Vascular effects of sildenafil in patients with pulmonary fibrosis and pulmonary hypertension: an *ex vivo/in vitro* study

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**ABSTRACT**

Sildenafil improves the 6-min walking distance in patients with idiopathic pulmonary fibrosis (IPF) and right-sided ventricular systolic dysfunction.

We analysed the previously unexplored role of sildenafil on vasoconstriction and remodelling of pulmonary arteries from patients with IPF and pulmonary hypertension (PH) *ex vivo*. Pulmonary arteries from 18 donors without lung disease, nine IPF, eight PH+IPF and four PH patients were isolated to measure vasodilator and anti-contractile effects of sildenafil in isometric organ bath. Ventilation/perfusion was explored in an animal model of bleomycin lung fibrosis.

Sildenafil relaxed serotonin (5-HT) pre-contracted pulmonary arteries in healthy donors and IPF patients and, to a lesser extent, in PH+IPF and PH. Sildenafil inhibited 5-HT dose-response contraction curve mainly in PH+IPF and PH, but not in healthy donors. Sildenafil did not impair the ventilation/perfusion mismatching induced by bleomycin. Pulmonary arteries from PH+IPF patients showed a marked expression of phosphodiesterase-5 and extracellular matrix components. Sildenafil inhibited pulmonary artery endothelial and smooth muscle cell to mesenchymal transition by inhibition of extracellular regulated kinases 1 and 2 (ERK1/2) and SMAD3 phosphorylation.

These results suggest an absence of direct relaxant effect and a prominent anti-contractile and anti-remodelling role of sildenafil in PH+IPF pulmonary arteries that could explain the beneficial effects of sildenafil in IPF with PH phenotype.

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[Sildenafil prevents pulmonary vasoconstriction and remodelling in patients with pulmonary hypertension and fibrosis](http://ow.ly/XU3ig)
Introduction

Idiopathic pulmonary fibrosis (IPF) is a fatal disorder with a median survival time of 2.5–5 years after diagnosis. Pulmonary hypertension (PH) is recognised as a severe complication of IPF associated with poor survival [1]. Recent epidemiological studies reported a 32–84% prevalence of PH in patients with IPF, depending on the stage of IPF progression [1]. PH as a complication of IPF is associated with reduced exercise ability, as evidenced by decreased 6-min walking distance (6MWD) and increased oxygen desaturation.

The pathogenesis of PH in patients with IPF is characterised by vascular muscularisation in the early stages, followed by fibrous vascular atrophy and pronounced intimal fibrosis [2]. Blood vessels may participate in the genesis of IPF, and similar disruptions in cytokines have been described in patients with IPF and PH. The latter suggests that these disorders share pathogenic features and that one may influence, generate or perpetuate the other. Different cellular processes have been described during pulmonary artery remodelling of PH-associated IPF, including endothelial dysfunction [3], the endothelial-to-mesenchymal transition as a source of myofibroblasts [4], as well as pulmonary artery smooth muscle proliferation and transition to myofibroblasts [5].

Although PH is clearly associated with worse IPF outcomes, it remains unproven that treating PH with vasoactive therapies results in improvement. Although effective in patients with PH, endothelin-1 (ET-1) receptor antagonists failed in IPF clinical trials. Deleterious effects of vasodilators in hypoxic PH, such as PH-associated IPF, are attributed to increased oxygen desaturation mediated by the propensity to worsen ventilation/perfusion (V’/Q’) matching by dilating vessels that are perfusing poorly ventilated lung units [6]. In this regard, the phosphodiesterase type 5 (PDE5) inhibitor sildenafil improved pulmonary haemodynamics by maintaining V’/Q’ ratios in the short term in a cohort of patients with PH secondary to lung fibrosis, suggesting a favourable profile of sildenafil for treating IPF [7]. STEP-IPF trial investigators treated patients with severe IPF (diffusing capacity of the lung for carbon monoxide (DLCO) <35%) with sildenafil. Sildenafil did not meet the primary outcome measure of an improved 6MWD compared with placebo in the overall study population; however, small but significant improvements were observed in secondary measures, including improved DLCO, arterial oxygenation, dyspnoea symptoms and quality of life compared with placebo [8]. Sildenafil significantly improved 6MWD (by 99.3 m) in a subgroup of patients with IPF and right-sided ventricular systolic dysfunction that was associated with improved quality of life. Sildenafil may have acted in this group by addressing PH [9]. Sildenafil ameliorates right ventricle hypertrophy and fibrosis, as well as pulmonary vascular remodelling, in animal models of bleomycin-induced pulmonary fibrosis associated with PH by improving nitric oxide synthase coupling and reducing reactive oxygen species signalling [10, 11]. Sildenafil also reduces endothelial dysfunction and inhibits human pulmonary artery smooth muscle cell (HPASMC) proliferation in cell models [12, 13].

