Online Methods Supplement

Altered Lipid Domains Facilitate Enhanced Pulmonary Vasoconstriction Following Chronic Hypoxia

Charles E. Norton, Laura Weise-Cross, Rosstin Ahmadian, Simin Yan, Nikki L. Jernigan, Michael L. Paffett, Jay S. Naik, Benjimen R. Walker, and Thomas C. Resta

METHODS

Animals and Chronic Hypoxia (CH) exposure

All protocols and procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center (Albuquerque, NM). Male Sprague-Dawley rats (250-350g, age 3-4 months; Harlan Industries, Indianapolis, IN) were used for all experiments. Rats were housed in a hypobaric chamber for four weeks with barometric pressure at ~380 mmHg. Age matched control rats were housed in similar cages at ambient atmospheric pressure (~630 mmHg; Albuquerque, NM).

Smooth Muscle Cell Caveolae Number

Lungs were exposed by sternotomy in anesthetized rats. Heparin (100 U) was injected into the right ventricle, and the pulmonary artery was cannulated with a 13-gauge needle stub. The lungs were perfused at a pressure of 12 mmHg with 100 ml of PSS with 4% (wt/vol) albumin, 10^{-4} M papaverine, and 1,000 U of heparin. 100 ml of fixative (PBS containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 10^{-4} M papaverine; Sigma) was then perfused through the lungs. Samples of lung tissue were then fixed for transmission electron microscopy with 3% formaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed in reduced osmium tetroxide (1% OsO_4 and 0.5% potassium ferrocyanide), en bloc, stained with 1% uranyl acetate, dehydrated, and embedded in epoxy resin.

Caveolae were identified as 50-100 nm diameter invaginations into the cell membrane. Caveolae numbers were counted on smooth muscle cells in arterial sections (~150 um) and divided by the membrane length using Image J software (National Institutes of Health; Bethesda, MD, USA). A total of 34 images encompassing 254 μm of membrane were analyzed from arteries of 3

control rats, whereas 25 images encompassing 113 μm of membrane were analyzed from 3 CH animals.

Isolation of Pulmonary Arteries

Lungs were removed from rats anesthetized with pentobarbital sodium (200 mg/kg, i.p.) and a dissected pulmonary artery [~150 μm inner diameter (ID)] was cannulated in a vessel chamber (CH-1; Living Systems, St. Albans, VT, USA). All arteries were studied at 37°C and a transmural pressure of 12 Torr, as we have previously demonstrated that reactivity to KCl following CH is similarly augmented at 12 or 35 mmHg (1). All isolated arteries were studied under normoxic conditions for the pulmonary arterial circulation [physiological saline solution (PSS) containing (in mM) 129.8 NaCl, 5.4 KCl, 0.5 NaH_2PO_4, 0.83 $MgSO_4$, 19 $NaHCO_3$, 1.8 $CaCl_2$, and 5.5 glucose (all from Sigma-Aldrich, St. Louis, MO, USA) and equilibrated with a 10% O_2, 6% CO_2, and balance N_2 gas mixture]. Arteries were endothelium-disrupted by gently rubbing a single strand of moose mane through the lumen of the vessel to directly evaluate effects of CH on vascular smooth muscle (VSM) reactivity to KCl independent of endothelial influences, and disruption of endothelial cells was confirmed by lack of response to acetylcholine (ACh, 1 μM; Sigma-Aldrich) following uridine triphosphate (UTP, 5 μM; Sigma-Aldrich) constriction (2, 3).

Quantification of Membrane Cholesterol Content

Confocal microscopy was used to quantify VSM cholesterol content in isolated pulmonary arteries (described above) under control conditions and following cholesterol depletion with MβCD (10 mM) or cholesterol supplementation with a solution of cholesterol and MβCD (cholesterol: MβCD) at a molar ratio of 1:5 for 45 min at 37°C as previously described (5).

Pulmonary arteries were subsequently fixed in 2% paraformaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature followed by incubation with the fluorescent cholesterol marker filipin [50 μg/ml, Sigma (6)] for 90 min at room temperature in darkness. Filipin is a polyene antibiotic that binds to membrane cholesterol via hydrophobic interactions (10) and is inherently fluorescent. After two 5 min washes in PBS, arteries were mounted on slides with ProLong™ Gold Antifade (Invitrogen; Carlsbad, CA, USA) and slides were stored at -20°C until imaging. Imaging data were acquired on a line scanning confocal microscope (Zeiss LSM 510 AxioObserver; Göttingen, Germany) using 405 nm laser (excitation), a 420 nm long pass filter (emission), and a Plan-Neofluar 40X/1.3 NA oil immersion objective. Filipin staining from was quantified using NIH Image J. Mean fluorescence intensity was quantified from background subtracted images.

Effects of AP-Cav on membrane cholesterol: We additionally examined effects of the caveolin-1 mimetic peptide, AP-Cav, on membrane cholesterol levels by incubating arteries for 40 min with AP-Cav (10 μM; Sigma, also known as Cavtratin) or its scrambled control peptide (10 μM; AP-Scramb) and filipin (as described above). Following fixation, these vessels were treated with Sytox Green (1:10,000) for 15 min for identification of cell nuclei, then washed 3 × 5 min in PBS and mounted on slides with Fluoro-Gel mounting medium [Electron Microscopy Sciences, Hatfield, PA, USA]. Images were acquired using a HC PL APO CS2 63x/1.4 oil objective on a Leica confocal microscope (TCS SP8, Wetzlar, Germany) using a 405 nm laser (excitation) and recording emissions between 438 and 470 nm. Filipin staining was quantified using LAS X (Leica), and mean fluorescence intensity per vessel was calculated using the fluorescence intensity taken from 4 regions of interest.

