Synergy between Natriuretic Peptides and Phosphodiesterase 5 Inhibitors Ameliorates Pulmonary Arterial Hypertension

Reshma S. Baliga^{1,2*}, Lan Zhao^{3*}, Melanie Madhani², Belen Lopez-Torondel¹, Cristina Visintin², David Selwood², Martin R. Wilkins³, Raymond J. MacAllister¹, and Adrian J. Hobbs^{1,2}

¹Centre for Clinical Pharmacology, and ²Department of Pharmacology, University College London, London, United Kingdom; and

³Department of Experimental Medicine and Toxicology, Imperial College London and Hammersmith Hospital, London, United Kingdom

Rationale: Phosphodiesterase 5 (PDE5) inhibitors (e.g., sildenafil) are selective pulmonary vasodilators in patients with pulmonary arterial hypertension. The mechanism(s) underlying this specificity remains unclear, but studies in genetically modified animals suggest it might be dependent on natriuretic peptide bioactivity.

Objectives: We explored the interaction between PDE5 inhibitors and the natriuretic peptide system to elucidate the (patho)physiological relationship between these two cyclic GMP (cGMP)-regulating systems and potential of a combination therapy exploiting these cooperative pathways.

Methods: Pharmacological evaluation of vascular reactivity was conducted in rat isolated conduit and resistance vessels from the pulmonary and systemic circulation *in vitro*, and in anesthetized mice *in vivo*. Parallel studies were undertaken in an animal model of hypoxia-induced pulmonary hypertension (PH).

Measurements and Main Results: Sildenafil augments vasodilatation to nitric oxide (NO) in pulmonary and systemic conduit and resistance arteries, whereas identical vasorelaxant responses to atrial natriuretic peptide (ANP) are enhanced only in pulmonary vessels. This differential activity is mirrored *in vivo* where sildenafil increases the hypotensive actions of ANP in the pulmonary, but not systemic, vasculature. In hypoxia-induced PH, combination of sildenafil plus the neutral endopeptidase (NEP) inhibitor ecadotril (which increases endogenous natriuretic peptide levels) acts synergistically, in a cGMP-dependent manner, to reduce many indices of disease severity without significantly affecting systemic blood pressure.

Conclusions: These data demonstrate that PDE5 is a key regulator of cGMP-mediated vasodilation by ANP in the pulmonary, but not systemic, vasculature, thereby explaining the pulmonary selectivity of PDE5 inhibitors. Exploitation of this mechanism (i.e., PDE5 and neutral endopeptidase inhibition) represents a novel, orally active combination therapy for pulmonary arterial hypertension.

Keywords: guanylyl cyclase; cyclic GMP; nitric oxide; natriuretic peptides; neutral endopeptidase

Pulmonary arterial hypertension (PAH) is characterized by increased pulmonary arterial blood pressure, vascular remodeling of the pulmonary small arteries, right ventricular hypertrophy, and ultimately right ventricular failure (1, 2). Whether idiopathic PAH, familial PAH, or PAH associated with other diseases (e.g., congenital heart disease, HIV infection), the

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The morbidity and mortality associated with pulmonary arterial hypertension remains high; targeted therapy to exclusively dilate the pulmonary vasculature would improve current therapeutic options.

What This Study Adds to the Field

Atrial natriuretic peptide (ANP) mediated vasodilation is augmented by phosphodiesterase 5 (PDE5) inhibition in the pulmonary vasculature, suggesting that combined use of PDE5 inhibitors and natriuretic peptide receptor activation may act synergistically to alleviate pulmonary arterial hypertension.

disease leads to premature death, in large part due to the paucity of satisfactory treatments, particularly vasodilators that selectively oppose the excessive vasoconstriction observed in the pulmonary vasculature, and agents able to reverse vascular wall remodeling. This therapeutic insufficiency is also a consequence of uncertainty regarding disease etiology. Current treatment options include prostacyclin analogs (3, 4) endothelin receptor antagonists (5, 6), and phosphodiesterase 5 (PDE5) inhibitors (i.e., sildenafil) (7), but despite these advances, 2-year mortality remains around 15% (8). As a consequence, treatments are being combined with the goal of achieving synergy in the pulmonary vascular bed without augmenting systemic effects.

The biological activity of cyclic guanosine-3',5'-monophosphate (cGMP; the intracellular second messenger that mediates the vasodilator activity of nitric oxide [NO] and natriuretic peptides) is regulated by a family of PDEs that rapidly hydrolyze this cyclic nucleotide. In the vasculature, PDE5 is believed to be the predominant PDE isoform involved in degrading cGMP; PDE5 inhibition with sildenafil increases vascular smooth muscle cGMP levels and lowers systemic and pulmonary arterial pressure ($\overline{Ppa}$) under physiologic conditions in animals and humans (9, 10). In contrast, in animal models and in patients with PAH, PDE5 inhibition causes larger reductions in pulmonary compared with systemic vascular resistance, thereby exhibiting relative selectivity for the pulmonary vasculature (11–13).

