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Hyperoxia enhances VEGF release from A549 cells via post-transcriptional processes

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Abstract

Exposure of animals to hyperoxia decreases lung VEGF mRNA expression concomitant with an acute increase in VEGF protein within the epithelial lining fluid (ELF). The VEGF concentration in ELF is in excess of that found in the plasma, leading to the hypothesis that hyperoxia stimulates the release of VEGF protein from stores within the extracellular matrix. To test this hypothesis in a cell culture system, we exposed A549 cells to 95% O₂ for 48 hrs followed by recovery in room air (RA) for 24 hrs. We found that Ox increased VEGF protein 2- to 3-fold within the medium at 48 hrs of exposure and during recovery. Heparin clearing revealed the medium to contain a 50:50 mixture of the heparin-binding (VEGF₁₆₅) and heparin-non-binding (VEGF₁₂₁) proteins and Ox to increase both proteins equally. Transcriptional activation of VEGF appears unlikely to explain the increase in VEGF protein as full-length and splice variant VEGF mRNA expression were unchanged by hyperoxia. Analysis of cell-associated VEGF proteins found that Ox increased the expression of VEGF₁₂₁ and VEGF₁₆₅ proteins. Blocking binding sites with exogenous heparin enhanced VEGF protein in the medium from RA grown cells while heparinase digestion of bound VEGF revealed a greater reserve of VEGF protein in RA cells. Collectively these findings indicate that hyperoxia enhances the expression of VEGF_{121/165} proteins and facilitates the release of VEGF₁₆₅ from cell-associated stores. Increases in VEGF in ELF may represent an adaptive response fostering cell survival and type II cell proliferation in O₂-induced lung injury.

Keywords

vascular endothelial growth factor; acute lung injury; hyperoxia

INTRODUCTION

Pulmonary epithelial-mesenchymal cell interactions foster the mutual maturation and structural development of alveolar and vascular systems. Regulation of this complex paradigm involves numerous growth factors including the vascular endothelial cell growth factor family

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(VEGF A-D). VEGF is an endothelial cell mitogen, whose primary pulmonary repository is the alveolar type II cell [1]. VEGF-A is a 34-46 kD homodimeric glycoprotein existing in several isoforms secondary to post-transcriptional mRNA splicing of the native VEGF gene. The resulting splice variants differ in the presence or absence of sequences encoded by exons 6 and 7, which contain basic amino acid sequences with high affinity for heparin and heparan sulfate moieties [2]. Variable splicing within these regions results in numerous isoforms, the most common of which are 206, 189, 165, 145, and 121 amino acids in length. Secreted peptides containing both exons (VEGF₂₀₆ and VEGF₁₈₉) are tightly bound to extracellular matrix (ECM) and cell surface heparan sulfates, while those lacking one exon (VEGF₁₆₅ and VEGF₁₄₅) possess modest diffusion and heparan-binding potential. VEGF₁₂₁, which lacks both exons, is acidic and easily diffusible. Within the lung, VEGF proteins 189, 165, and 121 are preferentially expressed by alveolar epithelial cells [1].

Regulation of VEGF protein expression is complex, involving transcriptional, post-transcriptional, translational, and post-translational processes. Transcriptional control involves a promoter region, which is responsive to hypoxia, oncogenes, and growth factors. Stabilization of hypoxia inducible factor 1 α (HIF-1 α) permits formation of a heterodimer with HIF-1 β , which binds the hypoxia-response element and induces VEGF mRNA expression [3]. In transcripts containing a lengthy 5'-untranslated region (UTR) with complex secondary structure like VEGF, cap-dependent translation is inefficient [4]. To bypass this inhibition, VEGF contains two internal ribosome entry sites (IRES), embedded start codons remote from the 5'-terminus. Post-translational processing of VEGF proteins occurs through proteolytic cleavage. The plasmin cleavage product of VEGF₁₆₅ (VEGF₁₁₀) is markedly less mitogenic toward endothelial cells than the parent protein [5].

Suppression of any of these regulatory pathways during development can attenuate VEGF protein expression and severely hinder pulmonary morphogenesis. Rodent and primate models of hyperoxia display similar decrements in VEGF protein and mRNA [6-9]. During neonatal oxygen injury in the rabbit, VEGF mRNA isoform expression is not only suppressed, but the ratio of VEGF₁₈₉ to VEGF₁₂₁ mRNA is dramatically reduced [10]. Despite the diminished VEGF expression, hyperoxia often induces a paradoxical rise in VEGF protein obtained from epithelial lining fluid (ELF) [6,10]. Given that plasma concentrations of VEGF are barely detectable during the same period, transudation of plasma VEGF into the alveolar space appears unlikely [6].

