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A potential role for reactive oxygen species and the HIF-1 α -VEGF pathway in hypoxia-induced pulmonary vascular leak

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Abstract

Acute hypoxia causes pulmonary vascular leak and is involved in the pathogenesis of pulmonary edema associated with inflammation, acute altitude exposure, and other critical illnesses. Reactive oxygen species, HIF-1 and VEGF have all been implicated in various hypoxic pathologies, yet the ROS-HIF-1-VEGF pathway in pulmonary vascular leak has not been defined. We hypothesized that the ROS-HIF-1-VEGF pathway has an important role in producing hypoxia-induced pulmonary vascular leak. Human pulmonary artery endothelial cell monolayers (HPAEC) were exposed to either normoxia (21% O₂) or acute hypoxia (3% O₂) for 24 h and monolayer permeability, H₂O₂, nuclear HIF-1 α , and cytosolic VEGF were determined. HPAEC were treated with antioxidant cocktail (AO: ascorbate, glutathione, and α -tocopherol), HIF-1 siRNA, or VEGF soluble binding protein (sFlt-1) to delineate the role of the ROS-HIF-1-VEGF pathway in hypoxia-induced HPAEC leak. Additionally, mice exposed to hypobaric hypoxia (18,000 ft, 10% O₂) were treated with the same antioxidant to determine if *in-vitro* responses corresponded to *in-vivo* hypoxia stress. Hypoxia increased albumin permeability, H₂O₂ production, nuclear HIF-1 α , and cytosolic VEGF concentration. Treatment with an AO lowered the hypoxia-induced HPAEC monolayer permeability as well as elevation of HIF-1 α and VEGF. Treatment of hypoxia-induced HPAEC with either a siRNA designed against HIF-1 α or VEGF antagonist soluble fms-like tyrosine kinase (sFlt)-1 decreased monolayer permeability. Mice treated with AO and exposed to hypobaric hypoxia (18,000 ft, 10% O₂) had less pulmonary vascular leak than those that were untreated. Our data suggest that hypoxia-induced permeability is due, in part, to the ROS-HIF-1 α -VEGF pathway.

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Keywords

Hypoxia; pulmonary edema; reactive oxygen species; HIF-1 α ; VEGF; HIF

INTRODUCTION

Acute hypoxia induces pulmonary vascular permeability and contributes to forms of non-cardiogenic pulmonary edema such as high altitude pulmonary edema and acute respiratory distress syndrome [1,2]. Reactive oxygen species [3] have been implicated in the onset of a wide variety of vascular diseases associated with endothelial cell barrier function properties, such as atherosclerosis, ischemic reperfusion injury and acute respiratory distress syndrome [4–6]. However, hypoxia-induced ROS formation and its exact role in pulmonary vascular permeability are controversial [3,7–11].

Some previous reports suggest that during hypoxia, increasing ROS may contribute to the stabilization of hypoxia-inducible factor-1 α (HIF-1 α) [12–14]. During hypoxia, cytosolic HIF-1 α is stabilized by inhibition of oxygen-dependent enzymatic hydroxylation of a proline residue [3,15,16] and subsequently translocated to the nucleus where it binds constitutively expressed β -subunit (HIF1- β), forming HIF-1. HIF-1 binds hypoxia response elements on numerous genes encoding proteins necessary for responding to hypoxic stress such as erythropoietin, vascular endothelial growth factor (VEGF) and numerous glycolytic enzymes [5,15,17].

VEGF induces endothelial vascular permeability in a variety of organs [12–14]. During acute hypoxia, lung VEGF mRNA and protein content are increased in animal models (31,14), and have been correlated with pulmonary vascular permeability (16,8,9). Although ROS, HIF and VEGF have each been implicated in hypoxia-induced pulmonary vascular leak independently, the interaction between these key molecules to produce hypoxia-induced vascular leak has never been defined.

