INTRAUTERINE GROWTH RESTRICTION DECREASES PULMONARY ALVEOLAR AND VESSEL GROWTH AND CAUSES PULMONARY ARTERY ENDOTHELIAL CELL DYSFUNCTION IN VITRO IN FETAL SHEEP

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Running head: IUGR impairs lung alveolar and vascular growth

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ABSTRACT

Intrauterine growth restriction (IUGR) increases the risk for bronchopulmonary dysplasia (BPD). Abnormal lung structure has been noted in animal models of IUGR but whether IUGR adversely impacts fetal pulmonary vascular development and pulmonary artery endothelial cell (PAEC) function is unknown. We hypothesized that IUGR would decrease fetal pulmonary alveolarization, vascular growth and in vitro PAEC function. Studies were performed in an established model of severe placental insufficiency and IUGR induced by exposing pregnant sheep to elevated temperatures. Alveolarization, quantified by radial alveolar counts, was decreased 20% (p<0.005) in IUGR fetuses. Pulmonary vessel density was decreased 44% (p<0.01) in IUGR fetuses. In vitro, insulin increased control PAEC migration, tube formation, and nitric oxide (NO) production. This response was absent in IUGR PAECs. VEGFA stimulated tube formation and NO production also was absent. In control PAECs, insulin increased cell growth by 68% (p<0.0001). Cell growth was reduced in IUGR PAECs by 29% at baseline (p<0.01) and the response to insulin was attenuated (p<0.005). Despite increased basal and insulin stimulated Akt phosphorylation in IUGR PAECs, endothelial NO synthase (eNOS) protein expression as well as basal and insulin stimulated eNOS phosphorylation were decreased in IUGR PAECs. Both VEGFA and VEGFR2 also were decreased in IUGR PAECs. We conclude that IUGR fetuses are characterized by decreased alveolar and vascular growth and PAEC dysfunction in vitro. This may contribute to the increased risk for adverse respiratory outcomes and BPD in IUGR infants.

KEYWORDS: endothelial nitric oxide synthase, bronchopulmonary dysplasia, alveolarization, angiogenesis, intrauterine growth restriction
INTRODUCTION

Bronchopulmonary dysplasia (BPD) results from severe injury to the developing lung. BPD has traditionally been defined by the presence of persistent respiratory compromise, the need for supplemental oxygen, and an abnormal chest radiograph at 36 weeks corrected age (9; 65). The initial histological descriptions of BPD included significant airway changes, chronic inflammation, and edema. Distal lung parenchyma was characterized by heterogeneous disease with atelectasis, fibrosis, and emphysematous regions (9; 59; 65). Premature infants are now being supported at much earlier gestational ages and compared to the original descriptions of BPD, the lung appears more uniform with milder regions of injury but signs of impaired or arrested alveolarization and vascular growth (59; 77). Additionally, infants with BPD often have increased pulmonary vascular resistance and pulmonary hypertension (59; 77).

Intrauterine growth restriction (IUGR), which is characterized by a complex set of fetal nutritional and hormonal deficiencies including low insulin (18; 53; 54; 62-64; 67), has increasingly been recognized as an independent risk factor for the development of BPD and lung disease (2; 8; 8; 14; 23; 26; 27; 43; 57; 71; 73; 85). Even IUGR infants born at term, without prematurity, have worse respiratory outcomes (58); and more than twice as many IUGR infants require oxygen at 36 weeks corrected age despite having similar rates of acute respiratory distress syndrome (RDS) as normally grown infants born at the same gestational age (4).

One potential mechanism for these adverse outcomes is abnormal pulmonary vascular development and function. Recent data has demonstrated the importance of lung vascular
development and endothelial cell (EC) signaling for normal parenchyma and that impaired
angiogenesis can halt alveolarization (34; 44; 77; 81; 82). Vascular endothelial cell growth factor
A (VEGFA) signaling plays a central role in this process (24; 29; 30; 34; 41; 42; 44; 78; 79; 81;
82). Although intracellular signaling pathways in the EC for VEGFA and insulin overlap
significantly (32; 33; 35; 60; 61; 68; 72; 76; 80; 87; 88), the role of insulin in the pathogenesis of
BPD in IUGR infants is unknown.

Angiogenesis is a complex in vivo process that cannot be fully replicated in vitro. Despite this
limitation, several EC functions related to angiogenesis can be measured in vitro, such as
growth, migration, and branching. These processes are regulated by multiple extracellular
growth and angiogenic factors (3; 24). Akt coordinates EC function with these signals, including
insulin and VEGFA (60; 76). In particular, the serine 473 residue of Akt is phosphorylated in
response to VEGFA and insulin, which increases Akt activity, allowing Akt to phosphorylate
several downstream targets including endothelial nitric oxide synthase (eNOS) at ser1177 and
regulate angiogenesis (76). Phosphorylation at this site correlates highly with production of NO,
which, along with other factors, stimulates critical EC functions (60; 76).