The mechanism(s) underlying the pulmonary selectivity of PDE5 inhibition remain unclear. Although PDE5 inhibition abrogates functional and structural changes in many animal models of PH, this effect is blunted in the natriuretic peptide receptor (NPR)-A knockout mouse (14). This suggests that the mechanism of pulmonary selectivity of PDE5 inhibition is dependent, at least in part, on the bioactivity of natriuretic peptides (i.e., atrial natriuretic peptide (ANP) or brain natriuretic peptide [BNP]). This thesis is supported by data demonstrating

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*These authors contributed equally to this article.

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Correspondence and requests for reprints should be addressed to Raymond J. MacAllister, M.D., Ph.D., Centre for Clinical Pharmacology, University College London, The Rayne Building, 5 University Street, London WC1E 5JJ, UK. E-mail: r.macallister@ucl.ac.uk

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that inhibition of natriuretic peptide activity augments the development of pulmonary hypertension (PH), whereas increasing natriuretic peptide activity attenuates PH development (14–16). Indeed, we have shown previously that inhibitors of neutral endopeptidase (NEP; an enzyme that hydrolyzes and terminates the biological activity of endogenous natriuretic peptides [17]) reduce the severity of hypoxia-induced PH (18). Such observations suggest that, in PH, release of natriuretic peptides from the heart represents a cytoprotective mechanism that reduces $\overline{Ppa}$.

In the present study, we have explored the interaction between PDE5 inhibition and the natriuretic peptide system *in vitro* and *in vivo*, including an animal model of PH, to more fully elucidate the (patho)physiologic relationship between these two cGMP-regulating systems and the therapeutic potential of a combination drug treatment exploiting these cooperative pathways.

Some of the results of these studies have been previously reported in the form of abstracts (19, 20).

METHODS

Materials

N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (SPER-NO) was purchased from Calbiochem (Nottingham, UK). N^G -nitro-L-arginine methyl ester, sodium nitroprusside (SNP), acetylcholine (ACh), and rat ANP were purchased from Sigma Chemical Co (Dorset, UK). The stable prostaglandin endoperoxide analog 9,11-dideoxy-11 α ,9 α -epoxymethano-prostaglandin F_{2 α} (U46619) was purchased from Affiniti (Exeter, UK). Sildenafil was extracted from proprietary tablets obtained from the pharmacy at University College London Hospital. The NEP inhibitor ecdotril was the kind gift of Dr. Johannes-Peter Stasch (Bayer AG, Wuppertal, Germany).

Functional Pharmacologic Studies

These studies were conducted on animals that had not been exposed to hypoxia.

Thoracic aorta and pulmonary artery. Male rats (Sprague-Dawley; 200–250 g) were killed by cervical dislocation. The thoracic aorta and pulmonary artery (including first-order branches) were carefully removed and set up for isometric tension recordings, as described previously (21). Tissues were contracted with an approximate EC₈₀ concentration (80% of the maximal response) of U46619 and cumulative concentration–response curves were constructed to SPER-NO (1 nM–1 μ M) and ANP (1 nM–1 μ M). To investigate the functional activity of PDE5, concentration–response curves to SPER-NO and ANP were obtained in the absence (control) and presence of sildenafil (3 μ M; 30 min preincubation).

Mesenteric and pulmonary small arteries. Rat (Sprague-Dawley; 200–250 g; male) pulmonary (third/fourth order; diameter, $\sim$ 100 μ m) and mesenteric (third/fourth order; diameter, $\sim$ 180 μ m) small arteries were dissected and mounted in Mulvany-type myographs, as previously described (21). The vessels were stretched to 90% of the diameter achieved when under a transmural pressure of 15 mm Hg (pulmonary) and 100 mm Hg (systemic), respectively. Vessels were precontracted with U46619 and endothelial integrity assessed using ACh (1 μ M), as above. Concentration–response curves to SPER-NO (10 nM–10 μ M) and ANP (1 nM–1 μ M) were constructed in the absence and presence of sildenafil (3 μ M; 30 min preincubation).

