A physiological increase in insulin suppresses gluconeogenic gene activation in fetal sheep with sustained hypoglycemia

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ABSTRACT

Reduced maternal glucose supply to the fetus and resulting fetal hypoglycemia and hypoinsulinemia activate fetal glucose production as a means to maintain cellular glucose uptake. However, this early activation of fetal glucose production may be accompanied by hepatic insulin resistance. We tested the capacity of a physiological increase in insulin to suppress fetal hepatic gluconeogenic gene activation following sustained hypoglycemia to determine if hepatic insulin sensitivity is maintained. Control fetuses (CON), hypoglycemic fetuses induced by maternal insulin infusion for 8 wk (HG), and 8 wk HG fetuses that received an isoglycemic insulin infusion for the final 7 d (HG+INS) were studied. Glucose and insulin concentrations were 60% lower in HG compared to CON fetuses. Insulin was 50% higher in HG+INS compared to CON and 4-fold higher compared to HG fetuses. Expression of the hepatic gluconeogenic genes, *PCK1, G6PC, FBP1, GLUT2*, and *PGC1A*, was increased in the HG and reduced in the HG+INS liver. Expression of the insulin-regulated glycolytic and lipogenic genes, *PFKL* and *FAS*, was increased in the HG+INS liver. Total FOXO1 protein expression, a gluconeogenic activator, was 60% higher in the HG liver. Despite low glucose, insulin, and IGF1 concentrations, phosphorylation of AKT and ERK was higher in the HG liver. Thus, a physiological increase in fetal insulin is sufficient for suppression of gluconeogenic genes and activation of glycolytic and lipogenic genes in the HG fetal liver. These results demonstrate that fetuses exposed to sustained hypoglycemia have maintained hepatic insulin action in contrast to fetuses exposed to placental insufficiency.

Keywords: PEPCK, glucose, liver, fetus, insulin, gluconeogenesis
INTRODUCTION

Glucose is the primary fuel for fetal oxidative metabolism (11). Reduced maternal glucose supply to the fetus is a common feature in animal models of placental insufficiency with decreased placental nutrient substrate and oxygen transport capacity and maternal nutrient restriction, both resulting in intrauterine growth restriction (IUGR) (1, 16, 23, 40). Recent data have shown that the nutrient restricted fetal baboon liver, IUGR fetal sheep liver produced from placental insufficiency (PI-IUGR), and IUGR neonatal rodent liver have increased gluconeogenic gene expression and glucose production rates (14, 16, 27, 35-36). This is in contrast to normal fetal sheep which produce little glucose until just prior to birth (10, 12). Our laboratory has developed a sheep model of IUGR based on experimental restriction of maternal glucose supply to the fetus and resulting fetal hypoglycemia and hypoinsulinemia (hypoglycemic model, HG), independent of generalized placental insufficiency and other pathophysiologic hallmarks of marked IUGR, notably uteroplacental ischemia and fetal hypoxemia (5, 8-9, 32). Similar to the PI-IUGR fetal sheep, reduced maternal glucose supply to the fetus for the final 2-8 wks of gestation increases fetal glucose production rates and hepatic gluconeogenic gene activation (8-9, 26, 32). Here, we used our HG fetal sheep model to determine the specific effect of reduced glucose supply versus other deficiencies in PI-IUGR model (e.g., reduced amino acid supply, uteroplacental blood flow, and oxygenation) on the mechanisms involved in the induction of hepatic glucose production in the fetus. Understanding these fetal adaptations is important as persistence of these fetal phenotypes into postnatal life likely contributes to the increased risk for development of uncontrolled hepatic glucose production, hepatic insulin resistance, and type 2 diabetes (23, 34, 37, 39).

Little is known about the mechanisms involved in the activation of glucose production in the PI-IUGR or HG model. Both PI-IUGR and HG late gestation fetal sheep have reduced pancreatic insulin secretion and decreased plasma insulin concentrations. They also demonstrate increased plasma cortisol, glucagon, and norepinephrine concentrations (16, 32, 35). These
hormonal changes may prime the fetal liver for activation of glucose production. Both PI-IUGR and HG fetuses have increased hepatic phosphorylation of cAMP response element binding protein (CREB) and peroxisome proliferator-activated receptor gamma co-activator (PGC1α) gene expression, supporting the concept that increased counter-regulatory hormone-mediated cAMP activation may drive activation of glucose production (16, 32, 36). Also, we have found increased expression of nuclear factors, including FOXO1, and a lack of activation of the energy sensor, AMPK, in the PI-IUGR fetal sheep liver (16, 36). The role of these factors in the HG fetus with sustained hypoglycemia for 8 wk is unknown but is important to understand if similar or different mechanisms are responsible for increased glucose production in HG and PI-IUGR fetal sheep.

