Chronic Anemic Hypoxemia Increases Plasma Glucagon and Hepatic PCK1 mRNA in Late Gestation Fetal Sheep

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ABSTRACT

Hepatic glucose production (HGP) normally begins just prior to birth. Prolonged fetal hypoglycemia, intrauterine growth restriction, and acute hypoxemia produce an early activation of fetal HGP. To test the hypothesis that prolonged hypoxemia increases factors which regulate HGP, studies were performed in fetuses that were bled to anemic conditions (anemic, n=11) for 8.9 ± 0.4 days and compared to control fetuses (n=7). Fetal arterial hematocrit and oxygen content were 32% and 50% lower, respectively, in anemic vs. controls (p<0.005). Arterial plasma glucose was 15% higher in the anemic group (p<0.05). Hepatic mRNA expression of phosphoenolpyruvate carboxykinase (PCK1) was 2-fold higher in the anemic group (p<0.05). Arterial plasma glucagon concentrations were 70% higher in anemic fetuses compared to controls (p<0.05) and they were positively associated with hepatic PCK1 mRNA expression (p<0.05). Arterial plasma cortisol concentrations increased 90% in the anemic fetuses (p<0.05), but fetal cortisol concentrations were not correlated with hepatic PCK1 mRNA expression. Hepatic glycogen content was 30% lower in anemic vs. control fetuses (p<0.05) and was inversely correlated with fetal arterial plasma glucagon concentrations. In isolated primary fetal sheep hepatocytes, incubation in low oxygen (3%) increased PCK1 mRNA 3-fold compared to incubation in normal oxygen (21%). Together, these results demonstrate that glucagon and PCK1 may potentiate fetal HGP during chronic fetal anemic hypoxemia.

KEY WORDS: Glucose, Oxygen, PEPCK, Glycogen, Hepatocyte
INTRODUCTION

Glucose is the principal energy substrate for the fetus and is essential for normal fetal metabolism and growth. The transport of glucose from the mother to the fetus occurs by facilitated diffusion across the placenta. Therefore, the rate of fetal glucose uptake depends largely on the maternal arterial plasma glucose concentration (19).

Normally, transported maternal arterial glucose is the sole source of fetal glucose and there is no fetal hepatic glucose production (HGP) (20).

As part of the transition to extrauterine life, fetal HGP is activated just prior to delivery (13). At birth, infants have a period of reduced glucose intake when they are removed from their placental glucose supply and their mother’s milk is being established. Thus, to avoid hypoglycemia the neonate must rely on endogenous HGP.

Fetal sheep at 0.97 gestation have increased endogenous HGP as well as higher hepatic levels of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK, encoded by PCK1) and glucose-6-phosphatase (G6Pase, encoded by G6PC) compared to 0.95 gestation (11, 13). PEPCK catalyzes the rate-limiting step and G6Pase catalyzes the final step in gluconeogenesis. Increased perinatal transcription of PCK1 is activated by rising plasma concentrations of glucagon and glucocorticoids (13, 17, 36). There also is activation of glycogenolysis by increased secretion and plasma concentrations of glucagon and catecholamines around the time of birth (17, 36).

Increased gluconeogenesis and glycogenolysis maintain glucose homeostasis in the neonate by matching glucose utilization with increased HGP (36).

Normal fetuses have little if any HGP prior to 0.97 gestation (11, 13). Animal studies, however, have shown that certain conditions can increase factors which
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regulate fetal HGP. One of these conditions is decreased fetal glucose supply and fetal plasma glucose concentrations produced during maternal fasting and maternal hypoglycemia (11, 26, 50). Other conditions that also increase these factors and fetal HGP include acute maternal hypoxia, bilateral uterine artery ligation, umbilical cord compression, and intrauterine growth restriction from placental insufficiency (PI-IUGR) (5, 15, 25, 34, 38, 40, 48). A characteristic common to these conditions is fetal hypoxemia, or low fetal blood oxygen concentration. Studies specifically testing the impact of low blood oxygen concentration and/or low PO$_2$ on factors regulating fetal HGP are limited to acute hypoxemia, intermittent hypoxemia, or hypoxemia coupled with hypoglycemia such as PI-IUGR (5, 15, 40, 44, 48, 50). The effect of chronic sustained fetal hypoxemia without hypoglycemia on the factors which regulate HGP has not been studied.

The goal of our study was to test the impact of experimental chronic anemic hypoxemia on factors which regulate fetal HGP, such as fetal arterial glucose, lactate, and hormone concentrations; as well as hepatic mRNA expression of key gluconeogenic enzymes and hepatic glycogen content. We hypothesized that chronic fetal hypoxemia would increase plasma arterial glucagon, cortisol, and hepatic $PCK1$ mRNA expression and decrease hepatic glycogen content. To test this hypothesis, we induced prolonged experimental fetal anemic hypoxemia by reducing the fetal blood oxygen carrying capacity through daily bleeding of the fetus.
MATERIALS AND METHODS

Fetal Sheep Model of Chronic Anemic Hypoxemia

Studies were conducted on pregnant Columbia-Rambouillet sheep carrying a singleton fetus at the Perinatal Research Center in Aurora, CO with approval of the Institutional Animal Care and Use Committee, University of Colorado School of Medicine. This center is accredited by The Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and is compliant with the Animal Welfare Act and Public Health Service Policy. At 120 ± 0.3 days gestational age (dGA, term ~148 dGA), fetal catheters were surgically placed into the descending aorta and femoral vein via pedal incisions and into the common umbilical vein via one of the umbilical veins. Maternal catheters were placed into the femoral artery and vein via a groin incision. Surgery was performed with appropriate anesthesia and pain control as previously described (47).

