Effects of hyperoxia on VEGF, its receptors, and HIF-2α in the newborn rat lung

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Hosford, Gayle E., and David M. Olson. Effects of hyperoxia on VEGF, its receptors, and HIF-2α in the newborn rat lung. Am J Physiol Lung Cell Mol Physiol 285: L161–L168, 2003. First published March 7, 2003; 10.1152/ajplung.00285.2002.—Signaling through the hypoxia inducible factor (HIF)-VEGF-VEGF receptor system (VEGF signaling system) leads to angiogenesis and epithelial cell proliferation and is a key mechanism regulating alveolarization in lungs of newborn rats. Hyperoxia exposure (>95% O2 days 4–14) arrests lung alveolarization and may do so through suppression of the VEGF signaling system. Lung tissue mRNA levels of HIF-2α and VEGF increased from days 4–14 in normoxic animals, but hyperoxia suppressed these increases. Levels of HIF-2α and VEGF mRNA were correlated in the air but not the O2-treated group, suggesting that the low levels of HIF-2α observed at high O2 concentrations are not stimulating VEGF expression. VEGF164 protein levels increased with developmental age, and with hyperoxia to day 9, but continuing hyperoxia decreased levels by day 12. VEGFR1 and VEGFR2 mRNA expression also increased in air-exposed animals, and these, too, were significantly decreased by hyperoxia by day 9 and day 12, respectively. Receptor protein levels did not increase with development; however, O2 did decrease protein to less than air values. Hyperoxic suppression of VEGF signaling from days 9–14 may be one mechanism by which alveolarization is arrested.

alveolarization; oxygen; septation

Hypoxic injury in developing lungs leads to microvascular damage, diminished epithelial cell proliferation, and arrest of alveolarization. In 1983, Roberts et al. (30) studied the effect of 6 days of exposure of neonatal rats to >95% O2 on the microvasculature of the lung and showed a decreased density of small and large capillaries in the lung. Hyperoxia during the neonatal period in rats also interferes with the process of septation; alveolar number and internal surface area are decreased, and the parenchymal airspace is enlarged (4, 23, 33).

Vascular endothelial growth factor (VEGF) is a key regulator of lung development. It acts through two distinct tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/flk-1), and is essential for endothelial cell differentiation as well as the sprouting of new capillaries from preexisting vessels (angiogenesis; see Refs. 12 and 29). Angiogenesis has been shown to be necessary for the development of the alveoli in the newborn rat. Jakkula et al. (15) administered inhibitors of endothelial cell proliferation (thalidomide and fumagillin) to rat pups between days 3 and 14 and demonstrated decreased capillary density along with inhibited septation of the lungs. In the same study, VEGF action was blocked by the VEGFR2 receptor blocker SU-5416, and similar results were observed (15). Further evidence that VEGF and angiogenesis are associated with the development of the alveoli comes from a study showing that pulmonary cell stretch, which stimulates capillary and alveolar septal growth in an in vitro system, upregulated VEGF mRNA and protein expression (26).

VEGF and its receptors have previously been shown to respond to O2 in neonatal animals. Maniscalco et al. (21) showed that neonatal rabbits exposed to 100% O2 for 9 days had decreased VEGF mRNA abundance, decreased alveolar epithelial cell VEGF expression, and decreased VEGF immunostaining. In later work, it was demonstrated that the reduction in VEGF mRNA was mainly because of a decrease in expression of the VEGF189 splice variant (34). However, Watkins and colleagues (34) actually showed an increase in lavage fluid VEGF protein on days 4, 6, and 8 during exposure of neonatal rabbits to 100% O2 and only a decrease after 9 days of hyperoxia. It has further been demonstrated that the expression of VEGF and its receptors is decreased after exposure to >95% O2 for 48 h in the adult rat, but nothing is known of the response of VEGF receptors to hyperoxia in neonatal rabbits or rats (18).