Based on these data, we hypothesized that fetal pulmonary alveolarization and vascular growth
would be decreased in experimental IUGR and that pulmonary artery ECs (PAECs) from IUGR
fetuses would have decreased in vitro function and decreased signaling through the Akt/eNOS
pathway. In order to test our hypothesis we used a chronic placental insufficiency model of
severe IUGR that shares many of the fundamental fetal complications of human IUGR including
a reduction of fetal plasma arterial insulin concentrations by 55-70% (1; 5-7; 49; 74; 83; 84). We
found that IUGR decreased fetal pulmonary alveolarization, pulmonary vessel density, \textit{in vitro} PAEC function, and PAEC eNOS signaling.

**MATERIALS AND METHODS**

**IUGR Model**

Studies were conducted in pregnant Columbia-Rambouillet ewes in compliance with the Institutional Animal Care and Use Committee, University of Colorado Denver, at the Perinatal Research Center in Aurora, CO. This laboratory is accredited by the National Institutes of Health, the United States Department of Agriculture, and the American Association for Accreditation of Laboratory Animal Care. IUGR fetuses were created by exposing pregnant ewes to elevated ambient temperatures (40°C for 12h; 35°C for 12h) from 33.4±0.3 days gestation age (dGA) until 115.3±0.4 dGA, as previously described (term, 148 dGA) (14). After this exposure ewes were housed in normothermic environments (20°C) until sacrifice for collection of lung tissue and isolation of PAECs. IUGR fetuses (n=8) were compared to normally grown controls (n=14).

**Histology and Morphometric Analysis**

For seven IUGR and five control fetuses, one lung was inflated with 4% paraformaldehyde (wt/vol) in phosphate buffered saline (PFA-PBS) to a pressure of 30 cm H$_2$O, ligated while under pressure, stored in PFA-PBS overnight, and then transferred to 70% ethanol (vol/vol). Fixed lung tissue from was paraffin embedded, cut, and then stained with hematoxylin and eosin (H&E). Radial alveolar counts (RACs) were
performed according to the methods of Cooney and Thurlbeck, and Emery and Mithal as previously described (11; 19; 28). At least two different lung sections per animal were used with five to six fields of view evaluated for each section. We determined vessel density after factor VIII staining in two sections per animal, a minimum of five fields of view (FOV) per section, by counting the number of positively stained vessels per high-powered field (hpf, x100) as previously described (28). Sections of peripheral lung adjacent to the pleural surface were examined, and fields with large airways or major vessels were avoided. For both RAC and vessel density, the measurements made on each FOV for each animal were averaged to derive the mean number for that animal. The mean for each animal was then used for statistical analysis. The investigator quantifying RACs and vessel density were blinded to the identity of the sections at the time of analysis.

**Endothelial Cell Isolation and In Vitro Assays**

**Isolation and Culture**

Large proximal PAECs were isolated as previously described and EC phenotype confirmed by positive immunostaining for EC markers vWF, VE-cadherin, eNOS, ac-LDL; and negative staining for smooth muscle cell markers aSMC and desmin (24). Cells from passage four to six were used for each of the experiments, and cells from each animal were kept separate throughout all passages and for all experiments. Cells were maintained and propagated in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). Insulin used in all in experiments was Humulin R (Lily, Indianapolis, IN) and VEGFA for all experiments was human recombinant VEGFA (R&D Systems, Minneapolis, MN). Cells were propagated in 21%
oxygen. Functional in vitro assays were performed in 21% and/or 3% oxygen, to mimic fetal conditions. For each individual in vitro assay the choice of 21% and/or 3% oxygen was based on previously published data demonstrating optimal conditions (3; 24). Incubations in 21% oxygen were performed in incubators with room air equilibrated with 5% CO$_2$ and incubations in 3% oxygen were performed in incubators equilibrated with 3% oxygen, 5% CO$_2$, and nitrogen to balance. Incubators for 3% oxygen conditions remained undisturbed for the duration of the experiment to minimize exposure to increased oxygen concentrations (3; 24).

Migration

PAEC migration was measured with a scrape assay in cells from three IUGR and five control fetuses (15). PAECs were allowed to reach confluence in 6-well plates in DMEM with 10% FBS. Media was changed to serum free DMEM with 0.5% bovine serum albumin (BSA). A standardized scrape was made using a pipette tip. Images of the scraped area were obtained for reference. Immediately following the scraping, the wells were washed with PBS and then incubated in DMEM with 0.5% BSA overnight with or without insulin in 3% or 21% oxygen. After incubation for 12-24 hours, images of the scraped area were taken and overlaid with the original reference image. The number of cells migrating into the scraped area was counted by an investigator blinded to the animal group. Results for each set of cells were normalized to the amount of migration in basal media without additions and all conditions were run in duplicate for each animal.

Cell Growth
PAEC growth was assayed in three IUGR and four control fetuses. Fetal PAECs were plated at $1 \times 10^5$ cells/well into 6 well plates and allowed to adhere overnight in DMEM with 10% FBS in 21% oxygen. The following day (day zero), the cells were washed three times with PBS. DMEM with 5% FBS with or without insulin was then added and cells incubated in 21% oxygen. Media was changed on day three and cell counts were performed on days zero and five after removing cells with trypsin digestion. All conditions were run in triplicate for each animal. Growth studies with treatment were performed in DMEM with 5% FBS, based on previous studies that determined that this was the lowest serum concentration that supported fetal PAEC survival with some proliferation (24).

