Online Supplementary materials

Hypoxic vascular response and ventilation-perfusion matching in end stage COPD may depend on p22phox

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Short Title: p22phox in COPD

MATERIALS AND METHODS:

Human Lung samples

Human lung tissue samples were obtained from patients with COPD who underwent lung transplantation at the Department of Surgery, Division of Thoracic Surgery, Medical University of Vienna, Vienna, Austria. The protocol and tissue usage were approved by the institutional ethics committee (976/2010) and patient consent was obtained before lung transplantation. The patient characteristics identified included; age at the time of the transplantation, weight, high, sex, oxygen requirement, pCO_2, pO_2, mean pulmonary arterial pressure measured by right heart catheterization (RHC), pulmonary function tests, as well as the respiratory and medication requirements. The chest computed tomography (CT) scans, and pulmonary artery wedge angiograms were reviewed as available. Percent emphysema was calculated from thoracic computed tomography (CT) images in 18 patients using a fully-automatic in-house developed algorithm. The CT images were acquired with various CT scanners with slice thicknesses varying between 1-7 mm. Medium-soft to hard kernels were used for the image reconstructions. The lungs were identified as connected regions with an attenuation <-400 HU within the thorax. Percent emphysema was defined as the percentage of voxels with an attenuation <-950 HU within the lung segmentation [1]. The trachea and the larger airways were excluded in order to avoid counting the air-filled voxels within them.

Laser capture microdissection (LCM) and RNA extraction

Frozen lung pieces collected from healthy donors and COPD patients were cut into 10 µm sections, stained with hematoxylin for contrast, fixed with ethanol and air-dried on PEN-MembraneSlide (Leica, Wetzlar, Germany) for laser-assisted microdissection as described previously[2]. Pulmonary arteries (intima and media) of 50-300µm diameter and bronchi's of 50-500µm diameter were selected and marked and isolated with the Arcturus® LCM System. Captured vessels were immediately transfered into RNA lysis buffer and were snap frozen. RNeasy Micro Kit was utilized to isolate RNA (RNeasy Micro Kit, Qiagen, Hilden, Germany)[2,3]. For laser capture microdissection analyses, n = 6-10 samples per group were analyzed.

Animals and chronic hypoxic treatment

p22phox+/+ and p22phox-/- mice were purchased from Jackson Laboratory. The mice were identified initially in an ENU mutagenesis screen (C57Bl6/TYR or nmf 333). The p22phox-/- mice exhibit balance disorder [4]however they are viable, fertile and do not display any other behavioural abnormalities, such as eating disorders etc. For the chronic hypoxia-induced pulmonary hypertension model, 12-16 weeks-old male p22phox-/- mice and wild type littermate (p22phox+/+) controls were randomly grouped and placed in normobaric, hypoxic chambers (10% O_2) for 5 weeks. Control mice (p22phox-/- and p22phox+/+) were kept in normobaric normoxia (21% O_2). The oxygen content was monitored and controlled by an automated OxyCycler system (BioSpherix, Lacona, NY, USA) using constant gas buffering with N_2. Chambers were opened two times per week for feeding and cleaning. All experiments were approved by the local authorities according to the national regulations for animal experimentation (Austrian Ministry of Education, Science and Culture, BMWF-66.010/0076-II/3B/2011).