In vivo measurement of mean arterial blood pressure and right ventricular pressure (RVP). Male C57/BL6 mice (20–25 g) were anesthetized with 1.5% isoflurane and placed supine on a thermostatically controlled heating blanket (37°C). To measure mean arterial blood pressure (MABP), the left common carotid artery was isolated and a fluid-filled (heparin; 100 U/ml diluted in 0.9% saline), 0.28-mm-internal-diameter cannula (Crichtley Electrical Products Pty Ltd, Castle Hill, Australia) introduced into the artery. To measure RVP, the right jugular vein was isolated and a fluid-filled cannula introduced into the superior vena cava and then advanced into the right ventricle. Both MABP and RVP were measured using an in-line P23 XL transducer (Viggo-Spectramed, Oxnard, CA) and recorded onto a precalibrated PowerLab system (ADInstruments, Castle Hill, Australia). The right femoral vein was cannulated for drug administration. After 10 minutes

of stabilization, mice were given 50 μ l intravenous bolus injection of ANP (10 or 100 μ g/kg) or SNP (3 or 10 μ g/kg). To examine the effects of PDE5 inhibitor on MABP and RVP responses, 50 μ l intravenous bolus injections of ANP (10 or 100 μ g/kg) or SNP (3 or 10 μ g/kg) were administered to mice that had received sildenafil (1 mg/kg; 50 μ l intravenous bolus) 30 minutes previously. Mice were used for the *in vivo* studies to provide evidence that synergy between sildenafil and ANP occurs in the pulmonary vasculature of a second species.

Hypoxia-induced Pulmonary Hypertension

Male Sprague-Dawley rats (220 g at start of study) were divided into five groups: (1) normoxia control rats that received daily gavage with vehicle, (2) hypoxia control rats that received daily gavage with vehicle, (3) hypoxic rats that received sildenafil (30 mg/kg/d) administered in drinking water, (4) hypoxic rats that received ecdotril (60 mg/kg/d) delivered by gavage, and (5) hypoxic rats that received combination therapy of sildenafil (30 mg/kg/d) + ecdotril (60 mg/kg/d). Treatment with the appropriate drugs/vehicles was started 2 days before placement in a hypoxic chamber (10% O₂). The sildenafil dose was monitored by weighing drinking bottles daily, and adjusting the concentration to maintain dose if necessary.

In further experiments, to establish a dose–response relationship for the beneficial effects of the combination therapy in PH, essentially identical studies to those described above were conducted with lower doses of sildenafil and/or higher doses of ecdotril. These were (1) sildenafil (10 mg/kg/d) plus ecdotril (60 mg/kg/d) and (2) sildenafil (30 mg/kg/d) plus ecdotril (90 mg/kg/d).

Hemodynamic measurements. After 2 weeks of exposure to hypoxia, right ventricular systolic pressure (RVSP) and $\overline{Ppa}$ were recorded in anesthetized animals by catheterization of the jugular vein (as above; for $\overline{Ppa}$, the catheter was advanced from the right ventricle [RV] into the pulmonary artery). MABP was determined via catheterization of the carotid artery (as above). Animals were removed from the chamber individually and anesthetized immediately to minimize the time spent outside the hypoxic environment before hemodynamic measurement. The animal was then killed by anesthetic overdose, plasma was collected, the heart was removed, and individual chamber weights were measured to evaluate right ventricular hypertrophy (right ventricle:left ventricle plus septum [RV/LV+S] ratio). The left lung was fixed by inflation with 10% formalin in phosphate-buffered saline before paraffin embedding and sectioning. Plasma was collected by centrifugation (220 $\times$ g; 20 min) of whole blood and stored at -80°C for measurement of cGMP levels.

cGMP measurements. Plasma samples were diluted in ice-cold buffer (50 mM Tris-HCl, pH 7.5, 4 mM ethylenediaminetetraacetic acid) containing 3-isobutyl-1-methyl xanthine (IBMX) (2.5 mM) and cGMP was measured by radioimmunoassay (TRK500; Amersham Little Chalfont, UK) according to the manufacturer's instructions, as we have described previously (22). Tissue samples (lung biopsies) were homogenized on ice in buffer containing IBMX (2.5 mM, as above). Homogenates were assayed to determine protein content (bicinchoninic acid) [BCA] assay; Pierce Biotechnology, Rockford, IL), deproteinized by heating, and the supernatant assayed for cGMP as above.

ANP measurements. Plasma ANP concentrations were determined using a specific ¹²⁵I-ANP radioimmunoassay (Peninsula Laboratories, Belmont, CA), after extraction with SEP-PAK C₁₈ columns (Peninsula Laboratories), according to the manufacturer's instructions.

