Online data supplement

Role of tetrahydrobiopterin in pulmonary vascular remodeling associated with pulmonary fibrosis

Authors

Patricia Almudéver 1*, Javier Milara 2,3,4,5*, Alfredo De Diego 6, Ana Serrano-Mollar 5,7, Antoni Xaubet 5,8, Francisco Perez-Vizcaino 5,9, Angel Cogolludo 5,9 and Julio Cortijo 1, 2, 4, 5

1Department of Pharmacology, Faculty of Medicine, University of Valencia, Spain
2Clinical research unit (UIC), University General Hospital Consortium, Valencia, Spain
3Department of Biotechnology, Universidad Politécnica de Valencia, Spain
4Research Foundation of General Hospital of Valencia, Spain
5CIBERES, Health Institute Carlos III, Valencia, Spain
6Servicio de Neumología, Hospital Universitario y Politécnico La Fe
7Dept de Patología Experimental, Instituto de Investigaciones Biomédicas de Barcelona, Consejo Superior de Investigaciones Científicas
8Servicio de Neumología, Hospital Clínico, Instituto de Investigaciones Biomédicas Agustí Pi Suñer (IDIBAPS)
9Department of Pharmacology, School of Medicine, Universidad Complutense de Madrid

*Both authors contributed equally to this work

Corresponding Author: Javier Milara, PhD., Unidad de Investigación, Consorcio Hospital General Universitario, Avenida tres cruces s/n, E-46014 Valencia, Spain.
Phone: +34 620231549, Fax: +34961972145, E-mail: xmilara@hotmail.com
METHODS

Patients with Idiopathic Pulmonary Fibrosis

A total of 36 IPF patients (n=36 patients for plasma studies and n=17 for tissue studies) were included in the study. IPF was diagnosed according to the American Thoracic Society/European Respiratory Society (ATS/ERS) consensus criteria (1). Fibrotic lung samples were obtained at surgery for lung transplantation or by open lung biopsy for histological diagnosis of the disease. Matched plasma samples were obtained from peripheral venous blood of each patient. All pulmonary function tests were performed within 3 months before surgery. Inclusion criteria were defined as: 1) IPF patients free of symptoms of respiratory tract infection, and none received antibiotics perioperatively. 2) Patients with evident honeycombing and fibrosis. 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. Clinical data is described in table 1. The protocol was approved by the local research and independent ethics committee of the University General Hospital of Valencia (CEIC28/2008). Informed written consent was obtained from each participant.

Control subjects

Age matched normal control lungs (n=21) were collected from patients undergoing thoracic surgery for removal of a primary lung tumour. Normal lung was obtained from a non-involved segment, remote from the solitary lesion. Plasma samples were obtained from age matched healthy subjects (n=30) without any medical disease. The biopsies taken from control donor lungs showed normal architecture with few intra-alveolar macrophages and edema.
Animal Model

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 (2). Rat were housed with free access to water and food under standard conditions: relative humidity 55 ± 10 %; temperature 22 ± 3°C; 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 (3). This dose of bleomycin reproducibly generated pulmonary fibrosis in previous experiments (4). Sham 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 sepiapterin treatment. Based on pharmacokinetic data (not shown) twice daily oral doses of sepiapterina (10 mg/Kg/b.i.d) were administered via an intraesophageal cannula from day 1 to 21. Sepiapterin was prepared immediately prior to use. Sepiapterin was prepared with 0.4 % methocel/HCl (0.05 M) aqueous solution and ultrasounds in order to ensure complete dissolution. The control group received 0.4 % methocel/HCl (0.05 M) as vehicle. The account for experimental groups was estimated in a number of 10 rats (n=10): (i) saline serum + pharmaceutical vehicle; (ii) saline serum + sepiapterin (10 mg/Kg/b.i.d); (iii) bleomycin + pharmaceutical vehicle; (iv) bleomycin + sepiapterin (10 mg/Kg/b.i.d). With these doses of sepiapterin, no adverse effects were observed during the experiments. Results obtained for the group of saline serum + sepiapterin were identical to those of saline group. Therefore we did not
include because space restrictions in main manuscript. Rats were weighed each two
days as an indicator of animal well-being and mortality was recorded during the 21 days
of treatment. At the end of the treatment period (day 21), rats were sacrificed by a lethal
injection of sodium pentobarbital followed by exsanguination. After opening the
thoracic cavity, trachea, lungs and heart were removed en bloc. Lungs were weighed
and then processed for histological, biochemical or molecular biology studies. The right
ventricular (RV) wall of the heart was dissected free and weighed along with the left
ventricle wall plus septum (LV + S), and the resulting weights are reported as RV/LV +
S ratio to provide an index of right ventricular hypertrophy. Moments before sacrificing
rats, femoral artery was canulated and heparinized blood was collected for measurement
of plasma BH4, BH2, nitrites and nitrotyrosine.