Pulmonary overexpression of VEGF has been shown to induce widespread capillary leakage and endothelial cell discontinuity, suggesting that enhanced alveolar VEGF promotes the early pulmonary edema noted during respiratory distress syndrome (RDS) and acute lung injury (ALI) [11,12]. Recent information also supports a reparative, anti-apoptotic role for VEGF in the RDS and ALI [13-15]. In the fetus, appropriate levels of pulmonary VEGF are paramount to normal vascular and alveolar development [16]. Clinical reductions in lung VEGF mRNA and protein are associated with the development of bronchopulmonary dysplasia (BPD) in premature infants, a form of neonatal chronic lung disease characterized by a reduction in alveolar number and microvascular density [17,18]. Conceptually, hyperoxia-induced VEGF secretion could have both positive and negative effects in the oxygen-exposed newborn lung, promoting cellular survival and propagating capillary leak into the alveolar space. Understanding the mechanism whereby enhanced VEGF secretion occurs, therefore, is essential to designing therapies aimed at improving pulmonary outcomes while minimizing lung injury.

Although it has been speculated that hyperoxia-induced increases in ELF VEGF emanates from proteolytic release of matrix- and receptor-bound VEGF isoforms, scientific validation of this premise is lacking. Accordingly, we undertook the present study to determine if *in vivo* results

could be replicated in cell culture and to ascertain the nature of the hyperoxia-mediated increases in secreted VEGF. We hypothesized that hyperoxia would increase secreted VEGF through reductions in cell surface and ECM-bound VEGF₁₆₅ in the type II cell-like adenocarcinoma cell line, A549. Our findings indicate that augmented VEGF secretion originates from greater VEGF protein expression in conjunction with the enhanced release of bound VEGF.

MATERIALS AND METHODS

Reagents and Supplies

Antibodies were purchased from the corresponding suppliers: anti-human VEGF-A_{189/165/121} (A-20) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-human VEGF-A from Becton Dickinson/Pharmingen (San Diego, CA), anti-HIF-1 α from Cell Signaling Technology (Danvers, MA), anti-HIF-2 α from Novus Biologicals (Littleton, CO), and anti- β -actin from Sigma Chemical (St. Louis, MO). Medium was purchased from Cellgro/Mediatech, Inc. (Herndon, VA). Human VEGF-A Quantikine ELISA kits were purchased from R&D Systems (Minneapolis, MN). Human VEGF and β -actin primers and controls for RT-PCR were obtained in the Dual-PCR kit from Maxim Biotech, Inc. (South San Francisco, CA). Real-time primers were produced by the core facility at Dartmouth College of Medicine. RNeasy RNA extraction kit was purchased from Qiagen (Valencia, CA). Heparin Sepharose 6 Fast Flow beads, L-(4,5-³H)leucine and mouse/rabbit HRP-IgG were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Pre-cast gels, Trizol, and Taq DNA polymerase were purchased from Invitrogen Corp. (Carlsbad, CA) and chemiluminescence detection was received from Pierce (Rockford, IL). Reverse Transcription (RT) System kit was purchased from Promega (Madison, WI). SYBR Green Master Mix was obtained from Applied Biosystems (Foster City, CA). Microcon centrifugal filter tubes were purchased from Millipore (Bedford, MA). VEGF₁₂₁ and VEGFA peptides were obtained from GenWay Biotech, Inc. (San Diego, CA). The remaining reagents and chemicals were obtained from Sigma Chemical (St. Louis, MO).

Cell culture and conditions

The human lung adenocarcinoma cell line, A549 (ATCC, Manassas, VA), was grown in Ham's F12 supplemented with 10% FBS, 100 IU/ml of penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. Normal, human lung fibroblasts (passage 4-10) were grown in a 1:1 mixture of Minimal Essential Medium for cell suspension and Leibowitz 15 medium supplemented with 10% FBS, 100 IU/ml of penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. Cells were allowed to attach overnight and the following morning, medium was refreshed and plates divided into room air (RA) and hyperoxic groups (Ox). Plates in the RA group were returned to the incubator to grow in room air + 5% CO₂ while plates in the Ox group were loaded into a sealed, humidified chamber flushed twice daily for 15 minutes with a 95% O₂/ 5% CO₂ gas mixture (Billups-Rothenburg Inc, Del Mar, CA). Plates were removed at 24 and 48 hours for analysis. After 48 hours of exposure, RA and Ox plates were removed from the chambers, supplemented with fresh medium, and replaced in the incubator in room air for an additional 24 hours (Recovery, Rec). To study the ability of heparin to displace VEGF from the cell, 10 μ g/ml of sodium heparin was added to each well prior to start of the experiments.