Unfortunately, there are no approved targeted therapies for PH in patients with IPF. Although growing evidence suggests beneficial effects of sildenafil on PH associated with IPF, the direct effects of sildenafil on the pulmonary arteries of patients with PH-associated IPF must be determined. In this study, we analysed the vasodilator and anti-remodelling properties of sildenafil ex vivo in the pulmonary arteries of patients with PH associated with IPF. Furthermore, we evaluated the effect of sildenafil on the V’/Q’ ratio in a bleomycin animal model.

Materials and methods

A more detailed version of the methods outlined below can be found in the online supplementary material.

Patients

Pulmonary arteries were obtained from: 1) patients with PH-associated IPF (n=8); 2) patients with IPF without PH (n=9); 3) patients with PH without IPF (n=4); and 4) donor subjects without any lung disease (n=18). This protocol was approved by the local research and independent ethics committee of the University General Consortium Hospital of Valencia (CEIC26/2013). Written informed consent was obtained from each participant. Please refer to the online supplementary material for further details.

Preparation of pulmonary artery rings for functional studies

Pulmonary arteries were isolated and prepared as outlined previously [14]. Isometric tension of the arterial rings was recorded as we reported previously [14]. We employed two different protocols. 1) Relaxant protocol: cumulative concentrations of sildenafil were added to pre-contracted arteries following 1 µM serotonin (5-HT) treatment. The results are expressed as the percentage of sildenafil relaxation compared with maximal relaxation produced by 0.1 mM papaverine. 2) Contraction protocol: sildenafil (0.1–10 µM) was added to the organ bath 30 min before cumulative concentrations of 5-HT (0.1 nM to 10 µM) were added. Details are described in the online supplementary material.
**Real-time RT-PCR analysis**
Total RNA was obtained from the pulmonary arteries of patients from different groups. The relative quantification of different transcripts was determined using the $2^{-\Delta\Delta CT}$ method with glyceraldehyde phosphate dehydrogenase as the endogenous control and normalised to a control group, as described previously [15]. Details are described in the online supplementary material.

**Immunofluorescence and Western blotting**
Collagen type I (Col I), $\alpha$-smooth muscle actin (\(\alpha\)-SMA), phospho-extracellular regulated kinases 1 and 2 (ERK1/2), phospho-SMAD3, S100A4, and PDE5 were detected in human lung tissue or in pulmonary artery rings by immunofluorescence or Western blot as outlined previously [16]. Details are described in the online supplementary material.

**Animal studies**
Animal experimentation and handling were performed in accordance with the guidelines of the committee of animal ethics and well-being of the University of Valencia (Valencia, Spain). A single dose of 3.75 U·kg$^{-1}$ bleomycin was administered intratracheally [16]. In both the sham control and bleomycin groups, a single dose of 1 mg·kg$^{-1}$ sildenafil was administered intraperitoneally on day 21. The $V'/Q'$ ratio was evaluated 2 h after administration using small-animal computed tomography (micro-CT) and single-photon emission computed tomography (SPECT) (Oncovision® micro-CT-SPECT-PET Imaging System; Albira, Valencia, Spain) imaging with the radioisotopes diethylene-triamine-pentaacetate (DTPA)-$^{99m}$Tc for ventilation (10 mCi) and microaggregated albumin (MAA)-$^{99m}$Tc for perfusion (0.5–1 mCi), as outlined previously with modifications [17]. Details are described in the online supplementary material.

