Effect of chemical stabilizers of hypoxia-inducible factors on early lung development

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Groenman FA, Rutter M, Wang J, Caniggia I, Tibboel D, Post M. Effect of chemical stabilizers of hypoxia-inducible factors on early lung development. Am J Physiol Lung Cell Mol Physiol 293: L557–L567, 2007. First published June 1, 2007; doi:10.1152/ajplung.00486.2006.—Low oxygen stimulates pulmonary vascular development and airway branching and involves hypoxia-inducible factor (HIF). HIF is stable and initiates expression of angiogenic factors under hypoxia, whereas normoxia triggers hydroxylation of the HIF-1α subunit by prolyl hydroxylases (PHDs) and subsequent degradation. Herein, we investigated whether chemical stabilization of HIF-1α under normoxic (20% O2) conditions would stimulate vascular growth and branching morphogenesis in early lung explants. Tie2-LacZ (endothelial LacZ marker) mice were used for visualization of the vasculature. Embryonic day 11.5 (E11.5) lung buds were dissected and cultured in 20% O2 in the absence or presence of cobalt chloride (CoCl2, a hypoxia mimic), dimethyloxalylglycine (DMOG; a nonspecific inhibitor of PHDs), or desferrioxamine (DFO; an iron chelator). Vascularization was assessed by X-gal staining, and terminal buds were counted. The fine vascular network surrounding the developing lung buds seen in control explants disappeared in CoCl2- and DFO-treated explants. Also, epithelial branching was reduced in the explants treated with CoCl2 and DFO. In contrast, DMOG inhibited branching but stimulated vascularization. Both DFO and DMOG increased nuclear HIF-1α protein levels, whereas CoCl2 had no effect. Since HIF-1α induces VEGF expression, the effect of SU-5416, a potent VEGF receptor (VEGFR) blocker, on early lung development was also investigated. Inhibition of VEGFR2 signaling in explants maintained under hypoxic (2% O2) conditions completely abolished vascularization and slightly decreased epithelial branching. Taken together, the data suggest that DMOG stabilization of HIF-1α during early development leads to a hypervascular lung and that airway branching proceeds without the vasculature, albeit at a slower rate.

LUNG DEVELOPMENT OCCURS IN A RELATIVELY HYPOXIC ENVIRONMENT IN UTERO (32). This may be beneficial for vascular growth since several angiogenic factors are induced by low oxygen (18, 35). Indeed, culturing early embryonic lungs in a low oxygen environment stimulates vascular development (49) and enhances VEGF gene expression (1). Other reports have shown that hypoxia affects lung branching morphogenesis (18, 19). Recent studies have demonstrated that fetal oxygen tension also stimulates branching of the Drosophila tracheal system (34). In most mammalian cells, the response to hypoxia is mediated through hypoxia-inducible factor-1 (HIF-1) (20, 35). The HIF-1 complex consists of one of three α-subunits (HIF-1α, HIF-2α, or HIF-3α) and a β-subunit known as aryl hydrocarbon receptor nuclear translocator (ARNT) (41). The regulation of HIF by oxygen occurs through modifications of the α-subunit, whereas the β-subunit is oxygen insensitive. Under normoxic conditions, proline residues 402 and 564 of the α-subunit are rapidly hydroxylated by prolyl hydroxylase domain enzymes (PHD1-3) allowing recognition of the α-subunit by the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex and subsequent proteosomal degradation (36, 40). In addition, hydroxylation of the asparagine 803 residue in the transactivation domain of HIF-α by an asparagyl hydroxylase, called factor-inhibiting HIF (FIH)-1, inhibits the recruitment of transcriptional coactivator proteins (34, 30). Under hypoxic conditions, PHDs are degraded by SIAH (seven in absentia homolog) E3 ubiquitin ligases (10, 39). The degradation of PHDs allows the α-subunit of HIF-1 to escape hydroxylation and accumulate in the nucleus, where upon binding to ARNT, it recognizes hypoxia response elements (HRE) within the promoter regions of hypoxia-responsive target genes. Numerous genes have HREs, and many of them seem to be involved in endothelial cell proliferation and survival (35).

