ONLINE DATA SUPPLEMENT

Adiponectin Decreases Pulmonary Arterial Remodeling in Mouse Models of Pulmonary Hypertension

Meiqian Weng[1,2], Michael J. Raher[3], Patricio Leyton[3], Terry P. Combs[4], Philipp E. Scherer[5], Kenneth D. Bloch[3], Benjamin D. Medoff[1,2]

METHODS

High-Dose OVA model of Pulmonary Hypertension (PH). We developed a new high-dose OVA model that induced pulmonary hypertension at normoxia in wild-type mice. For this model mice were immunized with intraperitoneal injections of 10 μg OVA (Sigma-Aldrich, St. Louis, MO) bound to 1 mg Alum (Sigma-Aldrich) in 0.5 ml PBS on day 1 and day 7. The mice were then challenged with intranasal injection of 2% OVA in 25 μl PBS or PBS alone every other day for 5 weeks starting on day 12. Mice were analyzed 24 hours after the last challenge.

Mouse analysis

Bronchoalveolar lavage (BAL) and removal of the lungs was performed as previously described (1). Briefly, BAL was performed 24 hours after the last challenge. Mice were anesthetized with a lethal injection of ketamine (100 mg/kg). The trachea was exposed and cannulated with polyethylene tubing. The lungs were lavaged with six 0.5 ml aliquots of PBS containing 0.6 mM EDTA. Lavage fluid recovered from the first 1 ml of instilled PBS/EDTA was collected separately from the rest of the BAL. Both BAL fractions were centrifuged and the pelleted cells from both fractions were pooled for analysis. The supernatant of the BAL recovered from the first 1 ml instilled was kept frozen at -80˚C for subsequent analysis. The live cells, as determined by trypan blue exclusion, were washed in PBS and enumerated in a hemocytometer. The differential cell count was determined by enumerating macrophages, neutrophils, eosinophils, and lymphocytes on cytocentrifuge preparations of the cells stained with Diff-Quick (Dade Behring, Newark, DE). At least 200 cells were counted on each sample. Cells recovered from the BAL were also stained with fluorescently labeled Abs to CD4, CD8 and CD69 (BD Biosciences, San Jose, CA) and analyzed by flow cytometry on an Accuri C6 (Accuri Cytometers, Ann Arbor, MI).

Histological Analyses

For histopathologic examination, lungs were flushed free of blood, inflated with 10% buffered formalin to 25 cmH_2O of pressure, and removed while inflated. Sections of paraffin-embedded lungs were stained with hematoxylin-eosin. Sections were also stained with antibodies to α-smooth muscle actin (Abcam, Cambridge, MA), according to the manufacturers' recommended protocol, for a quantitative analysis of vessel wall thickness as previously described (2). Briefly, 100 to 150 medium and small sized pulmonary arteries per mouse were analyzed. Wall thickness (%) of each examined vessel was calculated as follows: The external diameter of each vessel was measured using imaging analysis software (NIS Elements AR, Nikon). Then the distance between the endothelial component of the vessel wall and the adventitia at two diametrically opposed points of the vessel are measured and added together. This is divided by the external diameter of the vessel and multiplied by 100 to calculate the % wall thickness. Genotypes of mice were

blinded to examiners who performed the analyses.

Hemodynamic studies

RVSP was measured as previously described (3). Briefly, mice were anesthetized with ketamine (100 mg/kg) and fentanyl (250 μg/kg) intraperitoneally, then intubated and mechanically ventilated (10 μl/g, 110 bpm, FiO_2=1). Pancuronium (2 mg/kg) was administered, and a PE-10 polyethylene catheter was placed in the left carotid artery for continuous heart rate and systemic arterial pressure monitoring. Then, a 1F high-fidelity pressure catheter (SPR-1000, Millar Instruments Inc. Houston, TX) was advanced into the right ventricle via the jugular vein to measure right ventricular systolic pressure as an estimate of pulmonary arterial systolic pressure. All signals were recorded and analyzed using a data acquisition system (PowerLab with Chart, AD Instruments, Colorado Springs, CO). At the end of the study an arterial blood sample was taken while the animal was breathing room air and the partial pressure of oxygen was measured using a Chiron Diagnostic Blood Gas Machine (Siemens, Deerfield, IL).

Quantification of gene expression

RNA was purified from the lung and analyzed by quantitative RT-PCR (QPCR) as previously described (1). RNA was purified using a purification column (RNeasy, Qiagen, Valencia, CA). After a DNase step, 1 μg of RNA was converted to cDNA (Applied Biosystems, Warrington, UK). Samples underwent amplification in the presence of SYBR green (Applied Biosystems). The reaction was analyzed in real-time during amplification by the PCR machine (MX-4000, Stratagene, La Jolla, CA). Copy values for gene expression were generated by comparison of the fluorescence generated by each sample with standard curves of known quantities of target genes, performed by doing 10-fold serial dilutions of full-length cDNAs in the range of 20 million copies to 2 copies per QPCR reaction. Next the calculated number of copies was divided by the number of copies of the housekeeping gene β2-microglobulin. Primer sequences used were from the Massachusetts General Hospital (MGH) PrimerBank (pga.mgh.harvard.edu/primerbank/).

Preparation of lung protein extracts

The lungs were cut into pieces and homogenized by sonication in 1 ml of cold PBS containing protease inhibitors (Roche). Crude lysates were centrifuged at 900x g for 15 minutes to remove debris and nuclei. The supernatants were passed through a 0.2 μm pore size sterile Acrodisc® Syringe Filter (Pall Life Sciences, Port Washington, NY). The protein concentration of filtered supernatants was determined with the bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL) per the manufacturer's instructions.

APN binding Assay

PASMCs were incubated with purified APN (ALEXIS-Enzo Life Sciences, Farmingdale, NY) 10 μg/ml in cold PBS or cold PBS alone for 30 minutes. Cells were then washed with cold PBS and lysed in buffer containing 1% Nonidet P-40, 20mM Tris-HCl (pH 7.4), 150 mM NaCl, 2mM EDTA and protease inhibitor cocktail tablets. Lysates were homogenized, and protein contents were determined with the bicinchoninic acid protein assay. Equal amounts of protein were transferred onto a nitrocellulose membrane (Invitrogen) and blocked with Tris-buffered saline with Tween 20 (TBST) and 5% milk overnight at 4°C, followed by incubation for 1 hour at room temperature with anti-APN (R&D Systems, Minneapolis, MN) primary

antibody diluted in blocking buffer at 1:1000. Membranes were then washed with TBST for 30 minutes, followed by incubation for 1 hour at room temperature with secondary antibody (conjugated with HRP) diluted in blocking buffer at 1:1000 (R&D Systems).

References

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