We hypothesized that pulmonary vascular leak in acute hypoxia is mediated by ROS-induced HIF activation of VEGF and inhibition of the ROS-HIF-VEGF pathway would reduce pulmonary vascular leak.

Our approach was to expose human pulmonary artery endothelial cell monolayers (HPAEC) to acute hypoxia (3% O₂) for 24 h and measure H₂O₂, nuclear HIF-1 α , cytosolic VEGF and monolayer leak in comparison to normoxic HPAEC. We then treated the HPAEC with an antioxidant cocktail, HIF-1 α siRNA, and VEGF soluble binding protein (sFlt-1) to delineate the role of the ROS-HIF-VEGF pathway in hypoxia-induced HPAEC leak. We also treated mice exposed to hypobaric hypoxia (18,000 ft, 10% O₂) with the same antioxidant cocktail to determine if *in vitro* responses could be replicated *in vivo* following 24 hours of hypoxia. We found that 24 h of hypoxia (3% O₂) in HPAEC increased H₂O₂ production, nuclear HIF-1 α , cytosolic VEGF and permeability rate. Furthermore, treatment with antioxidants inhibited each of these variables *in vitro*, and decreased hypoxia-induced pulmonary vascular leak *in vivo*. *We used a series of interventions to demonstrate that ROS production in hypoxia was responsible for activation of HIF, leading to increased VEGF induced vascular leak in vitro and in vivo.*

MATERIALS AND METHODS

Animals

Male C56/BLJ6 weighing between 25–30 g and 10–12 weeks of age were obtained from a commercial vendor (Charles River) and housed in the University of Colorado Health Sciences Center's Center for Laboratory Animal Care (elevation = 5,280 ft). Animals were allowed access to food and water, ad libitum. The animals were kept on a 12 h day-night cycle. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at University of Colorado Denver School of Medicine.

Cell culture and experimental design

Human pulmonary artery endothelial cells (HPAEC) were obtained from Clonetics (Cambrex biosciences, CC 2530,) and cultured in endothelial cell basal media (Clonetics, EBM, Cambrex Biosciences, CC 3121). For all experiments HPAEC were used from passages 4–8, grown to confluence, and cultured under standard cell culture conditions (21%, 'normoxia') unless otherwise noted. For each experiment HPAEC were randomly divided into 4 groups: normoxia control (NX-CTRL); normoxia-treated (NX-TX); hypoxia control (HX-CTRL) and hypoxia treated (HX-TX). Cell treatments included: an antioxidant (AO) cocktail (ascorbate (0.2mM), glutathione (0.5 mM), and α -tocopherol (3.5 μ M)); HIF-1 siRNA (scrambled RNA used for control) or circulating VEGF receptor 1 binding protein, soluble fms-like tyrosine kinase (sFlt-1) receptor. The AO and sFlt-1 were added to the culture media 30 min prior to normoxic or hypoxic exposure (24 h) and HIF-1 siRNA cells were transfected 48 h prior to normoxic or hypoxic exposure (24 h). Data was collected from at least three separate cell culture preparations on three separate days (n=9).

Hypoxic incubation

Cell cultures were placed in a special humidified hypoxic chamber for 24 h as previously described [18]. Use of an antechamber ensured that once cells became hypoxic, they were never re-exposed to a normoxic environment. The chamber utilized a positive pressure system and was supplied with a gas mixture of 3% O₂, 5% CO₂, and balance of nitrogen. Culture media used in permeability tests was allowed to equilibrate to 3% O₂ before initiation of tests. All relevant permeability tests and preparation for hydrogen peroxide H₂O₂ production assays were conducted within the hypoxia chamber to avoid introduction of normoxia during tissue preparation.