Herein, we investigated whether chemical upregulation of HIF-1α expression and stability under normoxic (20% O2) conditions would increase vascularization and branching in early lung explants similar to that seen in explants cultured in low (3%) oxygen (1, 18, 19). Either cobalt chloride (CoCl2), dimethyloxalylglycine (DMOG), or desferrioxamine (DFO) was used (11, 15, 38). CoCl2, a transition metal, chemically mimics hypoxia, which stabilizes HIF-1α (28). It is a weak inhibitor of PHD1–3 and a strong inhibitor of FIH (25). In addition, it inhibits VHL binding to HIF-1α even when HIF-1α is hydroxylated (52). DMOG is a nonspecific 2-oxoglutarate (OG)-dependent dioxygenase inhibitor. The 2-OG dioxygenase family includes PHD1–3 and FIH-1 (15). Inhibition of the 2-OG dioxygenase family results in an upregulation of HIF-1α and an induction of oxygen-regulated gene expression (15). Recent studies have shown that PHD inhibition with DMOG enhanced HIF-1α and VEGF expression in lung cells (5). DFO, which removes intracellular iron, is known to stabilize HIF-1α (31). The enzymatic actions of PHDs are dependent on both molecular oxygen and iron (31). DFO inhibits FIH-1, partly
inhibits PHD3, and is a weak inhibitor of PHD1 (25). It does not inhibit PHD2 (25). The chemical treatments with CoCl₂, DMOG, and DFO to induce vascularization and branching were compared with SU-5416, a well-known inhibitor of VEGFR2 [kinase insert domain-containing receptor (KDR)/fetal liver kinase-1 (flk-1)] signaling and lung development (27).

MATERIALS AND METHODS

Mice. CD1 and Tie2-LacZ mice were obtained from The Jackson Laboratory, Bar Harbor, ME (42). In Tie2-LacZ transgenic mice, the 2.1-kb 5′ flanking region of the murine Tie2 promoter drives the expression of the bacterial LacZ reporter gene exclusively to endothelial cells (42). Cells transcribing the LacZ gene can be viewed by staining for β-galactosidase activity. The transgenic line was main-

Fig. 1. SU-5416 inhibits vascularization in early lung explants. Embryonic day 11.5 (E11.5) Tie2-LacZ lung explants were maintained in 2% O₂ with (C, D, G, and H) and without (A, B, E, and F) SU-5416. Vascularization was assessed after 24 h (A–D) and 144 h (E–H) by X-gal staining (B, D, F, and H). A, C, E, and G: unstained control explants. Blue color represents positive X-gal staining in the vessels.
tain on a CD1 background. All mouse protocols were in accordance with Canadian Council of Animal Care guidelines and approved by the Animal Care and Use Committee of the Hospital for Sick Children, Toronto, ON, Canada.

Whole lung organ culture. Lung buds were dissected from embryonic day 11.5 (E11.5) CD1 and CD1-Tie2-LacZ mouse embryos (day of vaginal plug is E0.5) and placed on a floating (8-μm Whatman Nuclepore polycarbonate) membrane (Integra Environmental, Burlington, ON, Canada). Explants were grown in DMEM plus 10% FCS (Gibco, Grand Islands, NY) and maintained in an atmosphere of 20% O2-75% N2-5% CO2 at 37°C. CoCl2 (100 μM; Sigma-Aldrich, Oakville, ON, Canada), between 500 and 1,000 μM DMOG (Frontier Scientific, Logan, UT), 10 μM DFO (Sigma-Aldrich), or 20 μM SU-5416 (VEGFR2 kinase inhibitor III, cat. no. 676487; Calbiochem, La Jolla, CA) was added to the medium at day 0. The explant experiments with SU-5416 were carried out in a hypoxic environment (2% O2-93% N2-5% CO2). Medium was changed every other day, branching was assessed every day, and vascular growth was assessed on days 2 and 4. Pictures were taken using a Leica microscope and a digital imaging system.