VEGF ELISA

Medium was removed from wells at specified times, centrifuged at 1000 $\times$ g to remove particulates, and frozen at -20°C. The remaining monolayers were trypsinized and the cells counted to normalize VEGF protein values. The VEGF ELISA was performed according to the manufacturer's specifications. Briefly, 200 μ l of medium were added to microplate wells containing a monoclonal VEGF_{165/121} antibody bound to the surface. Following incubation

for 2 hours, wells were washed three times and incubated for 2 hours with polyclonal VEGF-HRP conjugate. After washing, detection reagent was added for 20 minutes and plates immediately analyzed on a microplate reader (Tecan Sunrise, Mannedorf, Switzerland) at 450 nm with λ correction at 540 nm. VEGF values were derived from a standard curve of known concentrations of recombinant human VEGF₁₆₅. Each sample was analyzed in duplicate and averaged. To clear medium of heparin-binding proteins, 200 μ l of washed, heparin Sepharose 6 beads were incubated with 450 μ l of medium overnight at 4°C. Medium was separated from the beads by centrifugation and the ELISA performed as described above.

Real-time RT-PCR

RNA for real-time PCR was collected from trypsinized cells using Trizol and extracted with chloroform. The RNA was then precipitated in isopropanol, washed in 70% ethanol and vacuum dried. Pellets were reconstituted in RNase-free water and heated to dissolve. The amount and purity of the RNA was determined by spectrophotometry. Reverse transcription was performed using dNTP, oligo dt, AMV reverse transcriptase, and RNase inhibitor from a commercially available RT kit. Real-time PCR (DNA Engine Opticon, MJ Research) was then performed on 2 μ l of RT product using β -actin as control. RT product was mixed with SYBR Green Master Mix and VEGF primers (5'-TGTCTTGCTCTATCTTTCTT-3', lower; and 5'-CTTGCCTTGCTGCTCTACCT-3, upper'). Standard curves were run concurrently. Conditions for PCR were 2 minutes at 94°C, 15 seconds at 94°C, 1 minute at 60°C, 1 minute at 72°C, followed by 39 cycles of 15 seconds at 94°C, 1 minute at 60°C, 1 minute at 72°C. The specificity of the amplified PCR product was verified by the analysis of the melting curves, which were product-specific. Agarose gel electrophoresis of PCR product revealed a single band for VEGF and β -actin. A standard graph of C_T values obtained from serially-diluted genes was constructed for all reactions. C_T values were converted to gene copy number by instrument software and normalized to that of our control gene, β -actin. Changes in fold over baseline values for VEGF (T0) were then reported.

RT-PCR

RNA was collected using RNeasy according to the manufacturer's protocol. RNA quantity and purity were assessed spectrophotometrically. Synthesis of cDNA was conducted using 0.5 μ g of total RNA, RNase inhibitor, dNTPs, and reverse transcriptase, in RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 50 mM DTT) supplied in the Maxim Dual-PCR kit. PCR amplification was then performed using VEGF₁₈₉, VEGF₁₆₅, VEGF₁₂₁, and β -actin primers or VEGF and β -actin control cDNA from the kit. Equal volumes of PCR product were separated by electrophoresis on 1.5% agarose gels. Gels were then scanned on a Typhoon imager (GE Healthcare, Piscataway, NJ), digitized, and stored for analysis.

Protein synthesis

Protein synthesis was determined by measuring the incorporation of L-[4,5-³H]leucine (4 μ Ci/ml, final concentration) into protein after a 1-hour labeling period. Following incubation, monolayers were rinsed with ice-cold PBS prior to precipitation with 10% trichloroacetic acid for 15 minutes. Precipitated proteins were then dissolved in 0.5 N NaOH and the corresponding radioactivity measured by liquid scintillation counting.

