Online Methods Supplement

Augmented pulmonary vasoconstrictor reactivity following chronic hypoxia requires Src kinase and EGFR signaling

Charles E. Norton, Joshua R. Sheak, Simin Yan, Laura Weise-Cross, Nikki L. Jernigan, Benjimen R. Walker, and Thomas C. Resta

METHODS

Experimental Groups

Male Sprague-Dawley rats (body weight 250-350 g, age 3-4 months; Harlan Industries, Indianapolis, IN, USA) were exposed to 4 weeks chronic hypoxia (CH) by placement in a hypobaric chamber maintained at ~380 mmHg (inspired PO_2 ~70 mmHg) (1). The hypobaric chamber was opened three times per week to provide fresh food, water and clean bedding. Age-matched control rats were housed in similar cages under ambient pressure (~630 mmHg in Albuquerque, NM). A subset of animals was treated daily with the epidermal growth factor receptor (EGFR) antagonist gefitinib or vehicle (delivered in edible pills as detailed below: *Contribution of EGFR to Chronic Hypoxia-dependent Pulmonary Hypertension*). The hypobaric chamber was opened once per day to administer pills. All animals were maintained on a 12:12 hour light-dark cycle. All protocols were reviewed and approved by the Animal Care and Use Committee (University of New Mexico Health Sciences Center).

Isolated Pulmonary Artery Preparation: Assessment of Vasoreactivity and Vessel Wall $[Ca^{2+}]_i$

Endothelial Disruption, Cannulation and Pressurization of Small Pulmonary Arteries for Dimensional Analysis. Following CH or control exposure, rats were euthanized with an intraperitoneal injection of pentobarbital sodium (200 mg/kg) and the heart and lungs were exposed by thoracotomy. Both organs were excised and placed in ice cold 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)]. Lungs were pinned onto a dish with silicone rubber (Sylgard 184; Dow Corning, Midland, MI, USA) and small

intrapulmonary arteries (~150 μm, 4th or 5th order) without side branches were dissected from the surrounding tissue from the superior region of the upper left lobe of the lung. Isolated arteries were placed in a tissue chamber (CH-1; Living Systems, St. Albans, VT, USA), cannulated onto tapered glass micropipettes and tied in place with a strand of silk suture. These microcannulae were created from borosilicate glass tubes (Sutter Instrument, OD 1.2 mm, ID 0.69 mm) that were tapered to an approximate OD of 100 μm using a Sutter Instrument P-30 Vertical Micropipette Puller. After cannulation of the proximal end, the artery was gently flushed with PSS to remove blood from the lumen and a strand of moose mane was inserted into the distal end of the artery to disrupt the endothelium, allowing direct evaluation of effects of CH on vascular smooth muscle reactivity independent of endothelial influences. Following cannulation of the second end of the artery, the vessel was stretched longitudinally to approximate *in situ* length and pressurized with a servo-controlled peristaltic pump (Living Systems) to 12 mmHg. Arteries were studied under no-flow conditions. The absence of vessel leakage was confirmed by turning off the servo-control function and ensuring the vessel maintained pressure. Vessels with apparent leaks were discarded.

Isolated, cannulated pressurized vessels were transferred to the stage of a inverted Nikon Eclipse TS100 microscope for simultaneous measurement of changes in vessel diameter and vessel wall $[Ca^{2+}]_i$. This preparation allows for assessment of vascular smooth muscle function independent of circulating factors, innervation, or shear stress. Arteries were continuously superfused with PSS equilibrated with a gas mixture containing 10% O_2, 6% CO_2, and balance N_2 (37°C). A vessel chamber cover was positioned to permit this same gas mixture to flow over the top of the chamber bath. An Imaging Source camera was used to obtain red wavelength bright-field images of the vessels and dimensional analysis was performed by Ion Wizard software (Ionoptix, Milford, MA, USA) to measure inner diameter by edge detection. Endothelial disruption

was confirmed by lack of response to acetylcholine (1 μM; Sigma-Aldrich) following a uridine triphosphate (5 μM; Sigma-Aldrich) constriction (2, 3).

