Hypoxia upregulates VEGF expression in alveolar epithelial cells in vitro and in vivo

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Received 4 December 2001; accepted in final form 4 July 2002

Pham, Isabelle, Tokujiro Uchida, Carole Planes, Lorraine B. Ware, Robert Kaner, Michael A. Matthay, and Christine Clerici. Hypoxia upregulates VEGF expression in alveolar epithelial cells in vitro and in vivo. Am J Physiol Lung Cell Mol Physiol 283: L1133–L1142, 2002.—We investigated regulation of vascular endothelial growth factor (VEGF) expression by hypoxia in cultured and freshly isolated rat alveolar epithelial cells (AEC). In vitro, hypoxia increased VEGF mRNA and protein levels, with maximal stimulation at 0% O2 for 18 h. A similar upregulation of VEGF expression was found in alveolar epithelial type II (ATII) cells freshly isolated from rats exposed to 8% O2 for 24 h. In vitro, hypoxia-induced upregulation of VEGF mRNA was due to an increase in transcription, rather than an increase in RNA stability, inasmuch as the half-life of VEGF mRNA was unchanged. Upregulation of VEGF mRNA by hypoxia was mimicked by CoCl2 and desferrioxamine in normoxic AEC and was not prevented by inhibitors of reactive oxygen species, suggesting that hypoxic VEGF regulation involved an O2-dependent protein that requires ferrous ions but is independent of reactive oxygen species generation. In polarized ATII cells, VEGF protein was secreted at the apical and basolateral sides. Similarly, in rats, VEGF was secreted in bronchoalveolar lavage fluid. Hypoxia induced a twofold increase in VEGF protein at the apical side of ATII cells in culture and in bronchoalveolar lavage fluid. These findings suggest that release of VEGF synthesized by AEC may target not only endothelial cells but also other alveolar cells, including macrophages and epithelial cells.

VASCULAR ENDOTHELIAL GROWTH factor (VEGF), initially identified as vascular permeability factor, is an important growth and permeability factor for endothelial cells (8). VEGF is expressed in many tissues of the healthy adult rat and mouse, with the most abundant expression in the lung (18, 20). In normal lung, in situ hybridization studies have located VEGF transcripts at the luminal surface of alveolar walls, mostly in alveolar epithelial type II (ATII) cells (30). In accordance with the in vivo studies, VEGF transcripts were also detected in vitro in a human alveolar epithelial cell line (3). The transcripts correspond to the three major VEGF isoforms previously described: VEGF121 and VEGF165, which are secreted in a soluble form, and VEGF189, which remains associated with the cell surface or is primarily deposited in the extracellular matrix (11). Hypoxia is the best-characterized potent inducer of VEGF mRNA expression in the lungs and other tissues. In vivo, acute hypoxia induced a twofold increase in VEGF mRNA expression in the mouse lung; in the rat, chronic, but not acute, hypoxia upregulated lung VEGF mRNA expression (6). In contrast, hypoxic injury decreased and then increased VEGF expression during recovery in rabbit lungs (17).

The cellular and molecular mechanisms of hypoxia-induced upregulation of VEGF expression are not uniform and depend on the cell types. In a number of nonepithelial cells, such as cardiac myocytes, osteoblasts, and endothelial cells, the primary mechanism of hypoxic induction of VEGF mRNA is transcriptionally mediated (16, 21, 28). In this case, transcriptional activation of the VEGF gene is controlled by the specific binding of hypoxia inducible factor-1 (HIF-1) to the hypoxia-responsive element located in the 3′-flanking region of the gene. However, other data obtained in retinal epithelial and glioma cells indicate a modest role for transcriptional activation, demonstrating that hypoxic induction of VEGF mRNA occurs predominantly as a result of an increase in mRNA stability (12, 27). Moreover, the pathways by which cells sense low O2 concentration and transduce this signal to activate

http://www.ajplung.org
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the VEGF gene are not well characterized. A role for a putative O₂ sensor molecule has been suggested (16, 26), but recent studies propose that hypoxia stimulates mitochondrial production of reactive oxygen species (ROS), and this in turn activates HIF-1 and increases VEGF expression (5). Also the effect of hypoxia on VEGF release from polarized ATII cells has not been studied.