Immunoblotting

Cell monolayers were rinsed with PBS, trypsinized, and lysed in western lysis buffer at 2000 cells/ μ l [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, and 0.5 mM PMSF (final concentrations)]. Lysates were passed through a 28-gauge needle three times and supernatants collected following centrifugation at 1000 $\times$ g. Lysates

from whole cell extracts representing equal cell numbers were resolved on either 12% or 4-12% Bis-Tris gels. Resolved proteins were transferred to PVDF membranes and incubated overnight at 4°C with primary antibodies [VEGF_{189/165/121} (1:500), HIF-1 α (1:1000), HIF-2 α (1:1000), and β -actin (1:5000)] in 5% non-fat dry milk blocking buffer. Following rinsing, membranes were incubated with the corresponding HRP-conjugated secondary antibody (1:5000) and the signal detected by chemiluminescence. Values were normalized to β -actin expression.

Determination of VEGF isoforms within the medium was performed on cell culture medium that was volume-reduced by centrifugation in 10 kD exclusion columns. Following volume reduction, equal quantities of medium were separated and blotted as described.

Digestion of heparin-bound proteins

At each time point, medium was replaced with Ham's F12 without supplements and incubated in the respective environment for 60 minutes. Cells were then scraped from the wells, spun at 1000 $\times$ g for 5 minutes, washed in PBS, and re-pelleted. Heparinase III, at a concentration of 3.3 mU/100k cells (0.05 U/ml), was then added to each tube and incubated for 60 minutes at 37°C. Tubes were centrifuged to pellet cells and supernatants removed. Heparinase digests were then concentrated in Microcon centrifugal filter tubes to collect proteins with size greater than 10 kD. Equal volumes of concentrated digests were then blotted as described.

Statistics

All studies were performed a minimum of 3 times. Data were analyzed using 2- or -way analysis of variance. When a significant trend of oxygen, time, or an interaction of both was identified, post-hoc analysis using Fisher's LSD testing to determine individual differences was performed. Real-time data at baseline was used to determine the fold change in RA and Ox groups. Normalized values were then analyzed by non-paired, student's t tests with Bonferroni correction for multiple comparisons. Data are listed as mean $\pm$ standard error and the level of significance set at $p < 0.05$.

RESULTS

Hyperoxia increases secreted VEGF

In actively dividing A549 cells, exposure to 95% O₂ for more than 48 hours results in necrotic cell death [19]. For this reason, we exposed A549 cells to 48 hours of 95% O₂ followed by 24 hours of recovery in room air. Our preliminary studies illustrated that this strategy resulted in growth arrest during the 72-hour study period and minimal cell death as documented by stagnant cell number, markedly diminished incorporation of [³H]thymidine into DNA, and Trypan blue exclusion (not shown).

To explore the effect of hyperoxia on VEGF, we measured medium-derived VEGF with a commercially available ELISA. Control studies indicated that medium containing 10% FBS had undetectable levels of VEGF while incubation of A549 cells with 100 μ M desferoximine, which stabilizes HIF, increased medium-derived VEGF by 4.5-fold within 24 hours. In growing A549 cells, VEGF concentrations in the medium increased over time. In RA cells, VEGF increased from 24 to 48 hours, but remained constant during recovery. By comparison, hyperoxia lead to a time-dependent increase in secreted VEGF concentrations with respect to time and compared to RA, such that concentrations at 48 hours and during recovery were 2- to 3-fold higher than in RA cells (Fig 1A). To insure that our findings were not unique to the A549 cell line, we also exposed normal, neonatal lung fibroblasts to hyperoxia, cells previously shown to increase VEGF protein expression in response to activated factor VII and TGF- β [20,21] Because these cells are more resistant to oxygen-induced cell death, we exposed them to hyperoxia for the full 72-hour study period [22]. As was observed in the A549 cells,

hyperoxia markedly increased secreted VEGF over time and relative to room air-exposed cells (Fig 1B). Given the similar responses in the two cell lineages, we chose to further investigate the effects of hyperoxia on VEGF using only A549 cells.