Morphologic analysis. Transverse formalin-fixed lung sections were stained with van Gieson's elastic method. Pulmonary arterial muscularization was then assessed as previously described (23). Briefly, vessels of less than 100 μ m diameter were counted in each lung section, and defined according to degree of muscularization: fully muscularized (two distinct and continuous elastic lamina), partially muscularized (second elastic lamina not continuous [$<50\%$]), and nonmuscularized (single elastic lamina). At least 150 vessels were counted per section and the proportion of vessels in each category was expressed as a percentage of total vessels counted. Morphometric analysis was performed by two independent blinded examiners, with an interperson variability of less than 10%. Twenty-five muscularized arteries ranging from 25–100 μ m diameter from different fields were then imaged at $\times 400$ magnification by light microscopy from representative animals in each group.

Data analysis. For *in vitro* studies, relaxations are expressed as percentage of reversal of U46619-induced tone (mean $\pm$ SEM of “n”

animals). Curves were fitted to all the data using nonlinear regression (GraphPad Software, San Diego, CA) and the $-\log [M]$ of each drug giving a half-maximal response (pEC_{50}) was used to compare potency. Curves were analyzed using two-way analysis of variance (ANOVA) and $P < 0.05$ was taken as statistically significant. For *in vivo* studies, changes in MABP, RVP, $\overline{Ppa}$, RVSP, RV/LV ratio, and muscularization of arteries were analyzed by one-way ANOVA. Results are expressed as mean $\pm$ SEM, and $P < 0.05$ denotes significance.

RESULTS

Effect of PDE5 Inhibition on Responses to SPER-NO and ANP in Conduit Arteries

Thoracic aorta. Incubation of rat isolated aortic vessels with sildenafil (3 μ M) increased the potency of SPER-NO (pEC_{50} , 5.90 ± 0.09 and 6.61 ± 0.10 in the absence and presence of sildenafil, respectively; $P < 0.05$; Figure 1). In marked contrast, ANP was equipotent in the thoracic aorta in the absence (pEC_{50} , 7.33 ± 0.16) and presence of sildenafil (3 μ M; pEC_{50} , 7.51 ± 0.17 ; $P > 0.05$ vs. control; Figure 1).

Pulmonary artery. After incubation of rat isolated pulmonary artery with sildenafil (3 μ M), responses to SPER-NO were significantly enhanced (pEC_{50} , 6.30 ± 0.07 and 7.12 ± 0.10 in the absence and presence of sildenafil, respectively; $P < 0.05$; Figure 1). Sildenafil (3 μ M) also increased the relaxant potency of ANP in isolated pulmonary artery preparations (pEC_{50} , 8.74 ± 0.14 and 9.71 ± 0.09 in the absence and presence of sildenafil, respectively; $P < 0.05$; Figure 1).

Effect of PDE5 Inhibition on Responses to SPER-NO and ANP in Resistance Arteries

Mesenteric small arteries. After treatment of isolated mesenteric small arteries with sildenafil (3 μ M), responses to SPER-NO were significantly enhanced (pEC_{50} , 6.69 ± 0.08 and 7.26 ± 0.09 in the absence and presence of sildenafil, respectively; $P < 0.05$; Figure 2). In marked contrast, ANP had an equivalent relaxant potency in mesenteric small arteries in the absence and presence of sildenafil (3 μ M; EC_{50} , 8.71 ± 0.18 and 8.46 ± 0.18 , respectively; $P > 0.05$; Figure 2).

Pulmonary small arteries. SPER-NO and ANP produced concentration-dependent relaxations of U46619 precontracted pulmonary small arteries (pEC_{50} , 6.20 ± 0.05 and 9.72 ± 0.29 , respectively). Responses to both vasodilators were significantly enhanced in the presence of sildenafil (3 μ M; pEC_{50} , 6.86 ± 0.06 and 9.81 ± 0.18 , respectively; $P < 0.05$ vs. control; Figure 2).

Effect of PDE5 Inhibition on MABP and RVP Changes in Response to ANP and NO

MABP. As shown in Figure 3, both SNP and ANP caused dose-dependent decreases in MABP. After bolus administration of sildenafil (1 mg/kg), the hypotensive responses to SNP were significantly enhanced. In contrast, reductions in MABP in responses to ANP were not altered in the presence of sildenafil (Figure 3).

RVP. ANP and SNP decreased RVP in a dose-dependent manner (Figure 3). However, in the presence of sildenafil (1 mg/kg), RVP responses to both and SNP were significantly enhanced (Figure 3).

Hypoxia-induced PH *In Vivo*

Daily weighing of the animals revealed that, after an initial weight loss on initiation of gavage and hypoxia, all animals maintained or increased body weight throughout the study, suggesting good tolerance to the hypoxia, drugs, and handling (data not shown).

