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


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Thorn SR, Sekar SM, Lavezzi JR, O’Meara MC, Brown LD, Hay Jr, WW, Rozance PJ. A physiological increase in insulin suppresses gluconeogenic gene activation in fetal sheep with sustained hypoglycemia. Am J Physiol Regul Integr Comp Physiol 303: R861–R869, 2012. First published August 29, 2012; doi:10.1152/ajpregu.00331.2012.— 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 whether 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 days (HG+INS) were studied. Glucose and insulin concentrations were 60% lower in HG compared with CON fetuses. Insulin was 50% higher in HG+INS compared with CON and four-fold higher compared with 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.

PEPCK; glucose; liver; fetus; insulin; gluconeogenesis

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, 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 pathophysiological 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 wk 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 vs. 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-γ coactivator (PGC1A) 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 it is important to understand whether 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 glucose production, hepatic insulin resistance, and Type 2 diabetes (23, 34, 37, 39).
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 what the effect of insulin is on stimulating glycolytic or lipogenic pathways in the liver. These issues are important, in addition to effects on gluconeogenesis, to determine whether insulin action in the liver is maintained on multiple pathways or whether 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 the 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 3 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 5 days prior to initiation of fetal infusions. Ewes were kept in individual carts and given ad libitum diet of alfalfa pellets, water, and mineral supplements. All animal procedures were in compliance with guidelines of the U.S. 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 physiological measurements. Following recovery from the first surgery, the hypoglycemic group (HG; n = 9) received a continuous maternal infusion of intravenous insulin for 8 wk. 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/h (actual infusion rate adjusted for fetal weight at necropsy = 38.9 ± 2.8 mU·kg⁻¹·h⁻¹). 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 were analyzed for glucose and lactate concentrations (2, 16, 36). Plasma samples were stored and later analyzed for arterial insulin, IGF I, glucagon, norepinephrine, and cortisol (16–17, 22).

Liver tissue collection and analysis. After completion of the 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 previously (16). RNA was extracted from liver tissue, reverse transcribed, and used in real-time PCR, as described previously (36). Primers were designed and tested for real-time PCR (Roche LightCycler 480) for the following genes: fructose-1,6-bisphosphatase 1 (FBP1); F'-AACCGGGTCTCCAGATGGCAGC (HG/HG/H11001), gluconeogenic gene activation in hypoglycemic fetus (HG/HG/H11005); GLUT2 (F'-AGCTGCGCTTTGTCACGGGC, F'-GGCTGGCAGACGAGAAACCA, pyruvate carboxylase (PC): F'-GCACACATGTCGCTGTCGT, R'-AAGCTGGCGACTCCCCACAC, pyruvate kinase liver red blood cell isofor (PKLR); F'-TGCGGAGAAGCCCTGTGT, R'-CCAGAAGCGCTTTGCGACA, phosphofructokinase (PCK1), F'-TTGGTGGCTCATTCTGCGA, R'-CCAGAAGCGCTTTGCGACA, extraocular muscle phosphofructokinase (PFKL), F'-TGCGGAGAAGCCCTGTGT, R'-CCAGAAGCGCTTTGCGACA, fatty acid synthase (FAS), F'-GACACATCTCTTGGACGACCA, R'-TTGCAAACTTTTCCAGAACT, and sterol response element binding protein 1c (SREBP1C); F'-ATGATGTTTGGCCACAGCA, estrogen receptor-related transcript alpha (ERRA) were used as previously reported (36). Whole cell protein lysates were prepared from liver tissue and Western immunoblotting was performed (32, 36). Antibodies were obtained from Calbiochem to detect total glycogen synthase kinase 3-α/β (GSK3α/β), from Santa Cruz to detect hepatocyte nuclear factor 4α (HNF4α), from MP Biomedicals to detect actin, and from Cell Signaling to detect PKB (AKT), phosphorylated (P)-AKT (S473), P-AKT (T308), extracellular signal-regulated kinase (ERK1/2), P-ERK1/2 (T202/Y204), forkhead box protein 01 (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 (PDIFF option). Statistical significance was declared at P < 0.05.

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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 with 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 four-fold physiological 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 three groups. Arterial oxygen content was similar between CON and HG fetuses, but it 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 with CON fetuses. HG fetuses had lower insulin: glucagon, insulin:cortisol, and insulin:norepinephrine ratios compared with CON and HG+INS fetuses. IGF1 concentrations were 50% lower in HG and HG+INS fetuses. Fetuses in all three groups were of similar gestational age at the end of study. HG and HG+INS fetuses weighed 30% less compared with CON fetuses, and HG fetuses had a 40% lower 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 PCK1 (six-fold) and G6PC (eight-fold) gene expression compared with CON fetuses, consistent with previous studies in HG fetuses, demonstrating increased glucose production rates and PEPCK activity (26). Insulin infusion decreased both hepatic PCK1 and G6PC gene expression in HG+INS fetuses compared with levels observed in CON fetal livers. Similarly, the expressions of the gluconeogenic gene, FBP1, and bidirectional 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 four-fold in HG+INS fetal livers. Insulin treatment increased expression of the lipogenic gene, FAS, by twofold in HG+INS fetal liver. Hepatic expression of lipogenic transcription factor, SREBP1C, was not significantly different between groups, yet it 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 coactivator, PGC1A, was four-fold higher in the HG compared with 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 PGC-1α transcriptional target and regulator of mitochondrial biogenesis, was twofold greater in HG compared with CON fetal liver and remained higher in HG+INS liver despite the reduction in PGC-1α (Fig. 2A). Protein expression of CREB, a cAMP-dependent upstream regulator of PGC-1α, 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 three out of five fetal livers having a greater than five-fold increase in expression compared with 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. 3A). Phosphorylation of ERK was 15-fold greater in HG compared with CON fetal liver and was reduced in the HG+INS group (Fig. 3B). Phosphorylation of AKT at S473 was four-fold higher in the HG fetal liver compared with 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).