Hyperoxia increases the secretion of VEGF isoforms with and without heparin-binding capacity

The heparin and heparan-sulfate binding characteristics of the VEGF splice variants determines their solubility. Of the various isoforms, VEGF₁₂₁ is freely diffusible secondary to the absence of the heparin-binding region, while VEGF₁₆₅ remains partially bound to ECM forming a “reserve” of readily available protein. After volume-reducing the medium with fractional spin columns, we confirmed the presence of only two VEGF isoforms in the medium by immunoblotting (not shown). Further delineation of the contributions of the soluble VEGF isoforms was undertaken using affinity chromatography. Since the ELISA utilizes an antibody that recognizes only VEGF₁₆₅ and VEGF₁₂₁, we cleared the medium of heparin-binding moieties by prior assaying for VEGF. We observed that heparin clearing decreased VEGF secretion by approximately 50% in all groups regardless of the oxygen concentration or timing of the sample (Fig 2). Heparin-clearing had no impact on the time-dependent or hyperoxia-mediated increases in VEGF concentrations. This implies that medium-derived VEGF is a 50/50 mixture of VEGF₁₆₅ and VEGF₁₂₁ under both RA and Ox conditions.

Hyperoxia does not increase VEGF transcription

As an initial step toward deciphering the origin of hyperoxia-mediated increases in VEGF secretion, we explored the effects of hyperoxia on the transcriptional activation of VEGF. During hypoxia, expression of VEGF is enhanced by stabilization of HIF. To confirm the presence of intact HIF signaling, we immunoblotted whole cell lysates with HIF-1 α and HIF-2 α antibodies following treatment with desferoximine for 4 hours. As represented in Figure 3A, desferoximine significantly increased HIF-1 α and HIF-2 α protein expression. Despite the presence of intact HIF pathways, we were unable to detect HIF-1 α protein at 4, 24, and 48 hours of exposure or during recovery in either RA or Ox cells (Fig 3B). Although HIF-2 α protein was detectable at 4 hours through recovery, time failed to influence expression. The degree of HIF-2 α protein expression, however, was increased by hyperoxia, though only during recovery upon post-hoc analysis (Rec - RA: 0.70 $\pm$ 0.14 vs. 1.53 $\pm$ 0.25 arbitrary units, p <0.05; Fig 3B). The expression of VEGF mRNA derived by real-time PCR was not influenced by O₂ concentration at any time point as determined by t -tests with Bonferroni's correction (Fig. 3C). The lack of a hyperoxia effect was not secondary to an inability to induce VEGF mRNA expression, as incubation with desferoximine for 24 hours yielded a 4-fold increase in VEGF mRNA.

To exclude the possibility that post-transcriptional splicing increased expression of individual VEGF isoforms, we used RT-PCR to semi-quantitatively assess the levels of VEGF₁₈₉, VEGF₁₆₅, or VEGF₁₂₁ mRNA. As illustrated in Figure 3D, under basal conditions, all three isoforms were detected, with VEGF₁₆₅ and VEGF₁₂₁ displaying greater abundance than VEGF₁₈₉. When the A549 cells were exposed to hyperoxia, VEGF isoform mRNAs varied little from baseline or from RA cells.

Hyperoxia increases the expression of cell-associated VEGF proteins

After finding a lack of transcriptional regulation, we next sought to assess the translational processes involved in VEGF production. As an initial measure, we studied the impact of hyperoxia on global translation, by the measuring the incorporation of [³H]leucine into protein. We found that hyperoxia decreased global protein synthesis by 49%, 44%, and 28%, at 24 and 48 hours of exposure, and 24 hours of recovery, respectively (Fig 4). Due to the heparin and heparan sulfate binding capacity of VEGF, cell lysates generally represent intracellular and

ECM-bound isoforms. Using an antibody that recognizes VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉, we observed doublet bands separating at approximately 18 and 22kD under reducing conditions (Fig 5A). The migration of these bands was analogous to that of VEGF₁₂₁ and VEGF₁₆₅ protein monomers run as controls. Although not formally delineated, the doublets are likely to represent glycosylation of the individual monomers. Analysis of variance demonstrated that both time and hyperoxia increased VEGF₁₆₅ and VEGF₁₂₁ protein expression in A549 cells ($p<0.05$), but there was no interaction between these variables. Individual differences between RA and Ox cells were noted at 48 hrs and recovery for VEGF₁₂₁ and only during recovery for VEGF₁₆₅ (Fig 5B).

Hyperoxia alters VEGF binding

Cell membrane and ECM-associated VEGF constitutes a significant reserve of potentially releasable VEGF, particularly VEGF₁₆₅. To determine if hyperoxia alters bound VEGF pools, we incubated cell monolayers with sodium heparin prior to exposure to hyperoxia. This approach illustrated an effect of both time and hyperoxia on medium-derived VEGF (but no interaction). Specifically, we observed 30-40% more medium-derived VEGF in RA cells compared to Ox cells at each 24-hour time point (Fig 6A). Since VEGF₁₂₁ does not possess heparin-binding capacity, the observed increases are likely to represent VEGF₁₆₅ as the capture antibody does not bind larger VEGF species. We also digested heparin-bound VEGF from cells following exposure using heparinase III. Western blotting of volume-reduced digests demonstrated the presence of a single VEGF isoform. Curiously, this band was enhanced in cells treated with hyperoxia at 48 hours and 24 hours Rec. The combined results from these two experiments suggest that hyperoxia alters the concentration of membrane and/or ECM-bound VEGF.