Effect of Combination Therapy on Pulmonary Artery, Right Ventricular, and Mean Arterial Pressures

In untreated control rats, 2 weeks of 10% hypoxia produced markedly elevated $\overline{Ppa}$ (29.58 ± 1.3 mm Hg) compared with normoxia control animals (12.13 ± 1.7 mm Hg). Both sildenafil-treated (24.26 ± 0.7 mm Hg) and ecadotril-treated (25.45 ± 1.1 mm Hg) animals showed statistically significant reduction in $\overline{Ppa}$ compared with untreated hypoxic animals (Figure 4). The combination sildenafil + ecadotril group showed a further reduction (20.68 ± 0.9 mm Hg) in $\overline{Ppa}$ that was statistically different from both the sildenafil ($P < 0.05$) and ecadotril groups

Figure 1. Concentration response curves to atrial natriuretic peptide (ANP) and N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]-butyl]-1,3-propanediamine (SPER-NO) in rat aorta (left panels) and pulmonary artery (right panels) in the absence (open circles) and presence of sildenafil (3 μ M; 30 min incubation; solid circles). Relaxation is expressed as mean $\pm$ SEM percentage reversal of U46619-induced tone (* $P < 0.05$ vs. control by two-way ANOVA comparing the entire concentration range; ANP aorta, $n = 12$; ANP pulmonary, $n = 11$; SPER-NO aorta, $n = 8$; SPER-NO pulmonary $n = 11$).

Figure 2. Concentration–response curves to ANP and SPER-NO in rat mesenteric (left panels) and pulmonary (right panels) small arteries in the absence (open circles) and presence of sildenafil (3 μ M; 30 min incubation; solid circles). Relaxation is expressed as mean $\pm$ SEM percentage reversal of U46619-induced tone (* P < 0.05 vs. control by two-way ANOVA comparing the entire concentration range; ANP mesenteric, n = 9, ANP pulmonary, n = 7, SPER-NO mesenteric, n = 8, SPER-NO pulmonary, n = 5).

(P < 0.01; Figure 4). Similarly, 2 weeks of hypoxia produced a doubling of the RVSP in untreated hypoxic rats (38.72 ± 2.0 mm Hg) compared with normoxia control animals (18.8 ± 2.3 mm Hg). Single treatment with ecadotril (41.3 ± 1.8 mm Hg) failed to reduce RVSP, but the sildenafil (36.17 ± 1.8 mm Hg) and sildenafil + ecadotril group showed reduced RVSP (32.5 ± 1.4 mm Hg; P < 0.05 vs. sildenafil and ecadotril alone; Figure 4).

Effect of Combination Therapy on Cardiac Hypertrophy

There were no significant changes in total ventricle weight across the groups (Figure 5). Untreated hypoxic animals showed a significant increase in RV/LV+S ratio (normoxia, 0.30 ± 0.01 ; hypoxia, 0.51 ± 0.01). Treatment with sildenafil (0.39 ± 0.01) or

ecadotril (0.43 ± 0.01) significantly reduced RV hypertrophy. However, combination treatment of sildenafil plus ecadotril caused an additional reduction in RV hypertrophy (0.37 ± 0.01 ; P < 0.05 vs. hypoxia, sildenafil alone and ecadotril alone; Figure 5).

Dose–Response Relationship between PDE5 Inhibition and NEP Inhibition in Hypoxia-induced PH

To establish a dose–response relationship for the combination therapy in PH, we conducted additional experimentation to determine the efficacy of lower sildenafil and higher ecadotril doses in alleviating the hemodynamic aberrations and cardiac hypertrophy that underscore the disease. In this case, reducing the sildenafil dose to 10 mg/kg/day produced a smaller effect on

Figure 3. Changes in mean arterial blood pressure (MABP) (upper panels) and right ventricular pressure (RVP) (lower panels) in anesthetized mice in response to bolus injections (intravenous) of ANP (10 and 100 μ g/kg; left panels) and sodium nitroprusside (SNP) (3 and 10 μ g/kg; right panels) in the absence (open bars) and presence (solid bars) of sildenafil (1 mg/kg, intravenously; * P < 0.05 vs. corresponding control; n = 5 for each condition).

Figure 4. Mean pulmonary artery pressure (PAP; left panel) and right ventricular systolic pressure (RVSP; right panel) in control (normoxic) rats ($n = 6$) and animals exposed to 2 weeks of hypoxia (10% O_2 ; $n = 14$) in the absence and presence of sildenafil (30 mg/kg/d; $n = 14$), ecadotril (60 mg/kg/d; $n = 10$), or sildenafil plus ecadotril ($n = 20$). * $P < 0.05$ versus hypoxic control; # $P < 0.05$ versus sildenafil and ecadotril alone.