Arteries constricted with endothelin-1 (ET-1) (0.1-100 nM) or epidermal growth factor (EGF; 0.1-1,000 nM) were studied at a transmural pressure of 12 mmHg using a servo-controlled peristaltic pump (Living Systems). For pressure-dependent basal tone protocols, vessel inner diameter was measured in 10 mmHg pressure increments beginning at 5 mmHg and ending at 45 mmHg, with each pressure step lasting 5 min. Passive inner diameter was determined by repeating the pressure steps following one hour superfusion with Ca^{2+}-free PSS [containing (in mM): 129.8 NaCl, 5.4 KCl, 0.83 $MgSO_4$, 19 $NaHCO_3$, 5.5 glucose, 3 EGTA] and after flushing the lumen with Ca^{2+}-free PSS, followed by reestablishment of no-flow conditions. Basal arterial constriction was calculated as the difference in internal diameter between Ca^{2+}-free and Ca^{2+}-replete conditions, expressed as a percentage of inner diameter in Ca^{2+}-free PSS at each pressure, as previously described (1).

Measurement of Vessel Wall $[Ca^{2+}]_i$. Following verification of endothelial disruption and a rinse period, arteries were loaded abluminally with the ratiometric, Ca^{2+} sensitive, fluorescent indicator fura-2 AM (Molecular Probes/Life Technologies; Carlsbad, CA, USA). Prior to loading, fura-2 AM (1 mM in anhydrous DMSO) was mixed 2:1 with a 20% solution of pluronic acid (Invitrogen; Carlsbad, CA, USA). This mixture was diluted in PSS, resulting in a final concentration of 2 μM fura-2 AM and 0.05% pluronic acid. Arteries were incubated in this solution for 45 minutes at room temperature while being equilibrated with a 10% O_2 gas mixture. Subsequently, vessels were rinsed for 20 minutes with aerated PSS (37°C) to wash out excess dye and facilitate hydrolysis of AM groups by intracellular esterases. Fura-2-loaded vessels excited alternatively at 340 and 380 nm with an IonOptix Hyperswitch dual excitation light source, and

the respective 510 nm emissions were collected by a photomultiplier tube. Background-subtracted fura-2 F_{340}/F_{380} emission ratios were calculated with IonOptix Ion Wizard software and recorded continuously throughout the experiment to measure vessel wall $[Ca^{2+}]_i$, simultaneously with diameter.

Vascular Smooth Muscle Ca^{2+}– Permeabilization. To directly assess mechanisms of myofilament Ca^{2+} sensitization in ET-1 treated arteries independent of changes in vessel wall $[Ca^{2+}]_i$, we clamped $[Ca^{2+}]_i$ by permeabilizing with the Ca^{2+} ionophore, ionomycin (3 μM, Sigma-Aldrich). Vessels were initially equilibrated in Ca^{2+} free PSS containing 3 mM EGTA (Sigma-Aldrich), and then the Ca^{2+} ionophore, ionomycin was added to permeabilize the smooth muscle to Ca^{2+}. Arteries were next equilibrated with PSS containing a calculated free Ca^{2+} concentration of 300 nM [containing (in mmol/L) 129.8 NaCl, 5.4 KCl, 0.5 NaH_2PO_4, 1.3 $MgSO_4$, 19 $NaHCO_3$, 6.8 $CaCl_2$, 5.5 glucose, 8.2 EGTA, 0.003 ionomycin (all from Sigma-Aldrich)]. This Ca^{2+} concentration was calculated using the K_d of EGTA for Ca^{2+} of 43.7 nmol/L and the K_d of EGTA for Mg^{2+} of 3.33 mM at 37°C and pH 7.4 (3), and we have previously demonstrated that 300 nM extracellular Ca^{2+} in Ca^{2+}-permeabilized arteries optimizes vasoconstrictor responsiveness to endothelin-1 with minimal effects on basal tone.

Isolated Pulmonary Artery Protocols

Role of O_2^-, H_2O_2, NADPH oxidase, Rac1, EGFR, matrix metalloproteases (MMPs), and Src Kinases in Pressure-Dependent Tone and ET-1-dependent Vascular Smooth Muscle (VSM) Ca^{2+} Sensitization. Small endothelium-disrupted pulmonary arteries were prepared for assessment of vasoconstriction and vessel wall $[Ca^{2+}]_i$ as described above. Basal tone was assessed in non-permeabilized arteries from CH and control rats in the presence of polyethylene glycol-superoxide dismutase (120 U/ mL; Sigma-Aldrich, St. Louis, MO, USA) (4), polyethylene glycol-catalase