This study was undertaken, first, to investigate the characteristics of hypoxic induction of VEGF in ATII cells in vitro and in vivo. The second objective was to evaluate whether the upregulation of VEGF expression was associated with an increase in protein secretion and to determine the fractional release by the apical and basolateral surfaces of ATII cells. We used an in vitro hypoxic model in which primary culture of rat alveolar epithelial cells was exposed to 5 or 0% O₂ as well as an in vivo rat model using alveolar epithelial cells freshly isolated from rats that had been exposed to 8% O₂ for 24 h. Finally, we measured VEGF levels in pulmonary edema fluid from patients with severe hydrostatic pulmonary edema to determine VEGF concentration in a clinically relevant condition and VEGF concentration in the edema fluid vs. the plasma collected at the same time.

**MATERIALS AND METHODS**

All studies were approved by the Committee on Animal Research.

**Cell Isolation**

Alveolar epithelial cells were isolated from pathogen-free male Sprague-Dawley rats (180–200 g) as previously described (24). Rats were injected with pentobarbital sodium (30 mg/kg ip) and heparin sodium (1 U/g iv). After a tracheotomy, the rat was exsanguinated. Solution II (40–50 ml), which contained (in mM) 140 NaCl, 5 KCl, 2.5 sodium phosphate buffer, 10 HEPES, 2 CaCl₂, and 1.3 MgSO₄ (pH 7.40 at 22°C), was infused through the air-filled lungs via the pulmonary artery to remove blood from the vascular space. The lungs were removed from the thorax and lavaged to total lung capacity (8–10 ml) eight times with solution I, which contained (in mM) 140 NaCl, 5 KCl, 2.5 sodium phosphate buffer, 10 HEPES, 6 D-glucose, and 2 EGTA, to remove macrophages and twice with solution II. Then lungs were filled with 12–15 ml of elastase solution (porcine pancreas, twice crystallized, 40 U/ml, prepared in solution II) and incubated in a shaking water bath in air for 10 min at 37°C; then additional elastase solution was instilled for another 10 min. The lungs were minced in the presence of DNase I, and 5 ml of fetal calf serum (FCS) were added to stop the effect of elastase. The lungs were then sequentially filtered through 150- and 30-μm nylon mesh. The filtrate was centrifuged at 130 g for 8 min. The cell pellet was resuspended in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM d-glucose at 37°C. The cell suspension was plated at a density of 10⁶ cells/cm² in 25-cm² bacteriological plastic dishes to aid in the removal of macrophages by differential adherence. After incubation at 37°C in a 5% CO₂ incubator for 1 h, the unattached cells in suspension were removed and centrifuged at 130 g for 8 min. The resulting cell pellet (70% purity, >95% viability, 8–10 × 10⁶ cells/rat) was plated at a density of 7–10 × 10⁵ cells/cm² in six-well culture dishes. Culture medium consisted of DMEM containing 25 mM d-glucose, 10 mM HEPES, 23.8 mM NaHCO₃, 2 mM l-glutamine, 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10 μg/ml gentamicin incubated in a 5% CO₂-95% air atmosphere. Culture medium was changed 24 h after isolation and then on alternate days.

**Exposure to Hypoxia and Incubation in the Different Stimuli**

Two days after the cells were plated, growth medium was removed and replaced by a thin layer of fresh medium (0.15 ml/cm²) with 10% FCS to decrease the diffusion distance of the ambient gas. Culture dishes were then placed in a humidified airtight incubator with inflow and outflow valves, and the hypoxic gas mixture (0% O₂-5% CO₂-95% N₂) was delivered at 5 l/min for 20 min. The airtight incubator was kept at 37°C for 3, 9, or 18 h, while control normoxic cells were placed in a 21% O₂-5% CO₂-74% N₂ humidified incubator for the same period of time. In additional experiments, cells were exposed to mild hypoxia (5% O₂-5% CO₂-90% N₂) or hyperoxia (50% O₂-5% CO₂-45% N₂) as described above over an 18-h period. PO₂ levels assayed in culture medium were ~30, 60, 140, and 360 mmHg for 0, 5, 21, and 50% O₂, respectively. pH in culture medium measured at the end of exposure was not significantly different under normoxic and hypoxic conditions. When indicated, alveolar epithelial cells were incubated in 100 and 250 μM CoCl₂ for 3, 9, and 18 h, in 250 μM desferrioxamine (DFX) for 18 h, and in the antioxidants 1 mM N-acetyl cysteine (NAC) and 10⁻⁷ M diphenylene iodonium (DPI) for 18 h. DPI is an inhibitor of mitochondrial respiration at complex I and abrogates the formation of ROS by the mitochondria (10). To evaluate the mechanisms of VEGF mRNA induction by hypoxia, cells were incubated in a normoxic (21% O₂) or hypoxic environment (0% O₂) for 12 h, actinomycin D (5 μg/ml) was added, and cells were incubated for an additional 2–8 h in normoxic or hypoxic conditions. Total mRNA or protein was extracted and analyzed for VEGF and actin mRNA or protein.