Haemodynamic assessment of RVSP and RV hypertrophy

p22phox-/- mice or p22phox+/+ littermates were anesthetized using isoflurane and then placed on a controllable heated pad to maintain body temperature at 38°C. The heart rate was continuously monitored by ECG for the entire duration of the experiment. Heart catheterization was performed under 2-4% constant isoflurane-anaesthesia, using the closed chest technique through a small incision in the submandibular area, as described previously [5]. The right ventricle was catheterized via the right jugular vein and pressure values were monitored continuously in order to determine the position of the catheter. Right ventricular systolic pressure (RVSP) was obtained. Next, the catheter was inserted into the right carotid artery where systemic blood pressure (SBP) was measured, and then advanced further into the left ventricle where LVSP pressures were recorded. Data was collected using SPR-671 1.4F catheters (Millar Instruments Inc., Houston, TX, USA) coupled to a Millar PCU-2000 pressure control unit and PowerLab 8/30 acquisition system (AD Instruments, Spechbach, Germany). Minute long recordings were analysed with Powerlab Pro Software (AD Instruments). After hemodynamic measurements, animals were euthanized by over-dose of Isoflurane. En-block lungs and hearts were perfused with PBS via the right ventricle with efflux through a small opening in the left atrium. The hearts were placed in PBS solution and kept on ice. After removing the atria from the ventricles, the right ventricle (RV) was removed from the left ventricle & septum (LV+S) and the separated regions were blotted dry and weighed to obtain the ratio of the right ventricle to the left ventricle plus septum (RV/(LV+S)) or to the animal´s body weight (RV/BW).

Hypoxic pulmonary vasoconstriction (HPV) in isolated perfused and ventilated mouse lungs

p22phox+/+ and p22phox-/- mouse lungs were prepared as described previously using the system from HSE Harvard Apparatus (March-Hugstetten, Germany)[6]. Briefly, lungs were perfused with 37°C sterile Krebs-Henseleit hydroxyethyl amylopectin buffer (Serag-Wiesner, Naila, Germany; 1 ml/min) containing Indomethacin (30 µM) and Nω-Nitro-L-arginine methyl ester hydrochloride (1 mM, both Sigma-Aldrich, Steinheim, Germany), and left atrial pressure was adjusted at +2.2 cmH_2O. After isolation, lungs were ventilated with negative pressure in a closed chamber, volume-controlled with a tidal volume of ~9 ml × kg body $weight^{-1}$, an end-expiratory pressure of -2 cmH_2O and a respiratory rate of 90 breaths × min^{-1}. Hyperinflation (-24 cmH_2O) was performed at 4-min intervals. Mean pulmonary arterial pressure (PAP) was continuously monitored and the difference in mean PAP (ΔPAP mean) was expressed in cmH_2O. After an initial steady-state period of 10 min, lungs were perfused with a flow of 0.5, 1.0, 1.5, and 2.0 ml/min for 30 s each to generate a four-point pressure-flow (P-Q) curve under normoxic conditions. Flow was then set to 1 ml/min, and, after another steady-state period of 4 min, mean PAP was recorded for ~1.5 h in response to a change from normoxic to hypoxic (1% O_2) ventilation[7]. A second and a third P-Q curve were generated during HPV phase I, 10 min after initiation of hypoxia and during HPV phase II, 88 min after initiation of hypoxia, respectively. Data were discarded from further analyses if lungs had signs of edema, hemostasis, atelectasis, or if maximal HPV phase I response was <2 cmH_2O. Lung tissue was weighed and subsequently dried in a 60°C oven for 48 h. The ratio of wet weight to dry weight was calculate[8] .

Measurement of the vascular tension development

Intrapulmonary arteries were mounted under the microscope in a wire microvascular myograph (Danish Myo Technology 620M, Aarhus, Denmark) using 40 µm diameter tungsten wire. The

myograph chambers were connected to force transducers for isometric measurements (PowerLab,ADInstruments Ltd UK). The chamber contained PSS (5 ml; pH 7.4) and was equilibrated with 5% CO2 in artificial air (20.9% O_2, 74% N_2). After the myograph had reached 37°C, basal tension of 2mN was applied and the arteries were allowed to stabilize for 45 minutes. To test the viability of the preparation, tissues were challenged three times with a maximally depolarizing stimulus of PSS containing 120 mM KCl (KPSS; isotonic replacement of NaCl by KCl) for 15 min. Tissues which exhibited a contractile force of <2 mN upon KPSS challenge were discarded. The response of the artery segments was measured as the change in force. The data on vasoconstriction are given as percentage (% constriction) of the contraction induced by 120 mM KCl and the relaxation as percentage (% relaxation) of the contraction induced by the thromboxane receptor agonist U46619.

