Hypoxic pulmonary vasoconstriction requires endothelial signal conduction via connexin 40

Liming Wang, Jun Yin, Hannah T. Nickles, Hannes Ranke, Arata Tabuchi, Julia Hoffmann, Christoph Tabeling, Eduardo Barbosa-Sicard, Marc Chanson, Brenda R. Kwak, Hee-Sup Shin, Songwei Wu, Brant E. Isakson, Martin Witzenrath, Cor de Wit, Ingrid Fleming, Hermann Kuppe, Wolfgang M. Kuebler

Online data supplement

Materials and methods

Animals. Male C57BL/6 mice were obtained from Charles River Laboratories (St Constant, QC). *Cx40*$^{-/-}$ mice of C57BL/6 background and corresponding *Cx40*$^{+/+}$ wild type mice were provided by Dr. Cor de Wit (University of Lübeck, Lübeck, Germany) and Dr. Brant Isakson (University of Virginia School of Medicine, Charlottesville, VA). *Tie2Cre*$^{+}$*Cx40*$^{fl/fl}$*ApoE*$^{-/-}$mice and corresponding control *Tie2Cre*$^{+}$*ApoE*$^{-/-}$ and *Cx40*$^{fl/fl}$*ApoE*$^{-/-}$mice were provided by Dr. Marc Chanson and Dr. Brenda R. Kwak (Hôpitaux Universitaires de Genève and Université de Genève, Geneva, Switzerland). $\alpha_{1G}^{-/-}$ mice and corresponding $\alpha_{1G}^{+/+}$ wild type mice were provided by Dr. Hee-Sup Shin (Korea Institute of Science and Technology, Seoul, Korea) and Dr. Songwei Wu (University of South Alabama, Mobile, AL).

Chemicals and reagents. Cx40 mimetic peptide gap27[40] and Cx43 mimetic peptide gap27[43] were provided by Dr Brant Isakson. Di-8-ANEPPS and Fura-2AM were obtained from Invitrogen (Carlsbad, CA). Goat anti-Cx40 antibody, goat anti-Cx43 antibody, FITC-conjugated goat anti-rabbit IgG, rabbit anti-cPLA$_2$ antibody, rabbit anti-K$_v$2.1, rabbit anti-p-Cx43 antibody and secondary antibodies were from Santa Cruz (Santa Cruz, CA); rabbit anti-K$_v$1.5 and rabbit anti-Cx43 antibody were from Abcam (Abcam; Cambridge, UK); rabbit anti-vWF antibody and Dako protein block serum from Dako Canada, Inc (Burlington, ON); biotin-conjugated horse anti-goat IgG from Vector Laboratories (Burlington, ON); Alexa 594-Streptavin from Invitrogen (Carlsbad, CA); and donkey FITC-conjugated anti-rabbit IgG from Jackson Immuno Research Laboratories (West Grove, PA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Isolated perfused mouse lung. Isolated perfused mouse lungs were prepared as previously described (E1;E2). In brief, mice of 20–30 g body weight (bw) were anesthetized by intraperitoneal injection of pentobarbital sodium (100 mg/kg bw) and placed in a 37°C

water-jacketed chamber (Typ 839; Hugo-Sachs, March, Germany). After tracheostomy, volume-controlled ventilation (MiniVent 845; Hugo-Sachs) was initiated with a tidal volume of 10 mL/kg bw, 90 breaths/min and a positive end-expiratory pressure of 2 cmH_2O. Normoxic ventilation was performed with a gas mixture of 21% O_2, 5% CO_2, and 74% N_2 (Praxair, Mississauga, ON). Following a midsternal thoracotomy, 10 U of heparin were injected into the right ventricle for anticoagulation. Two catheters (1 mm ID) were inserted into the pulmonary artery and left atrium, respectively, and lungs were perfused by Hanks' Balanced Salt Solution containing 5% bovine serum albumin and 5% dextran. Indomethacin (30 µMol/L) and N^G-nitro-L-arginine methylester (L-NAME; 1 mMol/L) were added to the perfusate to inhibit endogenous prostaglandin and nitric oxide synthesis, respectively (E2). Sodium bicarbonate was added to adjust the perfusate pH to 7.35 –7.45. After rinsing the lungs with ≥10 mL buffer, the perfusion circuit was closed and lungs were perfused at a constant flow rate of 50 mL·kg bw^{-1}·min^{-1} at a left atrial pressure (LAP) of 2 mmHg and 37°C by a roller pump (Ismatec, Glattbrugg, Switzerland). Pulmonary arterial pressure (PAP) and LAP were measured continuously via saline-filled membrane pressure transducers (Hugo-Sachs) connected to a side port of the inflow and outflow cannula, respectively. Pressure transducers were connected to a transbridge amplifier (Hugo-Sachs) and data were recorded at 150 Hz per channel on a personal computer using appropriate data acquisition software (Data Translation 4.0; Data Translation GmbH, Bietigheim-Bissingen, Germany).