**Hemodynamic Measurements**

21 days after bleomycin/saline administration, 6 surviving rats of each experimental
group were anesthetized with ketamine/medetomidine and measured for right
ventricular systolic pressure (RVSP) by right heart catheterization. The right jugular
vein was canulated with a small silicone catheter (BPE-T50 Polyethylene tubing for
22ga swivels; Salomon Scientific, CA, USA) containing heparin saline solution (10
UI/ml of heparin in 0.9% saline), to reach the RV under the guidance of the pressure
tracing. After 20 minutes of stabilization, RVSP was recorded using a miniature
pressure transducer (TSD104A, BIOPAC Systems, Inc., CA, USA) digitized by a
BIOPAC MP100 data acquisition system. The right carotid artery was canulated in
order to simultaneously measure systemic arterial pressure (SAP) with a transducer.

**Histological, Immunohistochemical and Immunofluorescence Studies**
Lung histology was conducted as previously reported (5). Tissue blocks (4 μm thickness) were stained with haematoxylin-eosin for assessment of the fibrotic injury and pulmonary artery remodeling, and with Masson’s trichrome (Sigma-Aldrich, Madrid, Spain) to detect collagen deposition. Severity of lung fibrosis was scored on a scale from 0 (normal lung) to 8 (total fibrotic obliteration of fields) according to Ashcroft (6). To determine the extent of pulmonary vascular remodeling, the degree of muscularization of intraacinar pulmonary vessels was determined. Lung sections (4 μm thickness) were stained with haematoxylin-eosin, and mouse monoclonal anti-α-smooth muscle actin (1:200 v/v) and analysed using a morphometric system (Olympus BH2 Research Microscope, Olympus America Inc, Center Valley, PA, USA) with the software package Image ProPlus 5.0 (MediaCybernetics, Silver Spring, MD, USA). In each animal, 25–40 intraacinar arteries with an external diameter between 20 and 50 μm were analysed. The measurements made include internal area (IA), the external perimeter (EP) and the external area (EA), defined by the outer edge of the smooth muscle layer. The absolute wall area (WA) was calculated with the following formula: WA=EA-IA (7). All measurements were made by the same observer. The pulmonary artery wall thickness was calculated by dividing the WA by the EP as previously outlined (7).

For immunohistochemical analysis of rat and human lungs, tissue was fixed and embedded in paraffin, cut into sections (4-6 μm) and incubated with mouse anti-rat/human α-SMA (cat. n°: A5228; Sigma), goat anti-rat/human SPR (cat. n°: sc-169414; Santa Cruz Biotechnology, inc), mouse anti- rat/human GCH-1 (cat. n°: sc-271482; Santa Cruz Biotechnology), rabbit anti-rat/human eNOS (cat. n°: RB-1711-PO; ThermoScientific) and rabbit anti- rat/human iNOS (cat. n°: RB-1605 NeoMarkers, Fremont, CA) for 24 h at 4°C. A secondary anti-rabbit goat or anti-mouse antibody
(1:100; Vector Laboratories, Burlingame, CA) with avidin-biotin complex/horseradish peroxidase was used for immunohistochemistry. The non-immune IgG isotype control was used as negative control and gave negative for all samples.

All stained slides were scored by a pathologist under a Nikon Eclipse TE200 (Tokio, Japan) light microscope and representative photographs taken (10 slices per patient) as previously outlined (8). Staining intensity for different antibodies was scored on a scale of 0–3 (0-negative, 1-weak, 2-moderate, 3-strong immunoreactivity). The percentage of cells positive for different antibodies within pulmonary artery was scored on a scale of 1–4 as follows: 1: 0–25% cells positive; 2: 26–50% positive; 3: 51–75% positive; and 4: 76–100% positive. The score of the staining intensity and the percentage of immunoreactive cells were then multiplied to obtain a composite score ranging from 0 to 12.