**In Vivo Experiments**

Male Sprague-Dawley rats (200–220 g) were exposed to hypoxia (8% O₂) for 24 h in an airtight Plexiglas chamber as previously described (34). Throughout the experiment, rats were given free access to food and water. The O₂ level was monitored with an O₂ analyzer, and soda lime granules were used to remove CO₂. After 24 h of exposure, animals were anesthetized with pentobarbital sodium (30 mg/kg), and ATII cells were isolated by enzymatic digestion as previously described. RNA extraction with TRIzol reagent or protein extraction with an appropriate buffer was performed on freshly isolated ATII cells for RNase protection assay and Western blot.

**RNase Protection Assay**

Cells cultured in 35-mm plastic dishes were lysed in a buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, and 0.5% sarcosyl and directly used for RNase protection assay as previously described (24). tRNA equivalent to 10¹⁶ cells or 20 μg of yeast tRNA (Boehringer) were cohybridized with 10⁶ cts per minute (cpm) for rat VEGF₁₆₄ and 5 × 10⁶ cpm for β-actin probes in 80% formamide, 40 mM PIPES (pH 7.4), 400 mM NaCl, and 1 mM EDTA at 50°C overnight. RNase digestion [RNase A (40 μg/ml) and T1 (2 μg/ml), Boehringer] was performed at 30°C.
for 60 min. Then digestion with proteinase K (125 μg/ml; Boehringer) was done at 37°C for 30 min. After phenol extraction and ethanol precipitation, protected fragments were separated by urea-polyacrylamide gel electrophoresis. Gels were fixed with 10% acetic acid and vacuum dried before exposure to Kodak X-Omat AR 5 film, and the signal was quantitated from the gel using direct radioactivity measurement with an Instant Image (Packard Instruments). Actin expression was used as an internal standard, because hypoxia did not significantly modify the level of actin mRNA. Results were expressed as the ratio of VEGF mRNA to actin mRNA.

Antisense RNA probes were synthesized using a T7 polymerase in vitro synthesis kit (Promega) in the presence of [γ-32P]UTP (15 TBq/mmol). Rat VEGF cRNA probe was synthesized from a cDNA insert (168–517) in pBluescript plasmid (provided by A. Ladoux). The probe was 350 nt long with a protected fragment of 250 nt. Mouse β-actin was synthesized using a cDNA insert in pGEM-3. The probe was 190 nt long with a protected fragment of 155 nt (nt 696–831).

**VEGF Protein Expression**

**Western blot analysis.** ATII cells in culture (30 μg protein) were analyzed for VEGF protein expression after lysis in Tris buffer containing 0.5% sodium deoxycholate, 0.1% SDS, and 1% NP-40. Normoxic and hypoxic samples were boiled for 5 min under denaturing conditions (2% β-mercaptoethanol) and resolved by 12% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and, after nonspecific blocking with milk (10% in 0.1% PBS-Tween), incubated with a polyclonal goat anti-VEGF (A-20, Santa Cruz Biotechnology) at 1:1,000 overnight at 4°C. For a positive control, 100 ng of recombinant VEGF1–147 were also probed. The membrane was then incubated with a peroxidase-conjugated avidin secondary antibody and visualized using a chemiluminescence kit (ECL). For quantification, membranes were also stained with an antibody against constitutive mouse β-actin. Autoradiograms were quantitated by densitometric analysis and expressed as percentage of normoxic control.

**ELISA measurements.** For in vitro experiments, ATII cells were grown to confluence onto polycarbonate membranes in medium supplemented with 10% FCS. After 4 days of culture, the chambers, which consisted of an apical part containing the filter membrane inserts with the cell monolayer and a basolateral portion, were washed, and the culture medium was replaced with serum-free medium. Cells were then exposed to normoxia (21% O2) or hypoxia (0% O2) for 18 h or transforming growth factor-β1 (TGF-β1, 10 ng/ml), and VEGF protein concentration was measured in the apical and basolateral samples of the culture medium. For in vivo experiments, rats were exposed to normoxia (21% O2) or hypoxia (8% O2) for 24 h. A bronchoalveolar lavage (BAL) was performed three times with 5 ml of PBS. BAL was centrifuged (1,000 g for 5 min), and VEGF was measured on the supernatant by an ELISA method using an anti-mouse anti-VEGF antibody (R & D systems, Minneapolis, MN) that specifically recognizes the VEGF165 isoform.

