Sildenafil Inhibits Hypoxia-Induced Pulmonary Hypertension

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Background—This study investigated the effect of the phosphodiesterase 5 inhibitor sildenafil on the pulmonary vascular response to hypoxia in humans and mice.

Methods and Results—In a randomized, double-blind study, sildenafil 100 mg or placebo was given orally to 10 healthy volunteers 1 hour before breathing 11% O₂ for 30 minutes. Pulmonary artery pressure (PAP) was measured with an indwelling right heart catheter. The acute 56% increase in mean PAP produced by hypoxia during placebo treatment (mean PAP [mean±SD mm Hg]: normoxia 16.0±2.1 versus hypoxia 25.0±4.8) was almost abolished by sildenafil (normoxia 16.0±2.1 versus hypoxia 18.0±3.6), with no significant effect on systemic blood pressure. In the isolated perfused lung of wild-type and endothelial nitric oxide synthase (eNOS)–deficient mice, sildenafil markedly blunted acute hypoxic pulmonary vasoconstriction. Wild-type mice dosed orally with the drug (25 mg · kg⁻¹ · d⁻¹) throughout 3 weeks of exposure to hypoxia (10% O₂) exhibited a significant reduction in right ventricular systolic pressure (placebo versus sildenafil: 43.3±9.9 versus 29.9±9.7 mm Hg, P<0.05) coupled with a small reduction in right ventricular hypertrophy and inhibition of pulmonary vascular remodeling. In eNOS mutant mice, sildenafil attenuated the increase in right ventricular systolic pressure but without a significant effect on right ventricular hypertrophy or vascular remodeling.

Conclusions—Sildenafil attenuates hypoxia-induced pulmonary hypertension in humans and mice and offers a novel approach to the treatment of this condition. The eNOS-NO-cGMP pathway contributes to the response to sildenafil, but other biochemical sources of cGMP also play a role. Sildenafil has beneficial pulmonary hemodynamic effects even when eNOS activity is impaired. (Circulation. 2001;104:424-428.)

Key Words: hypertension, pulmonary n hypoxia n pharmacology

Alveolar hypoxia leads to pulmonary vasoconstriction and subsequently structural remodeling of blood vessel walls, most pronounced in distal pulmonary arterioles. Hypoxia-induced pulmonary hypertension is common in people who live at high altitude, such as Kyrgyz highlanders. Hypoxia also plays a significant role in the pathogenesis of the pulmonary hypertension that accompanies chronic obstructive airway disease. Pulmonary hypertension is a serious clinical problem with significant morbidity and mortality, but apart from continuous oxygen administration, there is currently no satisfactory treatment for the condition.

Nitric oxide (NO) and the natriuretic peptides attenuate vasoconstriction and vascular remodeling in hypoxia-induced pulmonary hypertension but are difficult to administer as drugs over the long term. Their vasorelaxant and antimitogenic actions are mediated by cGMP and activation of cGMP-dependent protein kinases. Pulmonary vascular cGMP levels can also be elevated by inhibiting the phosphodiesterases (PDEs) responsible for cGMP hydrolysis in the lung. PDE5 is the major cGMP PDE subtype present in the pulmonary vasculature and is more abundant in the lung than in other tissues. This offers the possibility of relatively selective pulmonary vasodilatation with little systemic hypotension. Agents with PDE5 inhibitory activity reduce pulmonary artery pressure (PAP) in animal models, but there are few data in humans.

Sildenafil is an orally active, potent (IC₅₀ ~4 nmol/L), and selective PDE5 inhibitor, used in doses of 50 to 100 mg for the treatment of erectile dysfunction. We have examined its effects on hypoxia-induced pulmonary hypertension in healthy volunteers and mice. To evaluate the contribution of the NO-cGMP pathway to the effects of the drug, we used mice with targeted disruption of the gene encoding endothelial NO synthase (eNOS).

Methods

Acute Hypoxia in Humans

The effects of sildenafil 100 mg and placebo on the pulmonary vascular response to an acute hypoxic challenge were compared in...
10 male volunteers aged 18 to 27 years in a randomized, double-blind study. The volunteers attended the catheterization laboratory at the National Center of Cardiology in Bishkek (760 meters above sea level), Kyrgyz Republic, on 2 occasions, 1 week apart. All gave written informed consent and were judged to be healthy on the basis of medical examination and routine hematologic and biochemistry. The study was approved by the local hospital ethics committee in Bishkek and followed international guidelines for medical research on human subjects. On each occasion, a Swan-Ganz thermodilution catheter (Baxter Healthcare Ltd) was sited in the pulmonary artery via a jugular vein. Baseline measurements were made after 30 minutes’ rest, and then sildenafil or placebo (lactose) was given orally in a gelatin capsule with 100 mL of water. One hour later, the volunteers breathed via a mouthpiece connected to a Douglas bag containing 11% O₂. Measurements of PAP, systemic blood pressure, and heart rate were repeated after 30 minutes of hypoxia. Blood samples were taken in 1% 0.5 mmol/L EDTA on ice just before and at the end of 30 minutes of hypoxia and stored for cGMP analysis. Arterial oxygen saturation was measured by pulse oximetry (PROPAQ 102, Protocol Systems Inc).

