Early fetal hypoxia leads to growth restriction and myocardial thinning

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Ream M, Ray AM, Chandra R, Chikaraishi DM. Early fetal hypoxia leads to growth restriction and myocardial thinning. Am J Physiol Regul Integr Comp Physiol 295: R583–R595, 2008. First published May 28, 2008; doi:10.1152/ajpregu.00771.2007.—Hypoxia is necessary for fetal development; however, excess hypoxia is detrimental. Hypoxia has been extensively studied in the near-term fetus, but less is known about earlier fetal effects. The purpose of this study was to determine the window of vulnerability to severe hypoxia, what organ system(s) is most sensitive, and why hypoxic fetuses die. We induced hypoxia by reducing maternal-inspired O2 by 21% to 8%, which decreased fetal tissue oxygenation assessed by pimonidazole binding. The mouse fetus was most vulnerable in midgestation: 24 h of hypoxia killed 89% of embryonic day 13.5 (E13.5) fetuses, but only 5% of E11.5 and 51% of E17.5 fetuses. Sublethal hypoxia at E12.5 caused growth restriction, reducing fetal weight by 26% and protein by 45%. Hypoxia induced HIF-1 target genes, including vascular endothelial growth factor (Vegf), erythropoietin, glucose transporter-1 and insulin-like growth factor binding protein-1 (Igfbp-1), which has been implicated in human intrauterine growth restriction (IUGR). Hypoxia severely compromised the cardiovascular system. Signs of heart failure, including loss of yolk sac circulation, hemorrhage, and edema, were caused by 18–24 h of hypoxia. Hypoxia induced ventricular dilation and myocardial hypoplasia, decreasing ventricular tissue by 50% and proliferation by 21% in vivo and by 40% in isolated cultured hearts. Epicardial detachment was the first sign of hypoxic damage in the heart, although expression of epicardially derived mitogens, such as FGF2, FGF9, and Wnt9b was not reduced. We propose that hypoxia compromises the fetus through myocardial hypoplasia and reduced heart rate. Epicardium; myocardium; hypoxia-inducible transcription factor-1; human intrauterine growth restriction; insulin-like growth factor binding protein-1

Hypoxia is a normal part of fetal life in all vertebrates and plays a requisite role in development, driving vasculogenesis/angiogenesis, hematopoiesis, and chondrogenesis (24). However, excess hypoxia leads to developmental abnormalities and postnatal deficits (1, 8, 10) that can be chronic and pervasive. Therefore, the fetal response to hypoxia is important for fetal and postnatal well being.

In mammals, the fetus is persistently hypoxic compared with the adult. While normal adult arterial PO2 is 80–100 mmHg, the highest PO2 in the late gestation fetus is 22–32 mmHg, which is found in the umbilical vein just after the fetal blood passes the placenta (28, 57). In placental mammals, transient periods of increased hypoxia occur throughout gestation as a result of spontaneous uterine contractions, which, on average, occur hourly lasting 6–8 min and can reduce fetal vascular PO2 by 10–25% (32).

While restricted oxygen availability is normal and necessary in utero, excessive hypoxia has lasting negative consequences. Complications from fetal hypoxia/anoxia are among the top 10 causes of fetal death (1). Maternal factors, such as living at high altitude, hypertension, anemia, pulmonary disease, pre-eclampsia, drug abuse, and smoking contribute to fetal hypoxia (33). In humans, fetal hypoxia causes intrauterine growth restriction (IUGR) and low birth weight, and is associated with prematurity, infant mortality (33, 37, 67), and elevated risk of adult cardiovascular disease (6, 7, 87). High altitude hypoxia is thought to be responsible for the 100-gm decrease in birth weight associated with each 1,000-meter increase in elevation of maternal residence (43).

Work in chick and rodents suggests that hypoxia preferentially compromises early heart development (12, 27, 31). In mouse, two important steps in cardiac development begin around embryonic day 12.5 (E12.5). First, the coronary vasculature forms. Epicardial cells detach and migrate into the subepicardial space where they divide and differentiate into vascular smooth muscle, endothelial cells, and fibroblasts. This process requires VEGF, which is induced by hypoxia (70). Second, a wave of cell division forms the compact myocardium that increases ventricular wall thickness by fivefold between E11.5 and E14.5 (72). Mouse mutants that die between E12.5 and E15.5 show signs of congestive heart failure and have cardiac hypoplasia (29, 42, 52, 61), suggesting that cardiac function, cardiomyocyte proliferation, and fetal survival are critically linked during this stage of development. Most studies of fetal hypoxia focus on chronic hypoxia late in gestation, but little is known about its effects on the early mammalian fetus. Here we investigated the effects of hypoxia on midgestation (E11.5–E13.5) mouse fetuses, which are similar to human fetuses at 6 wk (term being 38 wk) and sheep fetuses at 4 wk (term being 21 wk), based on Carnegie stages of anatomical landmarks. Our main purpose was to determine the window of vulnerability to severe hypoxia, to determine what organ system(s) is most sensitive, and to develop a hypothesis as to why hypoxic fetuses die.

MATERIALS AND METHODS

Animals. Fetuses were obtained by mating 5- to 6-wk-old CD-1 mice (Taconic, Germantown, NY) or were bred from local stock that had a small contribution (<0.2%) of C57BL/6J in their genetic background. Females were examined each morning, and the presence of a vaginal plug was considered to be E0.5. At the time of dissection, females were anesthetized with isoflurane (Aerane; Baxter Laboratories, Deerfield, IL) and killed by cervical dislocation. The uterus was removed and placed into W3 buffer [120 mM NaCl, 5 mM KCl, 1 mM NaH2PO4, 20 mM HEPES, and 20 mM glucose (pH 7.3)] that was supplemented with 10 mM MgSO4. The fetuses were removed and determined to be dead or alive by the presence of a heart beat. Fetal weight was determined by weighing freshly dissected or fixed fetuses.

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There was no difference in the weights of fresh or fixed fetuses, so the data were pooled. Fetal protein content was determined by the Lowry method (Sigma-Aldrich, St. Louis, MO) using at least three fetuses per litter. When hypoxic fetuses were compared with normoxic fetuses (sometimes referred to as 0 h of hypoxia), fetuses were age matched so that hypoxia ended at the time indicated, and animals in both oxygen conditions were killed at the same gestational age. Animals were used in accordance with protocols approved by the Institutional Animal Care and Use Committee of Duke University Medical Center, Durham, NC.