Beginning at least five days after surgery, fetal sheep were bled (anemic, n=11) with isovolumetric replacement by 0.9% NaCl to anemic conditions for an average of 8.9 ± 0.4 days before umbilical blood flow and fetal glucose, lactate, and oxygen uptake measurements were performed. Anemic fetuses were compared to control fetuses who were not bled, but were otherwise treated the same as the anemic fetuses (CON, n=7). The goal fetal arterial oxygen content for the anemic group was 2.0 mmol/L as this is the arterial oxygen content measured in PI-IUGR sheep fetuses (7, 28, 47). The amount of blood removed daily was determined with a previously established formula taking into account fetal hematocrit and the goal arterial oxygen content (22).
Biochemical Analysis

Fetal and maternal arterial blood samples were obtained daily. Arterial plasma glucose and lactate concentrations were measured using Yellow Springs Instrument 2700 (Yellow Springs Instruments, Yellow Springs, OH) and blood hematocrit, pH, partial pressure of oxygen, partial pressure of carbon dioxide (CO₂) and oxygen-hemoglobin saturation were measured with the blood gas analyzer ABL 520 (Radiometer, Copenhagen, Denmark). Oxygen content of the blood was calculated by the ABL 520 analyzer. Fetal arterial plasma insulin was measured by enzyme-linked immunosorbent assay (ELISA) (Alpco, Windham, NH; intra- and interassay coefficients of variation = 5.6% and 4.7%, respectively; sensitivity=0.14 ng/ml) (37) and norepinephrine by high performance liquid chromatography (HPLC) (model 2475, Waters; intra- and interassay coefficients of variation = 9.2% and 9.0%, respectively; sensitivity=170 pg/ml) (47) throughout the study. Fetal arterial plasma cortisol was measured by ELISA (ALPCO Immunoassays; intra- and interassay coefficients of variation = 4.6% and 5.8%, respectively; sensitivity=1.0 ng/ml) and glucagon by radioimmunoassay (Millipore, Billerica, MA; intra- and interassay CV's: 4.8% and 11.7%; sensitivity=18.5 pg/ml) (37) on day 0 (baseline) and on the final day of the study before \textit{in vivo} metabolic studies were performed.

Umbilical Blood Flow and Nutrient Uptake Studies

Studies were performed on the final day of the experimental period to determine umbilical blood flow and glucose, lactate, and oxygen uptake. Due to catheter failure, complete metabolic studies were completed on seven animals in each study group. One
anemic fetus did not survive to necropsy following the metabolic study, but there were no other unplanned fetal deaths. Fetal umbilical blood flow was calculated using an ethanol tracer and the transplacental diffusion technique (32). Glucose, lactate, oxygen, and ethanol were measured from four blood draws obtained from the fetal umbilical vein and descending aorta after the ethanol tracer reached a steady state. During the study period, fetal blood removed was replaced with a fetal transfusion of heparinized maternal whole blood diluted with 0.9% NaCl to match the hematocrit of the fetal blood (47). Nutrient uptake by the fetus was calculated using the Fick principle (32). Fetal oxygen extraction efficiency was calculated with the equation:

\[
\text{Oxygen extraction} \% = \frac{[O_2]_v - [O_2]_a}{[O_2]_v} \times 100
\]

where \([O_2]_a\) is the fetal arterial oxygen content and \([O_2]_v\) is the umbilical venous oxygen content (4).

**Fetal and Fetal Liver Weight**

The day after the metabolic studies, the mother and fetus were euthanized (37). Fetal organs were collected and weighed. A portion of the right lobe of the fetal liver was snap frozen in liquid nitrogen and then stored at -80°C. One anemic fetus did not survive to necropsy following the metabolic study. Other than not surviving to necropsy, we did not detect anything unusual about this fetus and have included it in the *in vivo* analysis.

**Glycogen Content**

Glycogen content in the liver was determined as previously described (29).
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Results are expressed as milligrams glycogen per grams tissue (wet weight).

\textbf{Real-time Quantitative PCR}

RNA was extracted from pulverized liver tissue and reverse transcribed to cDNA. Quantitative PCR was performed as previously described (50). Previously validated quantitative PCR primers and assays were used for \textit{PCK1}, \textit{G6PC}, peroxisome proliferator-activated receptor-γ coactivator (\textit{PGC1A}), glucose regulated protein, 78kDa (\textit{GRP78}; Heat Shock 70kDa Protein 5 [\textit{HSPA5}]), DNA-Damage Inducible Transcript 3 (\textit{DDIT3}), pyruvate dehydrogenase kinase 4 (\textit{PDK4}), lactate dehydrogenase A (\textit{LDHA}), phosphofructokinase 1 (\textit{PFK1}), and endothelin 1 (\textit{EDN1}) (6, 16, 39, 48, 50). Additional primers were developed and validated for vascular endothelial growth factor A (\textit{VEGFA}; forward: TTGCCTTGCTGCTCTACCTT; rev: GGGCACACACTCCAGACTTT), erythropoietin (\textit{EPO}; forward: CCCAGACACCAAGGTAACT; rev: GAAAGATAGCTTCTACCTT), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1(\textit{PFKB1}), and the glucagon receptor (\textit{GCGR}; forward: ATGCTGTTCGTCATCCCCTG; rev: AAGCGCAGAATCCACCAGAA). The housekeeping genes \textit{GAPDH} and \textit{RPS15} were not different between groups and were used to normalize results for the genes of interest (14).