Hydrogen Peroxide (H₂O₂) measurement assay

As previously described, hydrogen peroxide production was measured as quantitative index of ROS generation (indirect indicator of the free radical O₂⁻) by an Amplex red assay kit [19]. Briefly Amplex red reagent [(10-acetyl-3,7-dihydroxyphenoxazine; Molecular Probes, (A-22188)] reacts with hydrogen peroxide in the presence of horseradish peroxidase (HRP) with a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. Resorufin has absorption and fluorescence emission maxima of 571 nm and 585 nm. HPAEC were seeded (~15,000 cell/well) in 96 well plates and grown to confluence over 48 h. After which cells were exposed to either standard cell culture conditions (normoxia) or hypoxia for 24 h in the presence or absence of AO as stated above. Following 24 h of exposure, media was removed and cells were rinsed once with warm Krebs solution (100 μ l) and replaced with Amplex red assay solution (100 μ l). The rise in hydrogen peroxide was measured over a 20 min period. Hydrogen peroxide production was normalized to the amount of DNA/well by the Cyquant assay kit (Invitrogen, #35007, Carlsbad CA). Data are represented as μ M min⁻¹ μ g DNA⁻¹.

Time course estimation for HPAEC permeability studies

To determine an initial time point to study the ROS-HIF-VEGF pathway in terms of hypoxia-induced permeability a time course study was conducted on bovine pulmonary endothelial cells (n=3 each time point). Hydrogen peroxide was measured, as described above, after 30 min, 1, 18, 24, 48 h of hypoxia. Compared to cells cultured in standard conditions hydrogen peroxide was increased at 18, 24 and 48 h (data not shown). We previously reported that permeability in BPAEC also increased at 24 h hypoxia [18], therefore, we chose 24 h hypoxia as the time point at which to investigate pulmonary vascular leak and the potential role of the ROS-HIF-VEGF pathway.

Permeability assay

HPAEC cell preparations were treated as stated in *experimental design* and the permeability rate was determined. Briefly, HPAEC were seeded (~100,000 cells/insert) on polystyrene filters (Costar Transwell, no. 3470, 6.5 mm diameter, 0.4-um pore size; Cambridge, MA). HPAEC were grown to confluence over 48 h. After which cells were exposed to 24 h of either standard cell culture conditions or hypoxia. Following 24 h exposure HPAEC were washed three times in serum-free media and FITC-labeled albumin (concentration as stated in figure legends) suspended in serum-free medium was added to the HPAEC monolayers (100 ul). The insert was placed in a new well of a 24-well plate containing serum free medium (0.6 ml to ensure that the fluid volume on either side of the inserts was equalized to avoid a hydrostatic gradient that might alter the rate of albumin flux). Measuring the rise in FITC-albumin in the lower well after 60 minutes assessed the transfer rate of albumin across the monolayer. FITC-albumin was quantified in a fluorescence spectrofluorometer (company brand) and reported as relative fluorescence units (RFU).

Western blot analyses for nuclear HIF-1 α

Nuclear protein from HPAEC was prepared using Clontech TransFactor Extraction Kit (Palo Alto, CA) and protein concentration was determined using a BCA protein assay (BCA protein assay kit, Pierce, Rockford Ill). Isolated nuclear proteins were fractionated by electrophoresis using a 4–12% Bis-Tris gel (Invitrogen™, Carlsbad, CA). The proteins were then transferred to a methanol-soaked polyvinylidene difluoride (PVDF) membrane using the semidry immunoblot method (Owl Model HEP-1 Panther Semi-Dry Electrobloetter - #HEP-3, Nunc, Rochester, NY). The membranes were probed with primary antibodies against HIF-1 α and β -actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA and Sigma, St. Louis, MO, respectively). Following phosphate buffered saline (PBS)-tween washes, membranes were incubated with HRP conjugated secondary antibodies and bands were visualized using SuperSignal® West Dura Extended Duration Substrate (Pierce Biotechnology, Inc., Rockford, Ill). Imaging was carried out using a UVP BioChem Imaging System and densitometry was performed using LabWorks 4.0 software. The ratio of the HIF-1 α to β -actin (used for protein loading control) was determined and a fold-difference for each treatment group was calculated using the HIF-1 α to β -actin ratio in NX-HPAEC without antioxidant therapy as the baseline.