Induction of hypoxia and hyperoxia. Pregnant females were placed in a normobaric environmental acrylic chamber through which O2 flow could be controlled. For short-duration experiments, animals in their home cages were placed in loosely closed plastic bags into which an air line with a valved flowmeter was inserted such that oxygen levels could be changed within 10 min. Hypoxia was induced with 8% O2-2% N2, which the pregnant dams tolerated; none died even after periods of hypoxia (48–72 h) longer than those used here. We chose 8% O2 based on our observations that higher levels of oxygen did not result in fetal death or noticeable fetal deficits after 24 h, and that lower levels of oxygen (7%) killed almost all fetuses in <24 h from E11.5 to E12.5 and from E12.5 to E13.5, the only two ages assessed. Hyperoxia was induced using 95% O2-5% CO2. Oxygen in the chamber was measured using an oxygen analyzer (Engineered Systems and Designs, Newark, DE). During hypoxia and hyperoxia, humidity and temperature were monitored in accordance with our Institutional Animal Care and Use Committee procedures. Gas flow was maintained at 0.1–0.2 l per minute.

Maternal food consumption and blood glucose. Pregnant CD-1 mice were subjected to 24 h of hypoxia (8% O2, balance N2) or maintained in normoxia from E11.5 to E12.5. Food (standard mouse chow pellets) was weighed at the beginning and end of the 24-h period. For the nutritional restriction group, dams were housed in normoxia without access to food from E11.5 to E12.5. Initial and final blood glucose was measured from the dam’s tail vein as described previously (5) using a commercial glucometer (One Touch Ultra; LifeScan, Milpitas, CA).

Histology. Fetuses were fixed overnight at 4°C in either 10% buffered formalin (VWR, West Chester, PA) or Bouin’s solution (Sigma-Aldrich) for pimonidazole staining. Tissue was then dehydrated through two changes each of 30% and 70% ethanol, with each wash lasting 24 h. Fetuses were paraffin-embedded by the Duke University Immunohistology Research Laboratory and cut in 10-μm sections onto Superfrost Plus slides (Fisher Scientific, Hampton, NH). Tissue sections were stained with hematoxylin and eosin according to the manufacturer’s instructions. Total RNA from each of three fetuses per litter was extracted overnight at 4°C in either 10% buffered formalin or Bouin’s solution (Sigma-Aldrich) using a RNA STAT-60 kit (Tel-Test, Friendswood, TX) according to the manufacturer’s instructions. Total RNA from each of three fetuses from different litters in the same O2 condition was then pooled and purified with an RNeasy Mini Kit (Qiagen, Valencia, CA). Three samples of RNA representing nine fetuses were collected for each O2 condition. Isolated total heart RNA was prepared from dissected E12.5 hearts. Three samples were collected for each condition, with each sample composed of at least 23 hearts from at least two litters. Purified RNA was treated with RQ-1 RNase-free DNase (Promega, Madison, WI) at 1 U/μg RNA to remove genomic DNA and first-strand cDNA synthesis (RT) was performed with an Ambion RevertAid First Strand cDNA Synthesis Kit (Ambion, Austin, TX), using 1 μg of total RNA per 20 μl reaction. Quantitative real-time PCR analysis was performed using an Applied Biosystems 7300 Real-Time PCR System (Foster City, CA). PCR reactions used 0.2 μl of first-strand cDNA in a final volume of 20 μl in triplicate using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Dilutions of cDNA were used that gave a cycle number at threshold values between 21 and 28 cycles, and each pair was optimized for annealing temperature.

Primers synthesized by Invitrogen (Carlsbad, CA) are listed in Table 1. The PCR cycling conditions were as follows: polymerase activation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s, annealing for 30 s at primer-specific temperatures (see Table 1), and extension at 72°C for 30 s; dissociation at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. Each assay included (in triplicate) a standard curve of three serial dilutions of cDNA pooled from normoxic and hypoxic fetuses. Data were obtained using a relative standard curve method for each gene, normalizing to ribosomal protein L6 (rpl6, GenBank accession no. NM_011290), which did not change with hypoxia. The PCR products were confirmed by visualization of an appropriate-sized product on an agarose gel.

Quantification of myocardial tissue area, luminal volume, and wall thickness. Ventricular myocardial tissue area of E12.5 fetuses was estimated using the threshold tool in the NIH ImageJ program (http://rsb.info.nih.gov/ij/). Hematoxylin and eosin-stained tissue sections were photographed at ×50 magnification (Axioskop Zeiss, Oberkochen, Germany) using a black-and-white charge-coupled device camera (RT CCD-1317-11; Princeton Instruments, Trenton, NJ) and image acquisition software (IPLab 3.5; Scanalytics, Rockville, MD). A region of interest was drawn around the ventricles in an 8-bit grayscale image of each section to be quantified. The image threshold was set to include all pixels representing ventricular compact myocardium, ventricular trabeculae, interventricular septum, and atrioventricular cushion (when present in the section). The number of pixels in the image representing myocardial tissue was then determined. A second region of interest was drawn to measure the volume of the ventricular lumens. Fixation did not control for the stage of contraction in which the heart was arrested and preserved, such that hearts could have been captured in systole or diastole. The ventricular tissue and lumen areas from each 10-μm section 60 μm apart throughout the length of the ventricles were summed to give measurements proportional to the total ventricular tissue area, total lumen area, total ventricular volume (tissue + lumen), and fraction of total volume represented by luminal area. Tissue area (myocardial muscle volume) data were normalized to the normoxic ventricular tissue area. Heart wall thickness was measured from digital images of the ventricular wall from matched sections at the level of the atrioventricular cushion. Wall thickness was determined at five positions at least 100 μm apart in the ventral-most right and left ventricles in each section. Left and right ventricular measurements were averaged for each fetus.

In vivo proliferation assay. In vivo proliferation was visualized by (+)-5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) incorporation into newly synthesized DNA. Pregnant females were housed in ambient O2 (21%) or in 8% O2 for 12 h (from E12.0 to E12.5) or 24 h (E11.5 to E12.5). On E12.5, females were injected subcutaneously at two dorsal, midthoracic sites with a total of 50 μg/g BrdU (40)
dissolved in lactated Ringers solution and returned to their respective 
O2 condition for 3 h, after which time the embryos were processed as 
described above, and tissue sections were stained according to Mor-
genbesser et al. (44), with the following modifications: sections were 
blocked for 30 min in 1% horse serum in PBS and stained with 
anti-BrdU mouse monoclonal antibody (BD Biosciences, San Jose, 
CA) diluted 1:200 in 1% horse serum in PBS overnight at 4°C. 
Sections were then washed in PBS, and biotinylated anti-mouse IgG 
secondary antibody (BA-2000; Vector Laboratories) was applied for 
1 h at room temperature diluted 1:500 in 1% horse serum in PBS. 
Slides were developed with 3,3′-diaminobenzidene according to the 
manufacturer’s instructions and counterstained for 20 min with Basic 
Fuchsin (Sigma-Aldrich).

