<u>Online data supplement</u>

Connexin 43 Is a Promising Target for Pulmonary Hypertension due To Hypoxemic Lung Disease

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Detailed Methods

Human pulmonary arterial cell cultures

Human pulmonary smooth muscle cells (PA-SMC) were obtained and cultured as previously described [1, 2]. Human pulmonary arteries were initially cut into several pieces (1–2 mm^2) and placed at the bottom of individual wells of six-well culture plates containing culture medium (DMEM–HEPES supplemented with 1% penicillin–streptomycin and 1% nonessential amino acids) enriched with 10% foetal calf serum. PA-SMC were then isolated with trypsin-EDTA and cultured in 75 cm^2 flasks (Costar) in a humidified chamber at 37°C (5 % CO_2), with the medium changed twice a week. Cells were characterized as smooth muscle cells both morphologically (typical "hills and valleys" morphology) and immunocytochemically using antibodies directed against calponin 1/2/3 and α-smooth muscle actin (Sigma-Aldrich). Immunostaining demonstrated the presence of a population of smooth muscle cells with approximately 95% purity. Cells were used experimentally at passage 3 to 6. All cell culture products were purchased from Invitrogen.

PA-SMC exposure to chronic hypoxia *in vitro* was performed as previously described [3]. PA-SMC in the control group ("Normoxia" - N) were exposed to a gas mixture containing 21 % O_2, 74 % N_2, and 5 % CO_2. Cells in the chronic hypoxia group (CH) were exposed to a gas mixture containing 1 % O_2, 94 % N_2, and 5 % CO_2 for 24 or 48 h in a tri-gas incubator (Heracell 150i, ThermoScientific, Saint Herblain, France).

Animal experiments

All animal studies conformed to the Declaration of Helsinki conventions for the use and care of animals. Agreement (number A33-063-907) was obtained from the French authorities and

all the protocols used were approved by the local ethics committee (Comité d'éthique de Bordeaux n° 50, protocol number APAFIS#9212-2017031018562273 v5). Genetically modified adult male CD1 mice (8 – 12 weeks) (Gja1^{tm1Kdr}; Jackson Laboratory, Bar Harbor, ME) were used and compared to their origin's strain mice. Mutation is due to the in-frame insertion of a promoterless neomycin (Neo) gene into exon 2 of Cx43 (Gja1) gene [4]. Gja1^{tm1Kdr} homozygous (Cx43$^{-/-}$) mice die at birth due to a severe heart defect [4]. Consequently, only heterozygous (Cx43$^{+/-}$) and wild-type (Cx43$^{+/+}$) mice were used for the study. Tails from such mice were used for genotyping as previously described [5]. PH was induced by exposing mice to chronic hypoxia (CH) in a hypobaric chamber (380 mmHg) during 21 days while control animals were kept under normobaric normoxia (N - room air). Chambers were opened three times a week for animal care and cleaning, and all animals had free access to food and water.

Hemodynamic measurements and assessment of right ventricular hypertrophy

Mean pulmonary arterial pressures (mPAP) were measured in mice as previously described [6]. Mice were anesthetized with an intraperitoneal injection of Exagon® (50 mg/kg of body weight, Centravet) and after thoracotomy, a heparin-filled hypodermic needle connected to a polyethylene catheter was placed into the right ventricular cavity by direct puncture of the right ventricle. Right ventricular systolic pressure was measured by use of a fluid-filled force transducer. The Fulton index or weight ratio: right ventricle (RV) / (left ventricle (LV) + septum (S)) was calculated to assess hypoxia-induced right ventricular hypertrophy.

Hemodynamic responses to carbachol

Mice were anesthetized with an intraperitoneal injection of Exagon® (50 mg/kg of body weight, Centravet). A tracheotomy was performed and animals were ventilated with a small-animal ventilator (Minivent type 845, Hugo Sachs Elektronik, Germany). Body temperature was maintained with a heating platform. Bosentan and carbachol were administered via a catheter in the jugular vein and right ventricular systolic pressure was measured as described in the previous section. We blocked the endothelin A and B receptors with the antagonist bosentan administered at 20 mg/kg and 5 minutes later, we administered the vasodilator carbachol at 10 µM. Right ventricular systolic pressure was recorded just before carbachol injection and up to five minutes after carbachol injection. The carbachol effect measured, was the maximal effect observed during the five minutes of recordings.

Measurement of right ventricular function by echocardiography

Anesthesia of mice was induced with isoflurane gas (3 %) and maintained with 1.5 % isoflurane in room air supplemented with 100 % O_2. Mice were laid in supine position on a heating platform with all legs taped. The chest of mice was shaved. To provide a coupling medium for the transducer, a pre-warmed ultrasound gel was spread over the chest wall. Transthoracic echocardiography was performed with the Vevo770 high-resolution imaging system equipped with a 40-MHz transducer (RMV 704). Pulsed-wave Doppler was used to measure pulmonary artery flow through the pulmonary valve. Pulmonary artery acceleration time (PAAT) and velocity-time integral (VTI) were recorded.