Analysis of HPV response. In isolated perfused mouse lungs, the hypoxic pulmonary vasoconstriction (HPV) response was quantified as absolute increase in PAP (ΔPAP) quantified 10 min after switching from normoxia to a hypoxic gas mixture containing 1% O_2, 5% CO_2, and 94% N_2 (Praxair). As ΔPAP tends to underestimate the extent of vasoconstriction due to the non-linearity of the lung vascular pressure-flow relationship, we additionally calculated the intrinsic vascular resistance R_0, i.e. the resistance that would exist if the lung vessels were at their respective diameter at zero vascular pressure, from nonlinear regression analyses of

pressure-flow curves for selected proof-of-principle experiments (E1;E2). In brief, lungs were perfused under normoxic conditions with flows of 25, 50, 75, and 100 mL·kg bw^{-1}·min^{-1} in randomized order for 30 s each to generate a four-point pressure–flow (P-Q) curve. LAP was adjusted to 2 mmHg at each flow rate, and PAP was measured at the end of each step. After ventilation was switched to hypoxia, a second P-Q curve was generated as described above.

P-Q curves generated under normoxic or hypoxic ventilation were analyzed by nonlinear regression analysis according to the distensible vessel model (E3) as follows:

$$\mathrm{PAP} = \frac{[(1 + \alpha \mathrm{LAP})^5 + 5\alpha R_0 Q]^{1/5} - 1}{\alpha}$$

where α is the vascular distensibility factor describing the relation between vessel diameter and pressure when the diameter is normalized to the diameter at zero pressure, and Q is the applied perfusate flow.

Oxygenation during regional and global hypoxia *in vivo*. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg bw) and xylazine (10 mg/kg bw), tracheotomized, intubated and ventilated with room air (tidal volume of 10 mL/kg bw, 90 breaths/min). A catheter was inserted into the left carotid artery. Ventilation-perfusion mismatch was induced as previously described (E4) by intratracheal instillation of 25 µl of saline causing partial occlusion of the larger airways. Arterial blood samples were obtained at baseline as well as 2 and 10 min after tracheal saline instillation, and blood gas analyses were performed (Rapid Lab 348; Chiron Diagnostics GmbH, Fernwald, Germany).

To address the effects of global hypoxia *in vivo*, mice were anesthetized and ventilated as above. Arterial oxygenation was monitored continuously by pulse oximetry (MouseOx; Starr Life Sciences, Oakmont, PA) while the inspiratory fraction of oxygen (FIO_2) was decreased stepwise from 0.21 in 0.01 increments. In case of a sudden SaO_2 decrement, a recruitment maneuver with 30 cmH_2O airway pressure applied for 5 s was performed to reverse potential atelectases.

Ventilation-perfusion matching *in vivo*. Mice were anesthetized by ketamine and xylazine as described above. A catheter was surgically introduced into the right jugular vein. After tracheotomy, a 20G peripheral venous catheter (Vasofix®, B. Braun Melsungen AG, Melsungen, Germany) was advanced into the left main bronchus and one-lung ventilation with room air (tidal volume of 6 mL/kg bw, 150 breaths/min; Minivent®, Hugo Sachs Elektronik, Marchstetten, Germany) was verified by visual confirmation of unilateral thorax movements. Five minutes after one-lung ventilation was established, regional lung perfusion was determined by the fluorescent microsphere technique as described (E5). In brief, 150 µL ($1.5x10^5$ beads; 15 µm diameter) of yellow-green fluorescent microspheres (FM) (Fluorospheres®; Life Technologies, Carlsbad, CA, USA) were injected via the right jugular vein, followed by 100 µL of 0.9% saline. Lungs and right kidney (as control for systemic shunting of FM) were harvested, weighed, and digested in 4 M KOH for 24 h. Digested organs were filtered through a 10 µm pore polycarbonate filter (Carl Roth, Karlsruhe, Germany). FMs on the filter membrane were collected by washing with 3 mL of 2% TWEEN20 in reverse direction. Filtrates and FM-solutions collected from filter wash were each incubated with 1.25 mL of 2-ethoxyethyl acetate (Cellosolve®) over night and sample fluorescence was measured in a fluorometer (Versafluor®, Bio-Rad, Munich, Germany) at an excitation of λ=490 nm and an emission of λ=520 nm. 2% TWEEN20 plus Cellosolve® served as blank value. Fluorescence was related to lung weight, and expressed for both, the ventilated and non-ventilated lung as percentage of total lung blood flow.