The fraction of BrdU-labeled cells was determined from at least 
2,000 cells representing three areas of the heart (interventricular 
septum and left and right ventricular walls) per fetus and averaged to 
determine the mean heart labeling index for each fetus. Because 
interstitial fibroblasts represent a very low percentage of cells in the 
myocardium prior to birth (56), the majority of cells labeled with 
BrdU in the heart are cardiomyocytes. Similarly, labeling indexes 
were determined from at least 200 cells per fetus in the midthoracic 
spinal cord and at least 300 cells per fetus at the rostral edge of the 
third ventricle of the brain. Three hours was chosen for BrdU labeling 
based on the observation that S-phase is significantly longer than 3 h 
in fetal rat and mouse neurotic tissue at a variety of ages (64), so that 
labeling would be confined to one generation of proliferating cells.

### In vitro proliferation assay.

Heart cultures were prepared essentially as described by Wildenthal (78). E11.5 hearts were dissected 
free of inflow and outflow vasculature and cultured on top of cell 
culture inserts (BD Falcon, San Jose, CA) in 12-well plates (BD 
Falcon) in 1.0 ml of medium 199 (GIBCO-Invitrogen, Carlsbad, CA) 
supplemented with 10% or 0.5% fetal clonal serum (HyClone, Logan, 
UT) for 24 h at 37°C in a variable-oxygen tissue culture incubator at 
21% or 2% O2 with 5% CO2. Hearts were in contact with the media 
below the insert but were not submerged. Hearts continued to beat, but 
did not experience physiological hemodynamic loads that would be 
present in vivo. A maximum of three hearts were cultured per insert 
and each litter was equally divided between normoxic and hypoxic

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### Table 1. Primers used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product Length, bp</th>
<th>Annealing Temperature, °C</th>
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<td>Bnip3</td>
<td>GGC GTC TGA CAA CTT CCA CT</td>
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<td>61</td>
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<td>Ep0</td>
<td>GAG GCA GAA AAT GTG ACG ATG</td>
<td>CTG CCA CCT CCA TTC TTC TC</td>
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<td>58.4</td>
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<td>Fgf1A</td>
<td>CCC TGA CAG AGA GGT TCA AC</td>
<td>GTG CTT TGT CCC ATC CAG G</td>
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<td>58.4</td>
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<tr>
<td>Fgf2</td>
<td>AGC GAC CAC AGC ACA CAT AC</td>
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<td>61</td>
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<td>Fgf9</td>
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<td>Hif1α</td>
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<td>55</td>
</tr>
<tr>
<td>Hif1β</td>
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<tr>
<td>Hif2α</td>
<td>CTA AGG AAG CAC AGG AAA TCC GTG</td>
<td>TGT GTC GCA AGG AAG CTG ATG</td>
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<td>Igf1 R</td>
<td>GTC GGG GTG GTG CTG TTG TCT C</td>
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</tr>
<tr>
<td>Igf2</td>
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<td>AGG TCC TTC TGG GAC TTG G</td>
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<td>Igfbp-1</td>
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<td>ATG TAT GGG ACG CAG CCT TC</td>
<td>115</td>
<td>58.4</td>
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<tr>
<td>Pkhd3</td>
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<td>AGG GTA GTC CCC ATT GTT GAA GGA</td>
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<td>Rpl6</td>
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<td>61</td>
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<tr>
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<td>ACA GGA CCG CTT GAA GAT G</td>
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<td>61</td>
</tr>
<tr>
<td>Wnt9b</td>
<td>AAG TAC AGC ACC AAG TTC CTC AGC</td>
<td>GAA CAG CAC AGG AGC CTG ACA C</td>
<td>166</td>
<td>61</td>
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Fig. 1. Fetal tissue PO2 reflects maternal oxygenation. Representative images of embryonic day 12.5 (E12.5) fetuses labeled with a hypoxia-reactive dye, pimonidazole (120 μg/g), and exposed for 3 h to hyperoxia (A; hyperoxia, 95% O2), normoxia (B; normoxia, 21% O2), or hypoxia (C; hypoxia, 8% O2) are shown. As maternal FIO2 decreased, dye binding increased, indicating reduced fetal tissue PO2. These data indicate that fetal PO2 can be manipulated by changing maternal oxygenation. All pimonidazole sections were stained at the same time. H, heart; L, liver; VB, vertebral bodies; DRG, dorsal root ganglia; SC spinal cord. Scale bar = 600 μm. The experiment was repeated on at least 3 litters per condition with at least 1 fetus examined per litter.
conditions. After 21 h, DNA synthesis was determined by a 3-h pulse of [3H]thymidine (Amersham Biosciences, Piscataway, NJ) with 2 Ci/ml added to the preexisting culture media. At the end of the culture period, individual hearts were washed three times in PBS and dissolved in 0.1 ml 1 N NaOH. Fifty microliters of the dissolved heart solution was precipitated with 1.0 ml of 10% trichloroacetic acid (Sigma-Aldrich), followed by a 10-min incubation period on ice. Samples were filtered through a glass fiber filter (Gelman Sciences, Ann Arbor MI) to retain the precipitated DNA, and the filters were dried before scintillation counting.

Apoptosis assay. Apoptosis was visualized by terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) using an ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions, with modifications. The TdT enzyme reaction solution contained 15% TdT enzyme and 45% reaction buffer in PBS, and the labeling incubation was carried out for 2 h. The total number of TUNEL positive cells in the ventricular myocardium not including the cushion and outflow tract myocardium, endocardium, and epicardium, was counted and averaged. At least two sections separated by a minimum of 90 μm were counted per animal.

Statistical analysis. Presented values are means ± SE. Data were analyzed using one-way ANOVA followed by a post hoc Student’s t-test. Statistical significance was considered to be P < 0.05.