**Statistical analysis**
The Kruskal–Wallis test followed by Dunn’s post hoc tests were used as nonparametric tests when more than two groups were compared in the human studies. The Mann–Whitney U-test was employed to compare two groups. Two-tailed paired t-tests were used to compare two groups of dependent samples in the animal and cell studies, and unpaired t-tests were used for independent samples. Multiple comparisons were analysed by a one- or two-way ANOVA followed by Bonferroni post hoc tests. Details are described in the online supplementary material.

**Results**

**Relaxant and anti-contractile effects of sildenafil on pulmonary arteries from patients with IPF**
Four different groups based on the clinical characteristics defined in table 1 were used in the experiments. Sildenafil induced concentration-dependent relaxation of pulmonary artery rings that was greater in the control and IPF groups (maximum relaxation effect of 41.3±11.6% and 37.2±15%, respectively) than that the PH+IPF and PH groups (9.8±5.8% and 9.4±4.2%; p<0.05 compared with the control and IPF groups; figure 1a–d). When the endothelium was removed, the maximal relaxation effect of sildenafil decreased in the control and IPF pulmonary artery rings to 20.8±10% and 20.1±10.5%, respectively, whereas the maximal relaxant effect of sildenafil remained similar in the PH+IPF and PH groups (figure 1c and d).

In other experiments, pulmonary artery rings were incubated with sildenafil (0.1–10 $\mu$M) 30 min before administration of cumulative doses of 5-HT (0.1 nM to 10 $\mu$M). Sildenafil did not significantly inhibit 5-HT-induced contractions in control donors (figure 2a). In contrast, 5-HT-induced contraction was significantly inhibited by sildenafil in a concentration-dependent manner in the IPF (maximal inhibition, 54.3±3%; p<0.05 versus control; figure 2b), PH+IPF (maximal inhibition, 68.3±4%; p<0.05 versus control; figure 2c), and PH arterial rings (maximal inhibition, 73.6%; p<0.05 versus control; figure 1d). Importantly, the inhibitory effect of sildenafil on 5-HT-induced contraction was significantly higher in the PH+IPF and PH arterial rings than those from patients with IPF (p<0.05).

**Characterisation of remodelling markers in pulmonary arteries from controls and patients with IPF with or without PH**
The pulmonary artery functional experiment results indicated that sildenafil had different relaxant/anti-contractile effects in pulmonary artery from control, IPF, and PH+IPF, which may be explained by differences in pulmonary artery remodelling components. Thus, mesenchymal/myofibroblast markers were studied in pulmonary arteries. \(\alpha\)-SMA, vimentin, Col I, 5-HT receptor 2A and transforming growth factor (TGF)-\(\beta\)1 expression were significantly upregulated in pulmonary arteries from patients with IPF and PH +IPF compared with those from controls, suggesting increased muscularisation, extracellular matrix deposition, and fibrosis (figure 3a). Furthermore, vimentin, Col I, 5-HT receptor 2A and TGF-\(\beta\)1 expression were higher in pulmonary arteries from patients with PH+IPF when compared with those from patients with IPF (figure 3a), which indicates their role in PH development. The endothelial markers
| Subjects n | 18 | 9 | 8 | 4 |
| Age years | 59 (42–71) | 61 (56–75) | 65 (52–73) | 45 (42–52) |
| Males/females | 15/3 | 12/5 | 25/11 | 3/1 |
| Smoking | | | | |
| Never smoked/smokers | 7/11 | 3/6 | 4/4 | 4/0 |
| Pack-years | 26 (6–32) | 28.3 (6–35) | 31 (9–38) | |
| FEV1 % pred | ND | 72.8 (58–101) | 73 (53–98) | 92 (91–95) |
| FVC % pred | ND | 70.2 (63–79) | 71 (48–76) | ND |
| TLC % pred | ND | 73.5 (45–89) | 66 (43–90) | ND |
| DLco % pred | ND | 50.1 (34–61) | 40.8 (20–61) | ND |
| Ground glass % | 0 | 19 (8–39) | 22 (9–35) | 0 |
| Honeycombing % | 0 | 28 (15–40) | 26 (12–39) | 0 |
| PaO2 mmHg | 94 (88–96) | 72.8 (58–101) | 73 (53–98) | 92 (91–95) |
| mPAP mmHg | 20.5 (16–22) | 43 (36–48) | 69 (42–75) | |