Chronic hypoxia experiments. *Cx40*$^{+/+}$ and *Cx40*$^{-/-}$ mice were housed at either normoxia (21% O_2) or hypoxia (10% O_2) for 5 weeks, at which time mice were anesthetized by intraperitoneal injection of a triple combination of fentanyl (0.05 mg/kg bw), medetomidine (0.5 mg/kg bw), and midazolam (5 mg/kg bw) as described (E6). Following tracheotomy and intubation, mice were ventilated with room air (tidal volume of 10 mL/kg bw, 90 breaths/min). A 1.4 F microtip

Millar catheter (SPR-1000; Millar Instruments Inc., Houston, TX) was introduced via the right jugular vein for measurement of right ventricular end-systolic pressure (RVESP). Following euthanasia by exsanguination, heart and lungs were excision *en bloc*. The ratio of right ventricular weight over the sum of left ventricular and septal weight (Fulton index) was determined as a measure of right ventricular hypertrophy. Lungs were fixed by intratracheal instillation of 4% paraformaldehyde and paraffin-embedded. Lung sections of 5 μm were cut, mounted on slides coated with 10% poly-L-lysin, and stained with hematoxylin and eosin. In each group, 800 to 1000 pulmonary arterioles with external diameters of 20-50 μm were analyzed, and categorized as muscularized (i.e., with a complete medial coat of muscle), partially muscularized (i.e., with only a crescent of muscle), or non-muscularized (i.e., no apparent muscle) as described (E7).

Real-time fluorescence imaging. Pulmonary endothelial membrane potential (E_m) and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were determined by real-time *in situ* fluorescence microscopy as previously reported (E8;E9). In brief, isolated perfused mouse lungs were prepared as described above and positioned on a vibration-free table under a custom-built upright intravital microscope setup. Endothelial fluorescence in subpleural pulmonary capillaries (6-15 μm in diameter) and arterioles (20-35 μm diameter) was excited by near monochromatic illumination from a digitally controlled galvanometric scanner (Polychrome IV; T.I.L.L. Photonics, Martinsried, Germany). Fluorescence emission was collected in 10 s intervals through an upright intravital microscope (Axiotech^{Vario} 100 HD; Zeiss, Jena, Germany) equipped with an apochromat objective (UAPO 40x W2/340; Olympus, Hamburg, Germany) and appropriate dichroic and emission filters (Zeiss, Jena, Germany) by a CCD camera (Sensicam; PCO, Kelheim, Germany) and subjected to digital image analysis (TILLvisION 4.01; T.I.L.L. Photonics). For imaging of endothelial membrane potential, lungs were perfused for 30 min with the membrane-localizing fast-response probe di-8-ANEPPS (5 μMol/L), followed by a 5 min wash with dye-free buffer to remove residual intravascular dye. Fluorescence images were recorded at an excitation wavelength of λ=440 nm

and emission wavelengths of λ≥530 nm. Calibration of the di-8-ANEPPS fluorescence signal was performed by lung perfusion with buffers containing K^+ concentrations of 5.9, 7.5, 10, 12 or 14 mMol/L as described (E9), and the resulting endothelial membrane depolarization was calculated according to the Nernst equation. For quantification of endothelial $[Ca^{2+}]_i$, membrane-permeant Fura-2AM (5 μM) which de-esterifies intracellularly to impermeant Fura-2, was infused into lung microvessels for 60 min followed by wash-out with buffer as described (E8). Fluorescence images were recorded at excitation wavelengths of λ=340, 360 and 380 nm, and emission of λ=510, and endothelial $[Ca^{2+}]_i$ was determined from the 340/380 ratio using a K_d of 224 nMol/L and appropriate calibration parameters as described (E10).

