Intrauterine Pulmonary Hypertension Impairs Angiogenesis *in vitro*: Role of VEGF-NO Signaling.

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Online Data Supplement

Methods

Isolation and culture of fetal ovine pulmonary arterial endothelial cells. All procedures and protocols were reviewed and approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center, Denver, CO. Pulmonary arteries were isolated from late-gestation fetal sheep (mixed-breed Columbia-Rambouillet pregnant ewes at 135 days gestation, term = 147 days) and from fetal sheep that had undergone partial ligation of the ductus arteriosus *in utero* 7-10 days prior to euthanasia (PPHN). In this model, ductal ligation causes sustained elevation in pulmonary artery pressure without a change in blood flow or oxygenation (3). The ewe and fetus were killed with high dose intravenous pentobarbital sodium. The left and right main pulmonary arteries were isolated by direct visualization, branching vessels were tied with silk suture, and the endothelial surface exposed to 0.1% collagenase for 5 minutes. The vascular surface was washed with D-valine media (Cellgro; Mediatech Inc, Herndon, VA) containing 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic agent (Cellgro; Mediatech Inc, Herndon, VA). Cells were separated from the remaining collagenase by centrifugation for 8 minutes at 1200 RPM. The supernatant was removed and the cell pellet resuspended in D-valine media. The pellet was dispersed, and cells were plated in 6 well dishes coated with 0.1% gelatin. All tissue culture plastic used in the below mentioned experiments were coated with 0.1% gelatin. After 24 hours, D-valine media was removed and replaced with Dulbecco's Modification of Eagles Medium (DMEM; Sigma, St. Louis, MO) with 10% FBS, 1% antibiotic-antimycotic agent and 1% L glutamine. Cells were allowed to grow to confluence in humidified incubators (Forma Scientific, Marietta, OH) at 37¡C in

room air, 5% CO_2. Endothelial cell phenotype was confirmed at each passage by positive immunostaining for von Willebrands Factor (vWF), eNOS, vascular endothelial (VE)-cadherin and VEGFR-2 (KDR); the positive uptake of diacetylated-low density lipoprotein and the absence of staining for desmin. Cells from passage 4 and 5 were used for each of the study experiments.

The effects of vascular endothelial growth factor (VEGF; 50ng/ml), S-Nitroso-N-acetylpenicillamine (SNAP (1mM), NO donor, Calbiochem) nitric oxide gas (10 ppm), SU5416 (a VEGF receptor inhibitor, 10 μM), nitro-L-arginine (LNA; a nitric oxide synthase inhibitor; 4mM) and combinations of these agents, on cell growth were compared between PAEC from normal and PPHN sheep. Doses for each agent were based on preliminary experiments (VEGF, NO gas, SNAP and LNA) and previously published data (SU5416)(31). For each agent the lowest dose of agent for which a given effect was seen was used. These studies were performed in DMEM supplemented with 5% serum, as this was the lowest serum concentration that supported fetal PAEC proliferation.

Effect of SNAP and NO gas on PAEC growth and tube formation. Preliminary experiments were performed to determine the effects of NO on endothelial cell growth and tube formation. We compared the effects of NO gas at 10ppm and SNAP, a NO donor at various doses (1mM, 5mM, 10mM), with single treatment or repeated administrations every 24 hours, and found NO gas be more effective than SNAP in stimulating endothelial cell growth. SNAP spontaneously degrades and releases NO

when kept in solution and is depleted after 24 hours. As our experiments on PAEC growth were performed over several days, NO gas was used to provide both a continuous and constant dose exposure. NO gas was less effective at enhancing tube formation when continuously administered at 10 ppm (data not shown). For this reason, NO gas was used to determine the effect of nitric oxide as well as SU5416 (10 μM) + NO on PAEC growth, while SNAP was used to determine the effect of nitric oxide as well as SU5416 (10 μM) + NO on tube formation.