For immunofluorescence, human pulmonary artery endothelial cells (HPAECs), human pulmonary airway tissue, or rat pulmonary airway tissue were washed three times with PBS and fixed (4% paraformaldehyde, 24 h, at room temperature). After another three washes with PBS, lung tissue was included in O.C.T™ compound (tissue-Tek, USA) and fridge at -80ºC to be sectioned in 14µm with a cryotome (leica, spain). The slices obtained were permeabilized (20 mM HEPES pH 7.6, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, 0.5% Triton X-100), blocked (10% goat serum in PBS) and incubated with the primary antibody mouse anti-human CD31 monoclonal antibody (IS610; dako, Madrid spain), rabbit anti-human VE-cadherin polyclonal antibody (VI514; sigma, Madrid spain), mouse anti-human α-SMA monoclonal antybody (A5228; sigma, Madrid spain) or rabbit anti-human collagen type I polyclonal antibody (234167; Calbiochem Darmstadt, Germany) overnight at 4ºC, followed by secondary antibody anti-mouse rhodamine (1:100, Molecular Probes) or anti-rabbit-FITC (1:100 Molecular
probes) and DAPI to mark nuclei. Slices were mounted using fluorescent mounting medium (Dako, Spain). Cells were visualized by fluorescence microscopy (×200; Nikon eclipse TE200 inverted microscope, Tokyo, Japan). IgG isotype controls gave always negative immunofluorescence signal.

**Determination of BH4/BH2, Nitrites and Nitrotyrosine**

To measure BH4 and BH2 plasma and pulmonary artery tissue levels, venous blood or isolated and homogenized pulmonary artery tissue were collected in EDTA tubes containing either 0.1% (w/v) dithioerythritol (DTE) as antioxidant. After exactly 3h at room temperature, it was centrifuged at 2000g, for 10 min, and plasma was stored at -80 °C as previously outlined (9). Total biopterin equals the combined sum of 7, 8-dihydrobiopterin (BH2), BH4 and fully oxidized biopterin. Differential oxidation mediated by iodine permits the measurement of BH4 concentration. Under acidic conditions BH4 and BH2 are oxidized to biopterin, while under basic conditions only BH2 is oxidized to biopterin, and BH4 undergoes side-chain cleavage to form pterin. The difference in biopterin content between the two oxidations represents the actual BH4 levels (10). In brief, the acidic oxidation was realized as follows: 90 μl of plasma and 10 μl of the internal standard (rhamnopterin 400 nM) were acidified by the addition of 20 μl HCl (1M) and 50 μl of I2/IK solution (1% (w/v) iodine in 2 % (w/v) potassium iodide). Samples were mixed and incubated for 1 h in the dark at room temperature. The reaction was stopped adding 10 μl of 5 % (w/v) ascorbic acid and 20 μl of water. The basic condition was identical to the preparation of the acidic condition with the exception that HCl was substituted by the addition of 20 μl NaOH (1M). Samples were added, mixed and incubated for 1 h in obscurity at room temperature followed by the addition of 10 μl of 5 % (w/v) ascorbic acid and 20 μl HCl (2M).
The HLPC system consisted of a Shimadzu LC-10ADvp isocratic pump, Shimadzu SIL-10ADvp auto-injector, Shimadzu RF10Avp fluorescence detector and Shimadzu SCL-10Avp controller. HPLC system control and data processing were performed by Shimadzu LCM Solutions software (Shimadzu, Tokyo, JP). The analytical method was validated in our laboratory in accordance to FDA Guidance for Industry (11). The separation was performed as described by Fiege et al (12). The stationary phase included precolumn and column Sherisorb ODS1 5µm (4.6 x 250 mm; Waters® Barcelona, Spain), and a mobile phase consisting in 1.5 mmol/L potassium hydrogen phosphate at pH 4.6 with 10% methanol, at a flow rate of 1 mL/min at room temperature. The fluorescence detector was set at 350nm (excitation) and 450nm (emission). Prior to inject into the HPLC system, samples were filtered through a 10000 MW microfilter (Multiscreen, Millipore®) at 3000g for 1h at 10ºC for physical desproteinisation. Quantification of BH4 and BH2 was made by interpolation to a standard curve of biopterin (1, 5, 10, 25, 50, 75 y 100 ng/ml).

