Increased insulin sensitivity and maintenance of glucose utilization rates in fetal sheep with placental insufficiency and intrauterine growth restriction

Sean W. Limesand,1 Paul J. Rozance,2 Danielle Smith,1 and William W. Hay, Jr.1

1Perinatal Research Center, Department of Pediatrics, University of Colorado Health Sciences Center, Aurora, Colorado; and 2Agricultural Research Complex, Department of Animal Sciences, University of Arizona, Tucson, Arizona

Submitted 17 July 2007; accepted in final form 23 September 2007

Limesand SW, Rozance PJ, Smith D, Hay WW Jr. Increased insulin sensitivity and maintenance of glucose utilization rates in fetal sheep with placental insufficiency and intrauterine growth restriction. Am J Physiol Endocrinol Metab 293: E1716–E1725, 2007. First published September 25, 2007; doi:10.1152/ajpendo.00459.2007.—In this study we determined body weight-specific fetal (umbilical) glucose uptake (UGU), utilization (GUR), and production rates (GPR) and insulin action in intrauterine growth-restricted (IUGR) fetal sheep. During basal conditions, UGU from the placenta was 33% lower in IUGR fetuses, but GUR was not different between IUGR and control fetuses. The difference between glucose utilization and UGU rates in the IUGR fetuses demonstrated the presence and rate of fetal GPR (41% of GUR). The mRNA concentrations of the gluconeogenic enzymes glucose-6-phophatase and PEPCK were higher in the livers of IUGR fetuses, perhaps in response to CREB activation, as phosphorylated CREB/total CREB was increased 4.2-fold. A hyperglycemic clamp resulted in similar rates of glucose uptake and utilization in IUGR and control fetuses. The nearly identical GURs in IUGR and control fetuses at both basal and high glucose concentrations occurred at mean plasma insulin concentrations in the IUGR fetuses that were ~70% lower than controls, indicating increased insulin sensitivity. Furthermore, under basal conditions, hepatic glycogen content was similar, skeletal muscle glycogen was increased 2.2-fold, the fraction of fetal GUR that was oxidized was 32% lower, and GLUT1 and GLUT4 concentrations in liver and skeletal muscle were the same in IUGR fetuses compared with controls. These results indicate that insulin-responsive fetal tissues (liver and skeletal muscle) adapt to the hypoglycemic-hypoinsulinemic IUGR environment with mechanisms that promote glucose utilization, particularly for glucose storage, including increased insulin action, glucose production, shunting of glucose utilization to glycogen production, and maintenance of glucose transporter concentrations.

Increased insulin sensitivity and maintenance of glucose utilization rates in fetal sheep with placental insufficiency and intrauterine growth restriction

Numerous human epidemiological studies (8, 36, 37, 62) have linked poor prenatal growth resulting from fetal nutrient deficiencies to diseases later in life that are associated with insulin resistance and the metabolic syndrome. The “thrifty phenotype” hypothesis was proposed by Hales et al. (37) to explain how poor nutrition in early life could initiate a sparing response through adaptations in insulin secretion, insulin action, and glucose metabolism to benefit perinatal survival. After birth, if nutrients are abundant but the thrifty phenotype adaptations are maintained, the individual might be at greater risk for developing increased fat production and storage. Increased cellular fat in turn produces insulin resistance, which is a common underlying feature of the metabolic syndrome. Such insulin resistance is associated with reduced intracellular signaling of protein kinases that regulate insulin action and lower glucose transporter concentrations in muscle and adipose tissues (65–67). In contrast, increased insulin sensitivity has been shown during early postnatal life in male offspring from protein-malnourished rat dams (67) and lambs that were intrauterine growth restricted (IUGR) from placental growth restriction (20). Taken together, these data (42, 64, 66) indicate that a thrifty response develops in utero and persists during early postnatal life but ultimately leads to impaired insulin action at later stages.