RESULTS

Fetal hypoxia can be induced by maternal hypoxia. To determine whether altering maternal oxygenation affects fetal PO2, we subjected pregnant dams at E12.5 to normoxic (21%), hypoxic (8%), or hyperoxic (95%) levels of inspired oxygen for 3 h after injection with an oxygen-sensitive dye, pimonidazole (Fig. 1). Pimonidazole crosses the placenta (35a) and is reduced by nitroreductases only in hypoxic cells to form covalent protein adducts (35a), which can be detected immunohistochemically. Hence, increased dye binding reflects de-

![Fig. 2. Sensitivity to hypoxia peaks at E13.5 and is proportional to the length of time in hypoxia. A: pregnant mice were placed in 8% oxygen for 24 h ending on the gestational day indicated in the figure. Fetuses were dissected and determined to be dead or alive by the absence or presence of a heart beat. *Data vs. E11.5, P < 0.05. †Data vs. E13.5, P < 0.05. Number of alive/total fetuses for each age group was 39/41 for E11.5, 55/77 for E12.5, 8/71 for E13.5, 14/52 for E15.5, and 23/47 for E17.5. Four litters were used at E11.5 and E17.5; 7 litters were used at E13.5, 6 litters at E12.5, and 5 litters at E15.5. B: pregnant mice were placed in 8% oxygen for varying lengths of time prior to E13.5 so that hypoxia was complete at E13.5. All fetuses were then dissected and determined to be dead or alive. *Data vs. 0 h of hypoxia, P < 0.05. †Data vs. 24 h of hypoxia, P < 0.05. Number of alive/total fetuses was 46/46 for 0 h, 32/35 for 12 h, 19/33 for 18 h, and 8/70 for 24 h. In B, 3 litters were used at 12 and 18 h; 4 litters were used at 0 h and 7 at 24 h. For both A and B, N = a litter, with all fetuses in a litter counted.

![Fig. 3. Hypoxia causes growth restriction. Pregnant dams were subjected to 8% O2 for varying lengths of time prior to E12.5, so that hypoxia was complete at E12.5. A: hypoxia caused a time-dependent reduction in fetal weight among live fetuses. *Data vs. 0 h of hypoxia (i.e., normoxic E12.5 fetuses), P < 0.05. N = mean fetal weight of 5 litters at 0 h, 6 litters at 12 h, and 4 litters at 24 h, using at least 3 fetuses per litter. B: hypoxia reduces fetal protein content. *Data vs. 0 h of hypoxia, P < 0.05. N = mean fetal protein content of 3 litters at 0 h, 3 litters at 12 h, and 3 litters at 24 h, using at least 3 fetuses from each litter.](http://ajpregu.physiology.org/ content/295/8/R586/F1)
creased tissue PO$_2$ (49). In normoxic fetuses, we found hypoxic sites in vertebral bodies and in focal areas in the heart, liver, and spinal cord (Fig. 1B). Increasing inspired maternal O$_2$ to 95% reduced pimonidazole binding (Fig. 1A), whereas reducing O$_2$ to 8% increased pimonidazole binding in most tissues (Fig. 1C).

### Early fetuses are selectively vulnerable to hypoxia

To determine whether hypoxia selectively affects fetuses at different gestational ages, dams were exposed to 8% O$_2$ for 24 h, and fetal survival was determined on various days of gestation (Fig. 2A). We found that fetuses experience a window of vulnerability at E13.5. Vulnerability was proportional to the duration of hypoxia and death required at least 12–18 h of hypoxia, as opposed to acute anoxia, which induces death within several hours. Overall, these data suggest that fetuses are most vulnerable to hypoxia in midgestation (E12.5–E14.5). Because vulnerability begins at E12.5, and by E13.5 viability was so reduced that obtaining sufficient numbers of animals was problematic, E12.5 was chosen for further investigation.

### Hypoxia causes growth restriction

Chronic hypoxia (days to weeks) is well-documented to cause growth restriction when late-term fetuses or neonates are examined (26, 43, 47). To determine whether hypoxia has the same effect in younger fetuses, we assessed wet weight and protein content of whole fetuses, we assessed wet weight and protein content of whole embryonic day 11.5 (hypoxia) or from normoxic dams were dissected, and RNA was prepared and used for different gestational ages, dams were exposed to 8% O$_2$ for 24 h, and fetal survival was determined on various days of gestation (Fig. 2A). We found that fetuses experience a window of vulnerability at E13.5. Vulnerability was proportional to the duration of hypoxia and death required at least 12–18 h of hypoxia, as opposed to acute anoxia, which induces death within several hours. Overall, these data suggest that fetuses are most vulnerable to hypoxia in midgestation (E12.5–E14.5). Because vulnerability begins at E12.5, and by E13.5 viability was so reduced that obtaining sufficient numbers of animals was problematic, E12.5 was chosen for further investigation.

### Hypoxia causes transcriptional changes typical of hypoxia-inducible transcription factor-1 activation

In adult tissues and cell lines, hypoxia is well known to stabilize hypoxia-inducible transcription factor-1 (HIF-1), which activates transcription of hypoxia-responsive genes (reviewed in Ref. 76). However, little is known about hypoxia-induced transcription in the early fetus. RNA levels for six well-known HIF-1 targets (76) increased after 6 h of hypoxia (Table 3), a time point before most effects of hypoxia were seen. The induced HIF-1 targets were involved in anaerobic metabolism (Pfkbp3, 3.6-fold; Glut1, 1.6-fold), vascularization (Vegfa, 3.3-fold), and cell death (Bnip3, fourfold). The largest induction was in IGF-binding protein-1 (IGFBP-1, 4.5-fold), which binds insulin growth factors, thereby blocking their growth stimulatory effects (38, 47, 67, 68). Interestingly, other genes involved in the IGF pathway [IGF1, IGF2, IGF1 receptor (IGF1R)] were not changed by hypoxia. Although HIF-1 targets are transcriptionally induced by hypoxia, HIF transcription factors themselves [Hif1α, Hif1β (ARNT), and Hif2α (EPAS1)] were not induced (Table 3).

### Hypoxia results in signs of congestive heart failure and myocardial thinning

Hypoxia led to signs of congestive heart failure (Fig. 4) with loss of yolk sac circulation (Fig. 4, C and E), edema, and congestion in the vasculature, heart (Fig. 4D), and liver (Fig. 4F). In some cases, hypoxia resulted in hemor-

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### Table 2. Maternal food consumption decreases with hypoxia, but blood glucose is unchanged

<table>
<thead>
<tr>
<th>Food Consumption</th>
<th>Blood Glucose</th>
<th>Blood Glucose</th>
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<tr>
<td>of Dam at E12.5</td>
<td>of Dam at E12.5</td>
<td>of Dam at E12.5</td>
</tr>
<tr>
<td>g</td>
<td>mg/dl</td>
<td>mg/dl</td>
</tr>
<tr>
<td>21</td>
<td>5.1 ± 0.4</td>
<td>141 ± 6</td>
</tr>
<tr>
<td>8</td>
<td>0.61 ± 0.6*</td>
<td>155 ± 5</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
<td>144 ± 9</td>
</tr>
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### Table 3. Fetal gene expression changes in response to hypoxia