Insulin is the primary hormone responsible for suppression of hepatic glucose production and gluconeogenic gene expression and activation of glycolysis and lipogenesis (28-29). Our recent data demonstrate differences in the capacity for acute hyperinsulinemia to suppress fetal glucose production between PI-IUGR and HG fetal sheep. Specifically, during a high dose acute hyperinsulinemic clamp, the PI-IUGR fetus maintained an increased rate of glucose production while glucose production was suppressed in the HG fetus made chronically hypoglycemic with 3 wks of restricted glucose supply from the mother (8-9, 35).

It is unclear from these previously published studies whether fetal exposure to hypoglycemia for a longer duration (i.e., 8 wk) would impair hepatic insulin action, which would be important to determine, as insulin-resistant glucose production has been observed to develop over at least this long of a period in the PI-IUGR fetus (35). Furthermore, none of these previous studies addressed the question of whether a longer and more physiological increase in insulin concentration could suppress hepatic gluconeogenic gene expression or the effect of insulin on stimulating glycolytic or lipogenic pathways in the liver. The latter are important, in addition to effects on gluconeogenesis, to determine if insulin action in the liver is maintained on multiple
pathways or if selective hepatic insulin resistance to suppression of glucose production has developed (3).

We hypothesized that reduced maternal glucose supply to the fetus over the last 40% of gestation, independent of placental insufficiency and reduced uteroplacental blood flow and fetal oxygenation, would increase fetal hepatic gluconeogenic gene expression and that a physiological increase in fetal insulin concentration would reverse and suppress this activation, indicating maintained hepatic insulin sensitivity. We also investigated the molecular mechanisms responsible for increased gluconeogenic gene expression in the HG fetus and effect of insulin on hepatic glycolytic and lipogenic gene expression as other indicators of hepatic insulin action. Our results demonstrate that a physiological increase in insulin is sufficient to suppress fetal hepatic gluconeogenic gene activation as well as activating glycolytic and lipogenic activity in response to reduced maternal glucose supply to the fetus.
MATERIALS AND METHODS

Animal model and study design

Experiments were conducted in Columbia-Rambouillet ewes with singleton pregnancies. An initial surgery was performed at 70.0±0.8 days gestational age (dGA; term = 148 dGA) to place maternal femoral venous and arterial catheters through a left groin incision. The ewe was allowed to recover at least three days prior to the initiation of experimental infusions described below. At 119.4±0.5 dGA, a second surgery was performed to place fetal catheters. After a midline laparotomy, a hysterotomy was performed and infusion catheters were placed into fetal femoral veins via hind limb pedal veins. Additional sampling catheters were placed into the fetal abdominal aorta via a hind limb pedal artery. All catheters were tunneled subcutaneously through a flank incision on the ewe and kept within a plastic pouch attached to the ewe’s skin. The catheters were flushed daily with heparinized 0.9% sodium chloride. Maternal infusions were continued while each ewe was allowed to recover from the second surgery for at least five days prior to initiation of fetal infusions. Ewes were kept in individual carts and given an ad libitum diet of alfalfa pellets, water, and mineral supplements. All animal procedures were in compliance with guidelines of the United States Department of Agriculture, the National Institutes of Health, and the American Association for the Accreditation of Laboratory Animal Care. The animal care and use protocols were approved by the University of Colorado Denver Institutional Animal Care and Use Committee.

Maternal and fetal treatments

Ewes were randomly assigned to one of two groups in a 1:2 ratio (CON:HG) upon arrival prior to surgery and any biochemical or physiologic measurements. Following recovery from the first surgery, the hypoglycemic group (HG; n = 9) received a continuous maternal infusion of intravenous insulin for eight weeks. Maternal arterial plasma glucose was measured at least twice daily and the insulin infusion was adjusted to achieve a 40% reduction in maternal glucose.
concentration (5, 8-9, 26). The control group (CON; n = 5) received a maternal saline infusion at rates matched to the insulin infusion rates in the HG group.

