ONLINE SUPPLEMENTARY MATERIAL

Title:

Vascular effects of sildenafil in patients with pulmonary fibrosis and hypertension

Authors

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MATERIAL AND METHODS

Patients

Peripheral human lung tissue was obtained from 4 types of patients (Thoracic Surgery and Pathology Services of the University General Consortium Hospital and University and Polytechnic Hospital La Fe, Spain): A) Patients with PH-associated IPF who were underwent surgery for organ transplantation program (n=8). B) Patients with IPF who were underwent surgery for organ transplantation program (represents pulmonary arteries with IPF and without PH) (n=9). C) Patients with PH without IPF who were underwent to lung transplantation (n=4). D) Donor subjects who death in traffic accident without the possibility of donating their lungs for transplantation (represents pulmonary arteries without PH and without IPF and were used as controls), without any lung disease (n=18).

IPF was diagnosed according to the American Thoracic Society/European Respiratory Society (ATS/ERS) consensus criteria [1]. All pulmonary function tests were performed within 3 months before surgery. After selection based on diagnosis criteria, all lung

tissue samples used for the study were checked histologically by using the following exclusion criteria: (1) presence of tumor, (2) respiratory tract infection.

The lungs taken from donor controls showed normal architecture with few intra-alveolar macrophages and edema. Clinical data is described in table 1. The protocol was approved by the local research and independent ethics committee of the University General Consortium Hospital of Valencia (CEIC28/2011). Informed written consent was obtained from each participant.

Preparation of pulmonary artery rings for functional studies

Pulmonary arteries were carefully dissected free of adjoining connective tissue and lung parenchyma as previously outlined [2]. The preparations were placed in cold Krebs–Henseleit's solution (mM): NaCl, 118; $NaCO_3H$, 24; KCl, 4.7; KH_2PO_4, 12; $MgSO_4$, 1.2; $CaCl_2$, 2.5; and glucose, 11.1; pH 7.35–7.45. Arterial segments with an external diameter of 2–3 mm were carefully dissected and cut into rings of 2–3 mm in length.

The endothelium was removed from some pulmonary artery rings, as described previously [3]. Arteries were used within 1–5 h post-surgery.

The rings were mounted in a 10 ml organ bath chamber (Pan-Lab,USA) under an initial load of 1–2 g, and the isometric tension was recorded with a transducer (Grass FT03 isometric force transducer; Grass Instruments, Quincy, MA, USA) connected to a PowerLab® data acquisition system (AD Instruments, Castle Hill, New South Wales, Australia), as we reported previously [4]. The tissues were allowed to equilibrate in Krebs-Henseleit's solution for 90 min at 37ºC, while being aerated with 5% CO_2 in O_2. Pulmonary artery rings that failed to reach an increase of tension of 0.5 g in response to KCl 80 mM were discarded [5].

After an equilibration period of 1 h at ~1.5 g of basal tension, preparations were contracted with 1 μM noradrenaline (NA) and relaxed with 1 μM acetylcholine (Ach), to confirm endothelium function. The tissues were washed until resting tone was reestablished, and then the artery rings were contracted maximally using KCl 80 mM to establish the maximal contractile response.

After rinsing and equilibration, increasing 5-HT concentrations (0.1 nM to 10 μM) was added, and the tension was expressed as a percentage of the maximal contraction with KCl. The EC_{70} of 5-HT was selected as 1μM for further pharmacological experiments (data not shown). Two different protocols were conducted:

A) *Relaxant protocol*: Control rings (those not exposed to sildenafil) maintained a plateau level about 10–15 per cent of 5-HT 1μM-induced tone over the time interval (approximately 90 min) needed to make cumulative concentration drug–response curves in the time-matched rings exposed to relaxant drugs. During the cumulative addition of sildenafil, successive concentrations were added only when the tissue reached maximum relaxation (usually 5–10 min). At the end of all experiments, papaverine (0.1 mM) was added to each organ bath to establish a reference for maximal relaxation as previously we outlined [6]. Cumulatively increasing concentrations of the PDE5 inhibitor sildenafil (from 0.1 nM to 10 μM) was constructed in increments of 0.5 log units. In other experiments, endothelium was removed to analyze the contribution of endothelium to the relaxant effects of sildenafil. Results were expressed as % sildenafil relaxation of maximal relaxation reached by papaverine 0.1mM.