**Tube Formation Assay**

The ability of fetal PAECs to form vascular structures *in vitro* was assayed by plating PAECs on EHS Matrigel (BD Pharmingen, San Jose, CA). PAECs from three IUGR and three control fetuses were seeded at a density of $5 \times 10^4$ cells/well in serum-free DMEM supplemented with insulin or VEGFA and each condition was tested in quadruplicate for each animal. PAECs were incubated for 12-18 hours under 3% oxygen conditions based on previous studies that determined tube formation was more robust in 3% compared to 21% oxygen (24). Branch-point counting was performed in blinded fashion under 10X magnification from each of four wells with 3-4 FOVs per well, as previously described (24).

**Nitric Oxide Production**
PAEC NO production from three IUGR and five control fetuses was determined using the DAF-FM NO indicator (Molecular Probes, Eugene, OR). NO was measured by fluorescence after a 1-hour incubation with insulin or VEGFA in 21% oxygen, as previously described (24). Each condition was run in triplicate.

**Western Blot Analysis**

For all insulin signaling and western blot experiments, PAECs from five PI-IUGR and five control fetuses were used. PAECs were grown to approximately 70-80% confluence and then washed three times with PBS. Serum free DMEM with 0.5% BSA and insulin was added to each well and plates were placed either in 3% or 21% oxygen to determine the interactive effects of insulin and oxygen. At 18 hours cell lysates were collected by washing PAECs twice with ice cold PBS followed by the addition of ice cold lysis buffer (NaCl 150 mmol/L, Tris, pH 7.4, 20 mmol/L, Nonidet P-40 1% v/v, EDTA 2 mmol/L, Na₄P₂O₇ 2.5 mmol/L, Glycerol 10% v/v, β-Glycerophosphate 20 mmol/L, phenylmethylsulfonyl fluoride 0.575 mmol/L, Sigma Mammalian Protease Inhibitor Cocktail 2% v/v, Sigma Phosphatase Inhibitor I and II 0.5% v/v, each). Samples were frozen at -80°C and then thawed for collection of proteins, which was accomplished by mixing samples with a vortex, disrupting cell membranes with a bath sonicator and then placing samples on an orbital rocker at 4°C for 60 min. The protein was separated from cellular debris by centrifugation at 21,000g for 30 min at 4°C. The supernatant was removed and the protein concentration was quantified with the BioRad DC Protein Assay (BioRad, Hercules, CA).
10 μg of protein were separated by polyacrylamide gel electrophoresis under reduced conditions (5% β-mercaptoethanol). Proteins were then transferred to a polyvinylidene difluoride membrane. Western blot membranes were blocked in PBS with 0.01% tween 20 (v/v, PBST; Sigma) and 5% nonfat dried milk w/v (NFDM) for one hour at room temperature, except membranes probed for insulin receptor β (IRβ) which were blocked with PBST and 5% NFDM (w/v) plus 1% BSA (w/v). The primary antibody for IRβ (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) also was diluted in this buffer. The following primary antibodies were diluted in PBST with 5% NFDM: eNOS (1:1000, conjugated to horse radish peroxidase, Santa Cruz Biotechnology) and β-Actin (1:100,000, Medimmune, Inc., Gaithersburg, MD). Other primary antibodies were diluted in PBST with 5% BSA: ser473 phosphorylated Akt (1:500, Cell Signaling Technology), Akt (1:1000, Cell Signaling Technology), and ser1177 phosphorylated eNOS (1:500, Cell Signaling Technology). Horseradish peroxidase (HRP) conjugated secondary antibodies were diluted in PBST with 5% NFDM and applied to membranes for one hour at room temperature and then washed, except for eNOS conjugated to HRP which was simply washed. Immunocomplexes were detected with Western Lighting Enhanced Chemiluminescence (Perkin Elmer Inc., Waltham, MA). Densitometry was performed using Scion Image software (Scion Corporation, Frederick, MD). All results were normalized to β-Actin to control for loading differences and two reference samples were analyzed on every membrane to control for differences in transfer efficiency. Results for incubations with 200 nM insulin are shown. Antibodies were stripped from the membranes with Restore Western Stripping Buffer (Pierce, Rockford, IL).

Measurement of VEGFA and VEGF receptor 2 (VEGFR2) was performed on PAEC lysates obtained from the zero insulin incubations. The procedure was the same as above with the following modifications. Membranes were blocked in 1% or 2% ECL Advance Blocking Agent.
(GE Healthcare, Piscataway, NJ) for VEGFA and VEGFR2, respectively. Primary and secondary antibodies were diluted in the same blocking buffer. The antibody to VEGF was diluted 1:500 (Santa Cruz Biotechnology) and the antibody to VEGFR2 was diluted to 1:2500 (Cell Signaling Technologies).