DISCUSSION

Coordinated temporal and spatial expression of lung VEGF is crucial for the successful development of endothelial and epithelial cells into functional gas exchange units [23]. Aberrant VEGF expression leads to lung dysmorphogenesis, producing poor septal formation and a generalized emphysematous architecture [24,25]. Beginning in the saccular phase of lung development, increasing amounts of VEGF are detected within the lung parenchyma [25]. Delivery prior to term has the potential to diminish parenchymal VEGF expression and signaling through the introduction of heightened oxidant stress to the alveolar epithelium. VEGF secreted into alveolar lining fluid increases steadily from birth through the first week of life in preterm infants [26]. Concentrations of ELF VEGF are inversely correlated to gestational age, with preterm infants possessing levels 4-fold greater than term infants [18, 27]. Because transition from the intrauterine to extrauterine environment obligates exposure to “relative” hyperoxia, enhanced ELF VEGF could represent a direct effect of the change in ambient O₂ concentration. Exposure of newborn rabbits and 10-day-piglets to >95% O₂ results in increased lung lavage VEGF-A concentrations within 4-5 days of exposure [6,10]. In adult rabbits, the response is more rapid and short-lived, occurring within the first 24 hours of exposure [10]. The results presented herein confirm the ability of hyperoxia to enhance VEGF secretion in cell culture and suggest that post-transcriptional events contribute to this process.

Exposure of adult and newborn animals to hyperoxia is consistently reported to diminish VEGF mRNA within the lung, making the paradoxical rise of VEGF protein within lavage fluid somewhat of an enigma [6,10,28]. Further complicating the VEGF story is the rapid activation of extracellular-regulated kinases 1/2, c-jun N-terminal kinase, and p38 MAPK by hyperoxia [29]. Stimulation of these would be expected to prevent HIF ubiquitination, thereby stimulating VEGF transcription. In the current study, we found that incubation of A549 cells in 95% O₂ fails to alter HIF-1 α protein expression at 4 hours and beyond, but increases HIF-2 α during

recovery only. These outcomes are consistent with those recently reported by Asikainen and colleagues, demonstrating that exposure of A549 cells to 24 hours of hyperoxia does not influence VEGF mRNA, HIF-1 α , HIF-2 α protein, or total cell-associated VEGF protein levels [30]. Augmented HIF-2 α protein expression in the absence of changes in VEGF mRNA expression during recovery may indicate that HIF-2 α accumulation is exclusively cytoplasmic. HIF-2 α protein has been shown to escape oxygen-dependent degradation in immortalized mouse embryonal fibroblasts, leading to the progressive accumulation of cytoplasmic rather than nuclear HIF-2 α [31]. Because our measurements of VEGF mRNA are likely to reflect “steady-state” levels, we cannot exclude the possibility that enhanced nuclear HIF-2 α levels induced a transient augmentation of VEGF transcription. Nonetheless, HIF stabilization cannot account for the increases in VEGF₁₂₁ observed at 48 hours of exposure. In total, therefore, interpretation of our findings does not support HIF stabilization as a major mechanism for the increases in cell-associated VEGF protein during hyperoxia.

Post-transcriptional modification of the native VEGF mRNA by alternate splicing could also influence the relative distribution of the expressed messages leading to selective upregulation of specific VEGF isoforms. In newborn rabbits, hyperoxia suppresses total VEGF mRNA expression but increases the proportion of VEGF₁₂₁ expressed relative to VEGF₁₈₉ and VEGF₁₆₅ mRNA [10]. Similar findings have been reported in premature, non-human primates treated with oxygen and mechanical ventilation [32]. Transcripts corresponding to each of the major splice variants, VEGF₁₈₉, VEGF₁₆₅, and VEGF₁₂₁, have been previously identified in A549 cells [33]. In the current study, exposure to hyperoxia failed to alter the expression of any isoforms over time or in comparison to cells grown in room air, demonstrating that alternate splicing cannot account for the increase in medium-derived VEGF.

