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ORIGINAL PRE-CLINICAL SCIENCE

# Right ventricular oxygen supply parameters are decreased in human and experimental pulmonary hypertension

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**BACKGROUND:** In pulmonary arterial hypertension (PAH), high right ventricular (RV) power output requires increased myocardial oxygen consumption. Oxygen supply, however, does not increase in proportion. It is unknown what cellular mechanisms underlie this lack of adaptation. We therefore determined oxygen supply parameters in RV tissue slices of deceased PAH patients and compared them with RV tissue of patients who died from left ventricular myocardial infarction (MI). Because autopsy tissue only reflects end-stage disease, rat models with stable and progressive pulmonary hypertension (PH) were studied as well.

**METHODS:** Myocardial tissue of 10 PAH and 10 MI patients was collected at autopsy. In rats, stable PH ($n = 6$) and progressive PH ($n = 6$) was induced by 40 or 60 mg/kg monocrotaline, respectively. Six rats were used as controls.

**RESULTS:** RV cardiomyocyte cross-sectional area was strongly increased in PAH compared with MI patients ($p < 0.001$), whereas capillary density decreased ($p < 0.01$). Rat data showed similar RV hypertrophy in stable and progressive PH, and RV capillary density was decreased in both ($p < 0.01$ and $p < 0.0001$ vs control rats, respectively). RV myoglobin protein content and functional concentration were reduced in both human and rat PH RVs. In rats, this results from a lack of increase in myoglobin mRNA transcription per cardiomyocyte nucleus.

**CONCLUSIONS:** All measured cellular oxygen supply parameters are decreased in the failing human and rat pulmonary hypertensive RV. In contrast to stable PH rats, compensatory adaptations do not occur in end-stage PAH, despite higher myocardial oxygen consumption.

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Results from pre-clinical studies strongly suggest that, in pulmonary arterial hypertension (PAH), disturbances of the balance between oxygen supply and demand of the right ventricular (RV) myocardium underlies the transition from RV adaptation to failure.[1–4] A clinical study in PAH patients revealed that RV myocardial oxygen consumption is significantly increased.[5,6] This is due to increased RV power secondary to high RV afterload but also due to decreased RV mechanical efficiency in the failing RV.[5,6] On the other hand, oxygen supply measured as myocardial perfusion per gram myocardial tissue is decreased in end-stage disease.[7,8] These results show that, especially in

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end-stage RV failure, oxygen consumption is increased but not compensated by an increased blood supply.[7–9] It remains unknown what adaptations in oxygen supply occur at the cellular level in oxygen supply parameters, such as capillarization and the intracellular oxygen transporter myoglobin. For this reason, we investigated these parameters in tissue of autopsy hearts from patients who died from end-stage right heart failure due to PAH. In addition, to investigate the change in these parameters from stable disease to RV failure, we made use of a stable, non-failing pulmonary hypertension (PH) rat model and an end-stage disease PH model.

## Methods

### Human autopsy material

Myocardial tissue was collected during autopsy of 10 PAH patients who died from end-stage right heart failure. Myocardial tissue from 10 patients who died from an acute left ventricular (LV) myocardial infarction (MI) was used for comparison. From all patients, both LV and RV tissue was sampled and frozen in liquid nitrogen and stored at −80°C. Written consent to obtain body material for research purposes was obtained simultaneously with consent for autopsy from the closest relative.

### Rat autopsy material

Rat tissue was obtained from animals previously used in a study with another research goal, which was done by Handoko et al.[10] The present study was approved by the local animal ethics committee. Monocrotaline (MCT) was injected subcutaneously in male Wistar rats to induce PH. Stable PH was induced with a dose of 40 mg/kg MCT ($n = 6$) and severe progressive PH resulting in RV failure was induced after injection of 60 mg/kg MCT ($n = 6$). Six healthy rats were used as controls. Three weeks after MCT injection or when heart failure (defined by $>10\%$ body weight loss per day for 2 consecutive days) was obviously present, cardiac function was measured with echocardiography before the rats were euthanized and the heart was harvested. The heart was perfused with Tyrode solution (120 mmol/liter NaCl, 5 mmol/liter KCl, 1.2 mmol/liter $MgSO_4$, 2.0 mmol/liter $Na_2HPO_4$, 27 mmol/liter $NaHCO_3$, 1 mmol/liter $CaCl_2$, 10 mmol/liter glucose and 20 mmol/liter 2,3-butanedione monoxime, equilibrated with 95%/5% $O_2$/$CO_2$ at 10°C and pH 7.6) to remove the blood, frozen in liquid nitrogen, and stored at −80°C.[11] Rat cardiac function data prior to killing have been described in detail by Handoko et al.[10] Stable PH rats had a preserved cardiac output, whereas the progressive PH rats developed right heart failure.[10]