Data are presented as medians (interquartile range), unless otherwise stated. IPF: idiopathic pulmonary fibrosis; PH: pulmonary hypertension; FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; TLC: total lung capacity; DLco: diffusion capacity of the lung for carbon monoxide; PaO2: arterial blood oxygen tension; mPAP: mean pulmonary artery pressure; NAC: N-acetyl-L-cysteine; ND: not determined; #: percentage of pulmonary parenchyma with ground glass on a computed tomography (CT) image; ¶: percentage of pulmonary parenchyma with honeycombing on a CT image; +: patients who received this treatment at the time of pulmonary biopsy.

**FIGURE 1** Relaxant effects of sildenafil on pulmonary arteries. Concentration-dependent relaxant curves of sildenafil on pulmonary arteries pre-contracted with 1 µM serotonin from control donors (n=10) and patients with idiopathic pulmonary fibrosis (IPF) (n=6), IPF plus pulmonary hypertension (IPF+PH) (n=6) and PH (n=4). Four arterial rings per patient were used. The pulmonary artery endothelium (E) was removed for some experiments to analyse the effect of endothelium on sildenafil relaxant activity. The results are shown as mean±SE. Two-way ANOVA followed by Bonferroni post hoc tests. *: p<0.05 versus pulmonary arteries without endothelium; #: p<0.05 compared with baseline tension.
endothelial nitric oxide synthase (eNOS), vascular endothelial (VE)-cadherin, vascular endothelial growth factor receptor (VEGFR) and factor VIII (FVIII) were downregulated in pulmonary arteries from patients with IPF and PH+IPF (figure 3a), indicating impaired endothelial function or a change in endothelial phenotype. Phosphorylation of ERK1/2 and SMAD3 was greater in pulmonary arteries from patients with PH+IPF; however, this phosphorylation was less in pulmonary arteries from patients with IPF compared with controls (figure 3b and c; p<0.05). PDE5 expression was upregulated in pulmonary arteries from patients with IPF and PH+IPF (figure 3a and c) and mainly located in endothelial cells and the intimal layer (figure 3c), whereas PDE5 was weakly expressed in pulmonary arteries from control donors.

Anti-remodelling effects of sildenafil on human pulmonary arteries from patients with IPF

In other ex vivo experiments (experimental design details can be found in the online supplementary material), pulmonary artery ring explants were cultured and stimulated with 5 ng·mL^−1 TGF-β1 for 72 h in the presence or absence of sildenafil (10 nM–1 μM). TGF-β1 (5 ng·mL^−1) treatment resulted in decreased expression of the endothelial markers VE-cadherin and eNOS. In contrast, this treatment increased the expression of PDE5 and the mesenchymal markers Col I and vimentin, but not α-SMA or TGF-β1 in pulmonary artery rings from control subjects (figure 3a–c). These changes were also observed in pulmonary arteries from patients with IPF, where the loss in endothelial and the increase in mesenchymal markers were higher, including significant upregulation of α-SMA and TGF-β1 (figure 3d–f). Sildenafil inhibited the effect of TGF-β1 on the loss of endothelial markers and upregulation of the pulmonary artery remodelling markers in pulmonary artery rings of the control and IPF groups at 10–100 nM, suggesting pulmonary artery anti-remodelling properties in patients with IPF.