After the fetal surgery, fetuses from ewes receiving an insulin infusion were randomly divided into two groups in a 1:1 ratio (HG:HG+INS). One group received a direct fetal insulin infusion for the final week of the study (HG+INS; n = 4). The insulin infusion was kept constant at 100 mU/hr (actual infusion rate adjusted for fetal weight at necropsy = 38.9 ± 2.8 mU/kg/hr). To maintain isoglycemia, fetal arterial plasma glucose concentrations were measured at least twice daily and a variable rate infusion of 33% dextrose (wt/vol in saline) into the fetus was adjusted accordingly. The other group received a direct fetal saline infusion matched at equal infusion rates to the combined insulin and dextrose infusion in the HG+INS group (HG; n=5). Fetuses in the CON group (n = 5) also received a direct fetal saline infusion at rates equal to those in the HG+INS group. Fetuses in the CON and HG groups had fetal arterial plasma sampled for glucose concentrations at a similar rate as the HG+INS group to ensure that equal blood volumes were withdrawn.

Analysis of blood samples

All fetal blood and plasma samples were collected from catheters in the abdominal aorta. The day prior to tissue collection fetal blood was collected for hormone and metabolite analyses. Blood samples were immediately analyzed for oxygen content and plasma samples for glucose and lactate concentrations (2, 16, 36). Plasma samples were stored and later analyzed for arterial insulin, insulin-like growth factor 1 (IGF1), glucagon, norepinephrine, and cortisol (16-17, 22).

Liver tissue collection and analysis

After completion of study, the ewe and fetus were euthanized. Fetuses were weighed and samples of the right lobe of the liver were collected immediately and snap frozen in liquid nitrogen. Liver glycogen content was measured as described (16). RNA was extracted from liver tissue, reverse transcribed, and used in real time PCR as described (36). Primers were designed and tested for real time PCR (Roche LightCycler 480) for the following genes: fructose-1,6-
bisphosphatase 1 (FBP1: F’- AACCGGGCTCCAGCATGACG, R’- ACGGGCCTTCTGCCCCTCTT), glucose transporter 2 (GLUT2: F’- AGCTGGCTTTTGCACGGGC, R’- GGCTGGCAGACGACAAAAC), pyruvate carboxylase (PC: F’- GCACAGCATGGGGCTGGCT, R’- AACTGGGCCAGGTCCCCAC), pyruvate kinase liver red blood cell isoform (PKLR: F’- TGGCGGGAAGCCCGTTGTC, R’- CCAGAAACGCGTTGGCCACA), phosphofructokinase liver (PFKL: F’- TGGTGCTCCATGCTGGGA, R’- GCAGGGCGTGATGCTGTGA), fatty acid synthase (FAS: F’- GACACATCTTTGAGCAGCA, R’- TTTGCGAATTTCCAGGAATC), and sterol response element binding protein 1c (SREBP1C: F’- ATGGATTGCACGTTCGAAG, R’- CGGGAAATTCGCTGTCTTG). Assays for PCK1, G6PC, PGC1α, and estrogen receptor related transcript alpha (ERRα) were used as previously reported (36). Whole cell protein lysates were prepared from liver tissue and western immunoblotting was performed (32, 36). Antibodies were from Calbiochem to detect total glycogen synthase kinase 3 alpha/beta (GSK3α/β); Santa Cruz to detect hepatocyte nuclear factor 4 alpha (HNF4α); MP Biomedicals to detect actin, and Cell Signaling to detect protein kinase B (AKT), phosphorylated (P)-AKT (S473), P-AKT (T308), extracellular signal-regulated kinase (ERK1/2), P-ERK1/2 (T202/Y204), forkhead box protein O1 (FOXO1), FOXO1 (T24), P-GSK3β (S9), AMP-activated protein kinase (AMPK), P-AMPK (T172), CREB, and P-CREB (S133). Equality of protein loading was verified by actin expression. Results were quantified on each blot and a ratio of phosphorylated to total protein expression was calculated.

**Statistical analysis**

Data were analyzed by ANOVA with fixed effects of group (CON, HG, HG+INS) using SAS (PROC MIXED). When the overall ANOVA was significant, individual group comparisons were made using least square means (PDFF option). Statistical significance was declared at \( P < 0.05 \).
RESULTS

Fetal characteristics

Late gestation fetal sheep (HG) from ewes that received insulin for 8 wk (from day 74±1 through day 133±1 during pregnancy) were hypoglycemic and hypoinsulinemic (60% lower) compared to control (CON) fetuses from ewes that received saline (Table 1). A third group of HG fetuses (HG+INS) were infused for the final 7 days with exogenous insulin to produce a 4-fold physiologic increase in plasma insulin concentration relative to HG (50% increase vs. CON) and with exogenous glucose to maintain glucose concentrations at levels found in HG fetuses to establish fetal hyperinsulinemia and hypoglycemia (Table 1). Lactate concentrations were similar among all 3 groups. Arterial oxygen content was similar between CON and HG fetuses, but was reduced by nearly 50% in HG+INS. Fetal arterial plasma concentrations of cortisol, glucagon, and norepinephrine were not statistically higher in HG and HG+INS fetuses compared to CON fetuses. HG fetuses had lower insulin:glucagon, insulin:cortisol, and insulin:norepinephrine ratios compared to CON and HG+INS fetuses. IGF1 concentrations were 50% lower in HG and HG+INS fetuses. Fetuses in all 3 groups were of similar gestational age at the end of study. HG and HG+INS fetuses weighed 30% less compared to CON fetuses and HG fetuses had a 40% lower in liver weight (Table 1). Hepatic glycogen content was similar between groups (Table 1).