We have developed a model of intrauterine growth restriction induced by placental insufficiency in fetal sheep that replicates all of the fundamental complications found in human pregnancies with marked fetal growth restriction (2, 13, 31, 73, 76, 79, 88), including abnormalities in umbilical artery Doppler velocimetry (69), hypoglycemia, hypoinsulinemia, and hypoxia (57, 70). These fetuses also have reduced beta-cell mass and insulin secretion due to decreased rates of beta-cell proliferation and insulin biosynthesis in the existing beta-cells (53, 55), contributing to the hypoinsulinemia. Despite the hypoglycemia and hypoinsulinemia in these fetuses, however, preliminary observations indicate that insulin sensitivity is increased (55) and proximal insulin-signaling factors in liver and skeletal muscle (insulin receptor substrate-1) are increased (75). However, these preliminary observations have not been confirmed, nor has their impact on fetal glucose metabolism been determined.

Therefore, in this study we measured fetal glucose uptake, utilization, oxidation, and production rates on a body weight-specific basis in IUGR fetal sheep that were nutrient deprived from placental insufficiency (both smaller placental size and reduced glucose and amino acid transport capacity). We also examined fetal glucose metabolism during acute hyperglycemia to determine whether reintroducing glucose could reverse the higher fetal glucose production found in the IUGR fetuses (normal fetal sheep have no measurable glucose production). To evaluate the maintenance of glucose utilization we determined tissue glycogen contents and glucose transporter concentrations. Furthermore, to explain the glucose production in the fetus, we determined the mRNA expression levels for hepatic gluconeogenic enzymes that are activated by phosphorylated cAMP response element-binding (CREB) protein.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Ovine model of intrauterine growth restriction. Pregnant Cheviot ewes carrying singletons were purchased from Ovis (Canton, SD) and managed in compliance with the Institutional Animal Care and Use Committee, University of Colorado Health Sciences Center, at the Perinatal Research Center, Aurora, CO, accredited by the National Institutes of Health, the US Department of Agriculture, and the American Association for Accreditation of Laboratory Animal Care. IUGR fetuses were created by exposing pregnant ewes to elevated ambient temperatures (40°C for 12 h, 35°C for 12 h) from 39 ± 2 days gestation age (dGA; means ± SD) until 96 ± 5 dGA as previously described (53–55, 88). Control fetuses were from pair-fed, gestational age-matched, normal, healthy pregnant ewes maintained at 25°C. Six of seven fetuses in the control treatment group completed in vivo studies. Of the six IUGR fetuses that survived treatment (70% survival of seven fetuses in the control treatment group completed in vivo described (53–55, 88). Control fetuses were from pair-fed, gestational gestation age (dGA; means SD) until 96 ± 5 dGA as previously described (53–55, 88). Control fetuses were from pair-fed, gestational age-matched, normal, healthy pregnant ewes maintained at 25°C. Six of seven fetuses in the control treatment group completed in vivo studies. Of the six IUGR fetuses that survived treatment (70% survival of seven fetuses in the control treatment group completed in vivo described (53–55, 88). Control fetuses were from pair-fed, gestational gestation age (dGA; means SD) until 96 ± 5 dGA as previously described (53–55, 88). Control fetuses were from pair-fed, gestational age-matched, normal, healthy pregnant ewes maintained at 25°C. Six of seven fetuses in the control treatment group completed in vivo studies. Of the six IUGR fetuses that survived treatment (70% survival of seven fetuses in the control treatment group completed in vivo described (53–55, 88). Control fetuses were from pair-fed, gestational age-matched, normal, healthy pregnant ewes maintained at 25°C. Six of seven fetuses in the control treatment group completed in vivo studies. Of the six IUGR fetuses that survived treatment (70% survival of seven fetuses in the control treatment group completed in vivo described (53–55, 88). Control fetuses were from pair-fed, gestational age-matched, normal, healthy pregnant ewes maintained at 25°C. Six of seven fetuses in the control treatment group completed in vivo studies. Of the six IUGR fetuses that survived treatment (70% survival of seven fetuses in the control treatment group completed in vivo.

Surgical preparation. At ~125 dGA, fetuses were surgically instrumented with indwelling catheters (22–24, 39, 52, 61). Fetal catheters were placed in the abdominal aorta via hindlimb pedal arteries, umbilical vein, and femoral veins via the saphenous veins. Maternal catheters were placed in the femoral artery and vein. All fetuses were subcutaneously tunneled to the ewe’s flank and kept in a plastic pouch. Ewes were allowed to recover for 5–7 days after surgery before fetal glucose uptake and metabolism was determined.