The transduction of an O2 signal to the level of gene expression requires the nuclear translocation and activation of redox-responsive transcription factors over specific ranges of PO2. VEGF expression is induced when most cell types are exposed to hypoxia as a result of increased transcriptional activation and mRNA stabilization of hypoxia-inducible factor-1α (HIF-1α; see Ref. 31). HIF-2α (or HIF-1α-like factor) has close sequence similarity to HIF-1α (10); however, the modes

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of expression of HIF-1α and HIF-2α vary greatly, as HIF-2α was first reported to be abundantly expressed in the adult lung of mice in the normoxic state, whereas HIF-1α is ubiquitous at much lower O2 levels (10). Both HIF-2α and VEGF mRNA were found to be highly expressed in alveolar epithelial cells in the parturient newborn lung, whereas the low levels of HIF-1α during gestation did not change. It is therefore postulated that HIF-2α regulates VEGF expression under normoxic conditions and during lung and vascular development. Recent experiments further demonstrated that loss of HIF-2α impaired lung maturation and created a subtle deficit in vascularization of the alveolar septa (7).

There may be differences in lung maturation between the rat and the rabbit; dexamethasone has been shown to inhibit septation in the neonatal rat; however, recent data show that there is no difference in lung structure after administration of dexamethasone to neonatal rabbits (19, 24). No experiments have as yet addressed the effects of O2 on VEGF expression or protein levels in the neonatal rat lung or VEGF receptor expression or levels in any model of inhibited alveolar development. The initial aim of these experiments, therefore, was to determine changes in VEGF, VEGFR1, and VEGFR2 mRNA expression and protein levels, both during the normal period of alveolar development in the neonatal rat lung and during exposure to a hyperoxic environment. Additionally, we studied one of the potential regulators of VEGF expression in this system, HIF-2α, which has been previously shown to respond to high O2 concentrations.

**METHODS**

**Animals**

Sprague-Dawley albino rat pups (Charles River Laboratories, St. Constant, Quebec, Canada) of both sexes were used. They were housed in the Health Sciences Animal Laboratory Service Department of the University of Alberta under veterinary supervision. The approval of the University of Alberta Animal Care Committee was obtained, and the guide-lines of the Canadian Council of Animal Care were followed in all experimental procedures. Dams were maintained on regular laboratory rodent pellets and water ad libitum and were kept on a 12:12-h light-dark cycle.

**O2 Exposure**

Parallel litters of randomly divided rat pups and their dams were placed in 0.14-m3 Plexiglas exposure chambers containing >95% O2 or 21% O2 (room air/normoxia), as previously published (4, 6, 23), from day 4 to day 14 of postnatal life. O2 concentrations were monitored daily (Vetronic O2 analyzer no. 5517, Temecula, CA). O2 and room air were filtered through barium hydroxide lime (Baralyme; Chemetron Medical Division, St. Louis, MO) to keep CO2 levels below 0.5% and through charcoal to remove odors. Temperature and humidity were maintained at 28°C and 75–80%, respectively. Chambers were opened for <15 min daily to switch dams between air and O2 environments and to clean dirty cages.

**Preparation of Lung Samples**

Pups from each exposure group were killed on days 4, 6, 9, 12, and 14 with an intraperitoneal overdose of pentobarbital sodium (100 mg/kg Euthanyl; MIC Pharmaceuticals, Cambridge, ON, Canada). Lung vasculature was washed by perfusion with 5 ml of ice-cold PBS injected in the right ventricle. The lungs were removed and snap frozen.