HIF siRNA experiments

A short dsRNA oligonucleotides for siRNA were designed from BLAST analysis, synthesized and purified by Dharmacon (Lafayette, CO) To insure effectiveness, two 21 nucleotide siRNAs (NNUGACUCAGCUAUUCACCAA and NNAGUUCACCUAGCCUAAUA) were designed and targeted against the most common isoforms of the HIF-1 α sequence. To control for a transfection effect, control cells underwent an identical protocol using a scrambled siRNA (luciferase) in place of the siRNA oligonucleotides designed against HIF-1 α . Cells were transfected according to manufacturers instructions (Invitrogen Oligofectamine Reagent protocol (Invitrogen™, Carlsbad, CAL, catalogue # 12252-001). Briefly, cells were rinsed with

PBS and media replaced with Opti-Mem I (Invitrogen™, Carlsbad, CAL) (330ul), HIF-1 siRNA oligonucleotides (150 ul; 20 uM each of siRNA), or scrRNA), and Oligofectamine Reagent (120 ul) and then incubated (RT, 20 min). Cells were then washed with PBS and cultured for 48 h in EBM containing 30% fetal bovine serum. Following incubation HPAEC were seeded (~150,000 cells/insert) on polystyrene filters and allowed to adhere for 24 h before being randomly assigned to either 21% or 3% oxygen exposure for determination of monolayer permeability. HPAEC were collected and nuclear proteins were isolated for Western blot analyses as described above.

Vascular endothelial growth factor quantification

Cytosolic proteins were assayed for VEGF by ELISA (DuoSet® ELISA Development System, R&D Systems, Minneapolis, MN). Briefly, capture antibody was coated onto 96 well-plates, incubated (RT, ON), washed with PBS/tween and blocked with 1% BSA/PBS. Cytosolic proteins were then added and incubated (RT, 2 h). After washing, detection antibody was added and plates were incubated (RT, 2 h). Plates were then incubated with streptavidin-HRP reagent (RT, 20 min), then with substrate solution (R&D Systems, Minneapolis, MN) (RT, 20 min, dark) and stop solution (2 N H₂SO₄) added prior to analyses by a spectrophotometer at 450 nm (Thermo Labsystems, Chantilly, VA, Multiskan Ascent Spectrophotometer and Ascent Software, Version 2.6).

In vivo methods

Osmotic pump placement—Micro-osmotic pumps (Alzet, Cupertino, CA, model 1007D) were placed subcutaneously as per manufactures protocol. Briefly, animals were anesthetized with ketamine: Rompon (xylozine) (75 mg/kg; 6 mg/kg I.P.) and a small incision (1 cm) were made just lateral to the dorsal midline. Osmotic pumps filled with 100 ul of either saline or AO solution (Na⁺ ascorbate, 20 mM; glutathione, 50 mM; and α -tocopherol, 350 μ M) were randomly assigned and placed subcutaneously. The AO concentration was calculated to deliver approximately the same concentration/24 h as used in our cell culture model once absorbed and diluted into the blood stream (Na⁺ ascorbate (0.2 mM), glutathione (0.5mM), and α -tocopherol (3.5 μ M).

Hypoxic exposure of mice—All animals were allowed to recover from surgery for 72 h prior to hypobaric hypoxic exposure. Afterwards the animals were divided into 2 sets for either pulmonary vascular leak or immunohistochemistry analyses. Each set of animals was further subdivided into either normoxia or hypoxia (18,000 ft; P_B ~380 mm Hg) treated and untreated groups, making a total of 4 groups per set (n = 8–10/group); normoxic saline-treated (NX), normoxic AO-treated (NX-AO), hypoxic saline-treated (HX) and hypoxic AO-treated (HX-AO).