Increased gluconeogenic gene expression is reversed with physiological increases in fetal insulin concentrations during sustained fetal hypoglycemia

We next measured the effect of hypoglycemia and insulin on expression of fetal hepatic gluconeogenic and metabolic genes (Fig. 1). HG fetuses had greater \textit{PCK1} (6-fold) and \textit{G6PC} (8-fold) gene expression compared to CON fetuses, consistent with previous studies in HG fetuses demonstrating increased glucose production rates and PEPCK activity (26). Insulin infusion decreased both hepatic \textit{PCK1} and \textit{G6PC} gene expression in HG+INS fetuses compared
to levels observed in CON fetal livers. Similarly, the expressions of the gluconeogenic gene, *FBPI*, and bi-directional high capacity and low affinity glucose transporter, *GLUT2*, were higher in HG fetal livers and lower in HG+INS fetal livers. The pyruvate metabolism genes, *PKLR* and *PC*, were similar between groups. Expression of the glycolytic gene *PFKL* was similar between CON and HG fetuses and increased by 4-fold in HG+INS fetal livers. Insulin treatment increased expression of the lipogenic gene, *FAS*, by 2-fold in HG+INS fetal liver. Hepatic expression of lipogenic transcription factor, *SREBP1C*, was not significantly different between groups, yet was positively correlated with *FAS* expression among all animals ($r^2 = 0.6, P < 0.001$).

**Molecular mechanisms for increased gluconeogenic gene expression during sustained fetal hypoglycemia**

To determine whether upregulation of gluconeogenesis is mediated through hormone induced cAMP activity, we measured the following gluconeogenic regulatory factors. Expression of the transcriptional co-activator, *PGC1A*, was 4-fold higher in the HG compared to CON fetal liver (Fig. 2a). Insulin suppressed *PGC1A* in HG+INS fetal liver to similar levels seen in the CON group (Fig. 2a). Expression of *ERRA*, a PGC1α transcriptional target and regulator of mitochondrial biogenesis, was 2-fold greater in HG compared to CON fetal liver and remained higher in HG+INS liver despite the reduction in PGC1α (Fig. 2a). Protein expression of CREB, a cAMP-dependent upstream regulator of PGC1α, was not different between groups, although variations in phosphorylation and total protein expression resulted in a ratio of phosphorylated protein that was numerically higher in the HG and HG+INS fetal livers (Fig. 2b). Protein expression of HNF4α also was variable in the HG group, with 3 out of 5 fetal livers having a greater than 5-fold increase in expression compared to the CON group (Fig. 2b).

We next evaluated expression and activation of factors involved in insulin and nutrient signaling. The activation and expression of AMPK protein was similar between groups (Fig.
Phosphorylation of ERK was 15-fold greater in HG compared to CON fetal liver and was reduced in the HG+INS group (Fig. 3b). Phosphorylation of AKT at S473 was by 4-fold higher in the HG fetal liver compared to CON fetal liver and tended to be similarly increased in the HG+INS liver ($P=0.07$ vs. HG) (Fig. 3c). Phosphorylation of AKT at T308 was similar between groups (Fig. 3c).

To determine if increased AKT phosphorylation had functional consequences, we measured expression and activation of its insulin-sensitive targets. Total expression of FOXO1 was 60% greater in HG fetal liver (Fig. 4a). Phosphorylation of FOXO1 (T24) was 20-30% greater in HG and HG+INS fetal livers, respectively, compared to CON. The ratio of phosphorylated FOXO1 protein was similar between groups (Fig. 4a). Expression and phosphorylation of mTOR and GSK3β were similar between groups (Fig. 4b,c).
DISCUSSION

Previous studies have shown that reduced maternal glucose supply to the fetus activates fetal hepatic glucose production and gluconeogenic gene expression. This adaptive gluconeogenic response may be beneficial to supply the developing fetus with additional glucose for oxidative metabolism and growth. Persistence of this glucose production may have detrimental effects on glucose homeostasis in postnatal life, unless insulin sensitivity to suppress glucose production is maintained, allowing the normal postprandial increases in insulin secretion after birth to reduce the glucose production. This study aimed to determine the potential molecular mechanisms responsible for the activation of gluconeogenesis in the HG fetus. We also tested the effects of a physiological increase in insulin on reversibility and suppression of fetal hepatic gluconeogenic gene activation in response to reduced maternal glucose supply.