**RT and Real-Time Quantitative PCR**

Total RNA was isolated using TRIzol reagent according to the manufacturer’s instructions (Life Technologies, Burlington, Ontario, Canada). Samples were further treated with DNase I (DNA-free; Ambion, Austin, TX) to ensure that no DNA contamination existed. Quality of RNA was assessed by formaldehyde agarose gel electrophoresis. RT. Total RNA (100 ng) was added to a reaction mixture containing 100 ng random nanomers (Stratagene, La Jolla, CA), 1× cDNA first-strand buffer, 500 mM DTT, 0.4 U/µl RNase inhibitor, 1 mM each deoxy-NTPs, and 0.75 µl Superscript II RT (GIBCO-BRL Life Technologies). Negative RT (no enzyme) and no-template (no RNA) controls were also included. The RT thermal cycle was 25°C for 45 min, 50°C for 45 min, and 85°C for 5 min.

Real-time PCR. For real-time PCR, total VEGF and HIF-2α were detected using SYBR-green. VEGFR1 and VEGFR2 were detected using a fluorescently labeled molecular beacon. Primers and fluorescent beacons used are shown in Table 1. Primers were purchased from Sigma Genosys (Oakville, Ontario, Canada), and the fluorescent molecular beacons were from Stratagene. Primers were optimized for annealing temperature and RT RNA concentration. The correct product size and sequence were then confirmed by electrophoresis and DNA sequencing, respectively.

The PCR mixture (50 µl total volume) consisted of 0.2 µM of each primer, 0.2 µM of each molecular beacon (VEGF receptors and cyclophilin only), 10× PCR buffer (including SYBR green for VEGF and HIF-2α; Perkin-Elmer-Applied Biosystems, Warrington, UK), 1.9 mM MgCl2, 0.2 mM each dNTPs, 0.04 U/µl Tag polymerase (GIBCO-BRL Life Technologies), and 2 µl cDNA. Amplification and detection were

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HIF-2α, hypoxia inducible factor. All primers and beacons are written 5’ to 3’. Fluorescent beacons have Texas red attached to the 5’-end and the quencher DABCYL attached to the 3’-end.
performed using the iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) with the following cycle profile: 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s, 56°C for 1 min, and 72°C for 30 s.

Analysis of real-time PCR results. The mRNA of day 4 animals was pooled and used as a control group to allow analysis between PCR plates. All results have been normalized to cyclophilin, a cytoskeletal protein, which is expressed constitutively in all tissues, and did not change during these experiments. The mean value was 2.13 ± 0.13 relative fluorescent units. Quantification was performed by determining the threshold cycle (C_T). C_T is proportional to the amplified starting copy number of cDNA (or RNA). All reactions were performed in triplicate and controlled by a no-template reaction. The quantity of mRNA was calculated by normalizing the C_T of genes of interest to the C_T of the housekeeping protein cyclophilin of the same RNA probe, according to the following formula: ΔC_T = C_T of interest mRNA − C_T of cyclophilin mRNA (27).

VEGF Immunoassay

Samples were homogenized in lysis buffer (50 mM Tris·HCl, 3 mM sucrose, 0.1% Triton X-100, and 1 mM protease inhibitor cocktail; Calbiochem-Novabiochem, La Jolla, CA), supernatant was removed, and protein content was estimated using a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

Assay was performed using the Quantikine M, mouse VEGF Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. This assay recognizes both the 164- and 120-amino acid residue forms of VEGF. Starting sample concentration was 2 μg total protein/μl; samples were diluted fivefold before analysis. Results are expressed as picograms VEGF per microliter total protein.

Western Immunoblotting

The presence and relative abundance of VEGFR1 and VEGFR2 were determined using Western immunoblotting, as described by Laemmli (20). Aliquots from lung homogenates (prepared as above) were diluted in reducing sample buffer (0.5 M Tris·Cl, 2% β-mercaptoethanol, 87% glycerol, 10% SDS, and 1% bromphenol blue). Protein (40 μg/well) was loaded in 6% polyacrylamide gels. Proteins were separated by electrophoresis, transferred to nitrocellulose membranes (Bio-Rad, Mississauga, Ontario, Canada), and then blocked for nonspecific binding in a 7% skimmed milk solution. Membranes were incubated with primary antibodies raised in rabbit against VEGFR1 and VEGFR2 (Alpha-Diagnostics) for 2 h at a 1:500 dilution. Membranes were washed and incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, Bio/Can Scientific, Mississauga, ON, Canada). After repeated washing, membranes were incubated with enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) and placed in a Fluor-X Max Imager (Bio-Rad, Mississauga, Ontario, Canada) where the image was captured, and bands were analyzed by densitometric analysis. Each gel contained a day 4 sample from a pool of extracted tissues, and results on all subsequent days were normalized to the densitometric value of this sample. Only normalized values from days 6–12 are shown in Figs. 1–4.