Sildenafil inhibited TGF-β1-induced pulmonary artery endothelial-to-mesenchymal transition and the transition of smooth muscle cells to myofibroblasts

Subsequent experiments were designed to explore the anti-remodelling effects of sildenafil on isolated and cultured human pulmonary artery endothelial cells (HPAECs) and HPASMCs from patients with IPF. Incubating the HPAECs with TGF-β1 changed their endothelial phenotype to a mesenchymal/myofibroblast...
phenotype characterised by loss of the endothelial markers VE-cadherin, VEGFR1, FVIII and eNOS, and an increase in the mesenchymal markers Col I, α-SMA and vimentin, as well as the expression of the profibrotic factors TGF-β1 and PDE5 (figure 5a). Sildenafil prevented these changes at a low concentration (10 nM) and maintained the endothelial phenotype. TGF-β1 increased the expression of Col I and vimentin in HPASMCs in a concentration-dependent manner; this effect was inhibited by sildenafil (figure 5b). Furthermore, sildenafil inhibited TGF-β1-induced HPASMC proliferation (figure 5c). Adding 5 ng·mL⁻¹ TGF-β1 to HPAECs and HPASMCs from patients with IPF increased the phosphorylation of ERK1/2 and SMAD3, which was inhibited by sildenafil (figure 6a and b). In parallel experiments, stimulating HPAECs and HPASMCs with 5 ng·mL⁻¹ TGF-β1 enhanced expression of Col I and the S100A4 fibrotic marker, which were inhibited by sildenafil, PD98059 (ERK1/2 inhibitor) and SIS3 (SMAD3 inhibitor). These results...
indicate that sildenafil partially mediates anti-remodelling effects on human pulmonary arteries from patients with IPF by inhibiting ERK1/2 and SMAD3. Sildenafil did not affect $V'/Q'$ mismatch in a rat model of bleomycin-induced pulmonary fibrosis associated with PH. Rats were instilled intratracheally with saline (control) or bleomycin on day 1 to induce pulmonary fibrosis and PH on day 21 as we outlined previously [16]. The $V'/Q'$ study began 2 h after administering 1 mg·kg$^{-1}$ sildenafil intraperitoneally. Ventilation images were taken after breathing DTPA-99mTc, and perfusion images were taken after perfusing MAA-99mTc. As expected, the $V'/Q'$ ratio was clearly impaired in the lungs of bleomycin-treated rats compared with that in control animals. Administering sildenafil on day 21 (once pulmonary fibrosis and PH were established) increased the perfusion signal slightly (figure 7b), but did not modify the $V'/Q'$ ratio (figure 7c).

Discussion
No specific therapy currently exists for PH associated with lung diseases, such as IPF. According to recent guidelines, vasodilators approved for pulmonary artery hypertension (PAH) are not recommended for patients with PH due to lung disease [18, 19]. However, a subset of patients with severe PH related to their lung disease, namely out-of-proportion PH, can be treated according to the recommendations for PAH [18]. The incompletely understood and likely complex pathogenesis, together with the small series attesting

**FIGURE 4** Effect of sildenafil on transforming growth factor (TGF)-β1-induced ex vivo pulmonary artery ring remodelling. Pulmonary artery rings from a–c) donor controls (n=4) and d–f) patients with idiopathic pulmonary fibrosis (IPF) (n=4) were incubated with sildenafil (10 nM to 10 µM) for 1 h and then stimulated with 5 ng·mL$^{-1}$ TGF-β1 for 72 h. Artery rings were homogenised to extract RNA for gene expression analysis or embedded in paraffin for immunofluorescence analysis (DAPI: 4',6-diamidino-2-phenylindol) as shown in e) and f). Scale bar=150 µm. White arrows indicate adventitial and smooth muscle layers. Yellow arrows indicate endothelial cells. Four arterial rings per patient were used. VE: vascular endothelial; eNOS: endothelial nitric oxide synthase; Col type 1: collagen type 1; α-SMA: alpha smooth muscle actin; PDE5: phosphodiesterase type 5; Sil: sildenafil; COL 1: collagen type 1. The results are shown as mean±SE. Two-way ANOVA followed by Bonferroni post hoc tests. *: p<0.05 compared with control; #: p<0.05 compared with TGF-β1.