MTT assay. Cell proliferation was determined using the Vybrant 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (V-13154; Invitrogen, Burlington, ON, Canada). The MTT assay involves the conversion of water-soluble MTT to an insoluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in the presence of mitochondrial dehydrogenase enzymes. Formazan is a blue precipitate that can be measured spectrophotometrically. In brief, explants were cultured for 48 h in DMEM plus 10% FCS with and without 10 μM DFO, 100 μM CoCl2, 0.2 mM EDTA, or 0.5 mM DTT. The conversion of MTT to formazan was allowed to continue for 4 h. The formazan was then solubilized by adding SDS-HCl (0.35 M SDS in 0.01 M HCl) to the medium, and incubation continued for another 16 h. The concentration of formazan was determined by measuring the optical density at 570 nm.

LacZ staining. LacZ lung explants were fixed (in 1% formaldehyde, 0.1% glutaraldehyde, 2.5 mM MgCl2, and 5 mM EGTA in 0.1 M sodium phosphate buffer, pH 7.8–8.0, for 45 min at 4°C), washed (in 2 mM MgCl2, 0.01% deoxycholate, and 0.02% Nonidet P-40 in 0.1 M sodium phosphate buffer, pH 7.8–8.0) four times for 30 min at 4°C, stained overnight at 37°C in X-gal staining solution [5 mM K4Fe(CN)6·3H2O,5 mM K3Fe(CN)6, 5 mM K3Fe(CN)6, 0.5 mM MgCl2, 2 mM MgCl2, 5 mM MnCl2, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and EDTA-free protease inhibitor mixture (Roche Diagnostics Canada, Laval, Quebec, Canada)]. Explants were washed with PBS, fixed in 4% paraformaldehyde in PBS overnight at 4°C, and stored in 70% ethanol. Whole mount LacZ staining revealed that incubation of E11.5 mouse lung explants with SU-5416, a cell-permeable selective ATP-competitive inhibitor of VEGFR2 (KDR/Flik-1), completely abolished vascular growth.

Inhibition of VEGFR2 signaling blocks vascularization and slightly decreases epithelial branching. We first investigated whether inhibition of VEGF/VEGFR2 signaling affected vascular growth and epithelial branching morphogenesis using CD1-Tie2-LacZ mice. Lungs were dissected at E11.5 and cultured in 2% oxygen to stimulate optimal vascular growth (49). Whole mount LacZ staining revealed that incubation of E11.5 mouse lung explants with SU-5416, a cell-permeable selective ATP-competitive inhibitor of VEGFR2 (KDR/Flik-1), completely abolished vascular growth.

RESULTS

Inhibition of VEGFR2 signaling blocks vascularization and slightly decreases epithelial branching. We first investigated whether inhibition of VEGF/VEGFR2 signaling affected vascular growth and epithelial branching morphogenesis using CD1-Tie2-LacZ mouse lungs. Lungs were dissected at E11.5 and cultured in 2% oxygen to stimulate optimal vascular growth (49). Whole mount LacZ staining revealed that incubation of E11.5 mouse lung explants with SU-5416, a cell-permeable selective ATP-competitive inhibitor of VEGFR2 (KDR/Flik-1), completely abolished vascular growth.

Fig. 2. SU-5416 reduces branching morphogenesis. E11.5 Tie2-LacZ lung explants were maintained in 2% O2 with and without SU-5416. Terminal lung buds were counted every day for controls (solid line) and at 48 and 96 h for explants treated with SU-5416 (filled bars). Data are means ± SE; n = 35 explants per time point, n ≥ 15 per SU-5416 treatment. *P < 0.05.
Development (absence of X-gal positive vessels) was already noticeable after 24 h of incubation (Fig. 1, B vs. D), whereas epithelial branching appeared unaffected (Fig. 1, A and C). After 48 and 96 h of incubation of lung explants with SU-5416, a small but significant decrease in epithelial branching was noticed compared with control explants (Fig. 2). Thus epithelial branching proceeded without vascular development, albeit at a lower rate (Figs. 1 and 2).