$cPLA_2$ translocation. Hypoxia-induced translocation of $cPLA_2$ in endothelial cells of the isolated perfused mouse lung and in cultured human pulmonary artery endothelial cells (PAEC; Lonza, Basel, Switzerland) was assessed by use of an indirect immunofluorescence assay as described (E11;E12). In brief, lungs were ventilated with normoxic (21% O_2) or hypoxic (1% O_2) gas for 10 min. For corresponding *in vitro* experiments, PAECs were incubated at normoxia (21% O_2) or hypoxia (1% O_2) for 10 min. Endothelial cell membranes were permeabilized *in situ* or *in vitro*, respectively, by infusion of 0.5% Triton X-100 for 4 min. Then, lungs were successively perfused or cells incubated, respectively, with a primary rabbit anti-$cPLA_2$ antibody (150 μg/mL) and a secondary FITC-conjugated goat anti-rabbit IgG (1:100) for 60 min each, followed by wash. Importantly, this membrane permeabilization not only permits cell loading with primary and secondary antibodies, but also causes loss of unbound cytoplasmic molecules such as non-activated $cPLA_2$ when cells are subsequently washed. Hence, no immunofluorescent staining for cytoplasmic $cPLA_2$ is detectable in unstimulated cells. However, in activated cells, staining occurs on the translocated fraction of $cPLA_2$ that has bound to the cell membrane or the nuclear envelope and thus, remains intracellular. Thus residual fluorescence is specific for activated, membrane-bound $cPLA_2$. Endothelial nuclei were counterstained *in situ* by HOECHST 33342 (5 μg/mL) (E11). Endothelial FITC (excitation: λ=480 nm; emission: λ≥530 nm) and

HOECHST (excitation: λ=350; emission: λ≥460) fluorescence in pulmonary arterioles of the isolated perfused lung were visualized and recorded as described above. Cultured PAECs were mounted with aqueous mounting medium containing anti-fade, and FITC and HOECHST fluorescence were imaged at appropriate wavelengths by confocal microscopy (Bio-Rad, Bio-Rad Laboratories Inc., Berkeley, CA).

EETs concentration in isolated perfused lungs. To determine the effects of hypoxia on pulmonary EETs generation, isolated mouse lungs were perfused as described above, and ventilated with normoxic (21% O_2) or hypoxic (1% O_2) gas for 10 min. Lungs were immediately removed from the chest, snap frozen and homogenized in liquid nitrogen. Samples were extracted twice into ethyl acetate, evaporated under nitrogen, and resuspended in methanol/water (vol. 1.1). The concentrations of 8,9-EET, 11,12-EET and 14,15-EET were determined with a Sciex API4000 mass spectrometer (AME Bioscience, Toroed, Norway) operating in multiple reaction monitoring (MRM) mode as described (E13), and expressed relative to lung weight.

Western Blot analyses. For protein analyses from fresh lung endothelial cells (FLEC), endothelial cells were separated by a magnetic bead immunosorting technique yielding an endothelial cell fraction of > 97% as determined by FACS analysis of CD31 positive cells and a cell viability of > 96% as previously described (E14). Rat pulmonary artery smooth muscle cells (PASMCs) and human umbilical vein endothelial cells (HUVECs) were generously provided by Dr. B. Fuchs (University of Giessen Lung Center, Germany) and Dr. A. Zakrzewicz (Charité – Universitätsmedizin Berlin, Germany), respectively. Aliquots from freshly isolated or cultured cells, or from whole lung homogenate were homogenized in phosphate buffered saline (PBS) containing protease inhibitor mixture (Complete Mini; Roche Diagnostics GmbH, Mannheim, Germany), 1 mMol/L phenylmethansulfonyl fluoride, and 1% Triton-100. Total protein concentration was determined by Bradford Protein Assay (Bio-Rad, Munich, Germany). Sample proteins (50 µg/slot) and a pre-stained protein-weight marker (Bio-Rad) were size-fractionated

by SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany). Membranes were blocked, washed, and incubated with matching primary antibodies for $K_v1.5$, $K_v2.1$, Cx43, or Ser-368 phospho-Cx43, respectively, and appropriate secondary antibodies. Protein bands were visualized by enhanced chemiluminescence (ECL; Perkin Elmer GmbH, Freiburg, Germany).

Immunofluorescence histology. For immunofluorescence staining for Cx40, Cx43 and von Willebrand factor (vWF), which served as endothelial marker, mouse lungs were isolated, inflated by a mixture of PBS and optimal cutting temperature compound (Tissue-Tek® O.C.T.™; Somagen Diagnostic, Edmonton, AB), embedded into a cryomold (Tissue-Tek, Somagen Diagnostic) and snap frozen in liquid nitrogen. 5 µm thick sections were cut. The sections were fixed in acetone for 10 min in -20°C, incubated in 0.3% hydrogen peroxide in methanol for 30 min to block endogenous hydrogen peroxidase, and washed by PBS. After preincubation in Dako protein block serum for 30 min, the sections were incubated overnight at 4°C with rabbit anti-von Willebrand factor (vWF) antibody (1:400), goat anti-Cx40 antibody (1:100), or goat anti-Cx43 antibody (1:100) respectively. After washing three times with PBS for 5 min, the sections were incubated at room temperature for 60 min with specific secondary biotin-conjugated horse anti-goat IgG, (1:200), Alexa 594-Streptavin (1:1000), or donkey FITC-conjugated anti-rabbit IgG (1:100) antibodies, followed by triple washing with PBS. Sections were counterstained by incubation with the nuclear dye Hoechst 33258 (1:5000) for 10 min, washed with PBS, mounted with aqueous mounting medium containing anti-fade, and imaged by confocal microscopy (Bio-Rad, Bio-Rad Laboratories, Inc, Berkeley, CA).