\textbf{Western Blotting}

Tissue lysates were prepared from pulverized frozen liver tissue, and Western immunoblotting was performed as previously described (50). PEPCK, G6Pase, hepatocyte nuclear factor-4α (\textit{HNF4α}), actin, protein kinase B (\textit{AKT}), phosphorylated AKT (S473/T308), adenosine monophosphate-activated protein kinase (AMPK),
phosphorylated AMPK (T172), cyclic AMP response element binding-protein (CREB),
phosphorylated CREB (S133) were used as previously described (47,50). The
membrane for glucose regulated protein 78 kDa (GRP78) was blocked for 1 hour in
Tris-buffered saline with 0.01% Tween 20 (TBST; Bio-Rad) and 5% wt/vol nonfat dried
milk. A monoclonal rat IgG anti GRP78 antibody (Santa Cruz Biotech; Santa Cruz, CA)
was diluted to 1:500 in TBST with 5% bovine serum albumin and placed on the
membrane overnight. Quantification of bands was performed by densitometry (Image J)
or Image Studio (LI-COR) and normalized to actin. For phosphorylated proteins a ratio
to the densitometry of the respective total protein was calculated.

**Primary Fetal Hepatocytes**

Primary fetal hepatocytes were isolated from four additional late gestation fetal
sheep as previously described (47). Briefly, a piece of the right lobe was flushed with
perfusion buffer and then digestion buffer containing collagenase. The digested tissue
was filtered, spun, and isolated hepatocytes were washed, plated and allowed to attach
for 4 hr in Dulbecco’s Modified Eagle Medium (DMEM; 1.1 mM glucose, 2 mM lactate, 2
mM glutamine, 1 mM pyruvate, 1X non-essential amino acids, 1X penicillin-
streptomycin) supplemented with 0.1 nM insulin, 10 mM dexamethasone, and 10%
FBS. After attachment, the cells were washed and incubated in non-supplemented
DMEM. The cells were then incubated in two different oxygen concentrations (21% vs
3% oxygen) and 5% CO₂ (balanced with nitrogen) with or without 100 µmol/L cyclic
Adenosine Monophosphate (cAMP) and 500 nmol/L dexamethasone for 24 hours (47).
RNA was then extracted from the hepatocytes, reverse transcribed and quantitative
PCR performed for *PCK1, PGC1A*, and *RPS15*.  

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Statistical Analysis

Statistical analysis was performed using SAS version 9.1 or GraphPad Prism 6.0. Results are expressed as mean ± SEM. A mixed models analysis of variance (ANOVA) was performed to determine effects of treatment group (control or anemic), time (days of treatment), and treatment-time interactions for in vivo measurements made more than once. Repeated measures made in the same animal or hepatocytes isolated from the same animals also were accounted for. If $p \leq 0.05$ in the ANOVA, individual means were compared by Fischer’s Least Squares Difference. Data obtained at only one time point were compared using Student’s t test or Mann-Whitney test. Statistical significance was declared at $p \leq 0.05$. 
RESULTS

Maternal and Fetal Biochemistry and Necropsy Data

Daily bleeding resulted in significant reduction in both the fetal arterial hematocrit and oxygen content beginning on day 1 in the anemic group ($p<0.001$; Figure 1A, B). At the end of the study period, when umbilical blood flow and fetal nutrient uptakes were measured, the blood hematocrit and oxygen content were 32% and 50% lower, respectively, in the anemic vs. control group. There were no differences in pH, partial pressure of CO$_2$, or arterial plasma lactate on any study day between groups (Table 1). There was a decrease in the partial pressure of oxygen and hemoglobin-oxygen saturation in both groups over time, but the groups were not different from each other (Figure 1C, D). By the end of the study, plasma glucose concentrations were 15% higher in the anemic vs. control group ($p<0.05$; Figure 2A).

There were no differences in maternal pH, partial pressure of oxygen, partial pressure of CO$_2$, oxygen-hemoglobin saturation (sO$_2$), oxygen content, hematocrit, glucose, or lactate concentration on any day of study (Table 1).

Fetal necropsy data are shown in Table 2. The anemic fetuses had heavier right ventricular weight compared to controls ($p<0.05$). There were no other differences between the groups.

Fetal Hormones

Fetal arterial plasma norepinephrine and insulin concentrations were not statistically different between the two groups (Figure 3A, B). Fetal arterial cortisol
Anemic hypoxemia increases \textit{PCK1} concentrations were increased 90\% on the final day in the anemic group compared to baseline ($p<0.05$; Figure 3C), but they were not different from the controls on the final day (Figure 3C). By the final day of the study, arterial glucagon concentrations in anemic fetuses were significantly increased compared to baseline and 70\% higher than controls ($p<0.05$; Figure 3D).