(250 U/ mL, Sigma) (5), or their vehicle controls. Vasoconstrictor responses to increasing concentrations of ET-1 (10^{-10}-10^{-7} M, Sigma) in Ca^{2+} permeabilized arteries and basal tone were examined in the presence of the NADPH oxidase inhibitor apocynin (30 μM) (6) the specific NADPH oxidase 2 inhibitor gp91ds-tat [also known as Nox2ds-tat (50 μM; Tufts, Boston, MA, USA)] or scrambled peptide (negative control; 50 μM; Tufts, Boston, MA, USA) (7), the Rac1 inhibitor NSC 23766 [50 μM; Cayman, Ann Arbor, MI, USA (chosen for its selectivity for Rac1 over RhoA)] (8), or their respective vehicles. Responses were also measured in the presence of the selective EGFR inhibitors AG 1478 (1 μM; Cayman Chemical, Ann Arbor, MI, USA) (9) and gefitinib (50 μM, Cayman) (10), the Src family kinase inhibitor SU6656 (10 μM, Cayman) (9, 11) the general MMP inhibitor GM6001 (15 μM; EMD Millipore; Billerica, MA, USA) (12) and inhibitors specific for ADAM-17 (TAPI-1, 10 μM; Santa Cruz, Dallas, TX, USA) (13), MMP 2 (MMP 2 inhibitor 3, 100nM, Millipore) (14), and MMP 9 (MMP 9 inhibitor 2, 10 μM, Millipore) (15) or appropriate vehicles. Effects of AG 1478 and SU6656 on ROK activity were assessed during vasoconstriction to the ROK activator, sphingosylphosphorylcholine (SPC, 1 μM) (16) in Ca^{2+} permeabilized arteries from control rats. Vessels were incubated with SPC for 20 min until arterial diameter stabilized. Fura-2 ratios were monitored throughout all experiments to monitor $[Ca^{2+}]_i$.

EGF-dependent Vasoconstriction. Vasoconstriction and vessel wall Ca^{2+} responses were measured in nonpermeabilized pulmonary arteries from CH and control rats stimulated with increasing concentrations of EGF. To confirm that the observed effects of EGF were mediated by EGFR, experiments were repeated in the presence of the EGFR inhibitor AG 1478. The contribution of Rho kinase and NADPH oxidase to EGF-induced vasoconstriction were determined with HA-1077 (10 μM, Sigma) (17) and gp91ds-tat respectively. The use of these

inhibitors has been extensively characterized by our laboratory and others (17-19). The selectivity of HA-1077 for ROK in this preparation is indicated by evidence that 10 μM HA-1077 blocks arterial constriction to the ROK agonist, SPC (Fig. 11), but is without effect on vasoconstrictor reactivity to either KCl (2) or ET-1 (20) in pulmonary arteries from control rats. Furthermore, HA-1077 at this concentration does not alter basal vessel wall Ca^{2+}, vasoconstriction to the PKC agonist, PMA (18), or VSM membrane potential in this preparation (2). The NOX inhibitory peptide gp91ds-tat is selective for the NOX 2 isoform over other vascular NADPH oxidases (19). The selectivity of gp91ds-tat is further supported by effects to prevent KCl-induced constriction or superoxide generation in pulmonary arteries from CH rats, but not in vessels from control animals (21). The Src kinase inhibitor SU6656 (9, 11) was also used to evaluate the role of Src kinases in EGF-dependent vasoconstriction.

Role of EGFR in ET-1-dependent O_2^- Production in Pulmonary Arterial Smooth Muscle Cells.

Pulmonary arterial smooth muscle cells were isolated from 2^{nd}-5^{th} order arteries from CH and control rats. Arteries were treated with papain (9.5 U/mL, Sigma), collagenase (1750 U/mL, Sigma), and dithiolthreitol (1 mM, Sigma) in reduced Ca^{2+} HBSS at 37°C for 30 min (22). Following cell disruption with fire polished glass Pasteur pipette tips, the cell suspension was placed on 25 mm glass cover slips and cultured for 3-4 days in Ham's F-12 media with 5% fetal bovine serum and 1% penicillin streptomycin in a humidified incubator set at a 5% CO_2-95% air balance at 37°C. Following treatment with ET-1 (10 nM), ET-1 + AG 1478, or standard PSS for 1 hour, cells were stained with dihyroethidium (DHE, 5 μM in 0.05% pluronic acid, Molecular Probes] and the nuclear stain TO-PRO®-3 (1:2,000; Molecular Probes) for 15 minutes at 37 °C and subsequently fixed in 2% paraformaldehyde. Fluorescent images were acquired with a 63X