**Statistical Analysis**

Statistical analysis was performed using SAS version 9.1 (58). All results are presented as mean ± standard error. Animal and morphometric data were compared using either the Student’s *t* test (parametric) or the Mann-Whitney test (nonparametric). Data from isolated ECs was compared with a mixed models ANOVA which included terms (as appropriate) for group (IUGR or control), insulin or VEGFA concentration, oxygen concentration, time, and a random animal term to account for repeated measures from the same animal’s set of cells. Individual means were compared with Fisher’s Least Significant Difference test and *p* values of 0.05 or less were considered significant. For comparisons between control and IUGR PAECs in experiments performed in only one condition, results were compared with Student’s *t* Test or the Mann-Whitney Test for non-parametric analysis.
RESULTS

Fetal Characteristics

Fetal gestational age, weight, lung weight, and sex are provided in Table 1. Although fetal weight and lung weight were both reduced in IUGR fetuses (p<0.0005), lung weight was not significantly reduced relative to fetal body weight.

Histology and Morphometric Analysis

IUGR lungs were characterized by striking decreases in alveolarization and vascularization. When quantified, RACs were significantly decreased by 20% in IUGR fetuses compared to controls (p<0.005, Figure 1). Pulmonary vessel density was decreased to a greater extent (44%) than RACs in IUGR fetuses (p<0.01, Figure 1).

In Vitro Endothelial Cell Function

Migration

PAECs from four normal fetuses were treated with various concentrations of insulin and incubated in 3% or 21% oxygen to determine a dose response curve for insulin and to test for interactive effects of insulin and oxygen. PAEC migration was not increased with any concentration of insulin tested in 21% oxygen. In contrast, insulin treatment caused dose related increases in PAEC migration when tested in 3% oxygen (p<0.01, Figure 2a). Migration was then
tested in PAECs isolated from five control and three IUGR fetuses in 3% oxygen with 200 nM insulin. In these conditions insulin stimulated migration increased 115% in control cells but did not increase migration in IUGR PAECs (p<0.05, Figure 2b).

**Growth**

Based on previously published data showing that cell growth is optimized in 21% oxygen, we tested PAEC growth at 21% oxygen in cells isolated from three control and four IUGR fetuses (3). Basal cell counts on day zero were the same for both groups. In normal PAECs insulin treatment caused a dose related increase in cell growth to a maximum of 68% above basal conditions with 100 nM (p<0.0001). Cell growth was reduced in IUGR PAECs by 29% at baseline (p<0.01) and the response to insulin was attenuated (p<0.005, Figure 3).

**Tube Formation Assay**

Based on previously published data showing that fetal PAEC tube formation assay is optimized in 3% oxygen, we measured tube formation by control (n=3) and IUGR (n=3) PAECs in 3% oxygen (3). Insulin treatment caused a dose related increase in tube formation in control cells (p<0.0001), but this response was completely absent from IUGR PAECs, as was VEGFA stimulated tube formation (p<0.0001, Figure 4).

**Nitric Oxide Production**

We tested insulin stimulated NO production in three sets of control PAECs in 21% oxygen based on previous data indicating that NO production is inhibited in 3% oxygen (3). Insulin
increased NO production similarly between concentrations ranging from 1-200 nM (p<0.05, Figure 5a). Insulin was then tested in three sets of IUGR PAECs and compared to five sets of control PAECs. Although NO production was increased in the control cells it was completely absent in IUGR PAECs (p<0.005, Figure 5b). We then tested VEGFA stimulated NO production in the same cells and found a similar difference between control and IUGR PAECs as seen with insulin (p<0.0001, Figure 5c).

**Insulin Stimulated NO signaling**

IUGR PAECs (n=5) incubated in 21% oxygen had significantly less eNOS protein than control cells (n=5, P<0.05, Figure 6a). Furthermore, not only was there significantly less eNOS and less phosphorylated eNOS at baseline, insulin failed to increase eNOS phosphorylation as it did in control PAECs (P<0.005, Figure 6b). Incubating PAECs in 3% oxygen significantly reduced eNOS in both control and IUGR PAECs (P<0.0001, Figure 6a), but eNOS remained lower in IUGR PAECs compared to controls (P<0.005, Figure 6a). In 3% oxygen, neither control nor IUGR PAEC’s responded to insulin with increased eNOS phosphorylation, though the total amount of phosphorylated eNOS remained significantly lower in the IUGR PAECs (P<0.0001Figure 6b).

In order to investigate mechanisms of eNOS resistance to insulin stimulation, we also looked at insulin stimulated phosphorylation of Akt, an upstream positive regulator of eNOS phosphorylation. Although Akt concentrations were the same in control and IUGR PAECs, Akt phosphorylation was increased in the IUGR PAECs when incubated in 21% oxygen, both under basal conditions as well as under insulin stimulated concentrations (p<0.005, Figure 7).
Incubating cells in 3% oxygen did not affect the total amount of Akt present, but reduced Akt phosphorylation in both control and IUGR PAECs (p<0.01). However, even under 3% oxygen conditions, Akt phosphorylation remained higher in IUGR PAECs compared to controls (p<0.005, Figure 7b).