The lack of transcriptional activation led us to investigate translational and post-translational mechanisms for the increase in secreted VEGF. Angiotensin II (ANG II) has been documented to augment VEGF protein expression without altering mRNA levels or mRNA stability via a mechanism involving reactive oxygen species and cap-dependent translation [34,35]. During exposure to hyperoxia, however, both global protein synthesis and cap-dependent translation are depressed [22]. Despite this, VEGF protein expression could be enhanced by a cap-independent mechanism involving either the AUG 1039 or CUG 499 start codons regulated by two IRES elements located in the 5'-UTR [36]. Bornes and colleagues have recently reported that tertiary interactions in mature VEGF mRNA regulate IRES activity and translational efficiency of individual VEGF isoform mRNAs [36]. In this manner, modulation of IRES activity could rapidly switch VEGF translation to favor specific isoforms in the absence of increased transcription or cap-dependent translation. The observation that hyperoxia enhances VEGF₁₂₁ and VEGF₁₆₅ proteins in the absence of increased mRNA is consistent with such a mechanism.

To determine if hyperoxia displaced VEGF from binding sites, we studied cells pre-incubated with 10 μ g/ml of sodium heparin. This concentration has been shown to decrease the binding of ¹²⁵I-VEGF to endogenous and transfected VEGF receptor 2 (VEGFR2/KDR) [37]. We found that exogenous heparin increased medium-derived VEGF from cells grown in room air, but not in those exposed to hyperoxia. Treatment of cells with heparinase to dissociate VEGF from heparin/heparan sulfate-binding sites revealed a reserve of VEGF in the hyperoxia-treated cells. As A549 cells are known to express both VEGFR1 and VEGFR2 protein, the results of the present study could reflect a shift in VEGF binding from receptors to heparin moieties [38]. Indeed hyperoxia has been shown to reduce VEGFR1 and VEGFR2 expression in the lungs of rats [39]. Given that hyperoxia also has the potential to alter heparin sulfate synthesis and ECM turnover, direct measurements of VEGF receptor expression and VEGF binding affinity will be required to clarify the current findings.

In addition to the increased expression of VEGF₁₂₁ and VEGF₁₆₅ proteins, changes in the proteolytic activity of the cellular microenvironment could also participate in the increase in secreted VEGF. Plasmin is known to cleave VEGF to yield VEGF₁₁₀ and the anti-angiogenic isoform, VEGF_{165b} [40]. Plasmin activity is regulated by urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1). Hyperoxia has been reported to decrease uPA in airway epithelial cells and to increase PAI-1 in retinal pigment epithelial cells, effects that would diminish plasmin activity [41,42]. Indeed a recent report found increased PAI-1/uPA ratios in premature infants with respiratory distress syndrome who were treated with O₂ [43]. Hence increases in secreted VEGF could be explained by a reduction in VEGF clearance. Alternatively, cleavage of ECM-bound VEGF by matrix metalloproteinases could generate soluble, biologically-active, VEGF isoforms distinct from those transcribed [44]. Such isoforms may go undetected by the ELISA yet still contribute significantly to the pathophysiology. Thus although our findings strongly suggest that hyperoxia increases VEGF₁₂₁ and VEGF₁₆₅ secretion, we cannot exclude the presence of additional VEGF isoforms within the medium.

Lastly, we must concede that the present study has limitations. First, A549 cells, while used extensively throughout the literature to model alveolar epithelial cells, are a cancerous cell line incapable of making functional surfactant. Hence their response to hyperoxia may not accurately portray that of human type II cells. Furthermore, enhanced VEGF protein in lavage fluid is derived primarily from experimental models where O₂ is delivered to induce lung injury in an otherwise normal lung. Although similar inspired O₂ concentrations are used to treat patients, the goal is to restore physiologic arterial O₂ tensions, not to exceed them.

Nevertheless, confirmation that hyperoxia increases secreted VEGF may have important clinical implications. For the oxygen-exposed preterm newborn, augmentation of specific isoforms of VEGF in lung fluid may promote lung growth thereby reducing the severity of alveolar and microvascular underdevelopment seen in BPD [7,45]. Alternatively, isoform-specific increases in VEGF in lung lining fluid may augment alveolar-capillary permeability generating pulmonary edema common to the pathology both RDS and ALI [45,46]. Additional studies will be needed to delineate the relative contributions of VEGF_{121/165} in a given clinical situation and to decipher their respective roles of each isoform to lung protection and injury.