objective on a Leica confocal microscope (SP5; Leica Microsystems; Buffalo Grove, IL, USA). Mean fluorescence intensity was averaged from 5 images/sample. Each image was thresholded using Image J (NIH, Bethesda, MD, USA) to select for positively stained areas above background (cells not treated with DHE). Cellular purity was >90%, as assessed by morphological appearance and immunofluorescence of anti-smooth muscle 22 alpha (SM-22α). We have previously demonstrated the specificity of DHE for O_2^- vs. H_2O_2 using this preparation (20, 22).

Contribution of EGFR to Chronic Hypoxia-dependent Pulmonary Hypertension

CH and control animals were treated daily for 4 weeks with pills containing the EGFR inhibitor gefitinib (30 mg/kg/day) (10) or vehicle pills (BioServ dough with 10% peanut butter). We then measured right ventricular systolic pressure (RVSP), right ventricular hypertrophy, vascular remodeling and hematocrit in these animals as previously described (23-25). Peak RVSP was measured as an index of pulmonary arterial pressure in anesthetized rats (2% isoflurane, 98% O_2). Following an upper transverse laparotomy, a 25 gauge needle, connected to a pressure transducer (P23 XL; Spectramed, Oxnard, CA, USA), was inserted through the diaphragm into the right ventricle, and the output amplified by an amplifier (Harvard Instruments, Holliston, MA, USA). Data were recorded, and heart rate was calculated with a computer based data acquisition system (AT-CODAS; DATAQ Instruments, Akron, OH, USA). After isolation of the heart, the atria and other surrounding tissue were removed from the ventricles. Sections of the heart were weighed and right ventricular hypertrophy was assessed as right ventricle to left ventricle plus septum weight (RV/LV+S; also known as Fulton's index) and right ventricle to total ventricular weight. Hematocrit (% red blood cells) was measured in blood samples collected in glass

microcapillary tubes after direct cardiac puncture at the time of lung isolation as an index of polycythemia.

Assessment of Arterial Remodeling and Muscularization. A median sternotomy was performed in anesthetized rats and heparin (100 U in 0.1 mL) was injected directly into the right ventricle. The pulmonary artery was cannulated with a 13 gauge needle stub and perfused with 250 ml of PSS containing 4% bovine serum albumin (BSA) and 100 μM papaverine with a microprocessor pump (model 7524-10; Masterflex, Vernon Hills, IL, USA) to maximally dilate the vasculature and clear the circulation of blood. The perfusion rate was gradually increased to 60 ml/(minute x kg body weight). Perfusate was pumped through a water-jacketed bubble trap maintained at 38°C before entering the pulmonary circulation. Experiments were performed with lungs in zone three conditions, achieved by elevating the perfusate reservoir until venous pressure was ~12 mmHg. Previous work from our laboratory suggests that maximal recruitment and thus maximal vascular surface area is achieved at this flow and venous pressure (26). The trachea was cannulated with a 17 gauge needle stub and following removal of excess air, airways were filled with fixative (0.1 M phosphate buffered saline containing 4% paraformaldehyde, 0.1% glutaraldehyde and 100 μM papaverine; pressure 25 cm H_2O). 250 ml of fixative was next perfused through the pulmonary vasculature. Lungs were immersed in fixative overnight, dehydrated with increasing concentration of ethanol, and then mounted in paraffin. 4-μm sections were cut and mounted onto Superfrost Plus slides (Fisher Scientific, Hampton, NH, USA). To measure the number of fully and partially muscularized arteries, sections were incubated with a rabbit anti-smooth muscle α-actin antibody (1:200, Abcam, Cambridge, MA, USA) overnight at 4°C. Smooth muscle α-actin was detected by a donkey anti-rabbit Alexa fluor 549 antibody (1:100, incubated for 3.5 hours; Jackson Research, West Grove, PA, USA). Sections were mounted with FluoroGel

(Electron Microscopy Sciences). Images were acquired using a 20X objective on a SP5 Leica confocal microscope. Twenty images from the right and left lobes were randomly collected per animal. Fluorescence images were digitally inverted using ImageJ to facilitate improved contrast and visibility of immunofluorescence. Numbers of fully and partially muscularized arteries were counted per animal as described previously (23). Measurements were made using a blinded analysis.