**Chemical induction of HIF.** Since low oxygen has been shown to stimulate VEGF expression in murine lung explants, most likely via HIF-1 (49), we investigated whether enhancing HIF-1 stability under normoxic (20% O2) conditions would promote vascular development and, thereby, epithelial branching. E11.5 lungs from CD-Tie2-LacZ mice were incubated with either CoCl2, DMOG, or DFO to stabilize HIF-1α. First, we determined the dose-toxicity curves of CoCl2 and DFO for lung explant growth and branching (Fig. 2). Based on these pilot studies, we used 100 μM CoCl2 and 10 μM DFO in the subsequent explant experiments. These CoCl2 and DFO concentrations have previously been shown to increase HIF-1α protein amounts in various cell (37, 48, 49) and tissue systems (14). Dose-toxicity curves showed that explants started to disintegrate when exposed to between 500 and 1,000 μM DMOG for more than 48 h (Fig. 4). Since DMOG has been reported to stabilize HIF-1α at concentrations of >500 μM (26, 53), we exposed explants in subsequent experiments to

![Fig. 3. A–G: dose-toxicity curves of cobalt chloride (CoCl2) and desferrioxamine (DFO) for lung explant growth and branching. E11.5 Tie2-LacZ lung explants were cultured for 3 days in 20% O2 with and without increasing dosages of either CoCl2 (B, D, and F) or DFO (C, E, and G). A: untreated control. Inset: freshly isolated E11.5 lung. B, D, and F: between 10 and 100 μM CoCl2. C, E, and G: between 1 and 20 μM DFO.](http://ajplung.physiology.org/)

500–1,000 μM DMOG for maximally 48 h. Lungs cultured with CoCl₂ showed a significantly decreased vascular LacZ staining after 48 h of culture compared with control lungs (Fig. 5, E, F vs. B, and C). Also, DFO treatment resulted in reduced vascular development (Fig. 5, G, H vs. B, and C). The fine vascular network surrounding the developing lung buds seen in control explants had disappeared in CoCl₂- and DFO-treated explants. The X-gal positive vessels along the trachea, main bronchi, and larger airways appeared to be unaffected, especially in the DFO treatment group. After 96 h of culture, vascular growth was further reduced in explants treated with CoCl₂ and DFO (Fig. 6, B and C vs. A). Epithelial branching was significantly reduced in explants treated with CoCl₂ and DFO (Figs. 3 and 6E). Addition of 50 μM FeCl₂ to DFO-treated explants reversed the inhibitory effect of DFO on vascular growth and epithelial branching (Fig. 7). Of note, FeCl₂ addition did not completely overcome CoCl₂-induced inhibitory effect (data not shown). Epithelial branching and vascular growth were not affected by low dosages (between 25 and 100 μM) of DMOG (Figs. 4, 6, and 8A). However,
concentrations of >500 μM DMOG stimulated vascularization while inhibiting epithelial branching (Figs. 4 and 8, A–C). The Vybrant MTT assay was performed to determine the effect of CoCl₂, DMOG, and DFO on explant cell growth. CoCl₂, DFO, and DMOG (>500 μM) treatment of lung explants markedly inhibited cell proliferation compared with control explants (data not shown). These findings agree with the branching morphogenesis results.

To determine the effect of the treatments on HIF-1α protein expression, nuclei were isolated from explants after 48 h of culture. Western blot analysis of nuclear lysates revealed that DFO and DMOG treatments increased HIF-1α protein content, whereas CoCl₂ had no effect (Fig. 9A). Next, we investigated the effect of treatments on HIF-1α, VEGF, and VEGFR2 (KDR/Flik-1) mRNA expression by real-time PCR. None of the treatments affected HIF-1α mRNA expression (Fig. 9B). All treatments increased VEGF mRNA expression while reducing VEGFR2 mRNA expression.