Pulmonary vascular leak—Mice were lightly anesthetized using isoflurane gas and Evan's blue dye (EBD) solution (50 ul; 30 mg/kg) was injected retro-orbitally. Mice were then euthanized with an overdose of isoflurane gas 10 min after EBD injection, then transcatheterially perfused with PBS. Heart and lungs were removed *en bloc*, and extrapulmonary airways and tissues were removed by further dissection. After weighing the right and left lungs separately, the right lung was frozen in liquid nitrogen for EBD extraction, while the left lung was allowed to dry until a stable weight was obtained for calculation of wet-to-dry weight ratios. Evans blue dye was extracted from lungs incubated for 24 h in formamide (100%, 60° C). The extracted dye was quantified in spectrophotometer by measuring the absorption coefficient at 620 nm against standards of EBD dissolved in formamide and normalized to the calculated dry weight of tissue. Units are expressed as ng/mg of dry tissue.

Lung fixation and Immunohistochemical staining for HIF and VEGF—Animals were euthanized with an overdose of isoflurane gas. The right atria was severed and the lungs were perfused free of blood with PBS (3 ml). Afterwards, the lungs were fixed with 10% buffered formalin (~1 ml) by airway inflation under constant pressure at 25 cm H₂O pressure, paraffin embedded, and sectioned to 5μ for immunohistochemistry staining.

Lung tissue was stained for HIF-1α or VEGF expression in a humidity chamber per supplier's instructions. Briefly, antigens were unmasked on serial lung sections and then subjected to either a HIF-1α primary antibody (1:50, Novus biologicals, Littleton CO, #100-105) or a VEGF primary antibody (1:100, Novus biologicals, Littleton CO, #SC7269). Lung sections were incubated with a biotinylated secondary antibody and an enzyme conjugate solution was applied (150 ul, HRP-streptavidin, Zymed Labs, location #50-209). This was followed by a substrate chromagen solution (AEC single solution, Zymed Labs, #00-1111). Lung sections were then counterstained with hematoxylin. As a control for non-specific secondary antibody binding, some lung sections were simultaneously subjected to the same protocol without the primary antibody.

Pulmonary vessels (outside diameter 100–300 μM) were assessed for presence of HIF-1α and VEGF on a Nikon TE2000-U inverted epi-fluorescent microscope. Bright field, phase-contrast and fluorescent digital deconvolution images were captured to a personal computer with a Spot Insight color Camera (Diagnostic Imaging, Sterling Heights, MI). Images were analyzed and processed with Metamorph 6.1 software (Molecular Devices, Sunnyvale, CA)

Statistics

For all groups, means ± S.E.M. are reported. Statistical comparison between groups were analyzed with a two-way (Treatment, exposure) analyses of variance (ANOVA). Post hoc analyses were completed with unpaired, 2-sided Students's *t*-test. Statistical analyses were performed using JMP (Version 5) statistical software package (SAS; Cary, North Carolina) with statistical significance set at $p \leq 0.05$.

RESULTS

The current study was designed to determine if 24 h of hypoxic exposure activates the ROS-HIF-VEGF pathway, thus inducing pulmonary endothelial leak in human pulmonary artery endothelial cell monolayers (HPAEC) and in a mouse model. The sequence of events was investigated using an antioxidant cocktail (AO), HIF-1 siRNA and VEGF inhibitor soluble fms-like tyrosine kinase (sFlt-1) to interrogate the sequential changes in H₂O₂ production, nuclear HIF-1α, VEGF and endothelial leak.

Antioxidant treatment decreased hypoxia-induced permeability in HPAEC

To evaluate the role of ROS in hypoxia-induced permeability in cultured bovine pulmonary artery endothelial cell monolayers, we treated HPAEC cultured monolayers with an antioxidant (AO) cocktail and measured permeability of FITC labeled albumin following 24 h hypoxia. Permeability increased significantly in HPAEC monolayers exposed 24 h of hypoxia (HX) (albumin flux $p = 0.01$ vs normoxic (NX) cells, Figure 1A). Antioxidant treatment had no effect on NX cells, but decreased permeability in HX cells ($p = 0.02$ treatment, Figure 1a).