$\overline{Ppa}$ and RV hypertrophy (Figure 6). Likewise, raising the dose of ecadotril to 90 mg/kg/day increased its activity in lowering $\overline{Ppa}$ (Figure 6). However, at all doses tested, combination therapy was more efficacious than either drug alone in reducing $\overline{Ppa}$ and, at the higher doses, RV hypertrophy (Figure 6). Importantly, all of the sildenafil and ecadotril combinations did not significantly reduce MABP (Figure 6).

Effect of Combination Therapy on Pulmonary Remodeling

Normoxia animals showed only a modest degree of pulmonary muscularization ($14.09 \pm 1.9\%$), which was significantly increased by 2 weeks of hypoxia ($74.07 \pm 3.1\%$). Treatment with sildenafil ($65.87 \pm 2.7\%$) and ecadotril ($69.83 \pm 1.2\%$) did not show significant attenuation of this hypoxia-induced muscularization. However, the combination of sildenafil plus ecadotril produced a significant decrease in the percentage of muscularized arteries ($64.79 \pm 1.5\%$; $P < 0.05$; Figure 7).

Effect of Combination Therapy on Plasma and Lung cGMP Concentrations

Sildenafil alone, and the combination of sildenafil plus ecadotril, significantly increased plasma cGMP levels compared with

hypoxic controls (Figure 8), whereas in lung biopsies only the combination treatment caused a significant rise in cGMP concentrations (Figure 8). Moreover, the increase in cGMP content was significantly negatively correlated to $\overline{Ppa}$ ($r = -0.39$, $P < 0.0001$) only in the combination (sildenafil + ecadotril) group (Figure 8).

Effect of Combination Therapy on Plasma ANP Concentrations

Ecadotril alone, and the combination of sildenafil plus ecadotril, significantly increased plasma ANP levels compared with hypoxic controls (Figure 8).

DISCUSSION

Herein we attempted to unravel the endogenous mechanisms underlying the relative pulmonary selectivity of PDE5 inhibitors in PH and used an animal model of hypoxia-induced PH to determine if this pulmonary-centric profile of activity can be exploited to improve drug treatment of the disease. First, we assessed *in vitro* and *in vivo* the relative responsiveness of isolated aortic and pulmonary conduit and resistance arteries to the vasodilators NO and ANP in the absence and presence of sildenafil. An important and unexpected pattern of reactivity emerged that clearly explains the pulmonary selectivity of PDE5 inhibitors: in the pulmonary conduit and resistance arteries (i.e., pulmonary artery and pulmonary small arteries), the vasodilator activity of ANP and the NO donor SPER-NO was significantly augmented in the presence of sildenafil. This is consistent with a cGMP-dependent mechanism of relaxation invoked by both mediators, which is physiologically curtailed by the activity of PDE5, as has been previously described (24). In contrast, in the systemic conduit and resistance vasculature (i.e., aorta and mesenteric small arteries), whereas relaxant responses to SPER-NO were augmented in the presence of sildenafil, the vasoactive potency of ANP remained unchanged during PDE5 inhibition. To determine whether the studies with isolated arteries were parallel *in vivo*, we assessed the responsiveness of MABP and RVP to soluble guanylate cyclase (sGC) and particulate guanylate cyclase (pGC) activation in anesthetized mice. In this series of experiments, both SNP (NO donor) and ANP caused dose-dependent decreases in MABP and RVP. After sildenafil treatment, RVP reductions in responses to both SNP and ANP were enhanced, mirroring the augmentation observed *in vitro*. In contrast, sildenafil treatment significantly increased the fall in MABP in response

Figure 5. Total ventricular weight (left panel) and right ventricle: left ventricle plus septum ratio (RV/LV+S; right panel) in control (normoxic) rats ($n = 6$) and animals exposed to 2 weeks of hypoxia (10% O_2 ; $n = 14$) in the absence and presence of sildenafil (30 mg/kg/d; $n = 14$), ecadotril (60 mg/kg/d; $n = 10$), or sildenafil plus ecadotril ($n = 20$). * $P < 0.05$ versus hypoxic control; # $P < 0.05$ versus sildenafil and ecadotril alone.

Figure 6. Mean pulmonary artery pressure (PAP; upper panel), right ventricle:left ventricle plus septum ratio (RV/LV+S; middle panel), and mean arterial blood pressure (MABP) (lower panel) in control (normoxic) rats ($n = 6$) and animals exposed to 2 weeks of hypoxia (10% O_2 ; $n = 14$) in the absence and presence of sildenafil (10 or 30 mg/kg/d; $n = 6$ and 14), ecdotril (60 or 90 mg/kg/d; $n = 10$ and 6), or sildenafil plus ecdotril (10 and 60 mg/kg/d [$n = 6$], 30 and 60 mg/kg/d [$n = 20$], or 30 and 90 mg/kg/d [$n = 6$]). * $P < 0.05$ versus hypoxic control; # $P < 0.05$ versus sildenafil and ecdotril alone.

to SNP, but did not alter systemic blood pressure responses to ANP. This lack of effect of sildenafil on systemic vasodilator response to ANP (but not SNP) reiterated the observations made *in vitro* and confirmed the pulmonary-specific synergy between natriuretic peptides and PDE5 inhibitors. Indeed, a recent *in vivo* study has reported that, although sildenafil does augment the systemic hypotensive effect of ANP, this effect is mild compared with the concomitant pulmonary dilatation, and therefore severe systemic hypotension should not be a problem with this combination (25).