**Assay of Alveolar Epithelial Monolayer Permeability**

After ATII cells were seeded onto polycarbonate membranes, monolayers were obtained after 4 days of culture. Cells were exposed to normoxia (21% O2) or hypoxia (0% O2) for 18 h. At the end of exposure, the chambers, consisting of an apical part containing the filter membrane inserts with the cell monolayer and a basolateral part, were washed twice with culture medium. DMEM (2 ml) without serum was added to the basolateral chamber, and fluorescein isothiocyanate (FITC)-conjugated Dextran 20 (FITC-dextran; mol wt 20,000; 1 mg/ml) in 1 ml of DMEM without serum was added to the apical side. The appearance of FITC-dextran in the basolateral chamber was measured after different time periods in small aliquots of the basolateral buffer. Results were expressed as the ratio of dextran concentration determined after different incubation times to the total concentration of FITC-dextran added to the upper chamber at the start of the experiment. Throughout the experiment, chambers were kept at 37°C, and care was taken to ensure that fluid levels in the apical and basolateral chambers were equal.

**Measurement of Alveolar Epithelial Permeability in Vivo**

In vivo, the flux of albumin across the alveolar epithelial barrier was evaluated by measuring 125I-albumin (the vascular protein tracer) in the air spaces of the lung. Briefly, after rats were exposed to normoxia (21% O2) or hypoxia (8% O2) for 21 h, 125I-albumin was injected intraperitoneally (5 μCi/ml). The animals were replaced in normoxia or hypoxia. After 3 h, rats were anesthetized and a tracheostomy was performed. A BAL, performed three times with 5 ml of PBS, was centrifuged, and the radioactivity of the supernatant was measured. The 125I-albumin counts in the air space samples were expressed as the ratio of plasma counts. This ratio provided a good index of equilibration of the vascular protein tracer into the air space compartment, as we and others have shown in other experimental studies of lung epithelial permeability (23, 34).

**Human Studies**

Human studies were approved by the Committee for Human Research at the University of California, San Francisco. Forty mechanically ventilated patients with severe hydrostatic pulmonary edema were identified. Undiluted pulmonary edema fluid was sampled using a 14-Fr tracheal suction catheter within 1 h after tracheal intubation, as previously described (33). Simultaneous plasma samples were obtained. All samples were centrifuged (3,000 g for 10 min), and supernatants were stored at −70°C until further use. The medical record of each patient was reviewed for pertinent clinical data, including cause of pulmonary edema, severity of hypoxemia, duration of mechanical ventilation, and hospital survival. VEGF levels were measured in thawed edema fluid and plasma samples by ELISA kit (R & D Systems) using a monoclonal mouse antibody to recombinant human VEGF165. Each edema sample was measured at least in duplicate, and the results were averaged.

**Materials**

All chemicals were purchased from Sigma Chemical (St. Louis, MO). Radioactive tracers were provided by Amersham (Aylesbury, UK). Culture media and reagents were obtained from Gibco-BRL (Cergy-Pontoise, France) and plastic ware from Costar (Cambridge, MA).

**Statistical Analysis**

For in vitro and animal studies, values are means ± SE, with n = 3–6 (each point for 1 animal) in separate cultures for in vitro experiments or n = 3–4 rats for in vivo experiments, and expressed as percentage of control. One-way variance analyses were performed, and, when allowed by the F value, results were compared by the modified least significant difference. For human studies, results are expressed as median (25th–75th percentile) or mean ± SD where appro-
RESULTS

Hypoxia Upregulates VEGF mRNA in ATII Cells in Vitro and in Vivo

Exposure of ATII cells to 0% O\textsubscript{2} (25 mmHg P\textsubscript{O\textsubscript{2}}) induced a time-dependent increase in VEGF mRNA levels that was significant after 9 h of exposure and peaked after 18 h, with a threefold increase from baseline (Fig. 1). The effect of hypoxia was also dependent on the level of hypoxia: mild hypoxia (5% O\textsubscript{2}, 75 mmHg P\textsubscript{O\textsubscript{2}}) for 18 h induced a slight increase in VEGF mRNA (150% of normoxic value), and 0% O\textsubscript{2} for the same period of time induced a 300% increase (Fig. 2). To evaluate whether VEGF expression is dependent on O\textsubscript{2} concentration, ATII cells were also exposed to 50% O\textsubscript{2} (340 mmHg P\textsubscript{O\textsubscript{2}}) for 18 h. Hyperoxia did not significantly change VEGF mRNA levels (Fig. 2).