Table 1. Fetal arterial parameters and growth characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>CON</th>
<th>HG</th>
<th>HG+INS</th>
<th>$P$ value(^a)</th>
</tr>
</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>
</tr>
<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>
</tr>
<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>3.1 ± 0.6(\pm)</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(^a)</td>
<td>8.2 ± 1.6</td>
<td>2.0 ± 0.5*</td>
<td>9.1 ± 3.3(\pm)</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(^b)</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(^a)</td>
<td>0.029 ± 0.005</td>
<td>0.012 ± 0.003(\pm)</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(\pm)</td>
<td>165 ± 31.1(\pm)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Fetal age, days</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(\pm)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Liver wt, g</td>
<td>137.4 ± 13.1</td>
<td>76.0 ± 5.4(\pm)</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>

Values are expressed as means ± SE. 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. *Molar ratio of insulin to hormone concentrations. *Overall ANOVA $P$ values are presented. **$P < 0.05$ vs. CON. †$P < 0.05$ vs. HG.
To determine whether 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/H11001 INS fetal livers, respectively, compared with CON. The ratio of phosphorylated FOXO1 protein was similar between groups (Fig. 4A). Expression and phosphorylation of mTOR and GSK3/H9252 were similar between groups (Fig. 4, B and 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, and 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/H11001 INS group) demonstrated maintained hepatic insulin action on genes regulating the suppression of hepatic gluconeogenesis (PCK1, G6PC, FBP1, GLUT2, and PGC1A). Hepatic insulin sensitivity for activation of glycolysis (PFKL) and lipogenesis (FAS and 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 wk 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 insulin and its actions to suppress fetal hepatic glucose production.
GLUCONEOGENIC GENE ACTIVATION IN HYPOGLYCEMIC FETUS

Fig. 2. Effect of hypoglycemia and insulin on peroxisome proliferator-activated receptor-\(\gamma\) coactivator (PGC1A), cAMP response element binding protein (CREB) and hepatocyte nuclear factor-4\(\alpha\) (HNF4\(\alpha\)) 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 blot analysis in CON (open bars), HG (solid bars), and HG+INS (hatched bars) fetal liver samples using antibodies against phosphorylated (P)-CREB, total CREB, and HNF4\(\alpha\). 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. \(*P < 0.05\) vs. CON. \(†P < 0.05\) vs. HG.

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, HNF4\(\alpha\), 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 PGC-1\(\alpha\) 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 PGC-1\(\alpha\) 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 IGF I, HG fetuses had increased hepatic phosphorylation of AKT and ERK compared with CON fetuses. The increase in P-AKT, the increase in gluconeogenic gene expression, and the decrease in lipogenic gene expression in the HG fetus are 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, a 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 GSK3\(\beta\). 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 subsequent nuclear exclusion of FOXO1 is one example of how insulin inhibits gluconeogenesis at the molecular level, although re-
cent 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 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.

Fig. 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 blot analysis from control (CON, open bars; n = 5), hypoglycemic (HG, solid bars; n = 5), and HG fetuses receiving insulin (HG + INS, hatched bars; n = 4) samples using antibodies against phosphorylated (P)-AMPK, total AMPK (A), P-ERK, total ERK (B), P-AKT (S473 and T308), total AKT, and actin (C). 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 vs. CON. †P < 0.05 vs. HG.
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 1 wk 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 four-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 life span, especially if hepatic resistance to insulin-mediated suppression of hepatic glucose production is increased.

Fig. 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 blot analysis from control (CON, white bars; n = 5), hypoglycemic (HG, black bars; n = 5), and HG fetuses receiving insulin (HG+INS, hatched bars; n = 4) samples using antibodies against P-FOXO1 and total FOXO1 (A), P-GSK3β and total GSK3α/β (B), and P-mTOR, total mTOR, and actin (C). Results were quantified, and a ratio of phosphorylated to total protein was calculated for GSK3β, mTOR, and FOXO1. Overall ANOVA P value is shown and when significant (P < 0.05), individual comparisons were made. *P < 0.05 vs. CON.

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**Perspectives and Significance**

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glucose production persists across the life span (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.

ACKNOWLEDGMENTS

We thank David Caprio, Karen Trembler, Alex Cheung, Gates Roe, and Dan LoTurco at the University of Colorado for technical support. This research was supported by National Institutes of Health (NIH) Grants K01-DK-090199 to S. R. Thorn; K08-HD-060688, K01-DK-088139, and American Diabetes Association Junior Faculty Award 7-08-JF-51 to P. J. Rozance; R21-HD-04332 to L. D. Brown; and T32-HD-07186 to W. W. Hay Jr.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