Statistical analyses. Statistical analysis was performed by use of SigmaStat software (SigmaStat 3.0; Systat Software GmbH, Erkrath, Germany). Data are presented as means±SEMs. Statistical significance was determined by two-tailed Student *t*-test or one-way ANOVA as appropriate.

Non-linear regression analyses were performed by use of SigmaPlot software (SigmaPlot 9.0; Systat Software GmbH). Statistical significance was accepted at $P < 0.05$.

Study approval. The study was approved by the Institutional Animal Care and Use Committee of St. Michael's Hospital, Toronto, ON. All experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, 7th edition 1996).

References

E1. Urban N, Hill K, Wang L, Kuebler WM, Schaefer M. Novel pharmacological TRPC inhibitors block hypoxia-induced vasoconstriction. *Cell Calcium*. 2012;51:194- 206.

E2. Spöhr F, Busch CJ, Reich C, Motsch J, Gebhard MM, Kuebler WM, Bloch KD, Weimann J. 4-Aminopyridine restores impaired hypoxic pulmonary vasoconstriction in endotoxemic mice. *Anesthesiology*. 2007;107:597-604.

E3. Linehan JH, Haworth ST, Nelin LD, Krenz GS, Dawson CA. A simple distensible vessel model for interpreting pulmonary vascular pressure-flow curves. *J Appl Physiol*. 1992;73:987-994.

E4. Weissmann N, Dietrich A, Fuchs B, Kalwa H, Ay M, Dumitrascu R, Olschewski A, Storch U, Schnitzler M, Ghofrani HA *et al*. Classical transient receptor potential channel 6 (TRPC6) is essential for hypoxic pulmonary vasoconstriction and alveolar gas exchange. *Proc Natl Acad Sci USA*. 2006;103:19093-19098.

E5. Glenny RW, Bernard S, Brinkley M. Validation of fluorescent-labeled microspheres for measurement of regional organ perfusion. *J Appl Physiol*. 1993;74: 2585-2597.

E6. Tabuchi A, Mertens M, Kuppe H, Pries AR, Kuebler WM. Intravital microscopy of the murine pulmonary microcirculation. *J Appl Physiol*. 2008;104:338-346.

E7. Hoffmann J, Yin J, Kukucka M, Yin N, Saarikko I, Sterner-Kock A, Fujii H, Leong-Poi H, Kuppe H, Schermuly RT *et al*. Mast cells promote lung vascular remodelling in pulmonary hypertension. *Eur Respir J*. 2011;37:1400-1410.

E8. Kuebler WM, Parthasarathi K, Lindert J, Bhattacharya J. Real-time lung microscopy. *J Appl Physiol*. 2007;102:1255-1264.

E9. Song C, Al-Mehdi AB, Fisher AB. An immediate endothelial cell signaling response to lung ischemia. *Am J Physiol Lung Cell Mol Physiol*. 2001;281:L993- 1000.

E10. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly

improved fluorescence properties. *J Biol Chem*. 1985;260:3440-3450.

E11. Kuebler WM, Parthasarathi K, Wang PM, Bhattacharya J. A novel signaling mechanism between gas and blood compartments of the lung. *J Clin Invest*. 2000;105: 905-913.

E12. Schievella AR, Regier MK, Smith WL, Lin LL. Calcium-mediated translocation of cytosolic phospholipase A_2 to the nuclear envelope and endoplasmic reticulum. *J Biol Chem*. 1995;270:30749-30754.

E13. Michaelis UR, Fisslthaler B, Barbosa-Sicard E, Falck JR, Fleming I, Busse R. Cytochrome P450 epoxygenases 2C8 and 2C9 are implicated in hypoxia-induced endothelial cell migration and angiogenesis. *J Cell Sci*. 2005;118:5489-5498.

E14. Kerem A, Yin J, Kaestle SM, Hoffmann J, Schoene AM, Singh B, Kuppe H, Borst MM, Kuebler WM. Lung endothelial dysfunction in congestive heart failure: role of impaired Ca^{2+} signaling and cytoskeletal reorganization. *Circ Res*. 2010;106: 1103-1116.