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<th>Gene</th>
<th>Description</th>
<th>Function</th>
<th>Accession No.</th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Fold Increase</th>
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<td>Igfl</td>
<td>Insulin-like growth factor binding protein-1</td>
<td>Growth restriction</td>
<td>NM_008341</td>
<td>0.20 ± 0.06</td>
<td>0.89 ± 0.20*</td>
<td>4.5×</td>
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<tr>
<td>Bnip3</td>
<td>BCL2/adenovirus E1B 19 kDa interacting protein-1, NIP3</td>
<td>Apoptosis</td>
<td>NM_007960</td>
<td>0.32 ± 0.03</td>
<td>1.27 ± 0.09*</td>
<td>4.0×</td>
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<td>Pfkbp3</td>
<td>6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFK-2)</td>
<td>Glycolysis</td>
<td>NM_133232</td>
<td>0.22 ± 0.11</td>
<td>0.80 ± 0.16*</td>
<td>3.6×</td>
</tr>
<tr>
<td>Vegfa</td>
<td>Vascular endothelial growth factor A (VEGF)</td>
<td>Vascularization</td>
<td>NM_009505</td>
<td>0.42 ± 0.17</td>
<td>1.4 ± 0.23*</td>
<td>3.3×</td>
</tr>
<tr>
<td>Glut1</td>
<td>Glucose transporter isoform-1, (Slc2a1)</td>
<td>Glucose transport</td>
<td>NM_011400</td>
<td>0.57 ± 0.10</td>
<td>0.93 ± 0.05*</td>
<td>1.6×</td>
</tr>
<tr>
<td>Epo</td>
<td>Erythropoietin</td>
<td>Hemoglobin synthesis</td>
<td>NM_007942</td>
<td>0.53 ± 0.00</td>
<td>0.74 ± 0.04*</td>
<td>1.4×</td>
</tr>
<tr>
<td>Igfl</td>
<td>Insulin growth factor-1</td>
<td>Cell growth</td>
<td>NM_010512</td>
<td>0.37 ± 0.03</td>
<td>0.26 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>IgflR</td>
<td>Insulin growth factor-1 receptor</td>
<td>Cell growth</td>
<td>NM_010513</td>
<td>0.20 ± 0.02</td>
<td>0.23 ± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Igfl2</td>
<td>Insulin growth factor-2</td>
<td>Cell growth</td>
<td>NM_010514</td>
<td>0.59 ± 0.10</td>
<td>0.45 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Hif1a</td>
<td>Hypoxia inducible factor-1α</td>
<td>Transcription factor</td>
<td>NM_010431</td>
<td>0.35 ± 0.04</td>
<td>0.29 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Hif1a</td>
<td>Hypoxia inducible factor-2α (EPAS-1)</td>
<td>Transcription factor</td>
<td>NM_010137</td>
<td>0.23 ± 0.00</td>
<td>0.27 ± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>Hif2a</td>
<td>Hypoxia inducible factor-1β (ARNT)</td>
<td>Transcription factor</td>
<td>NM_007488</td>
<td>0.25 ± 0.02</td>
<td>0.22 ± 0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

E12.5 fetuses from dams placed in 8% O$_2$ for 6 h ending at E12.5 (hypoxia) or from normoxic dams were dissected, and RNA was prepared and used for quantitative RT-PCR (qRT-PCR) as described in MATERIALS AND METHODS. Gene expression is reported relative to that of a ribosomal protein gene, Rplp0, which did not change with hypoxia based on qRT-PCR and microarray analyses. Expression values are the means ± SE of 3 samples, each composed of 3 fetuses from different litters. *Data vs. normoxia, P < 0.05. NS, not significantly different between normoxia and hypoxia.

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### Hypoxia causes growth restriction under hypoxic conditions was due to hypoxia rather than reduced maternal food consumption.

Maternal food consumption and blood glucose are altered in some models of hypoxia (11, 16). Because fetal growth is dependent upon maternal nutrition, we determined maternal food consumption and blood glucose levels in normoxic and hypoxic females bearing E12.5 fetuses (Table 2). Food consumption decreased by 88% after 24 h of hypoxia, but blood glucose was not affected. To determine whether growth restriction was due to reduced maternal food consumption, pregnant dams were housed in normoxia without access to food. Starvation for 24 h did not change maternal glucose or fetal weight compared with normoxic, nonstarved controls, suggesting that the growth restriction under hypoxic conditions was due to hypoxia rather than reduced maternal food consumption.

Hypoxia causes transcriptional changes typical of hypoxia-inducible transcription factor-1 activation. In adult tissues and cell lines, hypoxia is well known to stabilize hypoxia-inducible transcription factor-1 (HIF-1), which activates transcription of hypoxia-responsive genes (reviewed in Ref. 76). However, little is known about hypoxia-induced transcription in the early fetus. RNA levels for six well-known HIF-1 targets (76) increased after 6 h of hypoxia (Table 3), a time point before most effects of hypoxia were seen. The induced HIF-1 targets were involved in anaerobic metabolism (Pfkbp3, 3.6-fold; Glut1, 1.6-fold), vascularization (Vegfa, 3.3-fold), and cell death (Bnip3, fourfold). The largest induction was in IGF-binding protein-1 (IGFBP-1, 4.5-fold), which binds insulin growth factors, thereby blocking their growth stimulatory effects (38, 47, 67, 68). Interestingly, other non-HIF targets in the IGF pathway [IGF1, IGF2, IGF1 receptor (IGF1R)] were not changed by hypoxia. Although HIF-1 targets are transcriptionally induced by hypoxia, HIF transcription factors themselves [Hif1α, Hif1β (ARNT), and Hif2α (EPAS1)] were not induced (Table 3).

Hypoxia results in signs of congestive heart failure and myocardial thinning. Hypoxia led to signs of congestive heart failure (Fig. 4) with loss of yolk sac circulation (Fig. 4, C and E), edema, and congestion in the vasculature, heart (Fig. 4D), and liver (Fig. 4F). In some cases, hypoxia resulted in hemor-
rhage into the yolk sac and/or pericardial cavity, causing loss of blood from the fetal body circulation. These signs were apparent to a lesser degree in live hypoxic fetuses (Fig. 4, C and D) compared with dead hypoxic fetuses (Fig. 4, E and F).

