ONLINE DATA SUPPLEMENT

Detailed Methods and Supplemental Results

Iloprost Reverses Established Fibrosis In Experimental Right Ventricular Failure

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**Complementary description of SU5416/hypoxia protocol:** S-D rats weighing 200g were injected subcutaneously with a single 20 mg/kg of SU5416 suspended in 0.5% (w/v) carboxymethylcellulose sodium, 0.9% (w/v) sodium chloride, 0.4% (w/v) polysorbate 80, and 0.9% (v/v) benzyl alcohol in deionized water, according to our protocol and previously published literature (1, 2). After SU5416 injection, rats will be placed in the hypoxia chamber for four weeks, as described above and published previously (1-3).

**Complementary description of Pulmonary artery banding**

Male Sprague-Dawley rats (weight 180-200 g) were anesthetized with isoflurane inhalation (5% and 2%, respectively, in oxygen-enriched room air). After intubation, the animals are mechanically ventilated with the use of a volume-controlled respirator. Positive endexpiratory pressure was maintained at 4 cmH2O. A left thoracotomy as performed, where the pulmonary artery was carefully dissected free from the aorta. A silk thread was then positioned underneath the pulmonary artery, while we also placed an 8-gauge needle is placed alongside the pulmonary artery. A suture was tied tightly around the needle, and the needle rapidly removed to produce a fixed constricted opening in the lumen equal to the diameter of the needle. After the banding, the thorax was closed in layers, and postoperative pain relief was obtained by applying buprenorphine (15μg/kg s.c.). For more information about the procedure please refer to Bogaard, et al. Circulation 2009 (3).
Complementary description of the inhalation protocol: Previous inhalation/exposure experiments showed that 0.5 ± 0.1% of each loaded dose was delivered to the airways of the rat lung (an average solute delivery efficiency of 0.5%). A piezoelectric vibrating mesh nebulizer, AeroNeb™ (Aerogen, CA) was used to generate 2-3 μm (volume median diameter) solution aerosols of iloprost or vehicle, at a rate of 0.3-0.4 ml/min into a small chamber from 1.5 mL dosing solution aliquots. Rats held in a restrainer were nose-only exposed to the aerosols for 5 min until dosing solution was no longer visible in the AeroNeb reservoir. During the exposure, a small animal ventilator (Harvard Apparatus, Holliston, MA) was used to propel and mix the aerosols at a rate of 60 cycles/min and 5 ml/cycle.

Hemodynamics: Mean pulmonary arterial pressure and mean left atrial pressure were used to estimate the pulmonary vascular resistance using the formula:

\[
PVR = \frac{mean \; pulmonary \; arterial \; pressure - left \; ventricular \; end \; - \; diastolic \; pressure}{cardiac \; output}
\]

Echocardiography
Transthoracic echocardiography was performed with a Vevo 2100 (Visual Sonics, Toronto, Ontario, Canada) to assess tricuspid annulus plane systolic excursion (TAPSE), cardiac output and to evaluate pulmonary velocity time integral, as described previously (2, 4). Ketamine and zylaxine were used to anesthetize the rats prior to echocardiography. TAPSE was assessed with M-mode Echo using right parasternal short-axis imaging view with the ultrasonic beam positioned perpendicularly to the sternum. CO was quantified by measuring the maximal diameter of pulmonary artery in diastole (PAd) from the short-axis view, as reported by Urboniene et al(5). Cardiac output was derived from the formula:

$$CO = SV \times heart\ rate, \ where \ SV = 3.14 \times (pulmonary\ artery\ diameter/2)^2 \times VTI\ of\ the\ pulmonary\ artery.$$  