Cell growth. Fetal PAEC from normal and PPHN lambs were plated at 2 x 10^5 cells/well and allowed to adhere overnight. Cells were plated and grown in DMEM media supplemented with 10% FBS. Cells were grown in 3% oxygen, as preliminary experiments, as well as previous studies in our lab have shown improved fetal PAEC function in low oxygen tension conditions that mimic the intrauterine environment (22). Daily cell counts were performed for 5 days using a Beckman Coulter cell counter, after removing cells from wells by 0.25% trypsin/0.53mM EDTA digestion.

Stimulatory effects of VEGF and NO on cell growth. PAECs were seeded at a density of 2 x 10^5 cells/well in 6 well dishes. Cells were allowed to adhere overnight and baseline counts performed on day 1. Cells were allowed to proliferate to day 3 and at this time point, cells were treated with VEGF or NO gas. This time point was chosen for stimulation, as at day 3 differences in growth were seen between normal and PPHN cells. Cell counts were then performed on day 5 (after

2 days of treatment), and comparisons were made between treated and untreated cells.

Inhibitory effects of SU5416 and L-NA on cell growth. Cells were seeded at a density of 2×10^5 cells/well and allowed to adhere overnight. Baseline counts were performed on day 1 prior to treatment with SU5416 and L-NA. Counts were performed on day 3 and comparisons were made between treated and untreated cells after 2 days of treatment.

To assess the ability of NO gas to rescue SU5416 inhibition of cell proliferation, cells were seeded at a density of 2×10^5 cells/well. Cells were allowed to adhere overnight and baseline counts performed on day 1. Cells were then exposed to the combination of SU5416 and NO gas. Cell counts were then performed on day 3 and a comparison made between normal and PPHN PAECs with respect to cell number.

Tube Formation Assay: The ability of fetal PAECs to form vascular structures in vitro was assayed by plating PAEC's on EHS Matrigel. (BD Pharmingen, San Jose, CA) EHS matrigel was pipetted into 24 well tissue culture dishes (250 µl/well) and allowed to polymerize at 37¡C for one hour. PAEC from normal and PPHN animals were seeded at a density of 5×10^4 cells/well in serum free DMEM supplemented with and without VEGF (50ng/ml), SNAP (1µM), LNA (4mM), SU5416 (10 µM) and SU5416 (10 µM) with SNAP (1 µM) in combination. PAECs were incubated for 6 hours under 3% oxygen conditions, since maximal PAEC tube formation occurred at this time.

Branch point counting was performed in blinded fashion under 10X magnification from each of 4 wells, as previously described (23).

Western Blot Analysis: PAECs from normal and PPHN animals were grown on 150mm cloning dishes in DMEM supplemented with 10% serum. At 90% confluence, cells were washed with ice cold PBS x 2 and lysed in radioimmunoprecipitation (RIPA) buffer (PBS, 1% Nonidet P-40, 0.5%, sodium deoxycholate, 0.1% SDS, PMSF [10mg/ml], aprotinin [16 µl/ml], and sodium orthovanadate [1mM]). Cell lysate was then scraped off the dishes. The cell lysate was sonicated and frozen down. On thawing, the cell lysate was sonicated once more and centrifuged at 10,000 x g for 30 min at 4¡C. The supernatant was removed and protein content in the supernatant was determined by the BCA assay (Pierce Biotechnology Inc (catalog # 23225) Rockford, IL), using bovine serum albumin as the standard. Briefly, 20 _g of protein sample per lane was resolved by SDS polyacrylamide gel electrophoresis, and proteins from the gel were transferred to nitrocellulose membrane.