Statistical Analysis

Western immunoblotting results were calculated as a ratio to day 4 values for comparison, and PCR results used day 4 lungs as control values. All results were normally distributed and were analyzed by two-way ANOVA, where variance was distributed according to treatment and time. When a significant F value was found, Tukey’s post hoc test was used to determine significance. Statistical significance was achieved at P < 0.05.

RESULTS

VEGF

Changes in total mRNA levels for the angiogenic growth factor, VEGF, were examined. The primers used for the real-time PCR analysis amplified a sequence that was common to all VEGF splice variants. Expression of message for total VEGF tended to increase slightly between days 6 and 12 in the animals raised in room air (Fig. 1A), and this increase reached significance by day 14 (P < 0.05) where values were two times those of day 6 air-exposed pups. Exposure to O_2 abolished the increase in mRNA levels seen in the normoxic pups so that O_2-exposed pups exhibited significantly lower VEGF mRNA levels than those from lungs of normoxic pups on days 12 and 14 (P < 0.001; Fig. 1A).

An increase, similar to that of the total VEGF mRNA, was seen in VEGF protein mass between days 4 and 12 (P < 0.05). This increase was maintained through day 14 at 0.61 ± 0.04 pg/μg total lung protein in the lungs of normoxic pups (Fig. 1B). Contrary to the inhibition seen in total VEGF mRNA, O_2 exposure resulted in a biphasic effect on VEGF protein levels, stimulating an increase from day 4 (0.41 ± 0.03 pg/μg protein) to day 9 (0.57 ± 0.03 pg/μg protein; P < 0.001) and then a decrease on day 12 to lower than normoxic control values (0.40 ± 0.06 compared with 0.59 ± 0.02 pg/μg total lung protein, respectively; P < 0.001). This trend was maintained on day 14, although values were not significant at this age (Fig. 1B).

VEGFR1

We were interested in investigating whether O_2 exposure had an effect on the VEGF receptor mRNA expression or protein levels. VEGFR1 mRNA increased from day 6 to 14 when levels were three times higher than those of the day 6 group (P < 0.05; Fig. 2A). Rat pups exposed to hyperoxia inhibited this age-dependent increase in VEGFR1 mRNA as levels remained similar to day 6 throughout the course of the experiment. This resulted in VEGFR1 mRNA levels of the O_2-exposed group being significantly lower than the air-exposed group on days 9, 12, and 14 (P < 0.001; Fig. 2A).

Contrary to mRNA expression, protein levels of VEGFR1 did not change significantly between days 6 and 14 in normoxic-exposed pups (Fig. 2B). However, exposure of rat pups to a hyperoxic environment did cause a significant decrease in overall VEGFR1 protein levels (P < 0.05 between air- and O_2-exposed groups; Fig. 2B). A significant interaction between group (air/ O_2) and postnatal day (age) was not observed.
VEGFR2

Message transcribing for VEGFR2 protein increased between days 9 and 14 in animals exposed to a normoxic environment so that by day 14 mRNA levels were 2.3 times those of day 6 (P < 0.05; Fig. 3A). Exposure to O2 had a significant effect by day 12 when VEGFR2 mRNA levels decreased to one-half the day 6 air values and were only 27% of the mRNA levels of the day 12 air-exposed pups (P < 0.001). The effect of O2 exposure was even greater by day 14, when mRNA levels from the O2 group were merely 8% of the air group (P < 0.001; Fig. 3A).