\textbf{Fetal Metabolic Studies}

Umbilical blood flow, fetal glucose and lactate uptake rates, and the maternal to fetal arterial glucose concentration difference were not different between groups (Table 1, Figure 2B). The anemic fetuses had significantly lower whole-body oxygen utilization despite having a higher oxygen extraction compared to controls ($p<0.05$; Figure 4A, B). There was a significant correlation between fetal arterial oxygen content and fetal oxygen extraction, such that at low oxygen contents there was increased fetal oxygen extraction ($p<0.001$; Figure 4C).

\textbf{Hepatic \textit{PCK1}, \textit{G6PC}, and Glycogen}

Hepatic \textit{PCK1} mRNA was 2-fold greater in the anemic group compared to controls ($p<0.05$; Figure 5A). The tendency for higher levels of hepatic \textit{G6PC} mRNA in anemic fetuses was not statistically significant (Figure 5D). Fetal arterial glucagon concentrations were significantly correlated with both hepatic \textit{PCK1} and \textit{G6PC} mRNA levels ($p<0.05$; Figure 5B, E). Fetal arterial cortisol concentrations did not correlate with either hepatic \textit{PCK1} or \textit{G6PC} mRNA (Figure 5C, F). The hepatic protein expressions of \textit{PEPCK} and glucose-6-phosphatase were not different between the groups (Table 3). Hepatic glycogen concentration was significantly lower in the anemic fetuses compared
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to controls ($p<0.05$; Figure 5G) and was inversely correlated to fetal arterial plasma glucagon concentrations ($p<0.05$), but not fetal cortisol concentrations (Figure 5H, I).

\textbf{Cellular Nutrient Sensors and Intracellular Signaling}

Hepatic levels of mRNA and protein of genes associated with cellular nutrient sensing, selected for their responsiveness to acute oxygen deprivation (including HIF1\textalpha target genes: \textit{VEGFA, EPO, EDN1, PFK1, PFKFB1, PDK4, LDHA}), were largely unchanged by chronic anemic hypoxemia (Table 3). Similarly unchanged were intracellular signaling proteins AMPK, CREB and AKT. Chronic anemic hypoxemia reduced hepatic mRNA expression of the glucagon receptor (\textit{GCCR}) ($p<0.05$; Table 3).

\textbf{Fetal Hepatocytes}

The roles of oxygen and hormone stimulation on expression of genes involved in regulation of HGP were tested in primary fetal sheep hepatocytes. In response to 3\% oxygen, mRNA expression of both \textit{PCK1} and \textit{PGC1A} increased 2-3-fold compared to 21\% oxygen ($p<0.001$; Figure 6). Expression of \textit{PCK1} and \textit{PGC1A} also increased in response to cAMP + dexamethasone treatment ($p<0.005$; Figure 6).
DISCUSSION

In this study, we determined that low fetal blood oxygen concentrations as a result of chronic fetal anemia increases circulating glucagon concentrations which were associated with increased hepatic $PCK1$ mRNA and decreased hepatic glycogen content. These results show that fetal anemic hypoxemia is sufficient to stimulate and sustain increases in factors which are associated with activation of both gluconeogenesis and glycogenolysis. Furthermore, these results demonstrate that fetal hypoglycemia (a hallmark of PI-IUGR) is not necessary for this response, even though it can, by itself, lead to fetal HGP (21). Supporting this conclusion is our finding that exposure to either 3% oxygen or glucogenic activators (cAMP + dexamethasone) increases expression of $PCK1$ and $PGC1A$ in isolated fetal hepatocytes. This is the first study to test the impact of chronic (>24 hours) fetal anemic hypoxemia independent of placental insufficiency and fetal hypoglycemia on fetal metabolic and endocrine responses, thereby identifying novel mechanisms by which fetal oxygen concentrations can regulate fetal glucose metabolism.

To determine the fetal endocrine response to anemic hypoxemia we measured the concentrations of several hormones throughout the study. Fetal glucagon concentrations increased in the anemic group and correlated with both $PCK1$ and $G6PC$ mRNA. Glucagon has been previously shown to activate fetal HGP, but the concentrations of glucagon required were greater than 1,000 pg/mL, suggesting fetal hepatic resistance to glucagon relative to adults (10, 45). In our study, physiological concentrations of glucagon, 20-80 pg/mL, correlated with higher $PCK1$ and $G6PC$ mRNA levels. It may be that fetal anemic hypoxemia augments the physiological
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hyperglucagonemia leading to the transcriptional activation of PCK1, while in fetal normoxemia much higher concentrations of glucagon are required to stimulate fetal gluconeogenesis. Lower hepatic glucagon receptor mRNA levels in anemic fetuses is evidence of ongoing hepatic glucagon signaling, consistent with a dose dependent decrease in GCCR mRNA in primary hepatocytes following incubation with glucagon (1).