**Animals**

Mice deficient in eNOS (−/−) and the wild-type strain C57BL6/SV129 (+/+) were provided by Dr P.L. Huang. The animals for the present study were littermates bred from heterozygous mutants. Genotype was confirmed by analysis of genomic DNA by standard techniques. Animals were fed standard chow and water ad libitum.

**Isolated Perfused Mouse Lung**

The lungs of anesthetized (Hypnorm [fentanyl and fluanisone 0.25 mL/kg] and midazolam 25 mg/kg IP) mice were ventilated with air at a constant end-expiratory pressure (12 to 15 cm H₂O) and perfused in situ in the open chest with Dulbecco’s modified Eagle’s medium containing 4% Ficoll and 25 mmol/L HEPEPS at a flow rate of 2 mL/min with a nonpulsatile pump (Masterflex model 7519), as described previously. After 20 minutes, the ventilation mixture was changed to 2% O₂/5% CO₂/93% N₂ for 10 minutes and the rise in PAP recorded (HPV1). The ventilation gas was returned to air for 15 minutes, and sildenafil (final reservoir concentration 100 nmol/L) or vehicle was added to the perfusate. The hypoxic challenge with 2% O₂ was repeated, and the pressure response was recorded (HPV2).

**Chronic Dosing Study**

Wild-type and eNOS mutant mice were exposed to normal air or placed in a specially constructed normobaric hypoxic (PiO₂ 10%) chamber for 3 weeks. Sildenafil (25 mg · kg⁻¹ · d⁻¹) was administered in the drinking water, and the dose was monitored by daily weighing of the water bottle. At 3 weeks, right ventricular systolic pressure (RVSP) was measured via direct cardiac puncture in the anesthetized (as above) animal or tissues were collected directly for biochemical assay. The heart was removed, and individual chamber weights were recorded. The left lung was fixed by inflation with 10% formalin in PBS before in situ in the open chest with Dulbecco’s modified Eagle’s medium containing 4% Ficoll and 25 mmol/L HEPES at a flow rate of 2 mL/min with a nonpulsatile pump (Masterflex model 7519), as described previously. After 20 minutes, the ventilation mixture was changed to 2% O₂/5% CO₂/93% N₂ for 10 minutes and the rise in PAP recorded (HPV1). The ventilation gas was returned to air for 15 minutes, and sildenafil (final reservoir concentration 100 mmol/L) or vehicle was added to the perfusate. The hypoxic challenge with 2% O₂ was repeated, and the pressure response was recorded (HPV2).

**Morphological Analysis**

Transverse lung sections were stained with van Gieson’s elastic method. Serial sections were stained with a monoclonal antibody against α-smooth muscle actin (clone 1A, Sigma) and a Mouse-on-Mouse avidin-biotin peroxidase kit (Vector Laboratories). Peroxidase activity was visualized with diaminobenzidine, and sections were counterstained with Harris’ hematoxylin. The proportion of vessels (viewed under light microscopy and characterized by the presence of elastic laminae) with a diameter <50 μm and with immunoreactivity for α-smooth muscle actin (taken as evidence of muscularization) was expressed as a percentage of total vessels counted. At least 40 vessels were counted per section, 3 sections per mouse.

**cGMP Measurement**

cGMP was extracted from plasma with ethanol and dissolved in assay buffer according to the instructions in the radioimmunoassay kit (TRK500, Amersham). Frozen tissues were homogenized in 6% ice-cold trichloroacetic acid solution containing 0.5 mmol/L 3-isobutyl-1-methylxanthine. The homogenate was assayed for protein (Bio-Rad protein assay). After centrifugation at 2000g for 5 minutes at 4°C, the supernatant was washed with water-saturated diethyl ether 6 times. Samples were assayed for cGMP by radioimmunoassay (kit as above) and levels expressed as picomoles per milligram of protein.