**In vitro cell culture experiments:** Primary human cardiac fibroblast cells were cultured with fibroblast growth media (Lonza) containing no growth factors, cytokines, or supplements in culture flasks inside an incubator at 37°C with 5% CO₂ and 95% air. Cells were seeded in six-well culture plates and cultured to near confluence prior to treatment. For immunohistochemistry analysis, cells were plated in chamber-slides with an approximate cell density of 2000 cells/cm². rhTGFβ1 was previously activated with 4 mM HCL containing 1 mg/ml of bovine serum albumin. We used TGFβ1 because it has been reported to have the same effect of TGF-β2 inducing CTGF expression(6).
**Migration/in-vitro wound closure assay:** For this assay, the cells were seeded in 6-well plates and incubated until confluent cultures were generated. Cell layers were scraped with a 200-ul plastic pipette tip as described previously(7). The remaining cell culture was treated with either phosphate-buffered saline (PBS), iloprost at 1 ng/ml, recombinant human transforming growth factor (rhTGF-β1) at a dose of 2 ng/ml, or iloprost at dose of 1 μg/ml plus TGF-β1 at 2 ng/ml for 24 hrs. To quantify cell migration, phase-contrast images of the identical location in each culture dish were taken at 0, 12 and 24 hrs.

**Tissue preparation for molecular analysis:** Upon sacrifice, the right lung and the total heart were removed. The RV was carefully dissected from the rest of the heart to measure the RV/LV+S. RV weight over left ventricle and septum weight (LV/+S) weight is the standard assessment for hypertrophy, as previously reported(2, 3, 8). After dissection the lung, RV and LV+S tissues were frozen with liquid nitrogen until the isolation of mRNA and protein was performed. The left lung was inflated with 0.5% low-melting agarose at a constant pressure of 25cm H₂O, fixed in 10% formalin for 48 hours and used for histological evaluation of the lungs and IHC analysis, as reported previously(3, 9).

**Gene and protein expression analysis:** RV tissue will be homogenized with Triazol® (in lysing matrix-D impact-resistant 2ml tubes using a FastPrep®- 24 instrument (MP Biomedicals). Total RNA will be carefully isolated using an RNeasy (Qiagen) isolation kit according to manufacturer's protocol. Total RNA (1
μg) will be reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression will be measured by quantitative PCR. First strand cDNA will be diluted and then mixed with Power-SYBR® Green PCR Master Mix (Applied Biosystems), along with rat or human specific primers. All PCR reactions will be performed in a LightCycler480 PCR system (Roche Diagnostics), following manufacturer’s protocol. α-actinin-1 or 18-s were used as reference (house-keeping) genes as previously reported(10). Target-gene/reference-gene ratios from all groups were normalized to control (untreated) rats or cell control group.

The following are the nucleotide sequence of the primers used:

**Rat**

**18S**
Forward 5’-GCAATAACAGGTCTGTGATGCC-3’
Reverse: 5’-CACGAATGGGGTTCAACG-3’;

**CTGF**
Forward 5’-CTGTTCCAAGACCTGTGGGAT-3’
Reverse 5’-TTTGCCCTTCTTAATGTTCT-3’.

**COLA1A**
Forward 5’- GAACGGAGATGATGGGGAAG-3’
Reverse: 5’- CCAAACCCTGAAACTCTCG-3’;

**COLA3**
Forward 5’- AGAATGGGAGACTGGACCT-3’
Reverse: 5’- ATGCCTTGTAATCCTTGGA -3’;
VEGFA
Forward 5' - GCAGAAAGCCCATGAAGTGGT -3'
Reverse: 5' - TCTCATCGGGGTACTCCTGG -3';

Angiopoietin-1
Forward 5' - GTGGCCGTGTGGTTTTGAAC -3'
Reverse: 5' - GTGGCCGTGTGGTTTTGAAC-3';

Apelin
Forward 5' - GGAAATTTCGCAGACAGCGG -3'
Reverse: 5' - GGAGAGCCCTTCAATCCTGC -3';

MMP9
Forward 5'-ACCTGAAAACCTCCAACCT -3'
Reverse: 5'- TGCTTCTCTCCCATCATCTG-3';

MMP2
Forward 5'-GCACCACCGAGGATTATGA -3'
Reverse: 5' - GTTGCCAGAAAGTGAA-3';

TIMP2
Forward 5'- TGGACGTTGGAGGAAAGA-3'
Reverse: 5' - TCCCAGGGCAACAATAAAGT-3';

P62
Forward 5'- TCTTCTAAGGAAGTGGAC -3'
Reverse: 5' - AGCCTCTGGTGGGAGATG-3';