Fetal cortisol concentrations increased in the anemic fetuses, but also started lower than controls. By the end of the study, cortisol concentrations were not different between the two groups. These mixed results make it difficult to speculate about the role of cortisol in regulating hepatic PCK1 in this set of animals, despite the well-described role for cortisol in activating gluconeogenesis (12, 13, 38). Whether this represents another pathway partially responsible for the hepatic phenotype in anemic fetuses is not clear. In order to demonstrate the capacity of hormone signaling, as well as oxygen to increase PCK1 and G6PC mRNA, we performed in vitro studies with isolated fetal hepatocytes. cAMP + dexamethasone treatment increased PCK1 mRNA levels in these cells. Interestingly, although we did not detect increased hepatic PGC1A mRNA in the anemic fetuses, both 3% oxygen and cAMP + dexamethasone treatment in isolated hepatocytes increased PGC1A expression. Thus, oxygen clearly has the capacity to increase hepatocyte PGC1A expression, but in vivo fetal hypoxemia does not.

Since physiological increases in glucagon were associated with increased hepatic expression of gluconeogenic genes, we analyzed components of the cAMP-dependent glucagon-signaling pathway. PI-IUGR fetuses have activation of PCK1
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associated with increased glucagon, PGC1α (*PGC1A*) and phosphorylated CREB (29, 48). However, we found no change in *PGC1A* or phosphorylated CREB in the livers of anemic fetuses on the terminal day of the study. This may be due to three factors. First, levels may have been increased earlier in the study representing an acute fetal response to hypoxemia (2, 41). Second, there may be fundamental differences in the fetal response to chronic hypoxemia and PI-IUGR. Fetuses affected by PI-IUGR experience global nutrient restriction, including reduced oxygen supply and blood concentrations (47, 48). Further, this oxygen deprivation occurs throughout most of the latter half gestation, in contrast to the nine days of anemic hypoxemia tested in the current model. Factors other than fetal hypoxemia, to which the fetus is exposed for longer than nine days, may be responsible for increased *PGC1A* and phosphorylated CREB in PI-IUGR. Third, and likely related to the duration and global nutrient restriction noted above, PI-IUGR fetuses have a much more robust increase in *PCK1* and *G6PC* mRNA levels compared to the anemic fetuses (29, 30, 47, 48). The modest increase in *PCK1* and *G6PC* mRNA in the anemic livers compared to models of placental insufficiency is consistent with the similar level of hepatic protein expression of PEPCK. It is notable that while both PI-IUGR and anemic fetuses have increased hepatic *PCK1*, substantial differences in hepatic signaling are apparent, likely due to different hepatic nutrient (glucose and oxygen) supplies.

We did not find any differences in hepatic AMPK phosphorylation or GRP78, two proteins normally activated by acute nutrient deprivation and/or hypoxemia (Table 3) (18, 24). Three reasons may account for this lack of activation. First, although AMPK and GRP78 are activated by hypoxemia, the anemic animals did not have a significant
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decrease in their PO$_2$ compared to controls and hepatocytes might not have
experienced tissue hypoxia, which may be required to activate AMPK and GRP78 (18, 24, 48). Supporting the conclusion that the liver did not experience hypoxia is the lack of change noted in any hypoxia responsive genes, such as \textit{VEGFA, EPO, EDN1, PFK1, PFKBP1, LDHA, PDK4, DDIT3,} and \textit{HSPA5} (8, 9, 43). Second, although anemic fetuses had low oxygen content, they were not deprived of glucose or lactate, which also are important for activating AMPK and GRP78 (48). Lastly, most studies demonstrating activation of AMPK and GRP78 focus on acute oxygen deprivation, often \textit{in vitro} (18, 24).

Fetal oxygen utilization was significantly decreased in the anemic group compared to controls. This is despite higher oxygen extraction in the anemic fetuses. In previous reports (4, 52), fetuses were shown to be able to maintain normal oxygen utilization during graded reductions in oxygen supply produced experimentally by partial occlusion of the maternal terminal aorta causing uterine blood flow restriction. This increased oxygen extraction enabled normal fetal oxygen utilization during graded hypoxemia in this model. In contrast, the anemic fetuses in the present study were unable to maintain oxygen utilization despite increased fetal oxygen extraction. In the uterine blood flow restriction paradigm, the effect of acute hypoxemia on fetal oxygen consumption was tested (4, 52). In the current study we tested the effects over a more prolonged period (nine days). Indeed, in chronic hypoxemia due to placental insufficiency, fetal sheep also tend to have slightly lower oxygen consumption rates than normal (47).

We also have shown in the current studies that chronic fetal anemic hypoxemia
increased fetal glucose concentrations without a change in fetal insulin concentrations. This was not due to an increase in the rate of placental glucose transfer as a possible cause of elevated fetal glucose in anemia. Elevated fetal glucose concentrations in anemic fetuses were likely mediated by high fetal glucagon and cortisol concentrations. Even the slight, though not statistically significant, increase in mean fetal norepinephrine concentrations may have played a role (27, 30, 31). As the focus of the current study was on the factors which regulate fetal HGP, we did not determine the relative contribution of HGP and decreased fetal glucose utilization for the increase in fetal glucose concentrations.