We also measured the amount of insulin receptor in the cells and found that although insulin stimulation decreased the total amount of insulin receptor within the cells with 21% oxygen (p<0.0001), this effect was the same in both IUGR and control PAECs (Figure 8). Incubating cells in 3% oxygen reduced the amount of insulin receptor in both control and IUGR cells (p<0.05, Figure 8).

Finally, we measured the amount of VEGF and VEGFR2 in the cells without insulin treatment. In 21% oxygen IUGR cells had less VEGFA (Figure 9a, p<0.0001) and VEGFR2 (Figure 9b, p<0.05) compared to control cells. Incubation in 3% oxygen decreased both VEGFA and VEGFR2 in control cells relative to 21% oxygen (Figure 9a,b, p<0.0001), but not in IUGR cells.
DISCUSSION

This is the first study demonstrating PAEC dysfunction along with impaired alveolar and lung vascular growth in IUGR fetuses. We found that experimental placental insufficiency with IUGR causes a significant reduction in radial alveolar counts and vessel density indicating decreased alveolarization and vascularization in the fetal lung, respectively. Furthermore, we isolated fetal PAECs from control and IUGR sheep to assess in vitro function. First, we showed that insulin increased cell motility, growth, tube formation, and NO production in normal fetal PAECs. In comparison with control PAECs, PAECs from IUGR fetuses were characterized by significantly decreased in vitro function. These findings included decreased PAEC motility, growth, tube formation, NO production, eNOS protein expression, insulin stimulated eNOS phosphorylation, VEGFA protein expression, and VEGFR2 protein expression. Additionally, although we found decreased VEGFA, decreased VEGFR2, and no difference in the amount of insulin receptor or Akt between control and IUGR cells, there was a paradoxical increase in both basal and insulin stimulated phosphorylation of Akt at ser473 in IUGR PAECs. Overall, these results support the hypothesis that abnormal lung growth may be related to impaired PAEC function, which may contribute to respiratory disease and the increased rates of BPD noted in IUGR infants. These findings further support clinical observations that adverse respiratory outcomes due to IUGR are independent of the degree of prematurity (8; 26) or the presence or severity of RDS (4), and occur for IUGR infants born at term (58).

Most cases of IUGR in developed countries are due to placental insufficiency (69). These pregnancies often share common complications such as decreased placental and umbilical blood flow; decreased placental transfer of glucose, essential amino acids, and oxygen to the
fetus; and decreased fetal anabolic growth factors such as insulin (18; 53; 62-64). The extent to which any of these mechanisms contribute to the pathogenesis of respiratory disease in IUGR infants is unknown. In order to determine the relative contribution of these complications, animal models of IUGR must be used. The model of IUGR in these experiments is well established and shares many features with human placental insufficiency and IUGR including decreased placental and umbilical blood flow (6).

Our results suggest that a compromised intrauterine environment can disrupt normal fetal pulmonary development. However, the severity depends on the model species used as well as the type, timing, and degree of insult. The model of IUGR used in this study is severe with effects documented as early as 60% gestation (6; 7; 12; 22; 48; 83). Another sheep model of IUGR resulting from restriction of placentation sites (carunclectomy) is also characterized by abnormal alveolar development (50). The severity of disrupted alveolar development due to carunclectomy was not as profound as observed in our model, however, fetal growth is also more severely reduced in the model used in our current study (50). Uteroplacental embolization over the last 20% of gestation in pregnant sheep results in a thicker fetal pulmonary blood-air barrier and a significant reduction in postnatal alveolar development (50; 55). Increased airspace wall thickness has also been documented in a rat model of placental insufficiency achieved by bilateral uterine artery ligation performed at approximately 90% of gestation (66). Other studies have shown disrupted fetal alveolar development following maternal energy restriction in both rats and guinea pigs (39; 45). Furthermore, capillary surface area was reduced in the guinea pigs after maternal energy restriction (45).
Although differences exist among the above models, some generalities emerge. One is that across multiple studies the lung to body weight ratio is not reduced in IUGR (10; 39; 50; 66). Second, increased alveolar septal thickness appears to be the predominant histological lung lesion in models of IUGR with insults that occur later in gestation (55; 66). Disrupted alveolar development, as seen in our study, is associated with insults which begin earlier in gestation and are more prolonged (39; 45; 50).