B) Contraction protocol: To determine the effect of sildenafil on contractility, sildenafil (0.1μM-10μM) was added to the organ bath 30 min before the cumulative doses of 5-HT (0.1 nM to 10 μM). Sildenafil was maintained in the bath medium during the concentration-dependent curve of 5-HT.

Artery rings experiments were performed in pulmonary arteries from control, IPF, PH and PH + IPF patients.

Real time RT-PCR

Total RNA was obtained from pulmonary arteries of different patient groups and from primary endothelial and smooth muscle pulmonary artery cells by using TriPure® Isolation Reagent (Roche, Indianapolis, USA). Integrity of the extracted RNA was confirmed with Bioanalizer (Agilent, Palo Alto, CA, USA). The reverse transcription was performed in 300 ng of total RNA with the TaqMan reverse transcription reagents kit (Applied Biosystems, Perkin-Elmer Corporation, CA, USA).

The mRNA expression of indicated genes was selected to characterise cellular transformations. In this regard, endothelial to mesenchymal transition is characterized by the loss of endothelial marker expression such as endothelial (e)NOS (endothelial enzyme that produces NO release in endothelial cells), vascular endothelial (VE)-cadherin (adherent junction protein expressed in endothelial cells that maintains endothelial cell barrier integrity), vascular endothelial growth factor receptor (VEGFR; protein expressed in endothelial cells that promotes endothelial cell regeneration) and factor VIII (FVIII; coagulation factor expressed in endothelial cells whose expression is decreased when endothelial phenotype is loss), and the increase of myofibroblast markers such as extracellular matrix collagen type I (expressed in myofibroblast), vimentin and alpha smooth muscle actin (αSMA; cytoskeleton proteins that mediates cellular contraction that helps mesenchymal cells to migrate) [7].

Smooth muscle cell to mesenchymal/ myofibroblast transition is characterized by the increase of extacellular matrix collagen type I secretion and vimentin expression [8]. cDNA was amplified using assays-on-demand specific primers pre-designed by Applied Biosystems for alpha smooth muscle actin (αSMA; Hs00559403_m1), α1(I)-collagen

(collagen type I; cat. nº: Hs00164004_m1), vimentin (cat. nº: Hs 00958116_m1), TGFβ1 (Hs00998133_m1), PDE5 (Hs00153649_m1), eNOS (Hs01574659_m1), VE-cadherin (Hs00170986_m1), vascular endothelial growth factor receptor I (VEGFR1; Hs01052961_m1) and factor (F)VIII (Hs00252034_m1) genes in a 7900HT Fast Real-Time PCR System (Applied Biosystems) using Universal Master Mix (Applied Biosystems). Relative quantification of these different transcripts was determined with the 2-ΔΔCt method using glyceraldehyde phosphate dehydrogenase (GAPDH) as endogenous control (Applied Biosystems; 4310884E) and normalised to control group as previously described by our group [9].

Immunofluorescence

Collagen type I (Col I), αSMA and PDE5 in human lung tissue or in pulmonary artery rings were analyzed by immunofluorescence. To this end distal lung tissue from all type of patients included in this study were fixed in paraformaldehyde (4%) for 48 h and embedded in Tissue-Tek® OCT™ cryosectioning compound (Sakura Finetek Europe BV, Leiden). Blocks were cut into 10µm thick sections, permeabilized in Triton X 100 (0.1% in PBS) for 5 min, blocked in 10% goat serum in PBS and immunostained with rabbit anti-human collagen type I (Col I) antibody (cat. nº: PA1-26204; Affinity Bioreagents), mouse anti-human α-SMA (cat. nº: A5228; Sigma Aldrich, Madrid, Spain) and Rabbit anti-human PDE5 (cat. nº: P434; Cell Signalling, Boston, Massachusetts, USA), for 24 hours at 4ºC followed by a secondary FITC or rhodamine conjugated anti-mouse/rabbit IgG antibody and finally DAPI (2µg/ml) to mark nuclei (Molecular Probes, Leiden, The Netherlands). Lung tissue was inspected at excitation/emission of 485/525nm (FITC), or 570/590nm (rhodamine), or 350/470nm (DAPI) with a x400 magnification in a TE-200 epifluorescence microscope (Nikon Eclipse-TE-200, Tokyo, Japan).