Immunohistochemistry and tissue staining

Paraffin-embedded lung tissues were cut to 2µm thick sections and stained with Hematoxylin-eosin for initial assessment of the lung structure. Quantification of muscularised vessels in the lung were made as previously described [9]. Briefly, double immunohistochemical staining was performed using ZytoChem Plus AP-Fast Red Kit (Zymed Laboratories, Invitrogen, Carlsbad, CA) or ImmPACT ^{TM} VIP Kit (Vector Laboratories LTD, UK) according to the manufacturers' instructions. The following primary antibodies were used, anti-alpha-SMA (Everest Biotech, Upper Heyford, UK), anti-vWF (Dako, Glostrup, Denmark) and p22phox (Santa Cruz Biotechnology, Dallas, TX). Negative controls were performed with the omission of the respective primary antibodies. Immunostained slides were scanned with an Aperio slide scanner-microscope and images were captured with Image Scope software (Aperio, Oxford, UK). Vessel remodeling in lung sections

was quantified using semi-automated image analysis software (Visiopharm, Hoersholm, Denmark).

Quantitative RT-PCR

Real-time PCR was used for relative quantification of gene expressions in mouse lung tissue and hPASMCs, compared to the reference β2mg housekeeping gene. The analyses were performed in an ABI 7700 Sequence Detection System (Applied Biosystems) using 10 µl reaction mix containing cDNA samples, SYBR Green (Qiagen, Hilden, Germany), as well as forward- and reverse primers. The amplification protocol was 1× (50°C, 2 min); 1× (95°C, 6 min); 45× (95°C, 5s; 60°C, 5s; 73°C, 10s). The data for the amplification curves were acquired after the extension phase at 73°C. Specificity of the amplification reaction was confirmed by analysing the obtained melting curves. Primers used for the Qpcr NOX1:5-TGGCTAAATCCCATCCAGTC-3,5-CCCAAGCTCTCCTCTGTTTG-3,NOX2:5TCGCTGGAAACCCTCCTATG-3,5-GGATACCTTGGGGCACTTGA-3,NOX3:5-CGACGAATTCAAGCAGATTGC-3,5-AAGAGTCTTTGACATGGCTTTGG-3,NOX4:5-ACTTTTCATTGGGCGTCCTC-3,5-AGAACTGGGTCCACAGCAGA-3,p47:5-GTCCCTGCATCCTATCTGGA-3,5-TATCTCCTCCCCAGCCTTCT-3,p67F:5-CAGACCCAAAACCCCAGAAA-3,5-AGGGTGAATCCGAAGCTCAA-3,p40F:5-TTTGAGCAGCTTCCAGACGA-3,5-GGTGAAAGGGCTGTTCTTGC-3,NOXO1:5-TTCCTGATGCTCCATTGCTG-3,5-GGTTGGGATAAGGGCTCCTC-3andNOXA1:5-AGCTGCAGAGGTTCCAGGAG-3, 5-GATGTCTTGAGCCCCCTCTG-3.Each gene expression was measured in triplicates. The ΔCt values were calculated using the following formula: $\Delta Ct = Ct_{avg}$ (reference gene) – Ct_{avg} (target gene).