Fetuses exposed chronically for 8 wk to reduced glucose supply and low plasma glucose concentrations (HG group) had increased hepatic gene activation (PCK1, G6PC, FBP1, GLUT2, PGC1A), a novel finding that is consistent with previous reports that demonstrated increased fetal glucose production rates in HG fetuses exposed for 2-8 wk (8-9, 26, 32). A physiological increase in insulin concentrations for 1 wk in the chronically exposed HG fetus (HG+INS group) demonstrated maintained hepatic insulin action on genes regulating the suppression of hepatic gluconeogenesis (PCK1, G6PC, FBP1, GLUT2, PGC1A). Hepatic insulin sensitivity for activation of glycolysis (PFKL) and lipogenesis (FAS, SREBP1C) also remained intact (25, 28-29). While previous studies demonstrated normal insulin-mediated suppression of glucose production during an acute hyperinsulinemic clamp in the HG fetus exposed for 3 wks or less (8-9), it was unclear from previously published studies whether exposure to hypoglycemia for a longer duration (i.e., 8 wk) during gestation would impair hepatic insulin action, as observed in the PI-IUGR fetus (35). This is important to determine, because IUGR from placental insufficiency develops over at least this long a gestational period and the PI-IUGR fetus develops resistance to the ability of insulin to suppress fetal hepatic glucose production. Therefore,
differentiating these two types of IUGR, chronic hypoglycemia vs. placental insufficiency, would help point to mechanisms for increased GPR or hepatic insulin action that might be different between these two conditions. We now have shown that hepatic insulin action is maintained in the HG fetus during a chronic physiological infusion of insulin, as this infusion suppressed hepatic gluconeogenic gene activation and increased glycolytic and lipogenic gene expression. Thus, hepatic insulin resistance in the PI-IUGR model is due to more than reduced glucose supply and fetal hypoglycemia.

Concentrations of the counter-regulatory hormones, cortisol, glucagon, and norepinephrine, tended to be increased while insulin concentrations were reduced in the HG fetus, producing a hormonal combination (e.g., decreased insulin: counter-regulatory hormone ratios) favoring gluconeogenesis. Furthermore, expression of PGC1A and activation of one of its upstream regulators, CREB, and binding partner, HNF4α, also tended to be increased. Therefore, our results support increased cAMP-dependent activity as a potential mechanism for increased glucose production in the HG fetus (13, 41). A physiological increase in insulin in the HG+INS fetus was sufficient to suppress PGC1A and gluconeogenic gene expression in the presence of an elevated counter-regulatory hormone profile, consistent with insulin’s dominant effect on regulating hepatic glucose production (18). Increased expression of ERRA in HG fetal liver is consistent with increased PGC1α activity. A similar relationship is observed in the PI-IUGR fetal liver (16, 36). ERRA expression was further increased in the HG+INS group, however, which could be due to insulin-dependent effects on stimulating hepatic oxidative metabolism or insulin’s effects on regulating PGC1α function separate from its effects on PGC1A expression (15, 21, 30, 41). Also, despite low nutrient and glucose concentrations, AMPK phosphorylation was not increased in the HG fetal liver, consistent with our data in the PI-IUGR liver (36), indicating that both HG and PI-IUGR fetuses have developed metabolic adaptations to the low nutrient environment.
Interestingly, despite lower plasma concentrations of glucose and the anabolic hormones, insulin and IGF1, HG fetuses had increased hepatic phosphorylation of AKT and ERK compared to CON fetuses. The increase in P-AKT, increase in gluconeogenic gene expression, and decrease in lipogenic gene expression in the HG fetus is unexpected, given that AKT activation is normally associated with suppression of gluconeogenic gene expression and activation of lipogenesis (18, 29, 31). Increased AKT phosphorylation has been reported in models of hepatic insulin resistance (19). Our data, however, indicate that HG+INS fetuses have maintained hepatic insulin sensitivity on key genes in multiple metabolic pathways (e.g., gluconeogenesis, glycolysis, and lipogenesis). Although increased AKT phosphorylation at S473 is mTOR complex 2 (mTORC2) dependent (33), we found no change in mTOR protein expression or activation and no change in RICTOR mRNA, the component of mTORC2 (data not shown). Furthermore, despite increased AKT S473 phosphorylation in the HG fetal liver, we were unable to find subsequent changes in the phosphorylation of AKT targets, including mTOR and GSK3β. Thus, the significance of increased AKT and ERK activation in HG fetuses remains to be determined.