NO was quantitatively determined in plasma and in HPAEC supernatants using a commercially available NO assay kit (Calbiochem-Novabiochem, San Diego, CA) based on the enzymatic conversion of nitrate to nitrite (NOx) by nitrate reductase according to the manufacturer's instructions. Nitrotyrosine was determined in plasma and in HPAEC supernatants using a commercially available ELISA (Hycult biotech, HK501)

**Human Pulmonary Artery Endothelial cell isolation and in vitro experimental conditions**

Cellular experiments were performed in HPAECs isolated from pulmonary arteries of normal 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 described previously (13, 14), 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 were discarded.

For *in vitro* studies, HPAECs were stimulated with TGF-β1 (5ng/ml; Sigma) or ET-1 (100nM; Sigma) for the indicated times, replacing culture medium and stimulus every 24 h. The antioxidant N-acetyl-l-cysteine (1mM; NAC; Sigma), SIS3 (described as Smad3 inhibitor, 10µM; Sigma) and sepiapterin (1µM-100µM; Schircks Laboratories, Buechstrasse, CH) were added 30 min before stimulus and remained together with the stimulus. Monoclonal anti-human TGF-β1 mAb (4 µg/mL; anti-TGF-β1; R&D Systems, Madrid, Spain) was added 30 min before stimulus to block the active form of TGF-β1 present in the culture supernatant as previously outlined (15).
**Real Time RT-PCR**

Total RNA was isolated from rat lung tissue, primary HPAECs and pulmonary arteries from IPF patients by using TriPure® Isolation Reagent (Roche, Indianapolis, USA). The integrity of the extracted RNA was confirmed with Bioanalyzer (Agilent, Palo Alto, CA, USA). The reverse transcription was performed in 300 ng of total RNA with TaqMan reverse transcription reagents kit (Applied Biosystems, Perkin-Elmer Corporation, CA, USA). cDNA was amplified with specific primers and probes predesigned by Applied Biosystems for, collagen type I (cat. nº: Rn01463848_m1), connective tissue growth factor (CTGF; cat. nº: Rn00583793_m1), transforming growth factor beta 1 (TGF-β1; cat. nº: Rn00572010_m1), endothelin 1 (ET-1; cat. nº: Rn00561129_m1) iNOS (cat. nº: Hs01075529_m1), eNOS (cat. nº: Hs01574659_m1), GCH-1 (cat. nº: Hs00609198_m1), and SPR (cat. nº: Hs00268403_m1) and GAPDH (pre-designed by Applied Biosystems, cat. nº: 4308313/rat and 4352339/human) as a housekeeping in a 7900HT Fast Real-Time PCR System (Applied Biosystem) using Universal Master Mix (Applied Biosystems). Relative quantification of these different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using GAPDH as endogenous control (Applied Biosystems; 4352339E) and normalized to control group.

To determine the relative expression of the α-SMA, sm22-α, snail, slug, CD31, VE-cadherin and vegfr SYBR Green real-time PCR was used. Primer sequences are defined in table 1. The percentage primer efficiency and correlation coefficients of the SYBR Green primers was calculated and accepted for efficiencies 100 ± 10%. The amplification specificity for each RT-PCR analysis was confirmed by melting curve analysis. 4 µl of cDNA was added to 19 µl of reaction mixture containing 7 µl H$_2$O, 10 µl QuantiTect® SYBR® Green PCR Master Mix (Qiagen, UK) and 1 µl each of forward
and reverse primers (10µM) (Table 1). Relative quantification of transcript levels (compared to control groups) was determined by evaluating the expression as $2^{\Delta\Delta CT}$ as described above.