LC3B
Forward 5' - AGATCCCGTGATTATAG-3'
Reverse: 5’- AAGGCTTGGTTAGCATTG-3’;

**PARK2**
Forward 5’- TTCAACTCCAGCTATGGCT-3’
Reverse: 5’- GTTCCAGGTACAGTTCTG -3’;

**Human**

**18S**
Forward 5’- CCGGTACGCCCCTCTCC-3’
Reverse: 5’- CAGACGTTCGAATGGGTCGT-3’;

**CTGF**
Forward 5’- CGTGTGCACCGCCAAAGAT-3’
Reverse: 5’- ACGTGCACGTGGTTACTTGCA-3’;

**COLA1A**
Forward 5’- CCCCCCTGTTGCTACTGGTTT -3’
Reverse: 5’- GGGTCCAGCATTCCAGAGG-3’;

**COLA3**
Forward 5’- AAGGGTGAAGTTGGACCTGC-3’
Reverse: 5’- CGGGACCCATTTGCCTTTA -3’;

**Protein analysis:** For protein analysis whole cell lysate from isolated right ventricle will be prepared using RIPA buffer (Sigma), and the protein concentration was determined using BioRad Protein DC Protein Assay. Whole
cellular protein was separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was incubated with the pertinent primary and secondary antibodies, according to our standard western blot protocol (2). Blots will be developed and scanned for densitometric analysis.

**Zymography**

MMP-2 and MMP-9 activity were determined by gelatin zymography, as previously published (11). Briefly, 125 µg protein extracts (at a final concentration of 5 µg/µL) were mixed with 25 µL of SDS for a final concentration of 2.5 µg/µL. A total of 37.5 µg of protein extract (15 µL of sample/SDS) were electrophoresed on 10% SDS-Tris-glycine gels containing 1 mg/ml gelatin (Invitrogen, Sunnyvale, CA) at 100V, 450 Amps for 1 hr 40 minutes. The gels were renatured, developed, and stained with Comassie brilliant blue and destained as per standard protocol (Bio-Rad, Hercules, CA).

**Histological analysis**

**Fibrosis quantification:** To assess the degree of collagen deposition in RV tissue we used standard Masson trichrome staining according to manufacturers protocol and as previously reported (12). Four snapshots of Masson trichrome stained slides per animal were used to evaluate the amount of collagen deposition using a color deconvolution tool in Image J (National Institutes of Health 1997-2011, Bethesda, MD; [http://imagej.nih.gov/ij](http://imagej.nih.gov/ij)). We measured the total blue-positive area in each animal and then divided it over the total tissue.
area to calculate the total fibrosis percentage per snapshot. The value obtained
was averaged with the rest of the slides per groups and reported as mean+/-
SEM.

**Vessel morphometry:** The analysis of the number of open, partially occluded
and occluded vessels was performed based on the study published by Oka *et al*(13) and as previously published by our group(14). Briefly, we took 10
snapshots per slide of lung tissue previously stained with anti-vonWillebrand
antibody, to select for endothelial cells. Only vessels ranging from 20-100 μ.
Partial occlusion was defined as the presence of double-layer of endothelial cells
occupying 25-75% of the total vessel lumen as illustrated in **Figure 1B**. More
than 75% of lumen occlusion was considered as fully obliterated.

**Von-Willebrand Factor Immunohistochemistry:** Formalin fixed- paraffin
embedded lung sections (3 μm) were rehydrated, then enzymatic digestion
(proteinase K, 1:50, DAKO, Carpinteria, CA) for 6 min in PBS was performed,
followed by blocking of endogenous peroxidase (3% H₂O₂ in PBS) for 5 min and
blocking with 1% normal swine serum (NSS) for 15 min. The sections were
incubated with the primary rabbit anti-von Willebrand Factor antibody (DAKO)
1:500 in 1% NSS in PBS overnight at 4°C and with the secondary, anti- rabbit
biotin-conjugated antibody (1:1500, Millipore, Billercia, MA) in 1% NSS/ PBS for
1 h at room temperature, followed by 45 min HRP-conjugated Streptavidin
(1:200, Vector Laboratories, Burlingame, CA). Sections were developed using
DAB chromogen (DAKO), counterstained with Mayer’s Hematoxylin, dehydration
and mounting.
**Lectin perfusion**: To evaluate capillary rarefaction the rats were intravenously perfused with Texas Red® lycopersicon esculentum lectin (Vector Laboratories). Tomato lectin (Catalog No. TL1176) was purchased in solution with 1mM HEPES, o.15 M NaCl, pH 7.5, o.08% sodium azide o.1 mM Ca++ with a concentration of 1mm/ml. The tomato lectin was infused via jugular vein while the rats were sedated and under mechanical ventilation as reported previously(3).