Western blot

Western blot analysis was used to detect phospho-ERK1/2, Collagen type I, S100A4 and phospho-Smad3 in pulmonary arteries from different patients and in primary endothelial and smooth muscle pulmonary artery cells in *in vitro* experiments. Lung tissue/ cells were homogenised and lysed on ice with a lysis buffer consisting of 20mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 150mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol and 1 µg/ml pepstatin A supplemented by a complete protease inhibitor cocktail (Sigma Aldrich). The Bio-Rad assay (Bio-Rad Laboratories Ltd., Herts, UK) was used to quantify the level of protein in each sample to ensure equal protein loading. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to separate the proteins according to their molecular weight. Briefly, 20 µg of protein (denatured) mixed with 2x loading buffer (comprising 160mM Tris HCl (pH 6.8), 4% SDS, 20% glycerol, 1.4mM β-mercaptoethanol, 0.04% bromophenol blue) along with a molecular weight protein marker (Bio-Rad Kaleidoscope marker, Bio-Rad Laboratories), was loaded onto an acrylamide gel consisting of a 5% acrylamide stacking gel on top of a 12% acrylamide resolving gel and run through the gel by application of 100 V for 1 hour. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a wet blotting method. The membrane was blocked with 5% Marvel in PBS containing 0.1% Tween 20 and then probed with mouse anti-human phospho-ERK1/2 (cat. nº: M-9692; Sigma Aldrich), rabbit anti-human collagen type I antibody (cat. nº: PA1-26204; Affinity Bioreagents), Rabbit anti-human S1004A antibody (cat. nº Ab27957, abcam), rabbit anti-human phospho-Smad3 (cat. nº: PS1023; Calbiochem) antibodies, and total rabbit anti-human ERK1/2 antibody (Cell Signalling, Boston, Massachusetts, USA; catalogue no. 4695) or total rabbit anti-human Smad3 (cat nº: 566414; Calbiochem) as internal standards followed by the corresponding

peroxidase-conjugated secondary (1:10.000) antibody. The enhanced chemiluminescence method of protein detection using ECL-plus (GE Healthcare, Amersham Biosciences, UK) was used to detect labelled proteins. Quantification of protein expression was performed by densitometry relative to ERK1/2 or Smad3 expression using the software GeneSnap version 6.08.

Pulmonary artery "ex vivo" experiments

Pulmonary arteries from control and IPF patients were dissected in small rings (2–3 mm of external diameter and 2–3 mm of length) and cultured in EGM-2 endothelial cell culture medium supplemented with Single Quotes (Clonetics, UK), 10% FCS, 1% fungizone, and 2% streptomycin/penicillin for 4 h in an incubator with 5%CO_2 at 37ºC. Arteries were incubated with/ without sildenafil 10nM-1µM for 1 hour followed by the stimulation with TGFβ1 5ng/mL during 72 hours. TGFβ1 is one of the most well characterized pulmonary artery remodelling factor that increase the muscularization of pulmonary arteries by different mechanisms. First, TGFβ1 promotes differentiation of endothelial cells to mesenchymal/myofibroblast phenotype characterized by the increase of expression of cytoskeleton machinery (including alpha smooth muscle actin and vimentin) that helps cells to migrate to the intima and adventitia, and the increase of extracellular matrix components such as collagen type I which favour cell migration and connective tissue deposition. By the other hand, TGFβ1 increase smooth muscle cell proliferation and transformation to myofibroblast which increase the migratory capacity of myofibroblast into the intima and adventitia. This alterations increase the pulmonary artery remodeling reducing the internal diameter and increasing contractile capacity inducing pulmonary hypertension [8, 10]. Therefore, we selected TGFβ1 as stimulus to induce change of mesenchymal markers in pulmonary arteries.