**Statistics**

Differences in the response of healthy subjects to hypoxia were analyzed by repeated-measures ANOVA with drug as a within-subject factor and order as a between-subject factor. Results from the animal studies were tested for normality and analyzed with either a Student’s t test or Mann-Whitney U test as appropriate (SPSS, version 9.0). Results were expressed as mean ± SD or median with interquartile range. A P value <0.05 was taken as evidence of significance.

**Results**

**Acute Hypoxia in Humans**

The volunteers had normal PAP at rest. Inspiration of 11% O₂ for 30 minutes led to a pronounced fall in arterial oxygen saturation and a 56% rise in mean PAP, from 16.0±2.1 to 25.0±4.8 mm Hg, on the placebo day (Table). The fall in arterial oxygen saturation was the same but the pressor response was almost abolished by pretreatment with sildenafil (increase from 16.0±2.1 to 18.0±3.6 mm Hg). The small fall in systemic blood pressure on exposure to hypoxia was not affected by sildenafil. The rise in heart rate on the placebo day was significantly greater than on the sildenafil day. Plasma cGMP levels increased significantly (P<0.05) with hypoxia after sildenafil treatment (from 4.9±2.1 before hypoxia to 7.3±2.7 pmol/mL after 30 minutes of exposure) but not with placebo (3.1±1.5 before hypoxia and 4.6±2.2 pmol/mL at 30 minutes).

**Isolated Perfused Mouse Lung**

Ventilation with 2% O₂ led to a prompt and reproducible rise in PAP in both wild-type mice (HPV1 7.1±0.2 versus HPV2 8.1±1.9 mm Hg, Figure 1) and eNOS mutant mice (HPV1 6.7±1.5 versus HPV2 7.8±2.0 mm Hg). There was no
significant difference in HPV1 between wild-type and mutant mice. This pressor response was reduced by pretreatment with sildenafil in both genotypes (wild-type: HPV1 8.4 ± 0.9 versus HPV2 3.8 ± 0.5 mm Hg, \( P < 0.05 \), HPV2/HPV1 45%; mutant mice: HPV1 10.1 ± 2.4 versus HPV2 6.4 ± 1.9 mm Hg, \( P < 0.05 \), HPV2/HPV1 63%).

Chronic Mouse Studies

Right Ventricular Systolic Pressure
In wild-type mice, 3 weeks’ exposure to hypoxia (10% \( O_2 \)) produced a 2-fold rise in RVSP in the vehicle treatment group (21.4 ± 4.1 versus 43.3 ± 9.9 mm Hg, \( P < 0.01 \), Figure 2a). This effect was significantly attenuated by treatment with sildenafil (29.9 ± 9.7 mm Hg, \( P < 0.01 \)). In normal air, RVSP was higher in eNOS mutant mice than wild-type animals (21.4 ± 4.1 versus 26.7 ± 3.5 mm Hg, \( P < 0.05 \)) and increased further on hypoxic exposure (26.7 ± 3.5 versus 50.7 ± 9.6 mm Hg, \( P < 0.01 \)). There was 1 death in the hypoxic eNOS mutant placebo group. Sildenafil had no effect on the elevated basal RVSP in eNOS-deficient mice in normal air. The drug attenuated the rise in RVSP due to hypoxia, but levels remained above those recorded in sildenafil-treated animals in normal air (33.7 ± 6.9 versus 28.0 ± 7.7 mm Hg, \( P < 0.05 \)).

RV Hypertrophy
A significant increase in RV/body weight (1.38 ± 0.22 versus 1.59 ± 0.26, \( P < 0.05 \), Figure 2b) and RV/left ventricle (LV) plus septum weight (0.30 ± 0.03 versus 0.41 ± 0.06, \( P < 0.05 \), Figure 2c) was observed in wild-type mice exposed to 3 weeks of hypoxia, and this was partially inhibited by sildenafil (Figure 2b and 2c). Under normoxic conditions, RV weights of eNOS mutant mice were similar to those of wild-type controls. After exposure to hypoxia, these mice developed RV hypertrophy to the same extent as did wild-type animals (RV/body weight 1.14 ± 0.12 versus 1.60 ± 0.39, \( P < 0.05 \), RV/LV 0.26 ± 0.03 versus 0.37 ± 0.09, \( P < 0.05 \)). Sildenafil treatment had no effect on the development of RV hypertrophy in eNOS mutants (RV/body weight 1.62 ± 0.56 versus 1.60 ± 0.39; RV/LV 0.37 ± 0.12 versus 0.37 ± 0.09).