The above observations suggest that generation of cGMP by sGC and pGC (i.e., in response to NO and natriuretic peptides, respectively), at least in the systemic circulation, can be regulated independently and does not result in changes in a single cellular pool of cGMP. These findings fit well with previous reports revealing differential effects of pGC and sGC activation on functions within the same cells (26, 27). Indeed, recent studies have provided the rationale for such discrepant effects of the two cGMP-generating pathways (28, 29). First, it has been proposed that localization of GC enzymes within specialized structures (e.g., caveoli, vesicles, or endoplasmic

Figure 7. Percentage of muscularized vessels (right panel) in control (normoxic) rats ($n = 6$) and animals exposed to 2 weeks of hypoxia (10% O_2 ; $n = 6$) in the absence and presence of sildenafil (30 mg/kg/d; $n = 6$), ecdotril (60 mg/kg/d; $n = 6$), or sildenafil plus ecdotril ($n = 8$). * $P < 0.05$ versus hypoxic control. The left panel depicts representative light microscopic images ($\times 80$ original magnification) of pulmonary arteries from normoxic, hypoxic, and combination (sildenafil plus ecdotril)-treated animals; the hypoxic vessels exhibit a marked muscularization that is reduced in the presence of sildenafil plus ecdotril.

reticulum) acts as a barrier to limit the spread of cGMP, similar to mechanisms suggested for cAMP (30). Second, different PDE isoforms might be involved in preventing the diffusion of cGMP. These theses are supported by the observation that, in cardiac cells, the pGC/membrane cGMP pool is controlled exclusively by PDE2, whereas the soluble GC/cytosolic cGMP pool is controlled by PDE5 (28, 29). Our data suggest that not only does intracellular compartmentalization of cGMP occur in vascular smooth muscle but this differs between the systemic and pulmonary vasculature. Moreover, because the EC_{50} values (i.e., potency), and relative shift in the presence of sildenafil, for SPER-NO-dependent relaxation of both the pulmonary and systemic vessels are equivalent, this suggests that it is not simply greater PDE5 expression/activity in the pulmonary circulation (which has been suggested to occur in PH [31, 32]) that is responsible for the differential effect on ANP-dependent responses.

The mechanism or mechanisms responsible for regulation of ANP-dependent cGMP-mediated responses in the systemic circulation remain unclear and merit further attention. At present, it is reasonable to assume that an alternate PDE isoform fulfills this capacity. Possible candidates include PDE1 (regulates ANP-mediated dilatation in porcine aorta and has been linked to nitrate tolerance [33]), PDE2 (plays a key role in regulating cGMP production in the central nervous system [34], heart [28], and adrenal cortex [35]), and PDE3 (found in the heart, vascular smooth muscle, and platelets, and may link cGMP and cAMP pathways [36]). Whatever the case may be, this differential activity of PDE5 inhibitors on the vasoactive properties of ANP gives rise to the possibility of exerting pulmonary-specific dilation by combining a PDE5 inhibitor and a natriuretic peptide "enhancer" (i.e., exogenous

Figure 8. Cyclic guanosine-3',5'-monophosphate (cGMP) accumulation in the plasma (upper left panel) and lung (lower left panel), and ANP concentrations in the plasma (lower right panel) in control (normoxic) rats ($n = 6$) and animals exposed to 2 weeks of hypoxia (10% O_2 ; $n = 14$) in the absence and presence of sildenafil (30 mg/kg/d; $n = 14$), ecdotril (60 mg/kg/d; $n = 10$), or sildenafil plus ecdotril ($n = 20$). * $P < 0.05$ versus hypoxic control. Correlation between cGMP and mean pulmonary artery pressure (PAP) (upper right panel) in rats exposed to 2 weeks of hypoxia (10% O_2) in the presence of sildenafil (30 mg/kg/d) plus ecdotril (60 mg/kg/d; $n = 36$).

natriuretic peptide or drug increasing endogenous natriuretic peptide levels) and exploiting the synergistic activity of these two pathways, which is absent in the systemic vasculature.