Histological analysis also suggested that the heart is more sensitive to hypoxia than other organs. The ventricular myocardium was thin (Fig. 5C, orange circle on left ventricle), and the epicardium was detached (black arrowhead). Hypoxia slowed the maturation of the heart. Between E11.5 (Fig. 5A) and E12.5 (Fig. 5B), the normoxic compact myocardium increases in thickness, the interventricular septum becomes more compact, and the epicardium adheres more closely to the myocardium. These events were retarded in hypoxic E12.5 fetuses, as if growth was severely delayed from the time hypoxia was initiated. In addition, hypoxic E12.5 fetuses had a thinner compact myocardium and a loosely compacted (disorganized) septum compared with normoxic E11.5 fetuses, suggesting that hypoxia caused pathological changes in addition to simply slowing normal development.

We digitally quantified ventricular tissue area including the compact myocardium, trabeculae, interventricular septum, and atrioventricular cushions through the length of the ventricles in live E12.5 fetuses. We found that ventricular tissue area and wall thickness were inversely related to the time in hypoxia, such that after 18 h of hypoxia, ventricular mass was reduced by 45% and wall thickness by 55% (Fig. 5, D and E) compared with normoxic E12.5 fetuses. In some areas, the compact zone of the ventricular myocardium was only one or two cells thick.

Hypoxia also resulted in ventricular dilation, increasing lumenal area by 173% (Fig. 5F). Organs other than the heart appeared largely unaffected by 18–24 h of hypoxia.

**Hypoxia preferentially affects the epicardium and causes its detachment.** Because the heart was most affected by hypoxia based on histological inspection, we investigated constitutive and induced areas of hypoxia in the heart. Under normoxia, pimonidazole bound to the myocardium adjacent to the outflow tract (arrows in Fig. 6B) but was largely absent from other regions. After 3 h of hypoxia, pimonidazole binding modestly increased in the ventricular myocardium, with substantial increases in localized areas (Fig. 6C). The most robust increase in binding occurred in the epicardium (black circles in Figs. 6, D and E) and to a lesser extent in the endocardium. Interestingly, the first morphological defect induced by hypoxia was epicardial detachment from the underlying myocardium, which was present in varying degrees (mild detachment in Fig. 6E, severe detachment in Fig. 6G, compared with normoxic appearance in Fig. 6F) after 3 h of hypoxia in ~90% of the hearts examined.

**Hypoxia-induced myocardial thinning is a result of reduced proliferation.** Myocardial thinning could result from decreased cell proliferation or increased cell death. In normoxic E12.5 fetuses, we found that 31% of ventricular myocardial cells were labeled with BrdU (Fig. 7A and C, left), which agrees with reports that cardiomyocytes are rapidly dividing in midgestation (45). Twenty-four hours of hypoxia from E11.5 to E12.5 reduced the labeling by 21%, indicating that hypoxia...
reduces proliferation (Fig. 7B, left). Using TUNEL to label apoptotic cells, fewer than 0.1% of myocardial cells were labeled, and this low level did not change after 24 h of hypoxia. There were $9.7 \pm 2.18$ TUNEL-positive cells per tissue section obtained midway through the ventricles from normoxic fetuses, compared with $6.8 \pm 1.51$ labeled cells per section after 24 h in 8% O$_2$ (data are mean of at least 2 slides $\pm$ SE from each of 3 fetuses in each condition, using 1 fetus per litter).

The level of hypoxia in our experiments reduced proliferation selectively in the myocardium. In the same animals that were used for myocardial BrdU labeling, we saw no significant change in proliferation in the brain or spinal cord (Fig. 7C, middle and right), despite the fact that these sites are highly proliferative at E12.5 (64, 65). Approximately 40% of cells in the brain and 25% of cells in the spinal cord were labeled.
To determine whether hypoxia directly blocks proliferation as opposed to inducing maternal or fetal circulating factors such as IGFBP-1, we assessed DNA synthesis in isolated hearts in organ culture. DNA synthesis dropped by \( \frac{40}{100} \) regardless of serum concentration in the media (Fig. 7D), suggesting that serum growth factors could not overcome the effects of hypoxia. All hearts continued to beat in hypoxia, implying that hearts were still alive. However, hypoxia reduced heart rate to \( \frac{38}{100} \) and \( \frac{23}{100} \) in 10% and 0.5% serum, respectively, consistent with the known bradycardic effect of hypoxia on cardiomyocytes and tissue strips (58, 60, 69, 75). Interestingly, heart rate partially recovered to 72% and 48% of normal in 10% and 0.5% serum, respectively, when oxygen was restored. Hence, hypoxia directly affected both proliferation and heart rate in organ culture, and the known mitogenic effects of serum on isolated hearts (35) could not reverse the effect of hypoxia.

To assess how hypoxia might block proliferation, we determined the expression of genes that affect myocardial proliferation or survival using heart RNA extracted from fetuses subjected to 6 h of maternal hypoxia ending at E12.5. Three factors, FGF2, FGF9, and Wnt9b, have been identified as potential epicardially derived mitogens in vivo (35, 39). However, none of these was repressed by hypoxia (Table 4). In contrast, two known HIF-1 targets, Bnip3 and Vegf, were induced in the heart by hypoxia, indicating that the heart indeed mounts a transcriptional response to hypoxia like other tissues.

**DISCUSSION**

The data presented here demonstrate that the early fetus is particularly sensitive to induced hypoxia, which causes growth restriction, changes in gene expression, and cardiac-specific deficits that can lead to fetal death.

**Fetal Po2 and regions of constitutive and induced tissue hypoxia.** Reducing maternal inspired O2 (FiO2) induces fetal hypoxia in large mammals where blood gas levels can be measured by sampling fetal blood (28, 30), but its ability to affect Po2 and induce hypoxia in the early rodent fetus has not been assessed. In the adult, transcriptional responses (reviewed in Ref. 55) begin when tissue Po2 drops below 30—40 mmHg. Fetal Po2, on the other hand, may never exceed 30 mmHg even in freshly oxygenated blood, so that fetal hypoxia sensing and response must adapt its dynamic range if it is to respond to truly hypoxic insults. Our results indicate that the fetus does

Fig. 6. The epicardium is particularly affected by hypoxia. A: representative image of hematoxylin and eosin-stained sagittal section of normoxic E12.5 heart. B: pimonidazole binding of an adjacent section reveals areas of constitutive hypoxia in the heart of a normoxic fetus. C: pimonidazole binding increases after 3 h of maternal hypoxia ending at E12.5 (8% O2). Arrows in B and C point to pimonidazole binding in the outflow tract (OFT) myocardium. Pimonidazole binding in epicardium (circled) in normoxic (D) and hypoxic (E) E12.5 fetuses shows that hypoxia preferentially increased dye binding in epicardial cells and to a lesser extent in endocardial cells (green arrow). The red arrow points to red blood cells (RBC). Hematoxylin and eosin-stained sections of ventricular wall of normoxic (F) and hypoxic (G) E12.5 fetuses show hypoxia-induced detachment of the epicardium (black arrows) from the myocardium. Scale bars in A–C = 300 \( \mu \)m, D = 50 \( \mu \)m, E–G = 25 \( \mu \)m. These observations were made in at least 3 litters.
sense and respond to excess hypoxia at an early age. We cannot measure PO2 directly in the small E12.5 mouse, but our experimental conditions (8% maternal FIO2) are likely to result in a fetal PO2 of 10–14 mmHg, based on measurements in lambs using similar levels of maternal hypoxia (3, 13, 23, 54).