The model of anemia, or reduced oxygen carrying capacity, for hypoxemia was chosen because it did not allow fetuses to compensate by increasing total hemoglobin, and because we could isolate the effects of hypoxemia from hypoglycemia. Kitanaka and colleagues (23) showed that fetuses exposed to 28 days of hypoxemia via high altitude responded by increasing their hemoglobin and hematocrit over time to normalize arterial oxygen contents; these fetuses grew at normal rates, indicative of normal fetal nutrient metabolism. In contrast, our model allowed us to chronically match the mean oxygen content of PI-IUGR fetuses (2.0 mmol/L) (7, 28, 47). However, comparison of sheep models of human PI-IUGR fetuses exposes limitations of this study, including length and timing of hypoxemia. Studies done to model chronic PI-IUGR in sheep result in fetal hypoxemia beginning as early as day 90 in gestation (33). In the current study, fetal hypoxemia occurred on average for nine days at 0.85 gestation. In contrast, the chronically anemic fetal sheep is an appropriate model for human chronic fetal anemia, such as occurs in chronic feto-maternal hemorrhage and
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subacute placental abruption; and also may help model aspects of fetal hypoxemia observed in cases of twin-twin transfusion syndrome and diabetic pregnancy and some cases of post-dates pregnancies (3, 42, 46). Future studies to induce experimental anemia earlier in gestation are planned. The studies also are limited by the random allocation of all female fetuses into the control group. We chose to include all animals in the anemic group as we have never detected sex differences in outcomes reported (29, 38, 47, 48, 50). Furthermore, for the most important outcomes, $PCK1$ mRNA, $G6PC$ mRNA, and glucagon concentrations, the two highest values in the anemic group were females and the mean concentrations within the anemic group were higher for females than males.

**PERSPECTIVES AND SIGNIFICANCE**

In conclusion, we speculate that in pregnancies complicated by fetal hypoxemia, such as intrauterine growth restriction, low oxygen concentrations in conjunction with elevated glucagon play a role in activating factors associated with fetal hepatic gluconeogenesis and glycogenolysis resulting in HGP. These are important findings, as complications of pregnancy that lead to premature activation of fetal HGP predispose to later life development of hepatic insulin resistance, hyperglycemia, and ultimately Type II diabetes mellitus by unknown mechanisms (35, 49, 51).
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DISCLOSURES

The authors have nothing to disclose.
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**FIGURE CAPTIONS**

**Figure 1.** Effect of chronic fetal anemic hypoxemia on fetal biochemistry. A) Fetal arterial hematocrit was significantly lower in the anemic (open squares; n=11) fetuses compared to controls (closed circles; n=7) (*; *p*<0.001). B) Fetal arterial oxygen content was significantly lower in the Anemic fetuses compared to control (*; *p*<0.001). The partial pressure of arterial oxygen (PO$_2$; C) and the hemoglobin-oxygen arterial saturation (sO$_2$; D) decreased in both groups compared to their baselines (#; *p*<0.01), but the groups were not different from each other. For all panels the mean and SE are plotted, statistical analysis was by mixed models ANOVA.

**Figure 2.** Effect of chronic fetal anemic hypoxemia on fetal glucose. A) Fetal arterial plasma glucose concentrations were measured throughout the study. Glucose concentrations were significantly higher in the anemic fetuses (open squares; n=11) at the end of the study period compared to controls (closed circles; n=7; *p*<0.05 by mixed models ANOVA). B) There was no difference in fetal glucose uptake between groups when measured at the end of the study. Plotted values are means and SE. *p*<0.05 control vs. anemic.

**Figure 3.** Effect of chronic fetal anemic hypoxemia on fetal hormone concentrations. Fetal arterial plasma levels of norepinephrine (A) and insulin (B) were not significantly different between control (closed bars; n=7) and anemic (open bars; n=11) fetuses. C) Cortisol increased over the course of the study such that in the anemic fetuses it was significantly higher at the end compared to their baseline (*p*<0.05). D) Glucagon increased over the course of the study such that in the anemic fetuses it was
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significantly higher at the end compared to their baseline, as well as on the final day being higher than controls (*p*<0.05). Mean and SEs are plotted and statistics are by mixed models ANOVA. *p*<0.05 between indicated values.

**Figure 4.** Effect of chronic fetal anemic hypoxemia on fetal oxygen utilization and extraction. A) Whole-body oxygen utilization was significantly less in anemic (n=7) fetuses compared to controls (n=7; *p*<0.05). B) Fetal oxygen extraction was significantly greater in anemics (n=7) compared to controls (n=7; *p*<0.01). C) In both anemic (open squares) and control (closed circles) fetuses, oxygen extraction was correlated with arterial oxygen content such that at lower levels of arterial oxygen there was higher placental oxygen extraction. Groups were compared by Student’s t test. Mean and SEs are plotted.

**Figure 5.** Effect of chronic fetal anemic hypoxemia on hepatic *PCK1* mRNA, *G6PC* mRNA, and glycogen. A) Hepatic *PCK1* mRNA levels were higher in anemic fetuses (n=10) vs. controls (n=7) (*p*<0.05). There was a significant correlation between final day anemic (open squares) and control (closed circles) fetal arterial plasma glucagon and hepatic *PCK1* mRNA (B; *r*<sup>2</sup> = 0.52, *p*<0.05), but not with fetal arterial plasma cortisol concentrations (C). D) *G6PC* mRNA levels were not statistically different between groups. There was a significant correlation between final day anemic (open squares) and control (closed circles) fetal arterial plasma glucagon and *G6PC* mRNA (E; *r*<sup>2</sup> = 0.29, *p*<0.05), but not with fetal arterial plasma cortisol concentrations (F). G) Hepatic glycogen levels were greater in anemic vs. CON fetuses. There was an inverse
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correlation between final day anemic (open squares) and control (closed circles) fetal arterial plasma glucagon and hepatic glucagon (H; $r^2 = 0.52$, $p<0.05$), but not with fetal arterial plasma cortisol concentrations (I). Median and interquartile ranges are plotted for *PCK1* and *G6PC* mRNA, and mean and SE are plotted for glycogen. *$p<0.05$ vs. controls.*