In addition to decreased alveolarization and vessel density, we found striking evidence of PAEC dysfunction from IUGR sheep in vitro. This is the first demonstration of persistent in vitro PAEC defects following IUGR. These findings are interesting due to recent studies that have highlighted the importance of angiogenesis in the regulation of alveolarization during pulmonary development (34; 44; 77; 81; 82). Potential mechanisms are diverse, but are likely related to disruption of endothelial-epithelial cross talk, which may include disruption of VEGF signaling (34; 44; 78). Our findings include evidence that PAECs from IUGR sheep have decreased VEGFA and VEGFR2 as well as minimal responsiveness to exogenous VEGF signaling, as reflected by impaired NO production and tube formation in vitro. In addition, insulin, a potent angiogenic factor for normal fetal PAEC, has little effect on PAECs from IUGR sheep. Overall, these findings suggest that endothelial cell dysfunction may contribute to decreased vessel density and alveolarization in experimental IUGR. However, mechanisms through which IUGR alters lung endothelial cell function are unknown, and future experiments will study specific features of IUGR that may be responsible for endothelial dysfunction and abnormal pulmonary development in this model.
We also report that PAECs from IUGR sheep have decreased eNOS expression and decreased insulin-stimulated eNOS phosphorylation. Decreased NO production, along with decreased VEGF signaling, may contribute to decreased PAEC growth, tube formation and motility, as observed in other settings (24). Mechanisms that impair insulin stimulated eNOS expression and activity in IUGR are uncertain, but we report that this is not due to decreased insulin receptor. In fact, we found a paradoxical increase in insulin signaling through Akt, which is upstream of eNOS activation, as both basal and insulin stimulated ser473 phosphorylation of Akt is higher in the IUGR PAECs. Despite increased Akt activation, eNOS phosphorylation remains decreased. Phosphorylation and activation of eNOS by Akt is complex and several regulatory steps may be disrupted in our IUGR PAECs (16; 60; 76). Disruption of eNOS localization into cell membrant caveolae due to decreased acylation will prevent its phosphorylation by Akt. Following localization to the caveolae, increased binding to the protein caveolin would decrease eNOS phosphorylation. Furthermore, decreased interaction between the molecular chaperone heat shock protein 90 and eNOS will prevent Akt mediated eNOS phosphorylation. Finally, the balance between other kinases, such as calcium/calmodulin dependent protein kinase II, and phosphatases, specifically serine-threonine protein phosphatase 2A, will influence the total amount of eNOS serine 1177 phosphorylation (16; 60; 76). Further experiments will be necessary to test which one of these mechanisms, or others, are responsible for our findings. In addition, whether exogenous NO treatment could restore abnormal PAEC function and restore lung structure in experimental IOUGR is currently under investigation.

Potential limitations to our study include issues regarding the experimental model of IUGR. Currently, there are no animal models of IUGR that truly replicate all features of human IUGR. This is in part due to the fact that there are multiple causes of IUGR (69), and that the initial
factor leading to abnormal placental development and function usually is unknown. There are identified structural abnormalities in the placenta, especially reductions in arterial number, diameter, and degree of branching, which in severe IUGR are associated with increased placental vascular resistance due to decreased placental angiogenic and vascular growth factors (17; 25; 40; 46; 51; 52; 56; 75). As in human IUGR, the IUGR model used in this study is associated with early alterations in placental angiogenic and vascular growth factors and receptors (31; 70), which is followed by increased placental vascular resistance, decreased placental growth, decreased uterine and umbilical blood flow, decreased oxygen and nutrient transfer, hypoglycemia, hypoxemia, hypoinsulinemia, decreased insulin secretion, and asymmetric IUGR (13; 21; 47-49; 74; 84).

Another potential limitation of this study is that we used PAECs isolated from large pulmonary arteries but our morphometric analysis demonstrated decreased small vessels. Future experiments will measure the impact of IUGR on microvascular pulmonary ECs. Finally, we report abnormal PAEC function in vitro after IUGR, but angiogenesis is a complex processes requiring multiple cell types which cannot be fully replicated in vitro. This is underscored by the finding that although fetal angiogenesis occurs in low oxygen conditions relative to postnatal angiogenesis, not all in vitro assays measuring fetal PAEC function are optimal in 3% oxygen. Furthermore, the complexity of this process is also highlighted by the findings that some in vitro functional assays like migration and tube formation are optimized in 3% oxygen but VEGFA, VEGFR2, and eNOS expression and phosphophorylation are decreased in 3% oxygen relative to 21% oxygen. Classically, acute hypoxia stimulates VEGFA expression. However, VEGFA in our experiments was measured following an 18 hour incubation. We do not know if there was an initial increase in VEGFA in control cells incubated in 3% oxygen followed by a decrease. Clearly, VEGFA and NO are not the only factors regulating in vitro PAEC function and
angiogenesis. However, given decreased vessel density and decreased PAEC function measured by several assays in IUGR fetuses a pattern consistent with decreased fetal angiogenesis emerges.

The results of this study clearly show that severe chronic placental insufficiency results in abnormal fetal pulmonary development. Specifically, we found both decreased alveolarization and pulmonary vascularization. We also isolated PAECs from IUGR fetuses and demonstrated decreased basal, insulin stimulated, and VEGFA stimulated \textit{in vitro} function. This includes motility, growth, tube formation, and NO production. These defects are associated with decreased PAEC VEGFA, VEGFR2, eNOS, and eNOS phosphorylation despite higher than normal insulin signaling through Akt, demonstrating a significant block between Akt and eNOS. Taken together, these results show that the IUGR fetal lung is characterized by significant vascular and endothelial cell dysfunction, and we speculate that the endothelial dysfunction is responsible for impaired alveolarization. If persistent into postnatal life, these defects would explain the increased risk that IUGR infants have for worse respiratory outcomes and increased rates of BPD.
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FIGURE LEGENDS

Figure 1. Decreased Alveolarization And Vessel Growth In IUGR Fetal Lungs.
Representative micrographs from control (A) and IUGR (B) fetal lungs after immunohistochemical staining for vWF with H&E demonstrate alveolar simplification and decreased vascularization. Arrows indicate vWF+ vessels. C) Quantification by radial alveolar counts demonstrated significantly less alveolarization in IUGR lungs (p<0.005). D) Quantification of vessel density showed significantly less pulmonary vascularization in the IUGR fetuses (p<0.01). n=7 IUGR and n=5 control.