Antioxidants decreased hypoxia-induced H₂O₂ production by HPAEC

As an indication of ROS formation we determined HPAEC production of H₂O₂ at 24 h of either normoxia or hypoxia (3% O₂) in the presence or absence of antioxidant cocktail (AO). Compared to normoxia, hypoxia stimulated cells to increase H₂O₂ production ($P = 0.026$,

Figure 1B). Treatment with AO had no effect on NX cells, but significantly decreased H₂O₂ in HX cells after 24 h hypoxia ($P = 0.039$, Figure 1b).

Antioxidant treatment decreased nuclear HIF-1 α

To test the hypothesis that AO decreased hypoxia-induced permeability in HPAEC monolayers is associated with HIF-1 α inhibition, Western blot analyses for HIF1- α were performed on nuclear protein isolated from NX and HX HPAEC in the presence or absence AO. HX cells increased nuclear HIF-1- α 3.0 ± 0.5 (SEM) fold in relation to NX cells ($p = 0.006$, Figure 1c–d). The hypoxia-induced rise of nuclear HIF-1- α was lowered with AO treatment ($p = 0.04$, Figure 1c–d).

Antioxidant treatment decreased cytosolic VEGF protein concentration

During hypoxia, HIF-1 α enhances transcription of the gene encoding VEGF and VEGF mediates vascular permeability [5,12,15,20,21]. Therefore, we determined if cytosolic VEGF concentration was lowered in association with the AO-induced decrease of nuclear HIF-1 α in HPAEC during 24 h of hypoxia. In normoxic conditions, no VEGF was detectable, with or without AO. However, there was a dramatic rise in cytosolic VEGF during hypoxia that was completely abolished with AO treatment ($p < 0.001$, Figure 1c).

HIF-1 α siRNA decreased hypoxia-induced permeability in HPAEC

To determine if HIF-1 α played a role in HX-induced permeability we designed a silencing RNA (siRNA) to inhibit production of HIF-1 α protein. The siRNA successfully inhibited HIF-1 α production and the scrambled RNA (scrRNA) used for a control did not lower nuclear HIF-1 α (Figure 2a–b). There was no effect on HPAEC permeability in NX cells treated with either a control scrRNA or HIF-1- siRNA. However, permeability decreased following 24 h hypoxia, in HPAEC treated with siRNA designed against HIF-1 α compared to cells treated with a scrambled siRNA ($p < 0.001$ Figure 2c).

VEGF blockade with soluble sFlt-1 decreased hypoxia-induced permeability in HPAEC

To determine if hypoxia-induced permeability in HPAEC monolayers was influenced by VEGF, VEGF antagonist sFlt-1 (1 μ M) was administered to HPAEC during normoxia and hypoxia. After 24 h of sFlt-1 exposure HPAEC monolayer permeability was measured. There was no effect of sFlt-1 on HPAEC permeability during normoxia, however sFlt-1 decreased hypoxia-induced monolayer permeability ($p = 0.045$, Figure 3).

Hypoxia-induced pulmonary vascular permeability in mice is less with AO treatment

We then determined if the AO offered the same protection from hypoxia-induced pulmonary permeability *in vivo*. Mice (C57/BLJ) were exposed to high altitude (HA, 18,000 ft.) for 24 h and pulmonary vascular leak was measured by Evans blue dye (EBD). Hypoxia induced pulmonary vascular leak after 24 h, as expected, and AO moderated but did not completely eliminate pulmonary vascular leak in mice at high altitude ($p = 0.02$, Figure 4).

HIF-1 α and VEGF expression in pulmonary vessels is decreased with antioxidant treatment

To further assess the AO effect on HIF-1 α and VEGF in the pulmonary vessels immunohistochemistry (IHC) was performed in mice exposed to either ambient pressure or simulated high altitude. As expected, high altitude exposed mice showed greater HIF-1 α and VEGF expression compared to normoxic cohorts. Treatment with AO decreased HIF-1 α and VEGF expression (Figure 5a–b).