**Table 1.** Primers for SYBR Green real-time quantitative reverse transcriptase polymerase chain reaction.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>Gapdh</td>
<td>CACAACTGCTTAGCACCACCC</td>
<td>TCTTCTGGGTGGCAGTGATG</td>
</tr>
<tr>
<td>Slug</td>
<td>TGGAGTGGCGCCCCATTAGAAA</td>
<td>TGGAGTCAGGGCAAGAAAAA</td>
</tr>
<tr>
<td>Snail</td>
<td>CCCAGTGCGCTCGGACACTAT</td>
<td>CCAGATGAGCAATTGCGCAGC</td>
</tr>
<tr>
<td>α-SMA</td>
<td>TTTCCGCTGCCCAGAGAC</td>
<td>GTCAATATCAGACTCCATGATGCTGT</td>
</tr>
<tr>
<td>sm22-α</td>
<td>CCGGTTAGGCAAGGCTC</td>
<td>GCGCTCATGCCATAGGA</td>
</tr>
<tr>
<td>CD31</td>
<td>AAAGTCCGACAGTGGAAGCT</td>
<td>GGCTGAGAGCATTTCACA</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>GATGCAGACGACCCCACTGT</td>
<td>CCACGATCCTACCTACCTGGCC</td>
</tr>
<tr>
<td>VegfR</td>
<td>TCAGGCAGCTCACAGTCTAGA</td>
<td>ACTTGTCTGTGTATTCTCCAGGT</td>
</tr>
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**Western blot**

Western blot analysis was used to detect changes of TGF-β1, ET-1 and p-Smad3 in lung tissue and HPAECs. Lung tissue from rats and HPAECs were homogenized 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. 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, 10 µg proteins (denatured) along with a molecular weight protein marker, Bio-Rad Kaleidoscope marker (Bio-Rad Laboratories), were loaded onto an acrylamide gel
consisting of a 5% acrylamide stacking gel stacked on top of a 10% acrylamide resolving gel and run through the gel by application of 100 V for 1 h. Proteins were transferred from the gel to a polyvinylidene difluoride membrane using a wet blotting method. The membrane was blocked with 5% Marvel in PBS containing 0.1% Tween20 (PBS-T) and then probed with a rabbit anti-rat TGF-β1 (cat. no: 3709S; Cell Signaling Technology Inc., Barcelona, ES), mouse anti-rat ET-1 (cat. no: MA3-005; Thermo Scientific, IL, US) and rabbit anti-human phospho-Smad3 (cat. no: PS1023; Calbiochem), and normalised to total mouse anti-rat β-actin antibody (cat no: A1978; Sigma) or total rabbit anti-human Smad3 (cat no: 566414; Calbiochem) as housekeeping reference, 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 total Smad3 expression using the software GeneSnap version 6.08.

**DCF Fluorescence Measurement of Reactive Oxygen Species**

2′, 7′-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA, Molecular Probes, UK) is a cell-permeable compound that following intracellular ester hydrolysis is oxidized to fluorescent 2′, 7′-dichlorofluorescein (DCF) by O$_2^\cdot$ and H$_2$O$_2$, and can therefore be used to monitor intracellular generation of ROS (16). To quantify ROS levels, HPAECs were seeded to black walled, clear bottom 96 well plates, washed twice with PBS and incubated for 30 min with 50 µM H$_2$DCF-DA diluted in Opti-MEM in presence or absence of sepiapterin (1µM-100µM). Then, cells were again washed twice with PBS to remove remaining H$_2$DCF-DA and stimulated with TGF-β1 (5ng/ml) or ET-1 (100nM) for 30 min in presence or absence of sepiapterin. Five randomly selected fields per
condition were measured for fluorescent intensity using an epifluorescence microscope (Nikon Eclipse TE 200, Tokio, Japan) with filter set for FITC. Subsequent image capture and analysis was performed using Metafluor® 5.0 software (Analytical Technologies, US). Results were expressed as DCF fluorescence in relative fluorescence units (RFU).

**Statistics**

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 patients. In this case, data were displayed as medians, interquartile range and minimum and maximum values. When the comparisons concerned only 2 groups, between-group differences were analyzed by the Mann Whitney test. Results from animal and cellular *in vitro* mechanistic cell experiments (Figures 1-6 in main manuscript) 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. Statistical analysis was done on raw data considered as the gene expression corrected by the housekeeping GAPDH, protein expression corrected by the internal standards Smad3 as appropriate. Analysis of levels of BH4, BH4/BH2, nitrotyrosine, nitrites and DCF fluorescence were performed on raw data.
Online References