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A. **B.** **Figure 1.**

Hyperoxia increases secreted VEGF. VEGF in the medium was determined by ELISA and values corrected to cell number from the corresponding well. A. Histogram illustrating the effect of exposing A549 cells to 95% O₂ (Hyperoxia) or room air for 48 hrs and the allowing recovery in room air for an additional 24 hrs (+24 hrs Rec). B. Lower histogram shows the effect of exposing human neonatal lung fibroblasts to 95% O₂ or room air for 72 hrs. Columns represent mean (n=3) and bars standard error of the mean. *Denotes p<0.05 between hyperoxia and room air groups at same time point.

Figure 2.

Hyperoxia increases the secretion of VEGF isoforms with and without heparin-binding capacity. A549 cells were exposed to 95% O₂ (Ox) or room air (RA) for 48 hrs and then allowed to recover in room air for 24 hrs (+24 hrs Rec). Aliquots of medium were affinity purified with heparin-binding beads as described under MATERIALS AND METHODS. VEGF protein levels in the purified and unpurified samples were then determined by ELISA and corrected to cell number. Columns represent mean (n=3) and bars standard error of the mean. *Denotes p<0.05 between Ox and RA groups at same time point.

Figure 3.

Effect of hyperoxia on HIF-1 α or HIF-2 α protein and VEGF mRNA expression. A. The immunoblot is representative of HIF-1 α and HIF-2 α protein expression in A549 cells following treatment with desferoximine or exposure to room air (RA) or 95% O₂ (hyperoxia, Ox) for 4 hrs. B. Similar studies were conducted in cells exposed to RA or Ox at 24, 48 hrs, and 24 hrs Rec (+24 hrs). Histogram illustrates the effect of RA and Ox on HIF-2 α protein expression corrected to β -actin. Columns represent mean (n=3) and bars SEM. “*” signifies p<0.05 of control vs. hyperoxia during recovery (+24 hrs). C. Following exposure to RA or Ox, A549 cells were harvested, RNA collected, and real time PCR performed as described in MATERIALS AND METHODS. The figure displays fold change in VEGF mRNA expression

from baseline (T0 = prior to exposure) (n=3). D. Representative gel showing VEGF₁₈₉, VEGF₁₆₅, VEGF₁₂₁, β -actin mRNA transcripts derived by RT-PCR. Corresponding controls confirmed the presence and size of each isoform.

Figure 4.

Hyperoxia decreases global protein synthesis in A549 cells. A549 cells were incubated with [^3H]leucine during the final hour of each 24-hr period. Incorporation of label into protein was then determined on acid-insoluble extracts as described in MATERIALS AND METHODS. Results were expressed as dpm/1000 cells. Columns represent mean (n=4) and bars standard error of the mean. *Denotes $p < 0.05$ between Room air and hyperoxia groups at same time point.

Figure 5.

Hyperoxia increases the expression of cell-associated VEGF protein. A. Representative immunoblots illustrating the impact of hyperoxia on VEGF protein expression in A549 cells. Right blot shows migration pattern of full-length VEGF₁₂₁ and VEGF₁₆₅ peptides under identical reducing conditions. B. Histograms illustrating the effect of room air (RA) and hyperoxia (Ox) on VEGF₁₂₁ and VEGF₁₆₅ protein expression. Expression was assessed at 24 and 48 hrs of exposure, and following recovery for 24 hrs in room air (+24). Columns represent mean (n=6) and bars standard error of the mean. *Denotes p<0.05 of hyperoxia vs. room air at a given time point.

Figure 6.

Hyperoxia alters the binding of VEGF to heparin/heparan sulfate moieties. A. A549 cells were exposed to hyperoxia or room air in the presence or absence of 10 μ g/ml of sodium heparin. Following the exposure period, medium was removed and assayed for the presence of VEGF by ELISA. Values are expressed as a ratio of medium + heparin/medium alone. Heparin increased soluble VEGF content at each time in room air (RA) but not in hyperoxia (Ox) cells. Columns represent mean (n=3) and bars standard error of the mean. *Denotes $p < 0.05$ of Ox vs. RA at each time point. B. Representative immunoblot showing VEGF protein digested from cells with Heparinase III. Expression was assessed at 24 and 48 hrs of exposure, and following recovery for 24 hrs in room air (+24). Digests were prepared by adjusting Heparinase III concentration to cell number as described in MATERIALS AND METHODS.