DISCUSSION

Previously, we (49) have reported that low oxygen levels enhance epithelial branching morphogenesis and vascular development in E11.5 murine lung explants. We hypothesized that HIF-related pathways were involved, and, indeed, antisense knockdown of HIF-1α mRNA expression decreased epithelial branching morphogenesis and vascularization (49). Herein, we show that inhibition of VEGF/VEGFR2 signaling with SU-5416 completely abolished vascular growth. However, epithelial branching proceeded without vascular development, albeit at a slower rate. This indicates that HIF/VEGF signaling is important for vascularization but that other downstream targets of the HIF pathway play a role in epithelial branching. Also, it suggests that epithelial branching is not as tightly linked to vascular development in the early phase of lung development as previously thought (49). Galambos et al. (17) showed that later in gestation (>E16), loss of one VEGF allele led to a decrease in lung development, suggesting that vascularization plays a greater role in epithelial branching at later gestation. This is supported by studies in which a VEGFR2 inhibitor was administered postnatally (days 1–13) to rat pups. Treated animals had enlarged air spaces, decreased alveolar number, and decreased arterial density (27). Endothelial monocyte-activating polypeptide II (EMAP II), another anti-angiogenic agent, decreased vessel density and arrested airway epithelial morphogenesis in E14.5 lung allografts (43).

VEGF expression in murine lung explants is stimulated by low oxygen, most likely through a HIF-mediated mechanism (49). Stimulation of HIF protein via inhibition of PHDs has been reported to enhance expression of VEGF and platelet endothelial cell adhesion molecule (PECAM) both in vivo and in vitro (7, 8). Furthermore, it stimulated in vitro angiogenesis (5, 7). Intravenous treatment of preterm baboons with a PHD inhibitor (FG-4095) was shown to be effective for HIF protein stabilization (5, 7). Another study
showed that this treatment is associated with enhanced lung growth, improved oxygenation, and lung compliance (6). Thus the increase of HIF proteins goes hand in hand with improved postnatal lung development. In the present study, we observed that chemical stabilization of HIF-1α in early lung explants with the nonspecific PHD inhibitor, DMOG, increased vascularization but decreased branching. The latter is opposite to our previously reported effect of low oxygen on explant branching (49).

In explants treated with CoCl₂ and DFO, the fine vascular network surrounding the developing lung buds disappeared, whereas the vessels along the trachea, main bronchi, and larger airways remained intact. Although VEGF expression was upregulated, VEGFR2 expression was downregulated by both treatments. It is possible that VEGFR2 downregulation is responsible for the lack of peripheral vascularization since VEGF-VEGFR2 signaling is important for the sprouting of new vessels from existing ones, and VEGF is a potent mitogen for endothelial cells (for review, see Ref. 50). However, in explants treated with SU-5416, not only the finer vasculature disappeared, but also the larger more proximal vasculature. An explanation may be that SU-5416 is not solely a VEGFR2 inhibitor, but also a PDGF β-receptor inhibitor. VEGFR2 accounts for most VEGF effects on endothelial cells such as cell proliferation, NO and prostacyclin production, angiogenesis, and vascular permeability (23, 46). The PDGF β-receptor is activated by PDGF-B, which is a potent mitogen of vascular smooth muscle cells (VSMC) and is also involved in the migration of pulmonary VSMCs (12, 50a). Inhibition of both VEGF and PDGF pathways has been shown to be more effective than blocking VEGF alone, causing vessel regression in multiple models of neovascular growth (29). Thus the blockade of both receptors may explain the complete absence of vascularization seen in explants incubated with SU-5416.