In experiments to assess arterial wall thickness, lung sections were stained for elastin (Sigma Accustain Elastin Stain kit), and arteries were identified by the presence of an internal elastic lamina (24, 25). Vessels were examined with a 40x objective on a Nikon Optiphot microscope, and images were acquired with a digital charge-coupled camera (Photometrics CoolSNAP; Tucson, AZ, USA) and processed with MetaMorph software (Universal Imaging, Bedford Hill, NY, USA). Measurements were performed on 16-51 arteries (<50 μm outer diameter) and 4-25 arteries (50-100 μm outer diameter) per rat from five rats/group. These measurements were made using a blinded analysis. External and internal arterial diameters were calculated from the medial the medial and luminal circumferences, respectively. Arterial wall thickness was assessed by subtracting luminal radius from external radius and expressed as a percentage of external diameter according to the following formula: [(2X wall thickness)/external diameter] x 100 as previously described (24, 25).

Western Blotting

For all western blot experiments, arteries were homogenized as previously described (2). To obtain sufficient tissue, collected 2nd to 5th order pulmonary arteries (endothelium intact) were homogenized in a buffer solution [255 mM sucrose, 10 mM Tris HCl, 2 mM EDTA, 12 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin (Sigma)] and then centrifuged at 10,000 x g for

10 min at 4°C to remove insoluble debris. The supernatant was collected and protein concentration were determined by the Bradford method (Bio-Rad protein assay; Bio-Rad, Hercules, CA, USA). Pulmonary arterial lysates (30 μg of protein per lane) was separated on a SDS-Page (10% Tris/glycine; Bio-Rad) and transferred to polyvinylidenedifluoride membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline [with 0.05% Tween 20 (Bio-Rad)] for 1 hour at room temperature, then the membrane was probed with primary antibodies in Tris buffered saline containing 5% nonfat milk overnight at 4°C. After three washes in Tris buffered saline, protein-loaded membranes were incubated with Alexa-Fluor secondary antibodies (1:400) in Tris buffered saline with 5% nonfat milk for 1 hour, then washed three times in Tris buffered saline. Membranes were imaged on an Odyssey fluorescent imaging system (LI-COR; Lincoln, NE, USA). Bands were quantified by densitometry with ImageJ software (NIH).

Src Kinase Activation. Intrapulmonary arteries from CH and control rats were isolated and permeabilized to Ca^{2+} with ionomycin (37ºC; 30 min) to simulate vasoreactivity protocols. Following treatment with ET-1 (10 nM; 30 min) or vehicle, vessels were homogenized and a polyclonal anti-phospho Src kinase antibody (1:1,000; 2101S, Cell Signaling, Beverly, MA) was used to detect Src phosphorylated on the tyrosine 416 residue (27). Phosphorylated Src levels were normalized to total Src (1:1,000; 2108S, Cell Signaling) for comparison. Total Src kinase was normalized to β-actin (1:5,000; A1978, Sigma). Similar results were obtained when data were normalized to total protein as assessed by Coomassie staining (not shown).

MMP Expression. Untreated arterial homogenates were blotted and probed for MMP 2 (1:500; ab512125, Abcam) (28), MMP 9 (1:2,500; ab76003, Abcam) (28), and ADAM-17 (1:1,000; ab2051, Abcam) (29) to determine effects of CH on expression of these MMPs. Blots were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat IgG

(1:5,000; Bio-Rad) in TBS following chemiluminescence labeling (Amersham, ECL), then detected by exposure to film (Kodak). Expression levels were normalized to β-actin (1:5,000; ab8227, Abcam) on the same blot, and similar results were obtained when data were normalized to total protein as determined by Coomassie staining (not shown).

Calculations and Statistics

Vasoconstrictor responses were calculated as a percentage of baseline inner diameter while basal tone was calculated as percent change in inner diameter from Ca^{2+} free conditions. All data are presented as means ± standard error, and n refers to the number of animals in each group. A *t*-test, ANOVA (one- or two-way), or repeated measures ANOVA (one- or two-way) was used to make comparisons when appropriate. If differences were detected by ANOVA, individual groups were compared using the Student-Newman-Keuls test. A probability of $P< 0.05$ was considered significant for all comparisons.