Protein levels for VEGFR2 in the normoxic-raised pups did not demonstrate the increase observed for VEGFR2 mRNA (Fig. 3B). However, exposure to O2 did have a similar effect of decreasing protein levels. VEGFR2 mRNA levels of the hyperoxic-exposed pups were 60% of those from the air-exposed group by day 12 (P < 0.001); this decrease was maintained through day 14 (P < 0.001; Fig. 3B).
To identify a possible mechanism whereby VEGF mRNA is decreased after exposure of rat pups to hypoxia, O₂-induced changes in mRNA for the transcription factor HIF-2α were determined. HIF-2α mRNA followed a similar pattern to that of VEGF. There was an age-dependent increase in HIF-2α mRNA between days 9 and 14, with day 14 values increasing to 2.4 times those on day 9 (P < 0.05; Fig. 4A). Again, similar to total VEGF mRNA levels, O₂ exposure inhibited the increase in HIF-2α mRNA seen in the control animals; there was a significant difference between air and O₂ groups on days 12 and 14 (P < 0.001; Fig. 4A). By day 14, mRNA levels of hyperoxic-exposed pups were 62% of those in the normoxic-exposed animals (P < 0.001; Fig. 4A). There was a strong correlation between VEGF mRNA and HIF-2α mRNA in the normoxic pups; the correlation coefficient was 0.799 (P < 0.001; Fig. 4B). Interestingly, when the pups were exposed to a hyperoxic environment, this correlation was lost (correlation coefficient, 0.355).

**Fig. 3.** Time course and effects of >95% O₂ from days 6 to 14 on VEGFR2 mRNA expression (A) and VEGFR2 protein levels (B). Data are means ± SE; n = 5–6 lungs/group. Animals were exposed to air (open bars) or O₂ (filled bars). A typical Western immunoblot is shown (B). VEGFR2 mRNA expression increased with advancing age; by day 14, levels were 2.3 times those of day 6 pups (P < 0.05). O₂ exposure caused a significant decrease in VEGFR2 mRNA expression below that of the air group on days 12 and 14 (P < 0.001). VEGFR2 protein levels did not change with advancing age. O₂ exposure caused a decrease in VEGFR2 protein levels compared with the air group on days 12 and 14 (P < 0.001). *Significant difference between air and O₂ pups on the day indicated. #Significant difference from day 6 air values.

**HIF-2α**

To identify a possible mechanism whereby VEGF mRNA is decreased after exposure of rat pups to hypoxia, O₂-induced changes in mRNA for the transcription factor HIF-2α were determined. HIF-2α mRNA followed a similar pattern to that of VEGF. There was an age-dependent increase in HIF-2α mRNA between days 9 and 14, with day 14 values increasing to 2.4 times those on day 9 (P < 0.05; Fig. 4A). Again, similar to total VEGF mRNA levels, O₂ exposure inhibited the increase in HIF-2α mRNA seen in the control animals; there was a significant difference between air and O₂ groups on days 12 and 14 (P < 0.001; Fig. 4A). By day 14, mRNA levels of hyperoxic-exposed pups were 62% of those in the normoxic-exposed animals (P < 0.001; Fig. 4A). There was a strong correlation between VEGF mRNA and HIF-2α mRNA in the normoxic pups; the correlation coefficient was 0.799 (P < 0.001; Fig. 4B). Interestingly, when the pups were exposed to a hyperoxic environment, this correlation was lost (correlation coefficient, 0.355).