Having established the pulmonary-specific regulation of ANP-dependent responses by PDE5 inhibitors, we used a well-defined model of hypoxia-induced PH (11) to assess the efficacy of a combination treatment for PH based on a PDE5 inhibitor (sildenafil) plus an ANP enhancer. Endogenous ANP levels increase in hypoxia, possibly as a compensatory measure, although this is insufficient to prevent the development of PH (37). Administration of exogenous ANP has been shown to reduce hypoxia-induced PH (38). However, the short half-life (0.5–5 min) and negligible oral bioavailability make ANP a poor candidate for drug therapy. We therefore chose to increase endogenous ANP levels by inhibiting NEP activity with the selective drug ecdotril (39). NEP is a membrane-bound zinc metalloproteinase that degrades a number of biologically active peptides including ANP and endothelin (ET)-1 (40). Metabolic breakdown by NEP is one of two primary routes of degradation of natriuretic peptides (the second is via binding to NPR-C, internalization, and lysosomal breakdown [41]). Ecdotril is an orally active prodrug of thiorphan that has been shown to potentiate the antihypertensive, diuretic, and natriuretic effects of ANP (39).

Our results demonstrate a clear synergistic activity of sildenafil and ecdotril in alleviating many indices of disease severity in an animal model of (hypoxia-induced) PH. Using a dose of sildenafil that is similar to that used clinically, we demonstrate that monotherapy with a PDE5 inhibitor or an

NEP inhibitor partially reduces the hemodynamic and structural changes associated with the disease (11, 18); the magnitude of the protective effect of both drugs was akin to previous studies using a rat model of hypoxia-induced PH (18, 42, 43). However, in accord with our hypothesis based on pharmacologic observations made *in vitro* and *in vivo*, a combination treatment regime of sildenafil plus ecdotril has synergistic activity in PH over a range of doses (albeit at submaximally effective doses of the individual agents). This is true not only for a reduction in $\overline{Ppa}$ (and right ventricular systolic pressure) but for RV hypertrophy and pulmonary vascular remodeling as well. The added value of this dual therapy was substantiated by the observation that both plasma and lung cGMP concentrations were significantly elevated in the combination group only, and that efficacy (i.e., reduction in $\overline{Ppa}$) correlated with cGMP levels, suggesting that the combination treatment was targeting the cGMP production where it was required, the pulmonary vasculature; accordingly, the combination therapy had no significant effect on MABP. Our data therefore give credence to the idea that a combined PDE5 inhibitor plus NEP inhibitor regime may offer therapeutic benefit in the treatment of PH above and beyond sildenafil alone.

These findings are consistent with, and advance, previous studies demonstrating that ANP synergistically potentiates pulmonary vasodilation in sildenafil-treated rats exposed to acute hypoxia (44) and that the pulmonary selectivity of sildenafil is lost in NPR-A knockout animals (23). Interestingly, in the present study, plasma ANP levels in the presence of sildenafil alone (in contrast to both ecdotril and the combination treatment) were not significantly increased in response to hypoxia; as such, the beneficial effects of sildenafil in PH may not fully exploit the synergy between PDE5 inhibition and the natriuretic peptide system that we have identified (hence the added benefit of the NEP inhibitor). Undoubtedly, NEP inhibition will augment the biological activity of a number of vasoactive peptides, principally ET-1 and bradykinin. However, because ET-1 is a potent vasoconstrictor, and bradykinin produces vasodilation predominantly via activation of NO-dependent pathways, it is likely that the primary beneficial effect of NEP inhibition in PH is augmentation of endogenous natriuretic peptide levels.

In conclusion, these data suggest that the pulmonary selectivity of PDE5 inhibitors in PH is a result of the differential ability of PDE5 to regulate ANP-mediated dilation in the pulmonary, but not systemic, vasculature. This gives rise to the possibility of effecting organ- or vessel-specific alterations in the vasodilator activity of ANP, which may not only represent an important physiologic regulatory mechanism but also be of therapeutic benefit in PH. In accord, we demonstrate that the combination of natriuretic peptide augmentation (i.e., ecdotril) and PDE5 inhibition (i.e., sildenafil) selectively produces pulmonary vasodilation in the absence of systemic hypotension and this strategy is synergistically effective in improving several indices of disease severity in an animal model of PH. Indeed, a recent study has revealed synergistic activity between sildenafil and BNP in patients with PH (45), suggesting this phenomenon is likely to occur in the human vasculature. Thus, this drug combination could be a novel therapeutic avenue for PH, and given the availability of both agents as licensed drugs, there are realistic translational opportunities.

Conflict of Interest Statement: R.S.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.L.-T. does not have a financial relationship with a commercial

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