Measurement of pulmonary arterial reactivity

After mice euthanasia by cervical dislocation, a thoracotomy was performed, and after exsanguination, the left lung was rapidly removed and rinsed in Krebs solution containing 119 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 5 mM glucose. Intrapulmonary arteries were dissected free from surrounding connective tissues under binocular control, and arterial segments were mounted in a Mulvany myograph (Multi Myograph System, model 610M, J.P. trading) as previously described [7]. Arterial viability was assessed using physiological salt solution containing 80 mM KCl (equimolar substitution with NaCl). Contractile responses were obtained by constructing a cumulative concentration-response curve to endothelin-1 (ET-1; 0.1 to 100 nM), serotonin (5-HT; 0.001 to 10 µM) or phenylephrine (Phe; 0.001 to 30 µM). Relaxation was assessed by applying cumulative concentrations of carbachol (Carb; 0.01 to 1000 µM) on vessels pre-contracted with Phe 1 µM.

Measurements of pulmonary arterial medial wall thickness

Mice lungs were fixed in 4% paraformaldehyde, and dehydrated in increasing grade of ethanol. After delipidation with xylene, lungs were embedded in paraffin and cut into transverse sections (4 µm) which were stained with haematoxylin and eosin. Pulmonary vascular remodelling was assessed by measuring the percentage of wall thickness of the intra-acinar arteries with an external diameter of less than 80 µm (74.2 ± 4 µm). 3 to 10 vessels per mice were analysed in a blinded fashion using Image J software (NIH Image). The percentage of wall thickness (% wall thickness) was calculated as [(external wall areas) – (internal wall areas)] x 100/ external wall areas.

Immunofluorescent staining

Serial 4 µm mice lung or heart sections or 5 µm human lung sections were deparaffinised and rehydrated. For antigen retrieval, sections were either heated at 95°C for 20 minutes in citrate buffer (pH 6) for proliferative cell nuclear antigen (PCNA) immunostaining or incubated in pronase E 0.1 % (Sigma-Aldrich) for Cx43, von Willebrand factor (vWF), CD31 immunostainings or incubated in a retrieval buffer for immunostaining of CD45 (a pan leukocyte marker) in mice lung and/or heart sections and alpha-smooth muscle actin (α-SMA), lectin, Cx37, Cx40 and Cx43 in human lung sections. Unspecific protein binding was blocked with a blocking buffer composed of bovine serum albumin (BSA 5%) and goat serum (5 %) (Sigma-Aldrich) for PCNA, Cx43, vWF and CD31 in mice lung and/or heart sections and then incubated overnight at 4°C with specific primary antibodies followed by incubation with corresponding secondary fluorescent-labelled antibodies (Thermo Fisher Scientific). Details on the primary and secondary antibodies are shown in the supplemental table S1. Nuclei were stained with DAPI (Sigma-Aldrich). Plasma membranes were stained with WGA-FITC (W834, Invitrogen). Negative controls were performed in parallel by omitting primary antibodies. The percentage of PCNA positive cells in intrapulmonary arteries was assessed in five to ten vessels (with a mean diameter of 70 µm) per section. In addition, TUNEL assay was performed using the ApopTag® Red In Situ Apoptosis Detection Kit (S7165, Merck), according to the manufacturer's protocol. Mounting was performed using ProLong Gold antifade reagent (Thermo Fischer Scientific) or Dako fluorescence mounting medium (Agilent). Images were taken using an LSM700 confocal microscope (Zeiss) for human lung sections and CD45 labelling in mice lung sections, using a LSM880 Airyscan confocal microscope (Zeiss) for mouse lung CX43/WGA labelling and for all other fluorescent labellings a TCS SP8 confocal microscope (Leica) was used.

Western Blot analysis

Intrapulmonary arteries, left or right ventricles from mice of the different experimental groups were homogenized in RIPA buffer containing 1 % triton X-100 and protease and phosphatase inhibitors (Sigma-Aldrich). Human PA-SMC and PM-EC were homogenized and sonicated in RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). CD45 expression was performed on mice whole lung homogenates. Whole lung samples were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl pH 7.4, 1 % NP-40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM PMSF and protease and phosphatase inhibitors (Sigma-Aldrich). For all the Western Blotting experiments, the protein concentration was determined by using the Lowry method (Bio-Rad). Equal amounts of proteins (50 µg for human PA-SMC and PM-EC, 30 µg for human pulmonary arteries, 15 µg for mice intrapulmonary arteries, 25 µg for mice whole lung homogenates and 30 µg for mice right and left ventricles), were separated by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking, membranes were incubated overnight at 4°C with one of the specific primary antibodies (details on primary antibodies are shown in the supplemental table S1). Antibodies against β-actin, calnexin and GAPDH were used as loading controls. Membranes were then incubated with corresponding horseradish peroxidase-conjugated secondary antibodies. Immunoreactive proteins were detected by chemiluminescence with an ECL detection system (Substrate HRP Immobilon Western, Millipore). ChemiDoc XRS+ system (Biorad) was used for blot imaging and Image Lab software (Biorad) was used for analysis.