**Figure 6.** *PCK1* and *PGC1A* mRNA levels are increased in fetal hepatocytes incubated in 3% oxygen compared to 21% oxygen. 3% vs. 21% oxygen and the presence of cAMP + dexamethasone (Dex) both significantly increased mRNA expression of *PCK1* (A) and *PGC1A* (B). Statistics were by mixed models ANOVA.
Anemic hypoxemia increases *PCK1*

Table 1. *Maternal and Fetal Biochemistry, Umbilical Blood Flow, and Lactate Uptake at the End of the Study*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Anemic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal Data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.45 ± 0.01</td>
<td>7.42 ± 0.02</td>
</tr>
<tr>
<td>Blood PCO₂ (mmHg)</td>
<td>34.4 ± 1.0</td>
<td>37.7 ± 2.1</td>
</tr>
<tr>
<td>Blood PO₂ (mmHg)</td>
<td>96.0 ± 2.3</td>
<td>88.3 ± 1.5</td>
</tr>
<tr>
<td>Blood sO₂ (%)</td>
<td>96.4 ± 0.7</td>
<td>96.2 ± 1.0</td>
</tr>
<tr>
<td>Blood O₂ Content (mmol/L)</td>
<td>5.28 ± 0.25</td>
<td>5.61 ± 0.27</td>
</tr>
<tr>
<td>Blood Hematocrit (%)</td>
<td>27.8 ± 1.3</td>
<td>30.3 ± 1.5</td>
</tr>
<tr>
<td>Plasma Glucose (mmol/L)</td>
<td>4.04 ± 0.34</td>
<td>3.99 ± 0.19</td>
</tr>
<tr>
<td>Plasma Lactate (mmol/L)</td>
<td>0.94 ± 0.15</td>
<td>1.05 ± 0.19</td>
</tr>
<tr>
<td><strong>Fetal Data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.34 ± 0.01</td>
<td>7.34 ± 0.00</td>
</tr>
<tr>
<td>Blood PCO₂ (mmHg)</td>
<td>51.7 ± 1.0</td>
<td>52.6 ± 0.9</td>
</tr>
<tr>
<td>Plasma Lactate (mmol/L)</td>
<td>2.28 ± 0.21</td>
<td>2.46 ± 0.17</td>
</tr>
<tr>
<td>Umbilical Blood Flow (mL/min/kg)</td>
<td>156 ± 14</td>
<td>139 ± 11</td>
</tr>
<tr>
<td>Net Lactate Uptake (μmol/kg/min)</td>
<td>28.0 ± 2.4</td>
<td>23.5 ± 2.9</td>
</tr>
<tr>
<td>Maternal-Fetal Arterial Glucose Difference (mmol/L)</td>
<td>2.87 ± 0.28</td>
<td>2.93 ± 0.15</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. n=7 for control and n=11 for anemic except for Umbilical Blood Flow and Lactate Uptake (anemic n=7). Partial pressure of carbon dioxide (PCO₂); partial pressure of oxygen (PO₂); oxygen saturation (sO₂).
Anemic hypoxemia increases \textit{PCK1}