REFERENCES

1. Resta TC, Walker BR. Chronic Hypoxia Selectively Augments Endothelium-Dependent Pulmonary Arterial Vasodilation. *Am J Physiol* 1996;270:H888-H896.

2. Broughton BR, Jernigan NL, Norton CE, Walker BR, Resta TC. Chronic Hypoxia Augments Depolarization-Induced Ca^{2+} Sensitization in Pulmonary Vascular Smooth Muscle Through Superoxide-Dependent Stimulation of RhoA. *Am J Physiol Lung Cell Mol Physiol* 2010;298:L232-L242.

3. Jernigan NL, Walker BR, Resta TC. Reactive Oxygen Species Mediate RhoA/Rho Kinase-Induced Ca^{2+} Sensitization in Pulmonary Vascular Smooth Muscle Following Chronic Hypoxia. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L515-L529.

4. Fresquet F, Pourageaud F, Leblais V, Brandes RP, Savineau J, Marthan R, Muller B. Role of Reactive Oxygen Species and Gp91phox in Endothelial Dysfunction of Pulmonary Arteries Induced by Chronic Hypoxia. *Br J Pharmacol* 2006;148:714-723.

5. Dennis KE, Aschner JL, Milatovic D, Shmidt JW, Aschner M, Kaplowitz MR, Zhang Y, Fike CD. NADPH Oxidases and Reactive Oxygen Species at Different Stages of Chronic Hypoxia-Induced Pulmonary Hypertension in Newborn Piglets. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L596-L607.

6. Stolk J, Hiltermann TJ, Dijkman JH, Verhoeven AJ. Characteristics of Inhition of NADPH Oxidase Activation in Neutrophils by Apocynin, a Methoxy-Substituted Catechol. *Am J Respir Cell Mol Biol* 1994;11:95-102.

7. Rey FE, Cifuentes M, Kiarash M, Quinn M, Pagano PJ. Novel Competitive Inhibitor of NAD(P)H Oxidase Assembly Attenuates Vascular Superoxide and Sytolic Blood Pressure in Mice. *Circ Res* 2001;89:408-414.

8. Liu R, Juncos LA. GTPase-Rac Enhances Depolarization Induced Superoxide Production by the Macula Densa During Tubuloglomerular Feedback. *Am J Physiol Regul Integr Comp Physiol* 2010;298:R453-R458.

9. Zhang B, Peng F, Wu D, Ingram AJ, Gao B, Krepinsky JC. Caveolin-1 Phosphorylation Is Required for Stretch-Induced EGFR and Akt Activation in Mesangial Cells. *Cell Signal* 2007;19:1690-1700.

10. Dahal BK, Cornitescu T, Tretyn A, Pullamsetti SS, Kosanovic D, Dunitrascu R, Ghofrani HA, Weissmann N, Voswinckel R, Banat G, et al. Role of Epidermal Growth Factor Inhibition in Experimental Pulmonary Hypertension. *Am J Respir Crit Care Med* 2010;181:158-167.

11. Blake RA, Broome MA, Liu X, Wu J, Gishizky M, Sun L, Courtneidge SA. SU6656, a Selective Src Family Kinase Inhibitor, Used to Probe Growth Factor Signaling. *Mol Cell Biol* 2000;20:9018-9027.

12. Cheng G, Xiurong LWW, Xiangzhen L, Songbin F. IL-17 Stimulates Migration of Carotid Artery Vascular Smooth Muscle Cells in an MMP-9 Dependent Manner Via P38 MAPK and ERK1/2-Dependent NF-KB and AP-1 Activation. *Cell Mol Neurobiol* 2009;29:1161-1158.

13. Myhre G, Toruner M, Abraham S, Egan L. Metalloproteinase Disintegrin-Mediated Ectodomain Shedding of EGFR Ligands, Promotes Intestinal Epithelial Restitution. *Am J Physiol Gastrointest Liver Physiol* 2004;287:G1213-G1219.

14. Odenbach J, Wang X, Cooper S, Chow FL, Oka T, Lopaschuk G, Kassiri Z, Fernandez-Patron C. MMP-2 Mediates Angiotensin II-Induced Hypertension Under the Transcriptional Contrrol of MMP-7 and TACE. *Hypertension* 2010;57:123-130.