Sil**

The incompletely understood and likely complex pathogenesis, together with the small series attesting
to the utility of treating PH in patients with IPF, provide a foundation and basis for further consideration of randomised controlled trials of pulmonary vasoactive therapies for this patient phenotype. In this study, we analysed the effects of sildenafil on relaxation and anti-contractile and anti-remodelling properties in an ex vivo in vitro system of pulmonary arteries from patients with IPF with and without severe PH. Our findings

FIGURE 5 Effects of sildenafil on transforming growth factor (TGF)-β1-induced endothelial- and smooth muscle cell-mesenchymal transition.

a) Human pulmonary artery endothelial cells and b) human pulmonary artery smooth muscle cells (HPASMCs) were isolated from pulmonary arteries of patients with idiopathic pulmonary fibrosis (n=4). The cells were incubated with sildenafil (10 nM to 1 µM) for 30 min and then stimulated with TGF-β1 for 72 h or b) 48 h. Endothelial and mesenchymal marker gene mRNA expression was analysed. c) HPASMCs from patients with IPF were incubated with sildenafil for 30 min and then stimulated with TGF-β1 for 48 h in 96-well plates to measure cell proliferation with the bromodeoxyuridine (BrdU) assay. The results are shown as mean±SE of n=4 cell populations from patients with IPF. VE: vascular endothelial; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; VEGFR: vascular endothelial growth factor receptor; FVIII: factor VIII; eNOS: endothelial nitric oxide synthase; α-SMA: alpha smooth muscle actin; Col type I: collagen type 1; α-SMA: alpha smooth muscle actin; PDE5: phosphodiesterase type 5. Two-way ANOVA followed by Bonferroni post hoc tests. *: p<0.05 compared with control; #: p<0.05 compared with TGF-β1.
Clinical studies have reported that sildenafil (50 mg orally) causes acute pulmonary vasodilation in patients with severe IPF and PH (mean pulmonary arterial pressure, 42 mmHg), mainly in well-ventilated areas with available NO, without affecting hypoxic vasoconstriction or oxygen saturation, and thus without disturbing the V’/Q’ match [7]. The long-term STEP-IPF trial demonstrated that sildenafil improved 6MWD in a subset of patients with severe IPF and right-sided ventricular systolic dysfunction measured by echocardiography [9]. These positive results corroborate previous findings in small cohorts of patients with PH-associated with severe IPF [20, 21] but not in patients with IPF and mild to moderate PH [22].

In this study, we observed a direct vasodilatory effect of sildenafil in 5-HT-precontracted pulmonary arteries from healthy control patients and from patients with IPF, which was partially endothelium-dependent (40% with endothelium versus 20% maximal relaxation without endothelium). A well-known vasodilatory mechanism of sildenafil implicates the production of NO from eNOS in endothelial cells. The endothelial release of NO activates guanylate cyclase (GC) in pulmonary artery smooth muscle cells to produce cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG), which induce vasodilation [23]. However, an endothelial-independent vasodilatory effect of sildenafil was also described. For example, sildenafil induces endothelial-independent relaxation in vessels of humans [24] and piglets [25], as well as rat [26] pulmonary arteries. Moreover, sildenafil significantly reduced acute hypoxic pulmonary vasoconstriction in perfused lungs of eNOS-deficient mice, indicating that the effects of sildenafil occur even when eNOS is impaired [27]. We observed a small degree of vasodilation in 5-HT-precontracted pulmonary arteries from patients with PH+IPF and PH that was independent of the presence of endothelium, suggesting that the eNOS-NO endothelial axis is impaired in patients with PH+IPF, as described previously [28]. Synthesis of cGMP also occurs in response to natriuretic peptides after activation of particulate GC in pulmonary vascular smooth muscle cells [29]. Serum natriuretic peptides are elevated as a compensatory mechanism in patients with PH-associated IPF [30], which partially explains the increased pulmonary vasodilation after a single oral administration of sildenafil in patients with PH+IPF [7].