To assess the cellular determinants of oxygen supply, we measured the following parameters: cardiomyocyte cross-sectional area (CSA); capillary density; myoglobin protein content; and functional concentration and myoglobin mRNA levels. Furthermore, we measured p53 and vascular endothelial growth factor (VEGF) protein levels to gain more insight in the pathways that lead to changes in the oxygen supply determinants. Cryostat sections of 5-$\mu$m thickness were cut from the human myocardial tissue and rat heart apex before storage at −80°C.

*Cardiomyocyte cross-sectional area* (CSA) was determined in hematoxylin and eosin (H&E)-stained cryostat sections. CSA was measured in 20 cardiomyocytes, which were cut perpendicularly to their longitudinal axis.

*Capillaries* were demonstrated using von Willebrand factor staining. Due to freezing damage it was difficult to clearly demonstrate capillaries in the cryostat sections from PAH patients. Therefore, the staining was performed on paraffin-embedded tissue in PAH patients. From myocardial infarction (MI) patients and rat hearts, cryostat sections were used because there was less freezing damage and no paraffin-embedded slices were available from these groups. Cryostat sections were fixed with 4% formaldehyde before incubation, an then for 10 minutes in 1% Triton, 7 minutes in 3% $H_2O_2$ and 20 minutes in 3 ml 5% normal swine serum in phosphate-buffered saline. The paraffin sections were treated with citrate at 94°C before incubation with the normal swine serum. Then sections were incubated overnight at 4°C with anti-human von Willebrand factor (Dako Denmark A/S, Glostrup, Denmark). After washing in phosphate-buffered saline, the sections were incubated for 30 minutes with a secondary biotin-labeled antibody and for 30 minutes in avidin–biotin complex, followed by 10 minutes with 3,3′-diaminobenzidine staining. Capillaries were counted and expressed as number per cardiomyocyte and per area.

*Myoglobin, p53 and VEGF protein content.* Human myocardial tissue and rat ventricles were homogenized and protein analysis was performed with gel electrophoresis (4% to 15% acrylamide gels) and western blotting. Myoglobin protein was demonstrated with a primary polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary polyclonal goat anti-rabbit antibody (Dako, Denmark A/S). p53 was identified with primary mouse monoclonal antibody (Cell Signaling, Merck Millipore, Darmstadt, Germany) and secondary goat anti-mouse antibody (Calbiochem, Merck Millipore, Darmstadt, Germany). VEGF was demonstrated with primary mouse monoclonal antibody (Santa Cruz Biotechnology) and secondary goat anti-mouse antibody (Calbiochem) All signals were normalized to $\alpha$-actin with primary monoclonal anti-actin mouse antibody (Sigma Aldrich, St. Louis, MO) and secondary polyclonal goat anti-mouse antibody (Calbiochem). AIDA software was used for quantification of the signals (AIDA, 4.21.033; Raytest, Strau-Benhardt Germany).

*Functional myoglobin concentration* was calculated from myoglobin peroxidase activity in individual cardiomyocytes as described in detail elsewhere.[11,12] Briefly, cryostat sections were freeze-dried and vapor-fixed with paraformaldehyde for 60 minutes at 70°C. Then the sections were fixed for 10 minutes at room temperature in 2.5% glutaraldehyde in 0.07 mol/liter sodium phosphate buffer, and then incubated for 1 hour in a medium consisting of 59 ml of 50 mmol/liter Tris/80 mmol/liter KCl buffer with 25 mg ortho-toluidine (Sigma-Aldrich) in 2 ml 96% ethanol and 1.43 ml 70% tertiary-butyl hydroperoxide (Fluka Chemie, Buchs, Switzerland). The functional myoglobin concentration was calculated using sections cut from gelatin blocks containing known concentrations of equine myoglobin as standards.

*Interstitial space* was measured in sections incubated for succinate dehydrogenase activity as described in detail elsewhere.[13]

*Images* were obtained as described elsewhere[11] and analyzed with IMAGEJ, version 1.39a for Windows (National Institutes of Health, Bethesda, MD), taking the pixel:aspect ratio into account.

*Quantitative polymerase chain reaction* (qPCR), ribonucleic acid (RNA) extraction and real-time PCR analysis were carried out as described elsewhere.[14] Briefly, small parts (mean weight 38.9 mg) were dissected from the frozen rat hearts. Total RNA was