**Fig. 4.** Time course and effects of >95% O₂ from days 6 to 14 on hypoxia inducible factor (HIF)-2α mRNA expression (A) and correlation between VEGF and HIF-2α mRNA expression (B). Data are means ± SE; n = 5 lungs/group. Animals were exposed to air (open bars and open squares) or O₂ (filled bars and filled squares). HIF-2α mRNA expression increased with advancing age; by day 14 levels were 2.4 times those of day 9 pups (P < 0.05). O₂ exposure caused a significant decrease in HIF-2α mRNA expression below that of the air group on days 12 and 14 (P < 0.001). There was a strong correlation between VEGF and HIF-2α mRNA expression in normoxic-exposed pups [solid line; the correlation coefficient was 0.799 (P < 0.001)]. There was no correlation between VEGF and HIF-2α mRNA expression in hyperoxic-exposed pups (broken line, the correlation coefficient was 0.355).
DISCUSSION

In this study we replicated, in the newborn rat, the response of VEGF to hyperoxia as seen in the newborn rabbit. We further extended the current body of knowledge to include the effects of hyperoxia on HIF-2α, VEGFR1, and VEGFR2. The results of this study demonstrate that VEGF mRNA expression increases during the period of alveolar development and is reduced after exposure of neonatal rats to a hyperoxic environment during this critical time. VEGFR1 and VEGFR2 mRNA and protein were also decreased during exposure to a high-O2 environment. Expression of HIF-2α followed a similar pattern to, and correlated strongly with, levels of VEGF mRNA in normoxic-exposed pups. However, this correlation was lost after exposure to hyperoxia, suggesting that the low levels of HIF-2α observed at high O2 concentrations are not stimulating VEGF expression. To the best of our knowledge, this is the first demonstration that HIF-2α mRNA relative abundance decreases in response to a hyperoxic environment. Because it is well established that this hyperoxic exposure protocol inhibits alveolarization in the rat pup (4, 23), we postulate that signaling through VEGF receptors may be an important mechanism for postnatal lung development and that hyperoxic inhibition of this pathway may contribute to the observed reduction in septation of the alveoli.

Our work is in agreement with that of Maniscalco and colleagues (21) who showed that neonatal rabbits exposed to 100% O2 for 9 days from birth had decreased VEGF mRNA abundance (21) because of a significant decline in VEGF188 (34). In our experiments, although total VEGF mRNA was decreased because of hyperoxia, VEGF164 protein levels were found to increase on day 9 before decreasing below normoxic values on day 12. Interestingly, Watkins et al. (34) found a similar trend measuring total VEGF protein from lavage fluid of newborn rabbits exposed to 100% O2; they found increased VEGF on days 4, 6, and 8 before levels decreased to below those of air-exposed animals on day 9 (34). Because total mRNA decreased and protein levels of combined VEGF164 and VEGF188 initially increased in our experiments, it is likely that the 188 splice variant was decreased during the first 6 days of hyperoxic exposure.

VEGF expression is induced when most cell types are exposed to hypoxia as a result of increased transcriptional activation and mRNA stabilization of HIF-1α and HIF-2α (1, 31). This study demonstrates increasing levels of HIF-2α mRNA expression from day 9 to day 14 in the postnatal rat lung, which is in agreement with earlier work showing a concurrent increase in HIF-2α and VEGF in the neonatal mouse lung between days 5 and 14 (10). The cellular location of HIF-2α in fetal and newborn mice and adult rats is the alveolar epithelial cell (type II; see Refs. 7, 10, and 35) where it has been found in the nucleus and also in the pulmonary artery endothelium (35). Normobaric hypoxia (created by perfusion with carbon monoxide) is a strong stimulus for HIF-2α expression in adult rat lung (35). It is unknown how hyperoxia decreases HIF-2α levels, although the hypoxia-induced increased expression may be via hypoxia-dependent stabilization of the protein or through a multistep process leading to interaction with cAMP response element B-binding protein (9).