VEGF: Blots were blocked for 30 minutes in 5% nonfat dry milk dissolved in buffer 1 (10mM tris-hcl,150mM NaCl, 0.05% tween-20, PH 8.0) The VEGF blot was incubated for 1 hour in room air with (Santa Cruz Biotech SC152) (1:500) diluted in 5% nonfat dry milk in buffer 1. After washing, the VEGF blot was then incubated for 1 hour at room temperature with goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotech, SC2054). VEGF bands were then visualized by enhanced chemiluminenscence (ECL+ kit; Amersham Pharmacia Biotech, Buckinghamshire,

UK). VEGF was run as a control for easy detection of the VEGF band.

eNOS/VEGF-R2: Blots were blocked for 30 minutes in 2% ECL advance (Amersham Pharmacia Biotech, Buckinghamshire, UK) dissolved in PBS with 0.5% Tween 20. The KDR blot was incubated overnight at 4¡C with SC504 (1:250; Santa Cruz Biotech) diluted in 2% ECL advance. The eNOS blot was incubated for 1 hour in room air with BD610297 (eNOS/NOS III)(1:1000) diluted in 2% ECL advance. After washing, the KDR blot was then incubated for 1 hour at room temperature with goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotech, SC2054) and the eNOS blot incubated for 1 hour at room temperature with goat anti-mouse HRP conjugated secondary (Chemicon)(1:20000). eNOS and KDR were visualized using the ECL advance kit, identified by molecular weight as identified by the manufacturer for the protein of interest.

All blots were then stripped and reprobed with an antibody to B-actin (Sigma,St. Louis, A5316). Densitometry was performed using NIH Image (v1.61). Changes in protein expression were analyzed after normalizing for B-actin expression.

Nitric Oxide Assay: Determined using the DAF-FM Nitric Oxide indicator (*Molecular Probes, Eugene, OR #D-23844)*. 5×10^3 normal and PPHN PAECs were plated in a 96 well plate in DMEM with 10% FBS under 3% oxygen conditions. Cells were allowed to adhere overnight after which media was switched to DMEM with 0.5% FBS for 24 hours followed by serum free DMEM for 24 hours. Normal and PPHN PAECs were

then incubated with DAF-FM with and without VEGF (50ng/ml) in PBS for 1 hour. PBS was then removed from each well and NO production measured in response to VEGF stimulation using a microplate reader with fluorescence excitation and emission maxima of 495 and 515 nm, respectively. Comparisons were made between normal and PPHN cells with respect to NO production.

Proliferation/Apoptosis was assessed in normal and PPHN PAECs. Normal and PPHN PAECs were plated on chamber slides and fixed with 4% paraformaldehyde at 50% and 90% confluence for 15 minutes. Control slides for apoptosis were obtained by serum starving normal PAECs for 72 hrs or exposing normal PAECs to ultraviolet (UV) irradiation for 15 minutes to induce apoptosis. After serum starvation and UV irradiation slides were fixed with 4% paraformaldehyde for 15 minutes. Slides were then permeabilized with ice cold methanol in -20C for 15min. After 3 washes with PBS, slides were again permeabilized with 0.1% Triton-X. 3 further washes with PBS were performed and slides blocked with 2% horse serum for 1 hour. 3 more washes were performed with 0.1%BSA in PBS (PBSA) and the slides were then incubated with primary antibody Ki67 (1:250; Santa Cruz Biotech SC15402) and active Caspase-3 (1:250 Chemicon, Temecula, CA AB2362) for 1 hour. After 3 more washes with PBSA slides were incubated with secondary antibody Alexa-Flour goat anti-rabbit 488 (1:500) (Invitrogen Carlsbad CA #A11034) for 1 hour. Slides were washed 3 more times with PBSA and mounted with Vectashield Hardmount with DAPI (Carpinteria CA #H1500). Comparison was made between normal and PPHN PAECs.

Statistical analysis. Data are presented as means ± SEM, with data from all 4 animals in the normal and PPHN groups combined for analysis. Statistical analysis was performed with the Prism 4 software package (GraphPad Software, San Diego, CA). Statistical comparisons were made using analysis of variance for growth and tube formation assays with Bonferroni post test analysis. Unpaired t test was used for western blot analysis. $P < 0.05$ was considered significant.