Both, sildenafil and TGFβ1 were replaced each 24h. Artery rings were used to analyze the expression of mesenchymal and endothelial markers. Time point incubation for the TGFβ1 stimulus was selected based on time-dependent curves (24h-96h) showing a maximal production of collagen type I after 72h of exposure (data not shown).

Isolation and culture of human pulmonary artery endothelial cells and pulmonary arterial smooth muscle cells and *in vitro* experimental conditions

Cellular experiments were performed in primary human pulmonary artery endothelial cells (HPAECs) and human pulmonary artery smooth muscle cells (HPASMCs) isolated from pulmonary arteries of IPF lungs. Segments of pulmonary artery (2-3 mm internal diameter) were dissected free from parenchyma lung tissue, cut longitudinally, and digested with 1% collagenase (Gibco, UK) in RPMI-1640 culture medium for 30 min at 37°C. The digestion was neutralized by adding RPMI 1640 supplemented with 20% foetal calf serum (FCS), and the homogenate was separated by centrifugation at 1100 rpm. The pellet was resuspended, and cells were cultured in EGM-2 endothelial culture medium supplemented with Single Quotes (Clonetics, UK), 10% FCS, 1% fungizone, and 2% streptomycin/penicillin. The selection of HPAECs was performed as we previously described [2, 11, 12], modified to include the use of a commercially available Dynabeads CD31 endothelial cell kit (Dynal Biotech, Germany). Briefly, cells were trypsinized (0.25% trypsin), and the cell mixture was incubated with CD-31-coated Dynabeads for 30 min at 4°C with end-over-end rotation. After incubation, the HPAECs were collected using a magnetic particle concentrator (MCP-1; Dynal) and washed four times with cold phosphate-buffered saline (PBS)/bovine serum albumin (BSA). Clusters of purified HPAECs retained on the CD-31-coated Dynabeads were

separately resuspended in EGM-2 full growth medium supplemented with 10% FCS, 1% fungizone, and 2% streptomycin/penicillin.

The cells not retained on the CD-31-coated Dynabeads, i.e., HPASMCs, were cultured in DMEM supplemented with 10% FCS, 1% fungizone, and 2% streptomycin/penicillin to selectively separate the HPASMCs. For positive identification of HPASMCs, the cells were subcultured for one passage, and α-actin expression was examined using a monoclonal antibody against a-smooth muscle actin (1:100 dilution; Sigma); >95% of the cells were positively stained. The cells were incubated for 16 h in 1% FCS culture medium before each experiment and were returned to 10% FCS culture medium at the start of each experimental condition.

For *in vitro* studies, HPAECs or HPASMCs were stimulated with TGF-β1 (1-10ng/ml; Sigma Aldrich) for the indicated times, replacing culture medium and stimulus every 24 h. Sildenafil (10nM-1μM; pfizer), was added 30 min before stimulus and remained together with the stimulus. Cellular extracts were collected to analyze the expression of mesenchymal and endothelial markers as well as mechanistic pathways.

Cell proliferation assay

HPASMC proliferation was measured by colorimetric immunoassay based on BrdU incorporation during DNA synthesis using a cell proliferation enzyme-linked immunosorbent assay BrdU kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Cells were seeded at a density of 3x103 cells/well on 96-well plates and incubated for 24 hours. Cells were incubated with sildenafil at indicated concentrations during 30 min followed by TGFβ1 stimulation for 48 h. The 490 nm absorbance was quantified using a microplate spectrophotometer (Victor 1420 Multilabel Counter, PerkinElmer). Proliferation data refer to the absorbance values of

BrdU-labeled cellular DNA content per well. Stimulation is expressed as *x*-fold proliferation over basal growth of the untreated control set as unity.