Discussion

The main finding of this study was that hypoxia-induced leak (24 h) in HPAEC monolayers was associated with increased hydrogen peroxide (H_2O_2) production, nuclear HIF-1 α , cytosolic VEGF concentration and HPAEC permeability and inhibition of these factors reduced albumin leak. Furthermore, *in vitro* data were supported by data from *in vivo* experiments in which mice were treated with antioxidants and exposed to hypobaric hypoxia (18,000 ft, 10% O_2) and had less pulmonary vascular leak, HIF-1 α and VEGF expression than untreated mice.

The Amplex® Red method was used to measure H_2O_2 production, as an indirect indicator of superoxide ($O_2^{\cdot -}$) and showed a clear increase following 24 hours of hypoxia in BPAEC. Furthermore, the antioxidant cocktail, though non-specific in nature, fully reversed the detection of ROS. Though Amplex® Red may produce an artifact signal by the mobilization of NADH and glutathione [22], the reversibility of the hypoxic signal by AO suggest its predominant signal is due to its reaction with H_2O_2 . This is a controversial area as other investigators have reported both an increase in ROS, similar to our findings, [7,8] as well as a decrease in ROS in response to acute hypoxia [3,9,10]. The differences in these data may relate to the length of hypoxic exposure, oxygen tension during the actual measurements of ROS, or cell type studied. It is possible that hypoxia leads to an increase in ROS from different sources, as NADPH oxidase, mitochondria electron transport, xanthine oxidase and eNOS have each been implicated [3,4,11,16]. Studies are needed to identify the sources of H_2O_2 in hypoxia and link this source directly to HIF-1 stabilization in hypoxic HPAEC. This will be an important area of future investigations.

In our study we used endothelial cells derived from the pulmonary artery which may behave differently than microvascular endothelial cells [23]. Heterogeneity between endothelial cells and their responses to stressors in the local environment is becoming more widely recognized [24]. Since our antioxidant cocktail decreased hypoxia-induced pulmonary vascular leak as well as HIF-1 α and VEGF expression in pulmonary vessels *in vivo*, it suggests that our cell culture model is relevant to understand the mechanisms present in the hypoxic pulmonary vasculature.

Interestingly, the addition of siRNA against HIF-1 α maximally decreased monolayer permeability, and was more effective than either AO treatment or soluble VEGF receptor. It is not clear why the HIF-1 α siRNA would decrease permeability below basal rates. Our data suggest that AO treatment does not completely block HIF-1 α stabilization, which would account for its incomplete protection against hypoxia-induced permeability. This could be due to either incomplete scavenging of ROS or more likely an alternative non-ROS mediated mechanisms to regulate HIF stabilization. Treatment with the soluble VEGF receptor sFlt-1 also did not decrease HPAEC monolayer permeability at the same magnitude as siRNA treatment. It is likely that HIF-1 activation alters monolayer permeability through VEGF independent mechanisms such as altering the endothelial cell tight junction proteins zona occludins or claudin-1 [25].

HIF-1 binds the RTP801 promoter region of VEGF, enhancing production of VEGF protein [26]. In the current study hypoxia-stimulated HPAEC had elevated nuclear HIF-1 α in association with a rise in cytosolic VEGF. The hypoxia-induced rise of VEGF in HPAEC was abolished by AO treatment in association with lower nuclear HIF-1 α . This is in agreement with other studies that showed VEGF directly regulated vascular permeability in various organs [17,27], and may have a role in clinical conditions such as acute respiratory distress syndrome, sepsis, acute mountain sickness (AMS), and high altitude pulmonary edema (HAPE). [28–30].