In our experiments, both VEGFR1 and VEGFR2 were decreased during exposure of the neonatal lung to a high-O2 environment. However, these receptors are known to have different modes of action. Targeted disruption of VEGFR1 leads to mice with mature, differentiated endothelial cells but with large, disorganized vessels thought to result from overproduction of endothelial progenitor cells rather than vascular disorganization (13, 14). It is now thought that VEGFR1 plays a negative regulatory role by binding to VEGF (11). VEGFR2 knockout mice produce neither differentiated endothelial cells nor organized blood vessels and also possess no hematopoietic precursors (29, 32). This receptor is responsible for endothelial mitogenesis and migration as well as regulating vascular permeability (2). Our results suggest that endothelial cell differentiation and migration, as stimulated through the VEGFR2, and endothelial cell maintenance and possibly spatial organization, through VEGFR1, are both necessary for postnatal lung development. However, if, as suggested above, VEGF188 is diminished early in the experiments, decreased signaling through VEGFR1 may be an important early result of hyperoxic exposure, since VEGF188 is unable to bind VEGFR2 in its intact form but does bind VEGFR1. This early response could affect different aspects of alveolar development than later in development when all VEGF splice variants appear to be decreased. Indeed events do occur at different critical periods during alveolarization (23); maximal lung cell proliferation occurs during the first half of alveolarization, maximal endothelial cell proliferation peaks on day 7 (17), and the concentration of pulmonary arteries doubles between days 8 and 11 (25).

It has been shown that it is VEGFR2, rather than VEGFR1, that mediates the advanced lung maturation seen when VEGF is exogenously administered to premature mouse fetuses in vivo (7). Furthermore, the VEGFR2 receptor blocker SU-5416 caused decreased capillary density and inhibited septation, suggesting that angiogenesis occurring by VEGF signaling through VEGFR2 receptor is essential for the maturation of the neonatal lung. Because VEGF188 can be cleaved by urokinase-type plasminogen activator forming a peptide similar to VEGF165, which also has similar receptor binding and mitogenic properties (28), the early lower levels of VEGF188 may decrease signaling through VEGFR2 by this mechanism.

Recent evidence suggests that VEGF affects more than just endothelial cells. Overexpression of VEGF in the developing pulmonary epithelium of transgenic mice not only increased growth of pulmonary vessels but disrupted branching morphogenesis and inhibited type I cell differentiation as well (36). There is also evidence that VEGF may be involved in epithelial cell growth and proliferation in the human fetal lung in...
vitro (5). VEGFR2 protein is found in the distal airway epithelial cells of the midtrimester human lung, and exogenous VEGF added to explants caused increased epithelial proliferation and volume density (5). A recent study by Compernolle et al. (7) demonstrated that primary II cells expressed VEGFR2 transcripts and responded to VEGF by increasing their production of surfactant protein (SP)-B and SP-C. The same paper demonstrated that, as well as causing a subtle deficit in vascularization of the alveolar septa, loss of HIF-2α impaired lung maturation (7).

This study has important implications in the pathology of chronic lung disease of prematurity (CLD), in which its severest form develops into bronchopulmonary dysplasia (BPD). The pathology of BPD in recent years suggests that the condition is mainly the result of arrested development of the premature lung (16), resulting in a lack of alveolarization and dysmorphic vasculature. Therefore, the process of endothelial differentiation and organization in the alveolar microvasculature may be disrupted after premature birth and injury from O2 and ventilation. Recently, a study by Bhatt et al. (3) of premature human infants dying of respiratory distress syndrome (RDS) in a model of prematurity with decreased expression of VEGF and VEGFR1. Further work from the same laboratory demonstrated a failure of the normal increase in capillary density or the endothelial cell marker platelet endothelial cell adhesion molecule-1 in very premature baboons; VEGF and VEGFR1 were again decreased in this model of CLD (22).

We conclude that hypoxic exposure during the period of alveolarization in the rat pup causes decreased VEGF levels, possibly through decreased levels of the transcription factor HIF-2α, and decreased VEGF receptor levels. Because it is evident that the vascular growth is a fundamental part of normal alveolar development, we speculate that hypoxic-induced changes in VEGF may be an important element in the pathology of BPD.

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