Vasoreactivity and Vessel Wall $[Ca^{2+}]_i$

For intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurement, vessels were loaded with fura-2 AM for 45 min in darkness followed by a 20 min rinse with PSS (2, 3). Background-subtracted fura-2 F_{340}/F_{380} emission (510 nm) ratios were calculated with IonOptix Ion Wizard software (Milford, MA, USA) and recorded continuously throughout the experiment to measure vessel wall $[Ca^{2+}]_i$, with simultaneous measurement of ID from red wavelength bright-field images. To directly address mechanisms of myofilament Ca^{2+} sensitization independent of changes in vessel wall $[Ca^{2+}]_i$, we clamped vessel wall $[Ca^{2+}]_i$ by permeabilizing arteries with the Ca^{2+} ionophore, ionomycin (3 μM, Sigma) (3). Ca^{2+} permeabilized vessels were equilibrated with PSS containing a calculated free Ca^{2+} concentration of 300 nM. This concentration of Ca^{2+} was chosen to provide optimal vasoreactivity to KCl while having minimal effects on resting tone based on preliminary studies. $[Ca^{2+}]_i$ concentration is expressed as fura 2 F_{340}/F_{380} ratios.

Vasoconstrictor responses (% change in inner diameter) to increasing concentrations of KCl (30, 60, and 120 mM) were assessed in Ca^{2+} permeabilized arteries from CH and control rats in the presence of the cav-1 scaffolding peptide, AP-Cav (10 μM; 21st Century Biochemicals, Marlborough, MA, USA), its scrambled control peptide (AP-Scramb), cholesterol depletion [10 mM methyl-β-cyclodextrin (MβCD); Sigma], cholesterol repletion [1:5 ratio of cholesterol (Sigma) to MβCD (4)], and vehicle conditions. Preparation of cholesterol solutions was performed as previously described in our laboratory (5, 6). During these experiments, fura-2 ratios were monitored to confirm Ca^{2+} clamp. In non-Ca^{2+} permeabilized arteries, we measured vasoconstriction and vessel wall $[Ca^{2+}]_i$ responses to epidermal growth factor (EGF).

Role of caveolin-1 (Cav-1) and Cholesterol in Arterial O_2^- Production

For detection of O_2^-, arteries were prepared as described above and imaged on a Nikon Diaphot microscope. Fluorescence detection of dihydroethidium (DHE; 10 μM; Molecular Probes, Eugene, OR) oxidation was used as a measure of O_2^- levels in pressurized arteries from CH and control rats as described previously (1, 3). After establishing a baseline value, 60 m*M* KCl was administered and DHE fluorescence was measured over 14 minutes (1 image/ minute) until the response plateaued. Responses were measured in the presence of AP-Cav, MβCD, cholesterol repletion, or their respective vehicles as described above.

Western Blot Analysis

Caveolin-1 expression: Cav-1 expression was compared between intrapulmonary arteries of CH and control rats by western blotting as previously described (1). Cav-1 levels were measured with a cav-1 antibody [1:1000; 610406 , BD Biosciences, San Jose, CA, USA (7, 8)] followed by detection with an Alexa Fluor anti-mouse secondary antibody. Blots were imaged on an Odyssey fluorescence imaging system (LI-COR; Lincoln, NE, USA). Bands were quantified with ImageJ and Cav-1 expression was normalized to that of β-actin.

EGFR activation and expression: Intrapulmonary arteries from CH and control rats were isolated and incubated with EGF (100 nM; Sigma) or vehicle for 30 min at 37ºC. For some experiments, arteries were additionally treated with supplemental cholesterol (500 μM in MβCD), AP-Cav (10 μM) or AP-Scramb (10 μM). Vessels were then snap-frozen until tissue homogenization. Following homogenization, protein concentration was determined with a fluorometer (Qubit 4, Thermo Scientific; Waltham, MA, USA). Samples were loaded onto SDS-PAGE gels (Bio-Rad), transferred onto polyvinylidene difluoride membranes, and blocked in tris-buffered saline with 0.1% Tween-20 with 5% nonfat milk (Carnation) at room temperature for 1

hour. Blots were probed with primary antibodies with 0.1% milk at 4ºC overnight, and a horseradish peroxidase conjugated secondary antibody (1:3,000, Bio-Rad) for 1 hr at room temperature the next day. The following primary antibodies were used: anti-EGFR [1:500, Cell Signaling #2232, Lot 16], anti-phospho EGFR Y1068 [1:500, Cell Signaling #3777, Lot 13] (7, 9), and anti--actin (1:14,000, Abcam #ab8227, Lot GR3215936-1). Phospho-EGFR blots were stripped and reprobed for total EGFR where indicated. Bands were developed with enhanced chemiluminescence Western blotting substrate (Thermo Scientific) and densitometry quantification was performed using ImageJ. Blots were also stained with Coomassie to ensure equal protein loading.

Calculations and Statistics

Vasoconstrictor responses were calculated as a percent change in diameter from baseline ID. Data are expressed as means ± standard error, and n refers to the number of animals in each group, or number of images for analysis of caveolar number. A *t*-test, one-way ANOVA, two-way ANOVA, or one-way repeated measures ANOVA was used to make comparisons when appropriate. If differences were detected by ANOVA, individual groups were compared using the Student-Newman-Keuls or Bonferroni test. A probability of $P<0.05$ was considered significant for all comparisons.

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