Fig. 6. Cobalt chloride and DFO reduce branching morphogenesis. E11.5 Tie2-LacZ lung explants were maintained in 20% O₂ for 96 h with and without CoCl₂ and DFO. A–C: X-gal staining (A, control; B, CoCl₂; C, DFO; E, DMOG, 25 μM). E: terminal bud counts. Data are means ± SE, n = 25 explants per treatment. *P < 0.05.
Although upregulation of VEGF mRNA in the lung explants by all three treatments suggests stabilization of HIF-1α (49), we found that only DFO and DMOG increased nuclear HIF-1α levels. CoCl₂ is a strong inhibitor of FIH-1 but not PHDs (25). Recent studies have shown that FIH-1 regulates selectively the expression of a variety of HIF-induced genes, in particular VEGF (13). It is plausible that CoCl₂ inhibition of FIH-1 stimulated HIF-1α transactivation activity and, thereby, VEGF mRNA expression in the explants without increasing HIF-1α protein. As discussed above, the increase in VEGF expression by CoCl₂ and DFO did not lead to improved vascularization. Moreover, lung explant growth and branching was severely reduced. Both CoCl₂ and DFO interfere with binding of molecular oxygen to heme proteins, thereby mimicking hypoxia. It is possible that the hypoxia was too extensive and led to cell death. DFO and CoCl₂ have been shown to induce apoptosis in a variety of nonlung cells (3, 21, 44). However, no increase in apoptotic or necrotic cell death of fetal alveolar type II cells was reported when the cells were exposed to 3% O₂ (22). In the latter study, the fetal cells were exposed to 3% O₂ only for 24 h. To prevent oxygen depletion and cell death, cells exposed to very low oxygen levels downregulate ATP-consuming pathways to decrease oxygen demand. The reduction in oxygen consumption in A549 cells was, however, not reversible after the cells were exposed to hypoxia for 24 h (24). Thus the prolonged CoCl₂- or DFO-induced hypoxia likely triggered cell death in the lung explants. Together with the reduction in cell proliferation, this may explain the negative effect of both treatments on lung growth and branching. Moreover, CoCl₂ and DFO will not only act as hypoxia mimetics but interfere with all ferro-dependent reactions needed for proper vessel formation and airway branching. The importance of iron for proper lung development was demonstrated by the reversal of the inhibitory effect of DFO with excess iron. These additional effects of DFO may explain the discrepant results between DFO and DMOG. Both increased nuclear HIF-1α protein and VEGF mRNA expression, but only DMOG stimulated vascularization. It is worthwhile mentioning that DMOG has been shown to inhibit hydroxyproline synthesis in lung (9). This may explain negative effect of DMOG on branching as collagens have been shown to be important for proper lung branching (45). Recently, it has been reported that the specific PHD inhibitor FG-4095 stabilizes HIF-1α protein and stimulates the expression of angiogenic factors, including VEGF, in primate lung explants cultured under hyperoxic conditions (7). Unfortunately, its effect on early lung morphogenesis was not determined.
Fig. 8. DMOG stimulates vascularization but inhibits branching. A: E11.5 Tie2-LacZ lung explants were cultured for 72 h with various concentrations of DMOG. B: E11.5 Tie2-LacZ lung explants were cultured for 48 h with or without between 500 and 1,000 μM DMOG or DMSO concentration control. C: vessel density (ratio of vessel pixels to whole lung pixels). Data are means ± SE, n = 4. *P < 0.05.

Fig. 9. Effect of DMOG, cobalt chloride, and DFO on hypoxia-inducible factor-1α (HIF-1α), VEGF, and VEGF receptor 2 (VEGFR2) expression. A: nuclei were isolated from E11.5 Tie2-LacZ lung explants maintained in 20% O2 for 48 h in the absence or presence of either 10 μM DFO, 100 μM CoCl2, or 500 μM DMOG, and nuclear lysates were analyzed for HIF-1α using Western blotting. Data are representative of 2 separate experiments. B: RNA was extracted from Tie2-LacZ lung explants maintained in 20% O2 for 48 h with and without either 10 μM DFO, 100 μM CoCl2, or 500 μM DMOG, and mRNA expression was quantified by real-time RT-PCR. Data are means ± SE, n = 4. *P < 0.05.
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