Under normoxic conditions we see focal areas of low tissue oxygenation in E12.5 fetuses using the oxygen-sensitive dye pimonidazole, which reacts at tissue PO2 levels below 10 mmHg (20) (Fig. 1). Such regional binding has been seen by others 1) at sites of chondrogenesis, which are known to be hypoxic (53); 2) in the outflow tract myocardium, where hypoxia drives apoptotic remodeling in chick (62, 63); and 3) in the brain and spinal cord.

In response to maternal hypoxia, most fetal tissues show increased dye binding, but labeling is not homogeneous across cell types within an organ (20). In the heart, epicardial cells showed the most robust increase in dye binding, which may recapitulate the normal hypoxia-driven epithelial-mesenchymal transformation during which epicardial cells generate cardiac vasculature (17, 70). We conclude that fetal PO2 can be manipulated by controlling maternal inspired oxygen and that sites of constitutive and induced hypoxia may reflect regions in which hypoxia drives normal development and that the fetus can mount a HIF1-mediated transcriptional response.

Midgestational sensitivity to hypoxia. We have employed a model of hypoxia (8% O2 for 18–24 h) that would be considered moderate in intensity and duration compared with studies of mild, chronic hypoxia (weeks at high altitude) or acute, severe hypoxia (occluded uterine or umbilical blood flow). At a developmental stage comparable to E12.5 in the mouse, 1 day

![Figure 7](image-url)

Fig. 7. Hypoxia leads to reduced proliferation in vivo and in vitro in the myocardium, but not in brain or spinal cord. Representative images of in vivo (+)-5-bromo-2′-deoxyuridine (BrdU) labeling in ventricular myocardium of E12.5 fetuses after 0 h (A) or 24 h in 8% O2 (i.e., from E11.5 to E12.5; B). Note the myocardial thinning in B. IVS, interventricular septum. Scale bars = 50 μm. C: hypoxia reduced the fraction of proliferating cells as determined by BrdU incorporation in the heart (left), but had no effect in the brain (middle) or spinal cord (right). *Data vs. 0 h in the same tissue type, P < 0.05. For heart, n = 7 fetuses at 0 h, 8 at 12 h, and 6 at 24 h. For spinal cord, n = 3 fetuses for each time point. For brain, n = 4 for 0 h and 5 for 24 h. One fetus was used per litter. D: in vitro tritiated thymidine incorporation into isolated hearts determined after 24 h under 21% O2 (normoxia) or 2% O2 (hypoxic) conditions in the presence of 10% or 0.5% fetal clonal serum. Hearts were explanted at E11.5, divided between conditions, and cultured for 21 h before being labeled with thymidine for 3 h. N = number of hearts: 21% O2-10% serum = 18; 21% O2-0.5% serum = 22; 2% O2-10% serum = 19; 2% O2-0.5% serum = 22. Average incorporation per heart ranged from 1,040 cpm to 2,510 cpm depending on the culture conditions. *Data vs. 21% O2 in the same serum concentration, P < 0.05. Incorporation at 21% O2 was set to 100%.
in fetal mouse development approximates 1 wk of human gestation. The duration and intensity of hypoxia in our experiments has not been studied in humans (or other large mammals) at this early stage. However, a variety of conditions including smoking and maternal drug abuse, placental abnormalities, and anemia can result in moderate-to-severe and potentially prolonged fetal hypoxia. For example, cocaine exposure in E11 rats phenocopies the effects of hypoxia we report here, with bradycardia and growth restriction, likely through uterine vasoconstriction (21). Its effects are exacerbated by induced maternal hypoxia. In mature fetal sheep, cocaine caused fetal hypoxia as evidenced by a reduced fetal P O2 at birth (2).

**Growth restriction and thin myocardium.** We observed generalized fetal growth restriction resulting in decreased fetal wet weight and protein content after 24 h of hypoxia from E11.5 to E12.5. As expected, hypoxia reduced food consumption in dams (16), but there was no change in maternal blood glucose (Table 2). Studies in rats have shown that 24 h in hypoxia (10.5% O2) does not cause a significant change in blood glucose or blood insulin levels, although hypoxic rats do consume less food and lose weight, while longer bouts of hypoxia can reduce glucose levels (11). Because fetal blood glucose is linearly related to maternal glucose (9), we did not expect a change in fetal glucose in our studies. Importantly, when normoxic pregnant dams fasted for 24 h, fetal weight was unaffected (Table 2), indicating that hypoxia-induced UGR results from hypoxia rather than malnutrition, consistent with previous studies (16). Additionally, hypoxia-induced growth restriction is known to occur in chick embryos where maternal nutrition is not a factor (reviewed in Ref. 25).

Except for the heart, organs appeared smaller in hypoxic fetuses but were anatomically normal. Generalized growth restriction without anatomical abnormalities is typical of animals with reduced level of IGFs (4, 36). Involvement of IGF is consistent with our finding that Igfbp-1 RNA is highly induced (4.5-fold) after hypoxia (Table 3). IGFBP-1 binds to and inhibits IGF1 and IGF2 and has been linked to IUGR (47) due to multiple causes including living at high altitude (41, 43, 73). In vivo the cell cycle effects required changes in mRNA levels of Hif1 and Hif2 rather than an increase in hypoxia based on qRT-PCR. Expression values are the means ± SE of 3 samples, each composed of a pool of at least 23 hearts from 2-3 litters.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Function</th>
<th>Accession No.</th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Fold Increase</th>
</tr>
</thead>
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<td>Bnip3</td>
<td>BCL2/adenovirus E1B 19 kDa interacting protein-1, NIP3</td>
<td>Apoptosis</td>
<td>NM_009760</td>
<td>0.72 ± 0.007</td>
<td>1.99 ± 0.28*</td>
<td>2.7×</td>
</tr>
<tr>
<td>Vegfa</td>
<td>Vascular endothelial growth factor-A (VEGF)</td>
<td>Vascularization</td>
<td>NM_009505</td>
<td>0.43 ± 0.02</td>
<td>0.81 ± 0.07*</td>
<td>1.9×</td>
</tr>
<tr>
<td>Fgf1</td>
<td>Fibroblast growth factor-1</td>
<td>Cell growth</td>
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<td>0.76 ± 0.10</td>
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<td>Cell growth</td>
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<td>NS</td>
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<tr>
<td>Fgf9</td>
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<td>Cell growth</td>
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<td>Wnt9b</td>
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<td>Cell growth</td>
<td>NM_011719</td>
<td>0.44 ± 0.07</td>
<td>0.43 ± 0.04</td>
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</table>

*Data vs. normoxia, P < 0.05. NS, not significant.