FOXO1 is a transcriptional activator of gluconeogenic gene expression. Insulin mediated phosphorylation and nuclear exclusion of FOXO1 is one example of how insulin inhibits gluconeogenesis at the molecular level, although recent studies indicate the importance of other AKT-FOXO1 independent pathways (20). FOXO1 expression was 60% higher, while phosphorylation was only 10-20% higher, producing a lower ratio of phosphorylated and potentially more active FOXO1 protein that might contribute to increased gluconeogenic gene activation in the HG fetal liver.

These results in the HG fetus contrast with our recently published data in the PI-IUGR fetus that show hepatic resistance to insulin-mediated suppression of gluconeogenic gene expression (35), suggesting that factors other than fetal hypoglycemia in IUGR lead to hepatic insulin resistance. One of the major differences between the HG and PI-IUGR models is fetal
oxygenation status. Oxygenation is normal in the HG fetus, in contrast to the PI-IUGR fetal sheep, which has a ~50% reduction in fetal arterial blood oxygen content and PO2. Despite these differences, both HG and PI-IUGR fetuses have similar oxygen consumption rates (5, 26, 36, 38). Thus, lower oxygen values may have an effect on glucose production and insulin sensitivity in the PI-IUGR fetus, rather than the metabolic rate of oxygen consumption. Arterial oxygen content was lower in HG+INS group, a finding similarly reported in other fetal sheep studies with chronic insulin treatments (4, 24). Fetal oxygen consumption rates were not measured in this study. Future studies are needed to measure the effect of chronic insulin infusion on umbilical venous and arterial oxygenation, umbilical blood flow, and net fetal oxygen consumption rates. Despite reduced oxygenation in the HG+INS group, gluconeogenic expression was suppressed by insulin, in contrast to the PI-IUGR fetus which is hypoxic and has hepatic insulin resistance (35). Lactate concentrations and umbilical uptake rates are similar between CON and HG (8wk) fetuses (5). In contrast, the PI-IUGR fetus has higher lactate concentrations raising the possibility that lactate might drive glucose production in the PI-IUGR model but not the HG model (35-36). Moreover, expression of the pyruvate metabolism genes, PC and PKLR, were similar between CON and HG fetal livers. Hepatic glycogen content also was similar between CON, HG, and PI-IUGR fetuses, supporting gluconeogenesis rather than glycogenolysis as the source of hepatic glucose production (16, 36).

In this study, we chose to test the effects of a physiological increase in insulin concentration for one week in the HG fetus. Our goal was to produce insulin concentrations in the HG+INS group that were at least as high as those observed in the CON group. Final insulin concentrations were 50% higher in HG+INS group relative to CON group and 4-fold higher relative to HG group. Our results demonstrate that this physiological increase in insulin concentration is sufficient for suppression of gluconeogenic gene expression. Furthermore, given the lack of gluconeogenesis in normal fetuses (10, 12), we focused on the effect of insulin only in HG fetuses.
**Perspectives and Significance**

In summary, we found that a chronic physiological increase in fetal insulin concentration suppresses gluconeogenic gene expression and activates glycolytic and lipogenic gene expression in fetal sheep chronically exposed to reduced maternal glucose supply. These results have important implications for understanding the metabolic adaptations and molecular mechanisms that develop in a fetus of any species chronically exposed to reduced nutrient supply. Activation of glucose production in the fetus in response to low glucose supply and concentrations may be a beneficial adaptive response to maintain glucose supply to vital organs, particularly the brain and heart, as placental glucose supply is diminished. If persistent in neonatal and postnatal periods, however, this adaptation could have adverse consequences by promoting glucose production in excess of the capacity for glucose utilization, contributing to persistent hyperglycemia. Preterm and low birth weight neonates have high rates of gluconeogenesis that is not completely suppressed by glucose or insulin (6-7). Increasingly, studies are showing that the early induction of gluconeogenesis in fetal life, as seen during IUGR, preterm birth, and animal models of reduced maternal nutrient supply or maternal stress, produces persistent and detrimental effects across the lifespan, especially if hepatic resistance to insulin-mediated suppression of glucose production persists across the lifespan (23, 34, 37). Consequently, understanding the developmental mechanisms that determine fetal metabolic adaptations to low glucose supply, including induction of gluconeogenesis and its reversibility, is important for understanding how to reduce such adverse metabolic conditions and thereby improve later life outcomes by reducing the incidence of type 2 diabetes and other aspects of the metabolic syndrome in offspring who were IUGR.