To assess the effect of hypoxia on VEGF mRNA in vivo, rats were exposed to 8% O\textsubscript{2} for 24 h, and VEGF mRNA levels were compared in ATII cells freshly isolated from hypoxic and normoxic rat lungs. Hypoxia induced a threefold increase in the level of VEGF mRNA level compared with normoxia (Fig. 3). These results indicate that in vivo and in vitro exposure to hypoxia increased VEGF mRNA expression and that 0% O\textsubscript{2} exposure in vitro induced an equivalent stimulation to 8% O\textsubscript{2} exposure in vivo (Fig. 3).

Effect of Actinomycin D on Hypoxia-Induced VEGF mRNA and Transcription Rate

To investigate the mechanism of hypoxia-induced increase of VEGF mRNA, ATII cells were treated with actinomycin D for 3 h before they were exposed to hypoxia (0% for 18 h). Actinomycin D abolished the hypoxia-induced increase of VEGF mRNA (Fig. 4), suggesting that active RNA transcription was required for this effect. To test whether hypoxia was associated with change in mRNA stability, ATII cells were cultured in normoxic and hypoxic (0% for 18 h) conditions for 12 h, actinomycin D was added, and the cells were replaced in hypoxia or normoxia for 0–8 h. Figure 4 shows that actinomycin D inhibited new RNA transcription in normoxia and hypoxia and indicates that there is no difference in the stability (i.e., rate of degradation) of the existing VEGF over the time period of this study.

Effect of CoCl\textsubscript{2} and DFX on VEGF mRNA

The transition metal CoCl\textsubscript{2} and the iron chelator DFX have been shown to mimic hypoxic state in sev-
eral hypoxia-responsive genes (9). CoCl₂ is thought to substitute for iron in the porphyrin ring of the O₂ sensor, binding O₂ with less affinity, thereby locking it in a deoxygenated conformation. In ATII cells, CoCl₂ induced a concentration-dependent increase in VEGF mRNA levels: 100 μM CoCl₂ had no significant effect, whereas 250 μM CoCl₂ increased VEGF expression by threefold, reaching the level of stimulation induced by hypoxia (Fig. 5A). The CoCl₂-induced increase in VEGF expression was also time dependent, and although 3 h had no significant effect, the maximal induction was achieved at 9 h of exposure (Fig. 5B).

DFX mimics hypoxia by chelating iron. Figure 6 shows that VEGF expression in normoxic cells was increased to a similar level by DFX (250 μM for 18 h), CoCl₂ (250 μM), and hypoxia (0% O₂) for the same period of time.

Effects of Antioxidants on Hypoxia-Induced Increase in VEGF mRNA

The role of ROS generation in the induction of VEGF mRNA expression during hypoxia or CoCl₂ incubation was assessed by incubating ATII cells with antioxidants. In cells subjected to 18 h of hypoxia (0% O₂) and cells incubated in 250 μM CoCl₂ for 18 h, VEGF mRNA expression increased 2.5- and 2-fold, respectively. The antioxidant NAC, a scavenger of H₂O₂, or DPI, a mitochondrial ROS inhibitor, did not abolish the transcriptional response to hypoxia or CoCl₂, indicating that ROS generation was not required in hypoxia and CoCl₂ upregulation of VEGF mRNA (Fig. 7).

Effect of Hypoxia on VEGF Protein Synthesis and Secretion

VEGF protein measured by Western blot analysis showed that, in vitro, 0% O₂ hypoxia for 18 h induced a 2.5-fold rise in the quantity of VEGF in alveolar epithelial cells in culture (Fig. 8). To determine whether the hypoxia-induced increase in VEGF mRNA in vitro was associated with increased VEGF protein secretion, VEGF protein was measured in the culture medium. Exposure of ATII cells to hypoxia (0% O₂ for 18 h) induced a small but significant increase in VEGF₁₆₅ protein in the medium (3.7 ± 0.2 vs. 3.0 ± 0.1 pg/μg protein, P < 0.05). To evaluate whether the secretion of VEGF is polarized, VEGF protein concentration was measured in the medium of apical and basolateral compartments of ATII cells grown on filters and exposed to normoxia and hypoxia (0% O₂ for 18 h). Under normoxic conditions, the concentration of VEGF was 10-fold higher on the basolateral side than on the apical side.