Only the heart appeared grossly dysmorphic after hypoxia. We saw hypoxia-associated reductions in ventricular mass and wall thickness (Fig. 5), myocardial proliferation in vivo, and DNA synthesis in isolated hearts, which do not experience the in vivo physiological load (Fig. 7). Our organ culture experiments suggest that hypoxia acts directly on the E12.5 heart to limit cell growth and division rather than by altering circulating maternal or fetal factors. Reduced proliferation in other cell types and in cultured cells is a known consequence of hypoxia, as it has been shown to both lengthen and arrest the cell cycle (reviewed in Ref. 18). Based on our ventricular tissue area measurements, heart size appeared to be reduced to a greater extent than the whole body weight. This finding contrasts those from late gestation in rats where prolonged hypoxia increases the heart-to-body weight ratio in fetuses, neonates, and adult offspring of hypoxic pregnant dams (79, 81, 82).

Although there was no increase in apoptosis as assessed by TUNEL, there was an increase in Bnip3 in the heart, which mediates cardiomyocyte cell death in response to hypoxia (51). In vivo the cell cycle effects required > 12 h of hypoxia (Fig. 7), suggesting that arrest was not immediate. In contrast, just 3 h after the onset of hypoxia, the epicardium detached from the myocardium, which was the first visible effect of hypoxia (Fig. 6).

Epicardial detachment suggests a mechanism for reduced proliferation. The epicardium is a source of growth factors for the myocardium, such as FGF2, Wnt9b (39), and FGF9 (35). Although the levels of Fgf2, Fgf9, and Wnt9b RNA in the heart did not change after 6 h of hypoxia (Table 4), other epicardially-derived factors may be involved. Interference with the apposition of epicardium and myocardium by blocking migration of epicardial precursors (46) or by genetic mutations that interfere with epicardial adhesion, such as α4-integrin or VCAM1 (34, 83), reduce myocardial proliferation, perhaps by reducing the availability of mitogenic factors to the myocardium. Additionally, hypoxia could alter cell signaling and current propagation mediated by gap junctions; cardiomyocyte connexin 43 is dephosphorylated, and the number of gap junctions decreases with hypoxia (66, 86).

**Thin myocardium syndrome.** Hypoxic fetuses share many characteristics with mouse mutants that die in midgestation (E12.5–E14.5). These include signs of congestive heart failure with loss of yolk sac circulation, vascular congestion, edema, hemorrhage, and thin myocardium, characteristics which have been described as thin myocardium syndrome (TMS) (15, 29, 52). Additionally, ventricular thinning in hypoxic fetuses is...
associated with ventricular dilation (Fig. 5F), a classic feature of congestive heart failure (71). Similar to what we see in hypoxic fetuses, the hypoplastic myocardium observed in TMS mutant mice is due to reduced proliferation of ventricular myocytes, rather than increased cell death (35, 46, 59, 80).

A thinned myocardium leads to reduced cardiac performance in TMS mutants. TMS mutations in the retinoic acid receptor, in βARK-1 (GRK-2) and in Pax3 (splotch) have been shown to reduce ejection fraction by 86%, 80%, and 62%, respectively (14, 19, 29). Reduced ejection fraction would be expected to reduce the perfusion of peripheral tissue and increase end diastolic volume and/or pressure, resulting in vascular congestion and edema, symptoms of congestive heart failure. Additionally, a reduction in ejection fraction would be expected to cause hypoxia.

Ejection fraction and heart rate determine cardiac output, which is also sensitive to vascular resistance. Vascular resistance has been shown in older mammalian fetuses to increase in response to hypoxia, and this response becomes more robust with age (22, 28). In the chick, whose sympathetic nervous system is functional earlier than in the mouse fetus, hypoxia results in increased systemic vascular resistance and reduced left ventricular function, both of which would further compromise cardiac output (44a, 55a).

The experiments presented here are unique in the early age at which the fetuses were investigated, but pose some limitations. The primary finding that hypoxia-related death is due to cardiac deficits requires further exploration. Our morphological and histological evidence implies, but does not confirm, cardiac insufficiency. Cardiac thinning seems apparent, but our fixation technique did not control for the stage of contraction, so it is feasible that hypoxia could have lengthened diastole, thereby increasing the chance of fixation during ventricular relaxation. However, the reduced DNA synthesis and decreased BrdU incorporation support the conclusion of true myocardial thinning. Additionally, our model is limited in the timing, duration, and severity of hypoxia, making its translation to other species and stages of development difficult. For example, the mouse fetus responds to hypoxia with bradycardia, while decreased heart rate variability is one of the primary clinical indicators for intervention in a pregnancy complicated by hypoxia. Finally, we ascribe the changes we see to fetal effects of hypoxia. However, it is possible that maternal hypoxia induces changes in the dam that indirectly compromise fetal heart development, apart from fetal hypoxia per se, for example by altering maternal blood flow (43, 77), metabolism, and hormone production (11).

**Perspectives and significance**

Our data show that the young fetus senses and responds to hypoxia. Hypoxia induces growth restriction and transcription of HIF-1 targets and blocks heart development, leading to death with symptoms of congestive heart failure. In conjunction with our previous work showing that hypoxia acutely causes fetal bradycardia (48), we propose a mechanism for hypoxia-induced midgestational death. Because heart rate is a major factor determining fetal cardiac output, we propose that hypoxia-induced bradycardia compromises cardiac output and tissue perfusion, which exacerbates hypoxia. Prolonged hypoxia results in a thin and disorganized ventricular myocardium, which further compromises cardiac output. These events may feed a downward spiral ending in death due to congestive heart failure. Further study of fetal cardiovascular physiology using imaging technology would clarify the physiological progression to fetal demise. Additionally, IUGR is known to have detrimental effects in adulthood, so it would be of interest to assess the long-term outcome of fetuses that survive early hypoxic episodes.

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**GRANT**

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**REFERENCES**


