ONLINE DATA SUPPLEMENT

Right Ventricular Myocardial Stiffness in Experimental Pulmonary Arterial Hypertension: Relative Contribution of Fibrosis and Myofibril Stiffness

Silvia Rain, MD\textsuperscript{1,2}, Stine Andersen, MSc\textsuperscript{3}, Aref Najafi, MSc\textsuperscript{1,2}, Jacob Gammelgaard Schultz, MSc\textsuperscript{3}, Denielli da Silva Gonçalves Bos, MSc\textsuperscript{1,2}, M. Louis Handoko, MD PhD\textsuperscript{2,4}, Harm-Jan Bogaard, MD PhD\textsuperscript{1}, Anton Vonk-Noordegraaf, MD PhD\textsuperscript{1}, Asger Andersen, MD PhD\textsuperscript{3}, Jolanda van der Velden, PhD\textsuperscript{2,5}, Coen A.C. Ottenheijm, PhD\textsuperscript{2}, Frances S. de Man, PhD\textsuperscript{1,2}

Departments of \textsuperscript{1}Pulmonology, \textsuperscript{2}Physiology and \textsuperscript{4}Cardiology, VU University Medical Center / Institute for Cardiovascular Research, Amsterdam, the Netherlands; \textsuperscript{3}Department of Cardiology, Aarhus University Hospital, Aarhus, Denmark; \textsuperscript{5}ICIN – The Netherlands Heart Institute
EXPANDED METHODS AND RESULTS

Study design

We used rat RV trabecular tissue obtained from a previous study protocol. (1) 15 male Wistar Galas rats were used for this study (M&B Taconic, Ry, Denmark). The rats were handled according to the Danish national guidelines and experiments were accepted in agreement with the Danish law for animal research (authorization number 2012-15-2934-00384 Danish Ministry of Justice). Rats weighting 105±30g at start of the study protocol underwent pulmonary artery banding (n=10), where a lateral thoracotomy was performed in previously sedated, intubated and mechanically ventilated state (Abbott Scandinavia AB, Solna, Sweden – induction 7% 2:1 O2/N2O, maintenance 3.5% 2:1 O2/N2O) and a titanium clip of different diameters (0.5mm or 0.6mm) was introduced and closed around the pulmonary trunk. All rats received buprenophine (Termgesic, RB Pharmaceuticals, Berkshire) in order to relieve postoperative pain. Control rats were sham operated (n=5). The 0.6 mm clip led to mild RV dysfunction (n=5) and the 0.5mm clip led to severe RV dysfunction (n=5). After 7 weeks of pulmonary artery banding, all animals underwent hemodynamic assessment as described previously (1) and RV tissue was harvested for further analyses. RV trabecular samples were dissected from the RV free wall and immediately transferred to a 50% relax-glycerol solution containing 50% (vol/vol) Glycerol, relaxing solution (pCa = 9.0; 100 mM BES; 6.97 mM EGTA; 6.48 mM MgCl2; 5.89 mM Na2-ATP; 40.76 mM K-propionate14.50 mM creatine phosphate) and protease and phosphatase inhibitors (0.5 mM E64, 2.0 mM Leupeptine, 1 mM DTT and 0.5 mM PMSF) and placed for 12h on a roller bank at 4 °C. Subsequently, RV trabecular tissue was stored at -20 °C in a 50% relax-glycerol solution containing low concentrations of protease and phosphatase inhibitors (0.05 mM E64, 0.2 mM Leupeptine, 1 mM DTT and 0.5 mM PMSF). The rest of the RV was snap-frozen in liquid nitrogen and stored at -80°C.
**Force measurements on skinned muscle strips**

Thin muscle strips with an average length of 1mm and diameter of ~0.2mm were dissected respecting the longitudinal orientation of the fibers. The ends of the strips were attached to aluminum T clips and membrane-permeabilized in a relaxing solution containing 1% Triton X-100. The strips were mounted between a length motor (ASI 403A, Aurora Scientific Inc, Ontario, Canada) and a force transducer (ASI 315C-I, Aurora Scientific Inc) in the set-up (ASI 802D, Aurora Scientific Inc) and viewed on an inverted microscope (Zeiss Axio Observer A1).

The solutions used during the experiments were: 1) relaxing solution: pCa = 9.0 (100 mM BES; 6.97 mM EGTA; 6.48 mM MgCl₂; 5.89 mM Na₂-ATP; 40.76 mM K-propionate; 14.50 mM creatine phosphate), 2) pre-activating solution with low EGTA concentration (100 mM BES; 0.1 mM EGTA; 6.42 mM MgCl₂; 5.87 mM Na₂-ATP; 41.14 mM K-propionate; 14.50 mM creatine phosphate; 6.9 mM HDTA), and 3) activating solution: pCa = 4.5 (100 mM BES; 7.0 mM Ca-EGTA; 6.28 mM MgCl₂; 5.97 mM Na₂-ATP; 40.64 mM K-propionate; 14.5 mM creatine phosphate).