Rho kinase activity assay

Rho kinase activity assay was performed according to manufacturer´s instructions (Cell Biolabs Inc). Briefly, lung tissue slices from p22phox+/+ and p22phox-/- mice were placed in 1% hypoxia

or normoxia with pre-conditioned PSS solution supplemented with L-NAME and indomethacin for a period of 120 minutes. After treatment, lung tissues were snap-frozen in liquid nitrogen. The samples were lysed in 1x kinase buffer using a MagNA lyser homogenizer (Roche AG, Switzerland). 90µl of tissue lysates were mixed with 10µl kinase reaction buffer containing DTT and ATP, and this mixture was incubated at 37°C for 60 minutes in the provided ROCK Substrate Coated Plates. Phosphorylation of the substrate was detected by anti-phospho-MYPT1 antibody, measuring absorbance wavelength on 450 nm. All measurements were performed in duplicate. Results of the activity assay were normalized to the respective sample protein concentrations.

Measurement of phorbol myristate acetate (PMA)-induced ROS level

Mice were euthanized using isoflurane overdose followed by cervical dislocation. The lung vasculature was perfused with saline to remove blood residues, and airways were inflated via the trachea with a 1:1 mixture of OCT compound (Scigen Scientific Gardena.USA) and saline solution. Next, the lungs were snap-frozen in liquid nitrogen. The inflated frozen lungs were cut into 5µm thin sections allowing them to adhere on glass slides. These slices were incubated for 30 minutes at room temperature with saline solution. The specimens were treated with PMA for 15 minutes, followed by 30 minutes incubation with a ROS detecting dye dihydroethidium (DHE). After washing with saline solution 4-5 images/sample were obtained in 20X magnification with an Olympus inverted microscope with virtual slide system.

The levels of hydrogen peroxide were determined by changes in intracellular H2DCF-DA or DHE (Life Technologies, Vienna, Austria) fluorescence levels. single cell lung tissue homogenates were prepared by digesting the single lube of lung from p22phox+/+ or p22phox -/- mice, with Collagenase (200ng/ml) and DNAse (200ng/ml) for 40 min at 37°C. The tissue homogenate was

passed through cell strainer (100µm) for single cell suspension and if necessary erythrocytes were lysed with erythrolysis buffer (2.6mM NH4Cl, 0.09M KCO3, 0.6M Titriplex III).followed by treatment with and without PMA for 15 min. later Cells (10^5) were loaded with H2DCF-DA (10µM) for 45 min at 37°C. Fluorescence activation of H2DCF-DA was measured at 504-nm excitation and 529-nm emission; Blank (cell fraction without H2DCF-DA) readings were subtracted from loaded sample readings

Statistical analysis

Data of the study population are presented as median [interquartile range (IQR)]. Numerical values are given as means ± SEM for all other measurements. Statistical analysis was performed using GraphPad Prism 5 (Graph Pad Software Inc., USA). Intergroup differences were assessed by factorial analysis of variance with post hoc Tukey's test or Mann–Whitney U-test as appropriate. For comparison of dose-response curves or progressive measurements between groups two-way ANOVA was used. The Spearman test was performed for intergroup correlations. *P*-values denoted as *<0.05, ** <0.01, ***<0.001 were considered statistically significant.

Online Supplementary figure legends:

S Fig.1. Compartment-specific analysis of p22phox expression in bronchi via laser capture microdissection by quantitative real-time PCR (n = 8-10 in each groups).

S Fig.2. Correlation of the emphysema score of COPD patients who underwent lung transplantation with mean PAP (A) and DLCO (B). (n=18)

S Fig-3. Preserved potassium chloride (KCl), thromboxane A2 receptor agonist U46619 and sodium nitroprusside (SNP)-induced responses in pulmonary arteries of p22phox-/- mice. p22phox deficiency did not alter the (A) KCl or (B) U46619-induced contraction of isolated pulmonary arteries or (C) the SNP (10μM)-induced vasodilation of U46619-preconstricted pulmonary arteries. n=4 p22phox+/+ and n=4 for p22phox-/- .

S Fig.4. Lack of difference in the wet/dry ratio (A) or in the total weight (B) of lungs obtained from p22phox+/+ and p22phox-/- mice at the end of the HPV analyses. n=6 for p22phox+/+ and n=8 for p22phox-/-.

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