Pulmonary Vascular Morphology
Exposure to hypoxia was accompanied by an increase in proportion of muscularized pulmonary arteries in both wild-type and mutant mice (33% versus 81% in wild-type mice, \( P < 0.05 \); 21% versus 71% in mutant mice, \( P < 0.05 \), Figure 3). Sildenafil treatment attenuated the muscularization in wild-type hypoxic mice (sildenafil versus placebo, 47% versus 71%, Figure 3).**

Figure 1. Change in PAP in isolated perfused mouse lung. Lungs were ventilated with air, which was changed to 2% \( O_2 \) during hypoxic challenges (HPV1 and HPV2). Vehicle or sildenafil (100 \( \mu \)mol/L) was administered between HPV1 and HPV2 in control and sildenafil-treatment groups, respectively. Data are mean ± SD, \( n = 4 \) in each group. *\( P < 0.05 \) vs HPV1.

Figure 2. Effect of chronic dosing with sildenafil in mice. a, RVSP; b, ratio of RV to body weight (RV/BW); and c, ratio of RV to LV plus septum weight (RV/LV+septum) in mice treated with sildenafil (solid columns) or vehicle (open columns) during exposure to hypoxia (10% \( O_2 \)) for 3 weeks or kept in normal laboratory air. Data are mean ± SD. †\( P < 0.01 \) and *\( P < 0.05 \) vs vehicle-treated normal air group. #\( P < 0.05 \) vs vehicle-treated hypoxic group. \( n = \) number in each group.

Figure 3. Effect of sildenafil treatment on hypoxia-induced muscularization of pulmonary vessels (<50 \( \mu \)m). Percentage of pulmonary vessels <50 \( \mu \)m diameter stained for \( \alpha \)-smooth muscle actin in mice treated with sildenafil (solid columns) or vehicle (open columns) and exposed to hypoxia (10% \( O_2 \)) for 3 weeks or kept in normal laboratory air. Data presented as box and whisker plot. Median is marked inside box, and ends of box correspond to interquartile range (25th and 75th percentiles). *\( P < 0.05 \) vs vehicle-treated normal air group. #\( P < 0.05 \) vs vehicle-treated hypoxic group. \( n = \) number in each group.
Oral sildenafil produced dose-dependent reductions of PAP in awake lambs in which PAP was raised with the thromboxane analog U46619. There is 1 case report in the literature describing the beneficial effects of oral sildenafil on exercise capacity in a 4-year-old girl with primary pulmonary hypertension. The study reported here is consistent with and extends these reports in demonstrating that sildenafil preferentially reduces the pulmonary pressor response to hypoxia in humans.

The 56% increase observed in mean PAP with hypoxia in the present study is greater than the 20% to 30% rise reported in other studies, indicating a significant hypoxic stimulus. The hypoxic challenge was timed to coincide with peak plasma levels of sildenafil after oral dosing. Although we cannot exclude a direct effect of the drug on cardiac contractility, the demonstration that the drug is effective in the isolated perfused lung, where perfusion pressure is kept constant, supports a direct vasorelaxant action.

Consistent with inhibition of PDE5, treatment with sildenafil during hypoxia was associated with significant elevation of plasma cGMP levels in healthy volunteers and lung cGMP levels in wild-type mice. No significant change in cGMP levels was observed in the RV, a tissue with little or no PDE5 activity. It is likely that the attenuated RV response to hypoxia in the sildenafil-treated wild-type mice is secondary to the smaller rise in PAP rather than a direct effect of sildenafil on myocardial hypertrophy.

The major stimulants of pulmonary vascular cGMP production are NO and natriuretic peptides. NO is synthesized by NO synthases (NOS), and 3 isoforms are recognized: eNOS, inducible NOS (iNOS), and neuronal NOS (nNOS). The eNOS-NO-cGMP pathway is thought to assist in maintaining the low vascular tone characteristic of the healthy adult pulmonary circulation. In keeping with this, eNOS-deficient mice have a modestly elevated basal PAP compared with wild-type mice when housed in a normal atmosphere.

eNOS-derived NO also appears to attenuate the magnitude of pulmonary hypertension on exposure to mild-to-moderate hypoxia, but the importance of eNOS in limiting the response to severe hypoxia (FiO₂ <12%) is less clear. Comparisons of eNOS-deficient and wild-type mice exposed to severe hypoxia have variously shown greater pulmonary hypertension and remodeling, no difference, and even reduced remodeling in eNOS-deficient mice. In the present study, eNOS-deficient and wild-type mice demonstrated a similar pressor response to severe hypoxia in the isolated perfused lung and developed a similar degree of pulmonary hypertension, RV hypertrophy, and vessel muscularization during chronic hypoxia.