The integrity of the trabecular muscle strip was checked prior to the stiffness determination by activating the preparation. Thereafter the trabecular strip was transferred to a relaxing solution where it was stretched by 20% from the initial slack-length with a speed of stretch of 10% preparation length per second. Passive force was recorded at the end of stretch and divided by the corresponding strip cross-sectional area to normalize for variation in trabecular strip diameters (passive tension (kN/m²) = total RV myocardial stiffness). In order to determine the relative contribution of fibrosis and myofibrils to total RV myocardial stiffness, thick and thin filaments were extracted by immersing the muscle strips in relaxing solution containing 0.6M KCl (60 minutes at 20°C) followed by a relaxation solution containing 1M KI (60 minutes at 20°C). Subsequently, the muscle strips were transferred to fresh relaxing solutions and passive force development was measured again at the end of the 20% stretch and was assumed to represent fibrosis-mediated stiffness. Myofibril-mediated stiffness was determined as total RV myocardial stiffness minus fibrosis-mediated stiffness.
**RV fibrosis**

Absolute RV myocardial fibrosis content was determined on histological sections as previously described.\(^1, 6-8\)

Collagen I and III mRNA levels were quantified by quantitative real time polymerase chain reaction (qPCR, 7900 HT Applied Biosystem). RNA was isolated from snap-frozen right ventricle tissue (-80°C) using a commercial purification kit (NucleoSpin® RNA II, Macherey-Nagel) according to the manufactures instructions before RNA concentrations in each sample was determined using a spectrophotometer (Eppendorf® BioPhotometer). Total RNA was reverse transcribed into complimentary DNA (cDNA) (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific) using a standard protocol. qPCR was performed with Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific) using specific primers for the genes of Collagen 1 and 3. The house keeping gene GAPDH was used for normalization. Collagen I/III ratio was calculated for each sample.

Primer sequences:

GAPDH: TTAAGGGCATCCTGGGCTACACT (forward)
TTACTCCTTTGAGGCCATGTAGG (reverse)

Collagen 1: TCAAGATGGGTGCGCTTTACT (forward)
CATCTTGGTACGCGCATG (reverse)

Collagen 3: ATGAATTGGGATGCAACTAC (forward)
TCTAGTGCTCATCATCACA (reverse)

**Force measurements on skinned cardiomyocyte**

RV tissue samples isolated from the free wall and previously preserved at -80°C were defrosted in relaxing solutions at 4°C. Using fine dissection scissors and a rotatory manual mechanical homogenizer free membrane-intact cardiomyocytes were released from the tissue sample. The single cells were then membrane-permeabilized by adding Triton (1%) to the relaxing solution in order to wash out the lipid cellular membranes and gain access to the contractile apparatus.
To remove Triton, cardiomyocytes were washed six times with relaxing solution. A droplet of cells was then studied under the microscope and a single cell was chosen based on length, width and clear light-dark sarcomeric band structure (length: 50-100µm, width 15-30µm at rest in relaxing solutions). The single cell was attached with silicone adhesive between a force transducer and a piezoelectric motor. The cell was stretched to the desired sarcomere increasing from 1.8 to 2.4 µm length to which the cell responded by increasing its passive tension. The baseline cardiomyocyte stiffness was determined by performing a rapid 25% shortening of the cell length to which the passive tension dropped to 0. The difference between the pre- and post-shortening of the passive tension was used to derive cardiomyocyte stiffness. All measurements were performed in the relaxing solution mimicking the diastolic cytoplasmic calcium content. Individual force values were normalized for the cardiomyocyte width and depth recorded at 2.2µm sarcomere length.

A minimum of three cells per sample were used to determine diastolic stiffness and their average was used for further statistical analysis (Control n=3, mild RV dysfunction n=3 and severe RV dysfunction n=3 samples).(8)

Cardiomyocytes were further incubated in relaxing solution with PKA (Protein-Kinase-A Catalytic subunit from bovine heart, P2645, Sigma Aldrich) at 20°C for 40 minutes and passive tension was again recorded after PKA treatment.

**Titin isoform and phosphorylation**

To determine titin isoform expression and phosphorylation, frozen RV free-wall tissue samples of control rats, rats with mild RV dysfunction and rats with severe RV dysfunction were weighed and pulverized in liquid nitrogen using a mortar and a pestle. Tissue powder was solubilized using a 8M urea buffer with DTT and 50% glycerol solution and protease inhibitors (0.16 mmol/L Leupeptin, 0.04 mmol/L E-64 and 0.2 mmol/L PMSF).(8, 9)
Increasing sample volumes (3 - 4.5 - 6 – 7.5 - 9µL) were loaded on 1% agarose gels and stained with Coomassie Blue. The slope of the protein band intensity – volume loading was used for titin isoforms quantification. The N2BA/Total Titin ratio was calculated.(8) ProQ Diamond Phosphoprotein Stain was used to determine titin phosphorylation. Gels were fixed, washed, destained and stained with SYPRO Ruby to determine total protein amount. The ratio between phosphorylation (ProQ) and total protein content (Sypro) was used to quantify differences in titin phosphorylation.(8)

**Statistical analyses**

Statistical analyses were performed using Prism 5 for Windows (GraphPad Software Inc, San Diego, CA). P-values lower than 0.05 were considered significant. All data are presented as mean ± SEM.

All analyses were performed using one-way ANOVA with bonferroni post-hoc comparison between control, mild and severe RV dysfunction, unless stated otherwise. The effects of PKA incubation on single RV cardiomyocytes of rats with mild RV dysfunction and severe RV dysfunction were tested at a sarcomere length of 2.2 using a two-way repeated measures ANOVA followed by Bonferroni post-hoc test.
REFERENCES