Table 2. \textit{Fetal Necropsy Data}

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Anemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational Age (days)</td>
<td>134.3 ± 0.5</td>
<td>134.6 ± 0.4</td>
</tr>
<tr>
<td>Fetal Weight (g)</td>
<td>2911 ± 159</td>
<td>3043 ± 113</td>
</tr>
<tr>
<td>Fetal Sex (% female)</td>
<td>100%</td>
<td>55%</td>
</tr>
<tr>
<td>Crown Rump Length (cm)</td>
<td>48.1 ± 1.6</td>
<td>47.5 ± 1.6</td>
</tr>
<tr>
<td>Pancreas (g)</td>
<td>2.96 ± 0.25</td>
<td>3.08 ± 0.20</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>89.9 ± 7.2</td>
<td>108.2 ± 8.9</td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>18.75 ± 0.84</td>
<td>18.78 ± 0.72</td>
</tr>
<tr>
<td>Perirenal Adipose Tissue (g)</td>
<td>9.43 ± 0.72</td>
<td>10.98 ± 0.93</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>6.26 ± 0.41</td>
<td>5.63 ± 0.40</td>
</tr>
<tr>
<td>Adrenal Glands (g)</td>
<td>0.44 ± 0.06</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>Lungs (g)</td>
<td>108.0 ± 3.4</td>
<td>103.3 ± 3.2</td>
</tr>
<tr>
<td>Heart (total) (g)</td>
<td>20.27 ± 1.92</td>
<td>22.53 ± 0.93</td>
</tr>
<tr>
<td>Left Ventricle + Septum (g)</td>
<td>8.25 ± 0.50</td>
<td>8.45 ± 0.56</td>
</tr>
<tr>
<td>Right Ventricle (g)</td>
<td>3.69 ± 0.31</td>
<td>5.44 ± 0.47 *</td>
</tr>
<tr>
<td>Total # Placentomes</td>
<td>74.3 ± 4.0</td>
<td>83.9 ± 5.1</td>
</tr>
<tr>
<td>Total Placental Weight (g)</td>
<td>325.9 ± 35.5</td>
<td>347.8 ± 24.4</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. n=7 for control and n=11 for anemic for gestational age, weight and sex. N=10 for anemic for fetal measurements. Statistical significance ($p \leq 0.05$) is indicated by an asterisk (*).
Table 3. Expression of genes and proteins in the fetal liver.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>Anemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxisome Proliferator-activated Receptor-γ</td>
<td>1.00 ± 0.27</td>
<td>0.90 ± 0.34</td>
</tr>
<tr>
<td>Coactivator 1α (PGC1A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activating Transcription Factor 2 (ATF2)</td>
<td>1.00 ± 0.29</td>
<td>0.68 ± 0.20</td>
</tr>
<tr>
<td>Hypoxia Inducible Factor 1α (HIF1A)</td>
<td>1.00 ± 0.34</td>
<td>0.61 ± 0.23</td>
</tr>
<tr>
<td>Vascular Endothelial Growth Factor A (VEGFA)</td>
<td>1.00 ± 0.17</td>
<td>0.82 ± 0.20</td>
</tr>
<tr>
<td>Erythropoietin (EPO)</td>
<td>1.00 ± 0.36</td>
<td>1.54 ± 0.95</td>
</tr>
<tr>
<td>Endothelin 1 (EDN1)</td>
<td>1.00 ± 0.17</td>
<td>0.81 ± 0.06</td>
</tr>
<tr>
<td>Phosphofructokinase 1 (PFK1)</td>
<td>1.00 ± 0.27</td>
<td>1.59 ± 0.21</td>
</tr>
<tr>
<td>6-phospho-2-kinase/fructose 2,6 bisphosphatase 1 (PFKFB1)</td>
<td>1.00 ± 0.27</td>
<td>0.39 ± 0.07*</td>
</tr>
<tr>
<td>Lactate dehydrogenase A (LDHA)</td>
<td>1.00 ± 0.14</td>
<td>1.26 ± 0.42</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase 4 (PDK4)</td>
<td>1.00 ± 0.31</td>
<td>0.84 ± 0.41</td>
</tr>
<tr>
<td>DNA Damage Inducible Transcript 3 (DDIT3)</td>
<td>1.00 ± 0.11</td>
<td>0.85 ± 0.08</td>
</tr>
<tr>
<td>Heat Shock 70kDa Protein 5 (HSPA5)</td>
<td>1.00 ± 0.10</td>
<td>0.73 ± 0.07*</td>
</tr>
<tr>
<td>Glucagon Receptor (GCGR)</td>
<td>1.00 ± 0.11</td>
<td>0.77 ± 0.06*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>Anemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic Proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEPCK (to Actin)</td>
<td>1.00 ± 0.10</td>
<td>1.12 ± 0.10</td>
</tr>
<tr>
<td>Glucose-6-phosphatase (to Actin)</td>
<td>1.00 ± 0.27</td>
<td>0.88 ± 0.30</td>
</tr>
<tr>
<td>Total AMPK (to Actin)</td>
<td>1.00 ± 0.30</td>
<td>0.55 ± 0.14</td>
</tr>
<tr>
<td>T172 phosphorylated AMPK (to total)</td>
<td>1.00 ± 0.19</td>
<td>0.95 ± 0.13</td>
</tr>
<tr>
<td>Total CREB (to Actin)</td>
<td>1.00 ± 0.31</td>
<td>1.23 ± 0.24</td>
</tr>
<tr>
<td>S133 phosphorylated CREB (to total)</td>
<td>1.00 ± 0.28</td>
<td>1.17 ± 0.27</td>
</tr>
<tr>
<td>Total AKT (to Actin)</td>
<td>1.00 ± 0.09</td>
<td>1.02 ± 0.09</td>
</tr>
<tr>
<td>S473/T308 phosphorylated AKT (to total)</td>
<td>1.00 ± 0.10</td>
<td>0.98 ± 0.19</td>
</tr>
<tr>
<td>HNF4a (to Actin)</td>
<td>1.00 ± 0.12</td>
<td>1.05 ± 0.20</td>
</tr>
<tr>
<td>GRP78 (to Actin)</td>
<td>1.00 ± 0.37</td>
<td>0.70 ± 0.22</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. n=7 for control and n=10 for anemic. Statistical significance (p≤0.05) is indicated by an asterisk (*).
Figure 1. Effect of chronic fetal anemic hypoxemia on fetal biochemistry.
Figure 2. Effect of chronic fetal anemic hypoxemia on fetal glucose.
Figure 3. Effect of chronic fetal anemic hypoxemia on fetal hormone concentrations.
Figure 4. Effect of chronic fetal anemic hypoxemia on fetal oxygen utilization and extraction.
Figure 5. Effect of chronic fetal anemic hypoxemia on hepatic *PCK1*, *G6PC* mRNA and glycogen.
Figure 6. *PCK1* and *PGC1A* mRNA are increased in fetal hepatocytes incubated in 3% oxygen compared to 21%.

A

![Graph A](image)

B

![Graph B](image)