To pursue the role of eNOS further, we examined the hypoxic response of eNOS-deficient animals to sildenafil. It is clear from the isolated perfused lung and chronic hypoxia studies that sildenafil is able to reduce hypoxia-induced pulmonary hypertension in the congenital absence of eNOS, which indicates that sildenafil has beneficial hemodynamic effects even when eNOS activity is impaired. However, the reduction in PAP was not as great as that in wild-type animals, and there was no associated reduction in RV hypertrophy or vascular muscularization in sildenafil-treated hypoxic eNOS-deficient mice. Taken together, these data suggest integrity of the eNOS-NO-cGMP pathway is not essential for the hemodynamic response to sildenafil but is necessary for sildenafil to exert its full benefit in hypoxia-induced pulmonary hypertension.

**Discussion**

Exposure to hypoxia produces a rapid and sustained rise in PAP in humans and mice. Oral treatment with sildenafil 100 mg, a PDE5 inhibitor, markedly inhibits this rise in healthy volunteers without significantly affecting systemic blood pressure. The inhibitory effect is reproduced in the isolated perfused mouse lung. In addition, continued administration of sildenafil attenuates the increase in RVSP, RV hypertrophy, and pulmonary vascular remodeling in mice chronically exposed to hypoxia. eNOS-deficient mice demonstrate a partial response to sildenafil, which suggests that the eNOS-NO-cGMP pathway contributes to the response to the drug but that other cGMP synthetic pathways also play a role.

At least 10 families of PDEs have been described that are products of separate genes. Of these, PDE5, PDE6, and PDE9 are highly specific for cGMP hydrolysis, and PDE5 is largely responsible for cGMP metabolism in the lung. In early animal studies, dipyridamole and zaprinast attenuated hypoxia-induced pulmonary vasoconstriction and remodeling, but their clinical use is limited by their lack of selectivity. E4021 and E4010, selective PDE5 inhibitors, have been reported to reduce PAP in animal models of pulmonary hypertension but are not available for use in humans.

Sildenafil is a potent, selective, orally active inhibitor of PDE5, which is used in doses of 50 to 100 mg for the treatment of erectile impotence. In hemodynamic studies, 100 to 200 mg has a very modest systemic vasodilator action in healthy men, with transient effects on systemic blood pressure and cardiac index. However, sildenafil 40 mg IV (equivalent to 100 mg PO) reduced basal PAP by 27% in men with ischemic heart disease. Oral sildenafil produced dose-dependent reductions of PAP in awake lambs in which PAP was raised with the thromboxane analog U46619. There is 1 case report in the literature describing the beneficial effects of oral sildenafil on exercise capacity in a 4-year-old girl with primary pulmonary hypertension. The study reported here is consistent with and extends these reports in demonstrating that sildenafil preferentially reduces the pulmonary pressor response to hypoxia in humans.

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Because inhaled NO also selectively reduces hypoxic pulmonary vasoconstriction in humans, there is interest in the potential therapeutic benefit of combining NO with PDE5 inhibition. Interestingly, data from animal studies show that sildenafil neither augments nor prolongs the acute effect of NO on PAP in pulmonary hypertension. This lack of potentiation supports our interpretation that PDE5 inhibition is not entirely dependent on NO for acute vasodilatation.

Among the factors other than NO that might contribute to the hemodynamic response to PDE5 inhibition are the natriuretic peptides. Levels of these peptides are increased in pulmonary hypertension and attenuate the pulmonary pressor response to hypoxia. Atrial natriuretic peptide synthesis is upregulated in hypertension and attenuates the pulmonary pressor response to endogenous vasodilators. Levels of these peptides are increased in pulmonary hypertension induced by hypoxia. Atrial natriuretic peptide synthesis is upregulated in eNOS-deficient animals. This might account for the normal pulmonary hemodynamic response to PDE5 inhibition.

Potentiation supports our interpretation that PDE5 inhibition is not entirely dependent on NO for acute vasodilatation. It is possible that the natriuretic peptides may be more important in regulating pulmonary vascular tone in pulmonary hypertension, whereas local eNOS-derived NO may be more important in maintaining the normal structure of the distal un muscularized pulmonary vasculature. In this regard, the response to sildenafil of mice in which components of the natriuretic peptide pathway have been disrupted would be of great interest.

In summary, sildenafil has a significant inhibitory effect on hypoxia-induced pulmonary hypertension and vascular remodeling. The eNOS-NO-cGMP pathway contributes to this response, but sildenafil is not entirely dependent on this pathway. Sildenafil may be a useful orally active treatment for pulmonary hypertension secondary to hypoxia, but the safety and efficacy of chronic administration in humans need to be subjected to controlled clinical trials before it can be recommended for routine use.

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