In our study, the small degree of vasodilation observed in our ex vivo system of pulmonary arteries from patients with PH+IPF could be attributed to the lack of circulating natriuretic peptides and the lack of NO-enriched lung tissue in well-ventilated areas where sildenafil induced a selective increase in vasodilation [7]. This is the rationale for the recent synergism observed between the natriuretic peptide system and sildenafil in an animal model of lung fibrosis associated with PH [31]. Although we did not explore the role of natriuretic peptides in this study, we speculate that small local pulmonary artery production of natriuretic...
peptides may be partially responsible for the actions of sildenafil that we observed. Similar selective vasodilator properties were observed after acute administration of sildenafil in the bleomycin-induced pulmonary fibrosis animal model associated with PH, in which the $V'/Q'$ ratio remained unaltered.

$V'/Q'$ SPECT imaging is a well-established nuclear medicine technique that provides spatial information of respiratory gas exchange, ventilation of alveolar units and perfusion of the pulmonary capillary beds. Chronic pulmonary hypoxic diseases such as chronic obstructive pulmonary disease (COPD) or IPF have an impaired ventilation and perfusion of the lung units and can be monitored by the analysis of the inhaled DTPA-99mTc signal and perfused MAA-99mTc. However the use of this technique in preclinical studies is limited. To our knowledge there is only one study that observed an impaired $V'/Q'$ SPECT ratio in a rat model of COPD smoking between the 8th to 24th weeks of smoking [17]. In this work we observed an impaired $V'/Q'$ SPECT ratio at the end of the 21-day bleomycin-induced lung fibrosis procedure. Sildenafil weakly increased perfusion signal without affecting $V'/Q'$ ratio, confirming previous results in humans [7] and the utility of this technique to the preclinical in vivo study of vasodilators in hypoxic lung disorders such as IPF. However, the selective actions of sildenafil on well-ventilated areas should be demonstrated with repeated administration of sildenafil in an animal therapeutic protocol rather than in acute experiments, which is a limitation of this study and warrants future experiments.

Different results were observed for sildenafil in the anti-contractile protocol. Chronic administration of sildenafil is the optimal scenario in patients with severe PH+IPF; thus, sustained inhibition of PDE5 prevents the contractile effects of elevated vasoactive mediators, such as ET-1 and 5-HT, among others. In the present study, pre-incubation with 10 μM sildenafil minimally affected the 5-HT concentration-response curves (10% inhibition) in healthy PAs as shown previously by Ried et al. [32] using ET-1 as a vasoactive mediator [32]. In contrast to our results observed for healthy donors, 10 μM sildenafil resulted in nearly 50% inhibition of 5-HT-induced contraction in pulmonary arteries from patients with IPF; however, the effect of sildenafil was greater in pulmonary arteries from patients with
PH+IPF and PH. Differences in the anti-contractile effects of sildenafil can be partially explained by the different PDE5 expression levels among patients: PDE5 was overexpressed in pulmonary arteries from patients with PH+IPF (and to a lesser extent in patients with IPF) compared with that in healthy subjects. However, we cannot exclude that other mechanisms may be involved.

We selected 5-HT as a vasoactive stimulus because increased levels of 5-HT and active signalling have been described in patients with PH [33]. More recently, 5-HT receptor overexpression was reported in patients with IPF when compared with that in controls, particularly 5-HT receptor 2A in the pulmonary artery smooth muscle layer [34]. In this study, we confirmed increased 5-HT receptor 2A expression in PAs from patients with IPF and 5-HT receptor 2A was significantly upregulated in patients with PH+IPF. 5-HT receptor 2A initiates downstream signalling, including activation of RhoA/RhoA kinase (ROCK) and subsequent pulmonary vascular contraction. In this regard, the GcGMP/PKG pathway in smooth muscle cells can decrease pulmonary vascular contractility by decreasing Ca2+ sensitivity and phosphorylating and inactivating the RhoA protein [35]. Thus, overexpression of PDE5 by decreasing cGMP and PKG may increase the activity of the RhoA/ROCK pathway, which increases pulmonary vascular sensitivity to 5-HT, as suggested previously in pulmonary artery smooth muscle cells [36]. Thus, PDE5 overexpression in pulmonary arteries from patients with IPF and PH+IPF may partially explain why sildenafil selectively desensitised the contractile effects of 5-HT in these pulmonary arteries.