![Figure 3: Induction of VEGF expression by hypoxia in rat ATII cells in vitro (primary cultures at day 3) and in vivo (freshly isolated ATII cells). ATII cells in primary culture were exposed to 21 or 0% O₂ for 18 h. Freshly ATII cells were isolated from lungs of rats exposed to 21 or 8% O₂ for 24 h. RNase protection assays for VEGF and ß-actin were done on cell lysates or after RNA extraction. VEGF mRNA expression was normalized to corresponding ß-actin mRNA. Value determined in normoxic cells was set at 100%. Values are means ± SD (n = 4 for cultured ATII cells and n = 4 for freshly isolated ATII cells). *P < 0.001 vs. 21% O₂.](http://ajplung.physiology.org/)

![Figure 4: Effect of actinomycin D on VEGF mRNA transcription and stability under normoxia and hypoxia. A: ATII cells were treated with actinomycin D (5 μg/ml) 3 h before and during exposure to normoxia or hypoxia (0% O₂ for 18). B: ATII cells were incubated in a normoxic (21% O₂) or hypoxic environment (0% O₂) for 12 h, and actinomycin D (5 μg/ml) was added to normoxic or hypoxic cells. Cells were incubated for an additional 2–8 h in standard culture conditions. VEGF mRNA expression was normalized to corresponding ß-actin mRNA. Value determined at time 0 was set at 100%. Values are means ± SD (n = 4). For normoxic cells, y = −9.2x + 9.7; for hypoxic cells, y = −10x + 10. There was no significant difference between slopes (F = 0.11, P = 0.73). *P < 0.01 vs. 21% O₂.](http://ajplung.physiology.org/)
apical side. When the cells were exposed to hypoxia, VEGF protein concentration increased twofold in apical culture medium, whereas no change was detectable in the basolateral culture medium (Fig. 9). In vivo, VEGF protein was measured in the BAL fluid of normoxic and hypoxic rats (8% O2 for 24 h). Hypoxia induced a twofold increase in VEGF protein concentration in the BAL fluid of hypoxic rats compared with normoxic rats (Fig. 10). To determine whether the apical increase in VEGF secretion under hypoxia is related to an increase in the transfer of VEGF from the basolateral to the apical side from an increase in epithelial permeability, we measured permeability of epithelial cells in vitro and in vivo. The permeability of ATII cells cultured on Transwell filters, measured by the transfer of FITC-dextran from the apical to the basolateral side over 90 min, was zero in normoxia and hypoxia. In vivo, permeability of alveolar epithelium...
was not changed under hypoxia: 0.08 ± 0.06 vs. 0.05 ± 0.03 ml of alveolar 125I-labeled plasma equivalent per total lung in normoxic and hypoxic rats, respectively.

**Effect of TGF-β1 on VEGF Expression in ATII Cells In Vitro**

We also tested the effect of TGF-β1, another potent inducer of VEGF synthesis in ATII cells. TGF-β1 induced a 1.5-fold increase of VEGF mRNA expression in ATII cells, and, 18 h was less effective than hypoxia (0% O2 for 18). TGF-β1 also increased VEGF secretion at the apical side from 2.3 ± 0.1 to 3.3 ± 0.2 pg/μg protein without a change in basolateral secretion (7.3 ± 0.3 vs. 8.5 ± 1.0 pg/μg protein).

**Human Studies**

Because VEGF was increased in the BAL of rats exposed to hypoxia, we also studied VEGF levels in the undiluted pulmonary edema fluid of 40 patients with severe hydrostatic pulmonary edema and hypoxemia. All patients were mechanically ventilated with a mean arterial PO2-to-inspired O2 fraction ratio of 121 ± 76, consistent with severe hypoxemia. Median VEGF levels were 40-fold higher in edema fluid than in simul-
taneously sampled plasma, indicating local production of VEGF in the alveolar compartment (Fig. 11A). Median edema fluid VEGF levels were threefold higher in nonsurvivors than in survivors (Fig. 11B). Edema fluid VEGF levels were also higher in patients with <7 days of unassisted ventilation over the first 28 days: 1,658 (25th–75th percentile = 757–7,174) vs. 503 (25th–75th percentile = 146–1,837) ng/ml (P = 0.033).