15. Dufour A, Sampson NS, Li J, Rizzo RC, DeLeon J, Zhi J, Jaber N, Lui E, Zucker S, Cao J. Small-Molecule Anticancer Compounds Selectively Target the Homopexin Domain of Matrix Metalloproteinase-9. *Cancer Res* 2011;71:4977-4986.

16. Jernigan NL, Walker BR, Resta TC. Chronic Hypoxia Augments Protein Kinase G-Mediated Ca^{2+} Desensitization in Pulmonary Vascular Smooth Muscle Through Inhibition of RhoA/Rho Kinase Signaling. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L1220-L1229.

17. Davies SP, Reddy H, Cavano M, Cohen P. Specificity and Mechanism of Action of Some Commonly Used Protein Kinase Inhibitors. *Biochem J* 2000;351:95-105.

18. Broughton BR, Walker BR, Resta TC. Chronic Hypoxia Induces Rho Kinase-Dependent Myogenic Tone in Small Pulmonary Arteries. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L797-L806.

19. Csanyi G, Cifuentes-Pagano E, Ghouleh IA, Ranayhossaini DJ, Egena L, Lopes LR, Jackson HM, Kelly EE, Pagano PJ. Nox2 B-Loop Peptide, Nox2ds, Specifically Inhibits the NADPH Oxidase NOX 2. *Free Radical Biology and Medicine* 2011;51:1116-1125.

20. Jernigan NL, Naik JS, Weise Cross L, Detweiler ND, Herbert LM, Yellowhair TR, Resta TC. Contribution of Reactive Oxygen Species to the Pathogenesis of Pulmonary Arterial Hypertension. *PLoS One* 2017;12:e0180455.

21. Norton CE, Broughton BR, Jernigan NL, Walker BR, Resta TC. Enhanced Depolarization-Induced Pulmonary Vasoconstriction Following Chronic Hypoxia Requires EGFR-Dependent Activation of NAD(P)H Oxidase 2. *Antioxid Redox Signal* 2013;18:1777-1788.

22. Plomaritas DR, Herbert LM, Yellowhair TR, Resta TC, Gonzalez Bosc LV, Walker BR, Jernigan NL. Chronic Hypoxia Limits H_2O_2-Induced Inhibition of ASIC-1-Dependent Store-Operated Calcium Entry in Pulmonary Arterial Smooth Muscle. *Am J Physiol Lung Cell Mol Physiol* 2014;307:L419-L430.

23. Nitta CH, Osmond DA, Herbert LM, Beasley BF, Resta TC, Walker BR, Jernigan NL. Role of ASIC1 in the Development of Chronic Hypoxia-Induced Pulmonary Hypertension. *Am J Physiol Heart Circ Physiol* 2014;306:H41-H52.

24. Resta TC, Chicoine LG, Omdahl JL, Walker BR. Maintained Upregulation of Pulmonary ENOS Gene and Protein Expression During Recovery From Chronic Hypoxia. *Am J Physiol* 1999;276:H699-H708.

25. Resta TC, Kanagy NL, Walker BR. Estradiol-Induced Attenuation of Pulmonary Hypertension Is Not Associated With Altered ENOS Expression. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L88-L97.

26. Eichinger MR, Walker BR. Nitric Oxide and CGMP Do Not Affect Fluid Flux in Isolated Rat Lungs. *J Appl Physiol* 1996;80:69-76.

27. Taniguchi K, Xia L, Goldberg HJ, Lee KW, Shah A, Stavar L, Masson EA, Momen A, Shikitani EA, John R, et al. Inhibition of Src Kinase Blocks High Glucose-Induced EGFR Transactivation and Collagen Synthesis in Mesangial Cells and Prevents Diabetic Nephropathy in Mice. *Diabetes* 2013;62:3874-3886.

28. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savail R, Roth M, Sydykov A, Lai YL, Weissmann N, Seeger W, et al. Reversal of Experimental Pulmonary Hypertension by PDGF Inhibition. *J Clin Invest* 2005;115:2811-2821.

29. Brooke MA, Etheridge SL, Kaplan N, Simpson C, O'Toole EA, Ishida-Yamamoto A, Marches O, Getsios S, Kelsell DP. IRHOM2-Dependent Regulation of ADAM 17 in Cutaneous Disease and Epidermal Barrier Function. *Hum Mol Genet* 2014;23:4046-4076.