To understand further the effects of sildenafil in patients with PH+IPF, we analysed the molecular profile of pulmonary arteries. αSMA and vimentin gene expression was upregulated in pulmonary arteries from patients with IPF and PH+IPF compared with those from donor subjects, and higher expression was detected in pulmonary arteries from patients with PH+IPF according to the PH remodelling profile. Furthermore, Col I and the profibrotic growth factor TGF-β1 were overexpressed in pulmonary arteries from patients with IPF and PH+IPF and were more highly expressed in pulmonary arteries from patients with PH+IPF, reflecting gradual activation of muscularisation and fibrosis. These results suggest that the vascular remodelling pathways are activated before PH is clinically detected, indicating the progressive nature of vascular remodelling in patients with IPF.

A similar molecular profile was recently described in pulmonary arteries from patients with PH+IPF using genome-wide expression profiling [37]. This profiling revealed a reduction in lumen diameter accompanied by thickening of the media and intimal layers via an increase in extracellular matrix components and αSMA in the intima and downregulation of von Willebrand factor exclusively localised in the endothelium of pulmonary vessels [37]. In the present study, we also observed downregulation of typical endothelial markers in pulmonary arteries from patients with IPF and PH+IPF, such as VE-cadherin, VEGFR-1, and FVIII, whereas eNOS was only downregulated in pulmonary arteries from patients with PH+IPF, which may explain the endothelial-dependent relaxant effects of sildenafil in the pulmonary arteries from patients with IPF (figure 1b). We recently described loss of the endothelial phenotype and acquisition of the mesenchymal phenotype in endothelial cells from the pulmonary arteries of patients with PH+IPF [16]. Recent in vivo data demonstrated that pulmonary capillary endothelial cells may be a source of fibroblasts in patients with pulmonary fibrosis via endothelial-to-mesenchymal transition [4]. Therefore, a sildenafil-induced increase in the bioavailability of NO could improve pulmonary artery endothelial cell transformation and pulmonary remodelling. We previously observed that adding sepiapterin in vitro increased levels of tetrahydrobiopterin (BH4) coupling of eNOS to produce NO, and thus inhibiting the endothelial-to-mesenchymal transition induced by TGF-β1 [16]. Other studies have demonstrated that chronic inhibition of eNOS induced endothelial-to-mesenchymal transition in kidney endothelial cells [38].

In the present study, TGF-β1 increased the expression of mesenchymal markers and PDE5, and decreased the expression of endothelial markers in pulmonary artery rings from healthy donor subjects. A more robust molecular change was observed in patients with IPF, indicating sensitisation to vascular remodelling. In both cases, sildenafil attenuated the increase in mesenchymal marker expression and the decrease in endothelial marker expression. We isolated HPASMCs and HPAECs from the pulmonary arteries of patients with IPF to dissect the possible mechanisms. TGF-β1 decreased endothelial and increased mesenchymal marker expression in HPAECs, whereas TGF-β1 increased Col I and vimentin in HPASMCs, representing the myofibroblast transition. Sildenafil partially inhibited both the endothelial- and smooth muscle-to-mesenchymal transition by inhibiting ERK1/2 and SMAD3 phosphorylation, which are the main downstream signals of TGF-β1. Although apparently unrelated, previous studies have shown that NO inhibits TGF-β1/SMAD-regulated gene transactivation in a cGMP/PKG-dependent manner by directing proteasomal degradation of activated SMAD3 [39] or by sequestering SMAD3 [40]. ERK1/2 phosphorylation can also be inhibited by PKG, which explains the inhibitory effects of sildenafil observed here [36]. These findings are particularly relevant, as we observed hyperphosphorylation of ERK1/2 and SMAD3 in pulmonary arteries from patients with PH+IPF.
We have provided the first evidence of direct relaxant/anti-contractile and anti-remodelling effects of sildenafil on pulmonary arteries from healthy donors and patients with IPF or PH+IPF. Our findings may contribute to understand the beneficial effects of sildenafil in patients with IPF; particularly in those with severe PH. Future randomised controlled trials of sildenafil in this patient phenotype are warranted.

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References