**DISCUSSION**

The primary findings of this study can be summarized as follows. O₂ deprivation upregulates VEGF expression in cultured and freshly isolated alveolar epithelial cells, whereas hypoxia does not. In ATII cells, a hypoxia-induced increase in VEGF expression (1) depends most likely on increased mRNA transcription, rather than increased mRNA stability, 2) likely involves a hemoprotein but is independent of mitochondrial ROS generation, and 3) is associated with an increase in VEGF protein levels. In polarized ATII cells, which predominantly secreted VEGF at the basolateral side, hypoxia markedly enhanced VEGF secretion at the apical side without changing basolateral secretion. Also, very high levels of VEGF are present in the pulmonary edema fluid of patients with hypoxic respiratory failure from hydrostatic pulmonary edema. In the kidney and brain, which express VEGF mRNA under control conditions, hypoxia upregulates this expression (18, 19). Several previous studies have reported that the lung is a major site of VEGF synthesis in the mouse and rat, and immunohistochemistry studies have demonstrated that the synthesis occurred predominantly in alveolar epithelial cells (18, 20). In this study, VEGF mRNA was expressed under normoxic conditions in freshly isolated and cultured rat ATII cells. Exposure of cultured alveolar epithelial cells to hypoxia induced a time- and concentration-dependent increase in mRNA levels: a maximal stimulation was observed for prolonged exposure times (>9 h) and for severe hypoxia (0% O₂). In ATII cells freshly isolated from rats exposed to acute hypoxia (8% O₂ for 24 h), VEGF mRNA was increased, and, interestingly, the stimulation was equivalent to that observed in vitro after 6% O₂ exposure for 18 h. These results are in agreement with the observation of Marti and Risau (18) in the mouse showing that hypoxia (6% O₂ for 6 h) increased VEGF mRNA expression in ATII cells. However, our study did not agree with a previous study by Christou et al. (6) in which acute (15–48 h) moderate hypoxia (10% O₂) did not increase lung VEGF mRNA in the rat. This discrepancy may be related to the severity of hypoxia (i.e., 8 vs. 10% O₂) and to experimental differences: we analyzed isolated ATII cells, whereas only lung homogenates were analyzed by Christou et al.

The hypoxia-induced increase of VEGF mRNA in alveolar epithelial cells was likely related to transcriptional activation of the VEGF gene with no change in mRNA stability, because 1) the inhibitor of transcription, actinomycin D, inhibited hypoxic VEGF mRNA upregulation and 2) no significant difference was noted between the mRNA half-life of normoxic and hypoxic cultures. These data agree with findings in endothelial cells (21), cultures of cardiac myocytes, and osteoblasts (28), in which hypoxic induction of VEGF mRNA was abolished by actinomycin D. In contrast, Ikeda et al. (12) in glioma cells and Shima et al. (27) in human retinal epithelial cell lines reported that hypoxic induction of VEGF mRNA was due to a modest transcriptional effect but was primarily related to an increase in mRNA stability. Transcriptional upregulation of the VEGF gene by hypoxia has been shown to be controlled via an enhancer element, hypoxia responsive element, located in the 3'-flanking region of the gene, which binds specific transcription factors, HIF-1α and endothelial PAS domain protein-1 (EPAS-1), which are activated and stabilized under hypoxia. We and others have recently reported that the human cell line A549, which possesses the characteristics of ATII cells, constitutively expressed HIF-1α and EPAS-1 in normoxic conditions, the expression of which were increased by hypoxia (25, 31). Further studies will be done to determine whether a hypoxia-induced increase of VEGF...

![Fig. 11. Box plot summaries of VEGF in pulmonary edema fluid vs. plasma of 40 patients with severe hydrostatic pulmonary edema (A) and survivors vs. nonsurvivors of severe hydrostatic pulmonary edema (B). Box encompasses 25th–75th percentile, horizontal bar represents median, error bars show 10th–90th percentile.](image-url)
mRNA transcription in ATII cells occurs through HIF-1α and/or EPAS-1 induction.

The mechanisms by which ATII cells sense decreased O₂ concentration and transduce this signal to induce VEGF expression have not been well elucidated. In one model, the O₂ sensor involves an O₂-binding protein that requires ferrous ions, and in this hypothesis, CoCl₂ mimics hypoxia in normoxic cells by substituting for ferrous ions (9). This protein was initially believed to be a heme protein, but recent studies indicate that hydroxylase enzymes that require ferrous ions as cofactors and are involved in HIF-1 stabilization may also be good candidates (13, 14). A second model has emerged recently in which an increase of mitochondrial ROS generation during hypoxia may serve as an O₂ sensor (5). According to this hypothesis, mitochondrial ROS appear to directly activate HIF-1 and, thereby, increase VEGF expression. In this study, the hypoxic induction of VEGF mRNA likely involved an O₂-dependent protein that requires ferrous ions but is independent of ROS generation, because 1) CoCl₂ and DFX treatment of normoxic ATII cells mimicked the effect of hypoxia and 2) neither the mitochondrial antioxidant DPI nor the nonmitochondrial antioxidant NAC blocked the hypoxic induction of VEGF mRNA, and exposure to hyperoxia did not change VEGF mRNA expression. These results are in accordance with those recently reported by Vaux et al. (32), who showed that, in a human lung alveolar epithelial cell line that lacked a functional mitochondrial respiratory chain, the induction of HIF-1 and O₂-sensitive genes by hypoxia was unchanged.