Animal studies

Experimentation and handling were performance in accordance with the guidelines of the Committee of Animal Ethics and Well-being of the University of Valencia (Valencia, Spain). Rat studies used pathogen-free male wistar rats (Harlan Iberica®, Barcelona, Spain) at 12 weeks of age which are reported to mount a robust early inflammatory response followed by pulmonary hypertension and fibrotic remodeling secondary to bleomycin [13]. Rat were housed with free access to water and food under standard conditions: relative humidity 55 ± 10 %; temperature 22 ± 3ºC; 15 air cycles/ per hour; 12/12 h Light/Dark cycle. Rats were anaesthetized with ketamine/medetomidine and then a single dose of bleomycin at 3.75 U/kg (dissolved in 200 µL of saline) was administered intratracheally via the endotracheal route [14]. This dose of bleomycin reproducibly generated pulmonary fibrosis and PH in previous experiments [15]. Sham control treated rats received the identical volume of intratracheal saline instead of bleomycin. This procedure fixed experimentation day 1 and was synchronously coupled with the initiation of single sildenafil administration at day 21. In both sham control and bleomycin groups, sildenafil at 1mg/kg was administered via i.p. After 2h of administration it was evaluated the V/Q ratio using a micro-CT-SPECT.

In vivo imaging and quantitative analysis were performed in rat lug tissue using small-animal computer tomography (micro-CT) and single photon emission computed tomography (Albira micro-CT-SPECT-PET Imaging System (Oncovision®, Spain). First, rats were tracheally intubated through the oral cavity after anesthetization with

ketamine and xylazine (90 and 6 mg/kg, respectively). The animals were then ventilated (0.02 L/min, 125 strokes/min) on a rodent ventilator (model 683; Harvard Apparatus) with diethylene-triamine-pentaacetate 10 mCi (DTPA-$Tc99^m$) (Molipharma, Valencia, Spain) for 15 min. After DTPA-$Tc99^m$ delivery, the animals were removed from the ventilator and allowed to breathe freely. SPECT scans were acquired on an ALBIRA micro-SPECT system (Oncovision®) using pinhole collimators and a radius of rotation of 3.5 cm. After the ventilation SPECT scan, rats were injected with 0.5–1 mCi of MAA-$Tc99^m$ via the tail vein. Perfusion imaging entailed a SPECT scan. The relationship between ventilation and perfusion data was determined with PMOD™ software analyzing the intensity of radiation (arbitrary units) of each volume of interest (VOI) of the whole lung region selected of 256 image sections for each animal study corrected by the maximal activity. Corrected radiation intensities of ventilation (V) and perfusion (Q) studies were represented.

Analysis of results

Statistical analysis of results was carried out by parametric (animal and cellular studies) or non-parametric (human studies) analysis as appropriate. $P < 0.05$ was considered statistically significant. Non-parametric tests were used to compare results from human samples of control patients and IPF, PH and IPF+PH patients. In this case, data were displayed as medians, interquartile range and minimum and maximum values. When the comparisons concerned more than two groups, analysis of variance (Kruskal-Wallis test) was first performed. In the case of a global significant difference, between-group comparisons were assessed by the Dunn's post-hoc test, which generalizes the Bonferroni adjustment procedure. When the comparisons concerned only 2 groups, between-group differences were analyzed by the Mann Whitney test. Results from animal, *ex vivo* and cellular *in vitro* mechanistic cell experiments were expressed as

mean ± SE of n experiments since normal distribution for each data set was confirmed by histogram analyses and Kolmogorov–Smirnov test. In this case, statistical analysis was carried out by parametric analysis. Two-group comparisons were analysed using the two-tailed Student's paired t-test for dependent samples, or unpaired t-test for independent samples. Multiple comparisons were analysed by one-way or two-way analysis of variance followed by Bonferroni post hoc test.

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