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REFERENCES


FIGURE LEGENDS

Figure 1. Effect of hypoglycemia and insulin on metabolic gene expression in the fetal liver. Gene expression was measured using real time PCR in the late gestation fetal liver of control (CON, n=5), hypoglycemic (HG, n=5), and HG fetuses receiving insulin (HG+INS, n=4) as indicated. Overall ANOVA P-value is shown and when significant (P<0.05) individual comparisons were made. * P<0.05 versus CON. † P<0.05 versus HG.

Figure 2. Effect of hypoglycemia and insulin on PGC1α, CREB, and HNF4α expression. A. Gene expression was measured in the late gestation fetal liver of control (CON, n=5), hypoglycemic (HG, n=5), and HG fetuses receiving insulin (HG+INS, n=4) for PGC1A and ERRA. B. Protein expression was measured in whole cell extracts by western blotting in CON (white bars), HG (black bars), and HG+INS (dashed bars) fetal liver samples using antibodies against phosphorylated (P)-CREB, total CREB, and HNF4α. A representative blot detecting actin expression is shown. Results were quantified and a ratio of phosphorylated to total CREB protein was calculated. Overall ANOVA P-value is shown and when significant (P<0.05) individual comparisons were made. * P<0.05 versus CON. † P<0.05 versus HG.

Figure 3. Effect of hypoglycemia and insulin on AMPK, ERK, and AKT protein expression. Protein expression was measured in whole cell extract fetal liver samples by western blotting from control (CON, n=5; white bars), hypoglycemic (HG, n=5; black bars), and HG fetuses receiving insulin (HG+INS, n=4; dashed bars) samples using antibodies against (A) phosphorylated (P)-AMPK, total AMPK, (B) P-ERK, total ERK, (C) P-AKT (S473 and T308), and total AKT. A representative blot detecting actin expression is shown. Results were quantified and a ratio of phosphorylated to total protein was calculated for AMPK, ERK, and AKT (S473 and T308). Overall ANOVA P-value is shown and when significant (P<0.05) individual comparisons were made. * P<0.05 versus CON. † P<0.05 versus HG.
Figure 4. Effect of hypoglycemia and insulin on FOXO1, GSK3β, and mTOR protein expression. Protein expression was measured in whole cell extract fetal liver samples by western blotting from control (CON, n=5; white bars), hypoglycemic (HG, n=5; black bars), and HG fetuses receiving insulin (HG+INS, n=4; dashed bars) samples using antibodies against (A) phosphorylated (P)-FOXO1, total FOXO1, (B) P-GSK3β, total GSK3α/β, (C) P-mTOR, and total mTOR. A representative blot detecting actin expression is shown. Results were quantified and a ratio of phosphorylated to total protein was calculated GSK3β, mTOR, and FOXO1. Overall ANOVA P-value is shown and when significant (P<0.05) individual comparisons were made. * P<0.05 versus CON. † P<0.05 versus HG.
Table 1. Fetal arterial parameters and growth characteristics.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>HG</th>
<th>HG+INS</th>
<th>P-value(^a)</th>
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</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>22.1 ± 2.0</td>
<td>8.7 ± 0.9(^*)</td>
<td>7.6 ± 0.3(^*)</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Insulin (ng/mL)</td>
<td>0.45 ± 0.09</td>
<td>0.16 ± 0.03(^*)</td>
<td>0.66 ± 0.17(^†)</td>
<td>&lt; 0.05</td>
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<tr>
<td>Lactate (mM)</td>
<td>1.6 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>0.43</td>
</tr>
<tr>
<td>Oxygen content (mM)</td>
<td>3.6 ± 0.13</td>
<td>3.3 ± 0.26</td>
<td>1.8 ± 0.6(^**,(^†)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Glucagon (pg/mL)</td>
<td>34.3 ± 2.8</td>
<td>57.7 ± 9.6</td>
<td>50.2 ± 13.2</td>
<td>0.19</td>
</tr>
<tr>
<td>Insulin:Glucagon(^c)</td>
<td>8.2 ± 1.6</td>
<td>2.0 ± 0.5(^*)</td>
<td>9.1 ± 3.3(^†)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Cortisol (ng/mL)</td>
<td>4.0 ± 1.1</td>
<td>8.2 ± 3.3</td>
<td>8.0 ± 0.8</td>
<td>0.34</td>
</tr>
<tr>
<td>Insulin:Cortisol(^c)</td>
<td>7.8 ± 0.9</td>
<td>2.6 ± 1.2(^*)</td>
<td>5.5 ± 1.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Norepinephrine (pg/mL)</td>
<td>464.3 ± 18.9</td>
<td>549.3 ± 158.7</td>
<td>843.1 ± 177.2</td>
<td>0.16</td>
</tr>
<tr>
<td>Insulin:Norepinephrine(^c)</td>
<td>0.029 ± 0.005</td>
<td>0.012 ± 0.003(^*)</td>
<td>0.022 ± 0.004</td>
<td>0.05</td>
</tr>
<tr>
<td>IGF1 (ng/mL)</td>
<td>30.4 ± 6.7</td>
<td>13.2 ± 1.8(^*)</td>
<td>16.5 ± 3.1(^*)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Fetal age (d)</td>
<td>133.2 ± 1.1</td>
<td>133.0 ± 1.3</td>
<td>134.5 ± 0.9</td>
<td>0.64</td>
</tr>
<tr>
<td>Fetal wt (kg)</td>
<td>3.6 ± 0.1</td>
<td>2.2 ± 0.1(^*)</td>
<td>2.6 ± 0.2(^*)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Liver wt (g)</td>
<td>137.4 ± 13.1</td>
<td>76.0 ± 5.4(^*)</td>
<td>104 ± 13.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Liver glycogen (mg/g)</td>
<td>28.1 ± 3.4</td>
<td>22.4 ± 5.7</td>
<td>39.48 ± 12.3</td>
<td>0.30</td>
</tr>
</tbody>
</table>