VEGF protein is constitutively expressed by ATII cells in vitro (3) and in vivo (6). In this study, VEGF protein was detected in normoxia in ATII cells and in the culture medium, and hypoxia induced an increase of protein levels in the cells and in the medium. An interesting new finding was that in normoxia the bulk of VEGF secreted by ATII cells grown on filters was detected in the basolateral medium and that less VEGF secretion was detected in the apical medium. This observation agrees with a previous report showing that, in human fetal lung, VEGF was mainly located at the basement membrane of distal airway epithelial cells (1). A polarized secretion of VEGF has also been reported in human retinal pigment cells (2). The presence of VEGF protein in the BAL fluid of normoxic rats indicates that, in vivo, VEGF was secreted, at least in part, at the apical surface. High levels of VEGF in the alveolar compartment were also measured in humans with acute respiratory failure from hydrostatic pulmonary edema and severe hypoxemia. The functional role of VEGF secretion by alveolar epithelial cells remains unclear. It can be hypothesized that VEGF secreted at the basolateral side targets the capillary endothelium and acts through VEGF receptors located on endothelial cells (8, 29). However, an autocrine or paracrine regulatory role is not excluded, inasmuch as a recent study has reported the presence of functional VEGF receptors, neuropilin-1, which binds only VEGF₁₆₅, and kinase insert domain-containing receptor, on distal airway epithelial cells of human fetal lung explants (4). Addition of exogenous VEGF in these explants induced epithelial cell proliferation and increased the expression of surfactant proteins, whereas inhibition of VEGF receptors in the rat causes lung cell apoptosis and enlargement of the air spaces (15). Finally, because VEGF receptor-1 (Flt-1) has been reported to be present on monocytes/macrophages, the activation of which led to monocyte activation and chemotaxis (7), it could be hypothesized that apical secretion of VEGF may play a role in the recruitment of immune cells in the alveolar space.

Exposure to hypoxia induced consistent changes in vitro and in vivo: in both systems, apical secretion was increased. The increase of VEGF in the apical compartment was not related to passage of VEGF from the basolateral to the apical side, because permeability of alveolar epithelial cells grown on filters, in vitro, as well as permeability of alveolar epithelium, in vivo, was unchanged after exposure to hypoxia. The observation that VEGF secretion increased only on the apical side during hypoxia is unexpected. Interestingly, TGF-β₁, a potent inducer of VEGF synthesis (3), also increased VEGF apical secretion, whereas no change occurred at the basolateral side. One explanation could be that, in our culture conditions, secreted VEGF remained partly bound to the extracellular matrix and that the value measured in the culture medium did not reflect the entire basolateral secretion. However, VEGF₁₆₅ is a soluble isoform, and the magnitude of the basolateral secretion in normoxia suggested that, if it occurred, binding of VEGF₁₆₅ to the extracellular matrix was small. Finally, a recent study has reported that inducible endoplasmic reticulum chaperone O₂-regulated protein, ORP 150, was expressed along with VEGF in human wound macrophages and was required to promote the intracellular transport and secretion of VEGF in a hypoxic environment (22). Thus it is possible that, in alveolar epithelial cells, hypoxia modifies VEGF protein sorting through the modifications of such proteins.

In conclusion, this study demonstrates that, in adult rat lungs, VEGF synthesis by ATII cells is induced by hypoxia, probably by a transcriptional mechanism, and further studies are needed to determine whether this occurs through HIF-1α or EPAS-1 induction. Second, the secretion of VEGF protein occurs at the apical and basolateral sides of ATII cells, suggesting that the targets of this growth factor may include the lung capillary endothelium and alveolar macrophages and perhaps even an autocrine effect on lung epithelial cells. The high concentrations of VEGF in the edema fluid compared with the plasma of hypoxic patients with severe hydrostatic pulmonary edema emphasize that VEGF is secreted in high quantities in the lung in a clinically relevant condition and that more studies are needed to understand the biological effects of VEGF in the lung under pathological conditions.
This work was supported by grants from Université Paris 13 and Fondation pour la Recherche Médicale and National Heart, Lung, and Blood Institute Grants HL-51856 and HL-51854.

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