**Chromatography Electrospray Ionization Tandem Mass Spectrometry**

6-keto-FG1α was measured from frozen heart samples as follows. Briefly the frozen heart tissues were thawed on ice and homogenized using an Omni TH tissue homogenizer to obtain a 10% (w/v) solution in PBS. 200 μl of the solution thus obtained was diluted 1ml of LCMS grade ethanol containing 10 ng of each internal standard and 0.05% BHT. The internal standards used was (d4) 6k PGF1α, (d4) 8-iso PGF2α, (d4) PGF2α, (d4) PGE2 (d4) and PGD2 (d4). The mixture thus obtained was agitated to homogeneity using a bath sonicator and the resultant suspension was incubated in the dark at 4°C for 5 hours with periodic sonication. Following incubation, the insoluble fraction was precipitated by centrifuging at 6000g for 20 minutes and the supernatant was transferred into a new glass tube. This supernatant was dried under vacuum followed by reconstitution in 100 μl of 50:50 EtOH:dH2O for quantitation via LC/MS/MS. A 30 minute reversed-phase LC method utilizing a Kinetex C18 column (150 x 2.1mm, 1.7μm) was used to separate the eicosanoids at a flow rate of 200μl/min at 50°C. The column was equilibrated with 100% Solvent A
[acetonitrile:water:formic acid (10:90:0.02, v/v/v)] for five minutes and then 10 µl of sample was injected. 100% Solvent A was used for the first minute of elution. Solvent B [acetonitrile:isopropanol (50:50, v/v)] was increased in a linear gradient to 25% Solvent B to 3 minutes, to 45% until 11 minutes, to 60% until 13 minutes, to 75% until 18 minutes, and to 100% until 20 minutes. 100% Solvent B was held until 25 minutes, then was decreased to 0% in a linear gradient until 26 minutes, and then held until 30 minutes. The eicosanoids were analyzed using a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP5500® ABSciex) via multiple-reaction monitoring in negative-ion mode. Eicosanoids were monitored using precursor → product MRM pairs. The mass spectrometer parameters used were: curtain gas: 30; CAD: High; ion spray voltage: -3500V; temperature: 500°C; Gas 1: 40; Gas 2: 60; declustering potential, collision energy, and collision cell exit potential vary per transition.

Online data supplement References


Figure legends

**Online supplement Figure 1.** A) Schematic representation of the in-house made system used to deliver aerosols to the rats. \( VDM \) = volume median diameter. B) Percentage of FITC-labeled Dextran 10 in the trachea and lungs. The loading dose of FITC-D10 was 6 mg. Data are presented as mean ± standard deviation, \( n=3 \).

**Online supplement Figure 2.** A) Hemodynamic analysis by conductance catheter inserted in the left ventricle demonstrated no reduction in left ventricular systolic pressure (LVSP) and no change in left ventricular contractility assessed by LVdp/dt (B).

**Online supplement Figure 3.** Trichrome-stained slides of RV tissue from iloprost treated rats at multiple levels of the right ventricle demonstrate consistently reduced collagen deposition.

**Online supplement Figure 4.** A) Correlation between tricuspid annulus plane systolic excursion (TAPSE) and connective tissue growth factor (CTGF) transcript levels fold-change.
Online supplement Figure 5.  A) Quantitative polymerase chain reaction analysis demonstrates significant reduction in the transcript levels of multiple genes encoding transcription factors and enzymes required for mitochondrial function. No change was seen after iloprost treatment.  B) Quantitative polymerase chain reaction analysis demonstrates significant increase in the transcript levels of fibrosis associated genes in both groups and no significant change after iloprost treatment.