Figure 2. Insulin Stimulates Cell Migration In Normal But Not IUGR PAECs. A) Insulin stimulated PAEC migration in 3% oxygen but not 21% oxygen (p<0.01). B) Insulin stimulated migration was completely absent in PAECs isolated from IUGR fetuses (p<0.05). Representative micrographs showing decreased insulin stimulated migration into the scraped area (denoted by the dashed line) in IUGR PAECs (D) compared to control PAECs (C). n=3 IUGR and n=5 control.

Figure 3. Basal And Insulin Stimulated Cell Growth Is Attenuated In PAECs From IUGR Fetuses. Cell counts were performed on day 5 and were found to be significantly increased by both 1 and 100 nM insulin in control cells when compared to basal conditions (n=4, #, p<0.0005). For IUGR cells, only incubation in 100 nM insulin was significantly different from basal conditions (n=3, #, p<0.05). For basal conditions and both insulin concentrations cell counts were lower in IUGR PAECs than for control cells (*, p<0.01).
Figure 4. Decreased Tube Formation By IUGR PAECs. Representative micrographs from control (A-C) and IUGR (D-E) PAECs following overnight incubation in basal conditions (A,D), or with insulin stimulation (200 nM, B,E) or VEGFA stimulation (50 ng/ml, C,F) are shown. For these micrographs, slides were incubated with 10% propidium iodide in PBS, though this was only done for presentation here and not for quantification of branch points. G) PEAC tube formation was quantified by branch point counting and is significantly increased in response to both insulin and VEGFA in control cells (n=3, #, indicates a significant increase within control cells only between basal conditions and insulin and VEGFA stimulated conditions, p<0.0001). PAECs from IUGR fetuses had decreased basal tube formation and no response to insulin or VEGFA (n=3, *, p<0.0001).

Figure 5. IUGR Cells Have Significantly Decreased Insulin Stimulated NO Production. A) Normal PEACs increase NO production in response to both insulin and VEGFA (p<0.05). B) Insulin (200 nM) stimulated NO production was completely absent in IUGR PAECs (p<0.005) C) VEGFA (50 ng/ml) stimulated NO production was also absent in IUGR PAECs (p<0.0001). n=3 IUGR and n=5 control.

Figure 6. IUGR Decreases PAEC eNOS Expression And Activity. A) eNOS was measured in control and IUGR PAECs following 18 hour incubations in 3% or 21% oxygen with or without insulin (200 nM). Incubation in 3% oxygen significantly decreased eNOS in control cells (Δ, p<0.05). IUGR cells had significantly less eNOS than control cells whether incubated in 21% oxygen (*, p<0.05) or 3% oxygen (*, p<0.005). Insulin had no effect on the total amount of eNOS protein. B) ser1177 phosphorylated eNOS was measured in the same samples. Insulin had a significant effect in control cells incubated in 21% oxygen only (♯,p<0.05). Insulin effects were absent when control cells were tested in 3% oxygen or in IUGR PAECs regardless of oxygen concentrations. There was significantly less phosphorylated eNOS in IUGR cells
compared to control cells with or without insulin in both 3% and 21% oxygen (*, p<0.005). C) Representative western blots. Data were compared with a mixed models ANOVA which included terms for group (IUGR or control), insulin, oxygen, and a random animal term to account for repeated measures from the same animal’s set of cells. n=5 IUGR and n=5 control.

**Figure 7. IUGR Increases PAEC Akt Phosphorylation.** A) Akt was measured in control and IUGR PAECs following 18 hour incubations in 3% or 21% oxygen with or without insulin (200 nM) and was the same in all conditions tested. B) ser473 phosphorylated Akt was measured in the same samples. Insulin had a significant effect in control cells incubated in 21% oxygen only (*, p<0.05). Insulin effects were absent when control cells were tested in 3% oxygen. IUGR cells had increased basal and insulin stimulated phosphorylation compared to control cells in 3% or 21% oxygen (*, p<0.005). There was significantly less phosphorylated Akt in both IUGR and control cells with 3% compared to 21% oxygen (Δ, p<0.01), though IUGR PAECs had a significant increase in phosphorylation in response to insulin in 3% oxygen (*, p<0.05). C) Representative western blots. Data were compared with a mixed models ANOVA which included terms for group (IUGR or control), insulin, oxygen, and a random animal term to account for repeated measures from the same animal’s set of cells. n=5 IUGR and n=5 control.