Soluble VEGF receptor, sFlt-1, decreased HPAEC permeability in the current study. sFlt-1 is an endogenous factor that binds VEGF circulating in the plasma and subsequently inhibits its biological effect [38]. VEGF and sFlt-1 contributed to endothelial cell barrier dysfunction [39–41] in human subjects and rodent models and correlated to the severity of illness in sepsis and ARDS [42]. Furthermore, treatment with exogenous sFlt-1 improved survival in models of sepsis and ARDS [43]. These data provide further support for the possibility that sFlt-1 could be used as a therapeutic agent not only for sepsis and ARDS, but also for high altitude pulmonary edema and other hypoxia-associated lung diseases.

In-vivo acute hypoxia-induced pulmonary vascular leak contributes to increased pulmonary endothelial permeability [14]. In the current study, mice exposed to hypobaric hypoxia experienced pulmonary vascular leak, while those treated with anti-oxidant did not. Furthermore, animals treated with anti-oxidant had less HIF-1 α and VEGF expression in pulmonary vessels compared to untreated cohorts. Taken together, these observations strongly support our *in vitro* data suggesting that the ROS-HIF-VEGF pathway modulates leak in pulmonary vascular endothelium.

HPAEC exposed to hypoxia increased H₂O₂ production, nuclear HIF-1 α , cytosolic VEGF, and monolayer permeability. Treatment with an anti-oxidant cocktail, HIF-1 siRNA or VEGF inhibitor, sFlt-1, decreased downstream mediators in the ROS-HIF-VEGF-pulmonary endothelial leak pathway and antioxidant therapy lowered hypoxia-induced pulmonary vascular leak after 24 hours *in vitro*. There are many potential therapeutic agents targeting the various steps in this pathway such as antioxidants [3], PHD inhibitors (HIF-1), and VEGF-trap agents that may be useful in decreasing pulmonary leak associated with adult respiratory distress syndrome, acute ascent to high altitude, and other acute hypoxic conditions. In conclusion, these data implicate the ROS-HIF-VEGF pathway in hypoxia-induced pulmonary vascular leak.

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Figure 1.

Human pulmonary artery endothelial cell monolayers were cultured for 24 h at 21% (normoxia, NX) or 3% (hypoxia, HX) in the presence and absence of an antioxidant cocktail and the following parameters were measured; **A)** monolayer permeability (50 μM FITC-Albumin), **B)** hydrogen peroxide production, **C)** nuclear HIF-1 α by western blot analysis, **D)** representative nuclear HIF-1 α western blot, **E)** cytosolic VEGF concentration and. * $p < 0.05$ vs. HX-AO; ** $p < 0.05$ vs. NX

Figure 2. Human pulmonary artery endothelial cell monolayers were cultured for 24 h at 21% (normoxia, NX) or 3% (hypoxia, HX) in the presence of HIF-1 α specific siRNA or a scrambled RNA (scrRNA) and the following parameters were measured; **A)** representative Western blot for HIF-1 α , **B)** densitometry analyses of Western blot for nuclear HIF-1 α , **C)** monolayer permeability (25 μ M FITC-Albumin). * $p < 0.05$ vs. HX with siRNA

Figure 3.

Human pulmonary artery endothelial cell monolayers were cultured for 24 h at 21% (normoxia, NX) or 3% (hypoxia, HX) in the presence and absence of soluble VEGF receptor (sFlt-1) and monolayer permeability (50 μ M FITC-Albumin), was measured after one hour. * $p < 0.05$ vs. HX with sFlt-1

Figure 4.

Mice were exposed to either ambient pressure (5,280 ft; 1690 m; $P_B \sim 630$ mm Hg) or hypoxia (18,000 ft., $P_B \sim 380$ mm Hg) in the presence or absence of antioxidant. **A)** Pulmonary vascular leak as determined by Evans Blue Dye analyses. * $p < 0.05$ vs all other groups

Figure 5. Immunohistochemical staining for HIF-1 α and VEGF. Panel A: Representative bright-field photomicrographs of pulmonary vascular HIF-1 α and VEGF expression, 40X, — 75 μ M; Panel B: Enlarged photomicrographs of boxed area in panel A. CTRL- control for non-specific antibody binding.