Quantitative real-time PCR

Total RNA of intrapulmonary arteries, right and left ventricles from mice was isolated by using the RNeasy plus micro kit (Qiagen) according to the manufacturer's instructions. Total RNA of human PA-SMC was isolated by using the Nucleospin RNA plus kit (Macherey Nagel) according to the manufacturer's instructions. cDNA of 1 µg ARN for human cells and 150 ng ARN for mice tissues were reverse-transcribed by using the qScript cDNA supermix reverse transcription kit (Quantabio). After reverse transcription, PCR was performed on the CFX Connect thermocycler (Biorad). 5 ng ADNc was amplified in duplicate by 40 cycles of 10 s at 95°C, 30 s at Tm 60°C for all primers (see table S2 for details on primers) using PerfeCTa SYBR Green supermix (Quantabio) and gene specific primers for Cx37, Cx40 and Cx43 (table S2 for details). The mRNA expression level of these genes of interest was determined using the comparative $2^{-\Delta\Delta Ct}$ method and normalized to the mRNA expression level of endogenous references by using geometric averages of 3 or 4 internal housekeeping genes (i.e. SDHA, GUSB, RPLPO and GAPDH for mice samples and GAPDH, HPRT1 and RPLPO for human samples – details on primers for housekeeping genes are shown in table S2) and according to Normfinder software [8].

Supplemental table S1

Protein	Source	Reference	Reactivity	Application	Dilution
α-SMA	Santa Cruz	Sc-32251	Mice – Rat - Human	Immunostaining	1:100
β-actin	Sigma-Aldrich	A3854	Human	Western Blot	1:5000
Calnexin	Santa Cruz	Sc-11397	Human	Western Blot	1:1000
CD45	Santa Cruz	SC-25590	Mice	Western Blot	1:200
CD45	Biotechne	BAM114	Mice	Immunostaining	1:50
CD31	Abcam	Ab24590	Mice – rat - human	Immunostaining	1:200
Connexin 37	Invitrogen	404300	Human	Immunostaining	1:200
Connexin 40	Tebu	Sc-20466	Human	Immunostaining	1:200
Connexin 43	Sigma-Aldrich	C6219	Human	Western Blot, immunostaining	1:400
Connexin 43	Sigma-Aldrich	C6219	Mice	Western Blot, immunostaining	1:2000 for immunostaining and 1:5000 for Western Blot
GAPDH	Santa Cruz	FL-335	Mice	Western Blot	1:1000
Lectin	Sigma	L8262	Human	Immunostaining	1:500
PCNA	Abcam	Ab29	Mice	Immunostaining	1:5000
von Willebrand Factor	Millipore	AB7356	Mice	Immunostaining	1:100

Supplemental table S2

Gene	Species	Genebank accession number	Sequence	Product size (bp)
mGja1 (Cx43)	mouse	NM_010288.3	CATCAGGGAGGCAAGCCAT TTACACACTTGCACACCCACAC	71
mGja4 (Cx37)	mouse	NM_008120.3	CTTACCCCAACCTCACCCTATG TCCTGTGAGGAGAGGGTTTGA	70
mGja5 (Cx40)	mouse	NM_001271628.1	TCTGACTCACCTGCCCATCTC CTCATTTATCTTTCCACCCAACAA	73
mGUSB	mouse	NM_010368.2	GAAGGCTGGCTCACAATTTAAGA GGTGGGTGCTAGGAATTGAATC	71
mSDHA	mouse	NM_023281.1	TACAAAGTGCGGGTCGATGA TGTTCCCCAAACGGCTTCT	74
mRPLPO	mouse	NM_007475.5	CTGAACATCTCCCCCTTCTCC GGGTTATAAATGCTGCCGTTGT	71
mGAPDH	mouse	NM_001289726.1	GCCAAAAGGGTCATCATCTCCG ATGAGCCCTTCCACAATGCC	179
hGJA1 (Cx43)	human	NM_000165.3	CGGGAAGCACCATCTCTAACT CGCTGGTCCACAATGGCTAGT	118
hGAPDH	human	NM_002046.6	CACATGGCCTCCAAGGAGTAA TGAGGGTCTCTCTCTTCCTCTTGT	75
hHPRT1	human	NM_000194.2	GGCAGTATAATCCAAAGATGGTCAA TCAAGGGCATATCCTACAACAAAC	130
hRPLP0	human	NM_001002.3	TCGTGGAAGTGACATCGTCTTT CTGTCTTCCCTGGGCATCA	74

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