**Figure 8. Insulin Receptor Expression Is Similar In Control And IUGR PAECs.** A) Insulin receptor was measured in control and IUGR PAECs following 18 hour incubations in 3% or 21% oxygen with or without insulin (200 nM). Incubation in 3% oxygen significantly decreased Insulin receptor in both groups (Δ, p<0.05). Incubation in insulin decreased the amount of insulin receptor in both groups in 21% but not in 3% oxygen (*, p<0.01). There were no differences between groups for insulin receptor. B) Representative western blots. Data were compared with a mixed models ANOVA which included terms for group (IUGR or control), insulin, oxygen, and a random animal term to account for repeated measures from the same animal’s set of cells. n=5 IUGR and n=5 control.
Figure 9. VEGFA and VEGFR2 Expression Are Decreased In IUGR PAECs. VEGFA and VEGFR2 were measured in control and IUGR PAECs following 18 hour incubations in serum and insulin free media in 3% or 21% oxygen. A) VEGFA was significantly decreased in IUGR cells incubated in 21% oxygen compared to control cells incubated in 21% oxygen (*, p<0.0001). Incubation in 3% oxygen significantly decreased VEGFA expression in control cells relative to 21% oxygen (#, p<0.0001). B) VEGFR2 was significantly decreased in IUGR cells incubated in 21% oxygen compared to control cells incubated in 21% oxygen (*, p<0.05). Incubation in 3% oxygen significantly decreased VEGFR2 expression in control cells relative to 21% oxygen (#, p<0.0001) C) Representative western blots. Data were compared with a mixed models ANOVA which included terms for group (IUGR or control), oxygen, and a random animal term to account for repeated measures from the same animal’s set of cells. n=5 IUGR and n=5 control.
Table 1. Fetal Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Gestational Age (days)</th>
<th>Fetal Weight (kg)</th>
<th>Lung Weight (g)</th>
<th>Lung:Body Weight (%)</th>
<th>Males (%)</th>
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<tbody>
<tr>
<td>Control (n=14)</td>
<td>132.8±0.9</td>
<td>3.26±0.12</td>
<td>125.3±13.1</td>
<td>3.95±0.2</td>
<td>31</td>
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<tr>
<td>IUGR (n=8)</td>
<td>132.0±0.7</td>
<td>1.81±0.19*</td>
<td>64.9±8.2*</td>
<td>3.54±0.1</td>
<td>50</td>
</tr>
</tbody>
</table>

* Indicates a significant difference between control and IUGR fetuses, P<0.0005
Reference List


55. Maritz GS, Cock ML, Louey S, Joyce BJ, Albuquerque CA and Harding R.  

56. Mayhew TM, Ohadike C, Baker PN, Crocker IP, Mitchell C and Ong SS.  


Figure 2.

A

B

C

D

* IUGR vs Control

(Migration

Insulin Stimulation)

(3, 32, 2% Oxygen)

Fold Increase Over

Basal

Insulin (mM)

4 3 2 1 0 1 2 3
Figure 3.
Figure 4A-F.

Control

A Basal

B Insulin

C VEGFA

IUGR

D Basal

E Insulin

F VEGFA
Figure 4G.
Figure 5.
Figure 6.

A

\[\Delta = \text{significant effect of oxygen in control cells only}; *= \text{significant effect of IUGR}\]

B

\[\Delta = \text{significant effect of oxygen}; *= \text{significant effect of IUGR}; #= \text{significant effect of insulin in control, 21\% cells only}\]

C

\[
\begin{array}{ccc}
21\% O_2 & 3\% O_2 \\
\hline
\text{Control} & \text{IUGR} & \text{Control} & \text{IUGR} \\
- & + & - & + \\
\text{Insulin (200 nM)} & \text{ph eNOS (ser1177)} & \text{eNOS} & \text{Actin}
\end{array}
\]
Figure 7.

A

![Graph A](image)

- Control
- IUGR

B

![Graph B](image)

Δ = significant effect of oxygen; * = significant effect of IUGR; # = significant effect of insulin in control, 21% and IUGR, 3% cells only

C

<table>
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<tr>
<th></th>
<th>21% O&lt;sub&gt;2&lt;/sub&gt;</th>
<th>3% O&lt;sub&gt;2&lt;/sub&gt;</th>
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<tr>
<td>Insulin</td>
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<tr>
<td>+</td>
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</table>

Insulin (200 nM)

ph Akt (ser473)

Akt

Actin
Figure 8.

A

Insulin Receptor
(Arbitrary Units)

21% Oxygen 3% Oxygen

Control IUGR Control IUGR

Δ = significant effect of oxygen; # = significant effect of insulin 21% oxygen only

B

21% O₂ 3% O₂

Control IUGR Control IUGR

Insulin (200 nM)

Insulin Receptor

Actin
Figure 9.

A

* = significant effect of IUGR in cells incubated in 21% oxygen only; ^ = significant effect of oxygen in control cells only;

B

* = significant effect of IUGR in cells incubated in 21% oxygen only; ^ = significant effect of oxygen in control cells only;

C

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IUGR</th>
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<tr>
<td>21% O₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3% O₂</td>
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VEGFA
VEGFR2
Actin
Table 1.

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