9±2.7%; Figure 6). Sildenafil at 75 mg · kg\(^{-1} \) · day\(^{-1} \) significantly reduced the extent of pulmonary artery muscularization when started before and continued during 14 days’ exposure to hypoxia (28.4±5.0% reduction; \( P<0.001 \)) and attenuated the vascular muscularization in established hypoxia-induced pulmonary hypertension (39.9±4.9% reduction; \( P<0.001 \)) when administered during the last 10 days of a 24-day exposure period (Figure 6).

Discussion
This study demonstrates that PDE5 is widely expressed in pulmonary vascular smooth muscle and accompanies the distal muscularization of pulmonary arterioles that is pathognomonic of hypoxia-induced pulmonary hypertension. PDE5 is the most abundant CGMP metabolizing enzyme in the lung and is thought to limit the vasodilator and antiproliferative effects of cGMP-mediated vasoactive factors, such as nitric oxide and the natriuretic peptides (particularly atrial natriuretic peptide and brain natriuretic peptide) on the pulmonary vasculature. Given this function and the presence of PDE5 in newly muscularized arterioles, PDE5 is an attractive target for the pharmacological manipulation of pulmonary vascular tone and structure.

In support of this approach, the PDE5 inhibitor sildenafil significantly attenuated the rise in PAP and vascular remodeling of pulmonary arterioles when begun before and continued during chronic exposure to hypoxia. Of greater significance in therapeutics, sildenafil also reduced PAP when initiated after 14 days of hypoxia, i.e., during ongoing hypoxia-induced pulmonary hypertension. This was associated with a significant reduction in the proportion of muscularized pulmonary arterioles compared with vehicle-treated hypoxic animals, which suggests that the PDE5 inhibitor partially...
The increase in natriuretic peptide levels in hypoxia-induced pulmonary hypertension is largely responsible for the increase in lung cGMP levels measured in this setting.18 An increase in PDE5 activity with hypoxia would limit this rise and facilitate an increase in pulmonary vascular resistance. Earlier studies of the effect of PDE5 inhibition on hypoxia-induced pulmonary hypertension in animals have focused on prophylactic treatment of the animal before exposure to hypoxia.19 In these experiments, hemodynamic response was assessed from a single measurement in the anesthetized animal at the end of the experiment. Telemetry permits the continuous measurement of PAP in the conscious animal and provides information about the dynamics of hypoxia-induced rises in PAP and the response to treatment.

Both the rise in PAP and the extent of muscularization of pulmonary arteries reached a plateau at ~10 to 14 days. Removal of the animal from the hypoxic chamber after 14 days resulted in a gradual return of PAP to normal, which indicates that the condition is potentially reversible over this time scale. This is a somewhat shorter time scale than suggested by previous studies by Fried and Reid,20 a difference that may reflect the difference in methodology used. Treatment with sildenafil before and during hypoxia produced almost complete inhibition of the rise in PAP at the higher dose. Initiation of treatment when pulmonary hypertension had developed did not return PAP to normal. In fact, both doses caused a smaller fall in PAP than seen with pretreatment, but reductions in PAP of similar or smaller magnitude with other drugs, such as prostacyclin and bosentan, are associated with significant clinical improvement in human studies.21 Interestingly, a similar reduction of distal muscularization was observed in both treatment protocols. It is possible, however, that hypoxia and the initial increase in PAP may have initiated other structural changes, such as adventitial fibroblast proliferation and extracellular matrix deposition in more proximal vessels, that contribute to the elevated PAP. Such remodeling may be more resistant to the effects of PDE5 inhibition, hence the lack of response to increasing the dose of sildenafil. These structural changes may limit the benefit from PDE5 inhibition in ongoing hypoxia-induced pulmonary hypertension.

The study itself has some limitations. For technical reasons, it was not possible to measure systemic artery pressure or cardiac output in the same conscious animals instrumented for PAP telemetry. In all studies to date, however, sildenafil has had little effect on cardiac output and systemic blood pressure and exhibited relative selectivity for the pulmonary circulation.22,23

In summary, the present study shows that PDE5 is expressed in hypoxia-induced remodeled pulmonary vessels, providing a target for PDE5 inhibitors in pulmonary hypertension. One such inhibitor, sildenafil, not only prevents hypoxia-induced pulmonary hypertension but is also effective in reducing PAP and pulmonary vascular muscularization once the condition has developed.

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References

Figure 6. Effects of sildenafil on pulmonary artery muscularization caused by chronic hypoxia. Muscularization of distal pulmonary arteries (≤25 μm external diameter [dia.]), expressed as ratio of muscular arteries displaying PDE5 immunoreactivity to total number of vessels. Muscularization of distal arteries was significantly reduced by treatment with sildenafil (75 mg · kg⁻¹ · d⁻¹) given before and continued during exposure to hypoxia (−4 to 14 days) and when started 14 days after hypoxia-induced pulmonary hypertension was established (day 14 to 24). Data points represent mean±SEM from 5 to 6 rats.

Reversed pulmonary vascular remodeling despite continued exposure to hypoxia.

Previous biochemical studies have reported that hypoxia increases cGMP PDE activity in proximal (internal diameter 0.2 to 5 mm) but not resistance (100 to 300 μm) rat pulmonary arteries.14 An increase in cGMP PDE activity has also been reported in an ovine model of fetal pulmonary hypertension.15 Although Black et al16 reported an increase in PDE5 protein expression in homogenates of whole lung, this was not observed by Hanson et al,15 who attributed the increase in activity to an increase in abundance of phosphorylated enzyme. Recent studies support the thesis that phosphorylation of PDE5 by cGMP-dependent protein kinase I is a major regulatory pathway for the control of PDE5 activity in smooth muscle cells.17 Small but pathophysiologically significant changes in PDE5 expression can be difficult to detect by Western blotting of whole lung homogenates, and biochemical activity studies on dissected intrapulmonary vessels can also be misleading. The present data clearly demonstrate extension of PDE5-immunoreactive smooth muscle cells into previously mainly nonmuscularized pulmonary arteries (<25 μm in diameter) with exposure to hypoxia. Indeed, PDE5 immunostaining effectively acted as a smooth muscle cell marker.

Several authors have suggested that an increase in PDE5 activity contributes to the pathophysiology of pulmonary hypertension.14 In vitro studies show that cGMP mediates vasorelaxation and inhibits vascular smooth muscle cell growth. Intrapulmonary cGMP concentrations are a balance between synthesis by smooth muscle cell guanylate cyclases and degradation by PDEs. Nitric oxide and the natriuretic peptides are thought to drive cGMP synthesis in the lung, and arguably, the increase in natriuretic peptide levels in hypoxia-induced pulmonary hypertension is largely responsible for the increase in lung cGMP levels measured in this setting.18

An increase in PDE5 activity with hypoxia would limit this rise and facilitate an increase in pulmonary vascular resistance.

Effects of sildenafil on pulmonary artery muscularization caused by chronic hypoxia. Muscularization of distal pulmonary arteries (≤25 μm external diameter [dia.]), expressed as ratio of muscular arteries displaying PDE5 immunoreactivity to total number of vessels. Muscularization of distal arteries was significantly reduced by treatment with sildenafil (75 mg · kg⁻¹ · d⁻¹) given before and continued during exposure to hypoxia (−4 to 14 days) and when started 14 days after hypoxia-induced pulmonary hypertension was established (day 14 to 24). Data points represent mean±SEM from 5 to 6 rats.


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