\(^a\) Fetal measurements at the end of study periods in control (CON, n = 5), hypoglycemic (HG, n = 5), and insulin infused HG (HG+INS, n = 4) fetuses. \(^b\) Ratio of glucose (mg/dL) to insulin (ng/mL). \(^c\) Molar ratio of insulin to glucagon concentrations. Means ± SE are shown. Overall ANOVA \(P\)-values are presented. \(^*\) \(P < 0.05\) v CON. \(^†\) \(P < 0.05\) v HG.
Fig. 1

The figure shows the expression levels of various mRNA markers under different conditions: CON (control), HG (high glucose), and HG+INS (high glucose plus insulin). The x-axis represents the conditions, while the y-axis represents the mRNA expression levels. The statistical significance is indicated by asterisks (*) and dagger symbols (†), with the p-values provided for each comparison.

- **PCK1 mRNA**: 
  - P<0.005 for HG compared to CON
  - HG+INS is intermediate

- **G6PC mRNA**: 
  - P<0.001 for HG compared to CON
  - HG+INS is intermediate

- **FBP1 mRNA**: 
  - P<0.05 for HG compared to CON
  - HG+INS is intermediate

- **GLUT2 mRNA**: 
  - P<0.05 for HG compared to CON
  - HG+INS is intermediate

- **PKLR mRNA**: 
  - P=0.41
  - HG+INS is intermediate

- **PC mRNA**: 
  - P=0.63
  - HG+INS is intermediate

- **PFKL mRNA**: 
  - P<0.001 for HG compared to CON
  - HG+INS is intermediate

- **FAS mRNA**: 
  - P<0.05 for HG+INS compared to HG

- **SREBP1C mRNA**: 
  - P=0.16
  - HG+INS is intermediate
Fig. 2

A

![Graph showing PGC1α mRNA expression with CON, HG, and HG+INS conditions.]

B

![Western blot images and corresponding bar graphs showing protein expression of P-CREB, CREB, HNF4α, and Actin in CON, HG, and HG+INS conditions.]

- **PGC1α mRNA**
  - CON: Baseline
  - HG: Increase
  - HG+INS: Decrease

- **ERRA mRNA**
  - CON: Baseline
  - HG: Increase
  - HG+INS: Increase

- **Protein Expression**
  - P-CREB
  - CREB
  - HNF4α
  - Actin

- **P-values**:
  - Actin: P=0.55
  - HNF4α: P=0.20
  - P-CREB: P=0.08
  - P-CREB ratio: P=0.45
Fig. 3

A

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
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B

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C

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<tr>
<td>Actin</td>
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</table>
Fig. 4

A

CON HG HG+INS

P-FOXO1

FOXO1

B

CON HG HG+INS

P-GSK3β

GSK3α/β

C

CON HG HG+INS

P-mTOR

mTOR

Actin

P-FOXO1

FOXO1

P-FOXO1 ratio

P-GSK3β

GSK3β

P-GSK3β ratio

P-mTOR

mTOR

P-mTOR ratio

* P < 0.005

* P < 0.05

= P = 0.15

P = 0.37

P = 0.10

P = 0.25

P = 0.16

P = 0.18

P = 0.28