Fetal glucose uptake and utilization. Fetal glucose uptake was calculated using the Fick principle and performed in conjunction with [1-14C(U)]glucose (New England Nucleotides; PerkinElmer Life Sciences, Boston, MA) as a glucose tracer to determine rates of glucose utilization and oxidation, as previously reported (23, 39, 40). Briefly, 1 h prior to baseline blood sampling, a bolus of 26.6 μCi [3H2O and 70.6 μCi [1-14C(U)]glucose in saline was administered, followed by a constant infusion of 0.49 μCi/min [3H2O and 1.31 μCi/min [1-14C(U)]glucose. Four arterial and umbilical vein blood samples were collected simultaneously during the basal steady-state period at ~35, ~25, ~15, and ~5 min prior to dextrose administration. The hyperglycemic clamp was initiated with a dextrose in water bolus (230 ± 22 mg/kg) into the fetus followed by a constant infusion of 33% dextrose in water to increase and maintain fetal arterial plasma glucose concentrations at 2.3 mmol/L. Fetal arterial plasma samples were collected between 5 and 60 min to clamp the fetal plasma glucose concentration. Fetal blood samples for the hyperglycemic steady-state period were collected at 60, 75, 90, and 105 min from the fetal artery and umbilical vein. Fetal blood concentrations of [3H2O, glucose, radiolabeled glucose, radiolabeled [14CO2, lactate, and oxygen were determined for the arterial and umbilical vein. All calculations were performed during steady-state conditions. Umbilical blood flow rate was calculated by the steady-state diffusion technique (23, 39, 59). Umbilical (net fetal) uptake rates of glucose, oxygen, and lactate from the uteroplacenta were calculated using the Fick principle [umbilical blood flow (ml/min) × umbilical venous-arterial substrate concentration difference (mg/ml or mmol/ml)]. During the hyperglycemic clamp period, total fetal glucose uptake rate was determined as the product of oxygen saturation and oxygen capacity. The pH, PO2, and hematocrit were determined for 39.1°C using an ABL 520 blood gas analyzer.

Whole blood collected in EDTA-coated syringes was centrifuged (14,000 g) for 3 min at 4°C. Plasma was aspirated from the pelleted red blood cells and stored at ~70°C for hormone and amino acid measurements. Plasma glucose and lactate concentrations were measured immediately using a YSI model 2700 Select Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin concentrations were measured by an ovine insulin ELISA (Alpco Diagnostics, Windham, NH).

Glucose transporter immunoblots. Immunoblots for glucose transporters (GLUT1) and GLUT4 were performed on fetal tissues as previously described (3, 4, 9). Fetal tissues were homogenized in ice-cold lysis buffer containing: 1% Nonidet P-40, 150 mmol/l NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 50 mmol/l Tris, pH 7.4, 1 mmol/l phenylmethanesulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The lysates were centrifuged for 15 min at 12,000 g and pellets discarded. The protein was stored at ~80°C for immunoblotting. Protein concentrations were determined with a Bradford DC protein assay (Bio-Rad Laboratories, Richmond, CA) prior to separation.

Equal volumes of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing 5% β-mercaptoethanol were added to 75 μg of the tissue protein extract and denatured for 5 min at 95°C. Protein extracts were separated by 10% SDS-polyacrylamide gel electrophoresis, (6) transferred to PVDF membrane (BioRad Laboratories), and blocked for 1 h in 5% nonfat dry milk and phosphate-buffered saline with 0.05% Tween 20 (PBST). Immunoblot detection of glucose transporters was accomplished with a rabbit anti-GLUT1 polyclonal antibody (1:5,000; Chemicon International, Temecula, CA) or rabbit anti-GLUT4 polyclonal antibody (1:1,000; Chemicon) in PBST containing 5% nonfat dry milk. Binding of the rabbit antiseraum was detected with anti-rabbit immunoglobulin G horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Bio-Rad Laboratories) using enhanced chemiluminescence-Plus (Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer’s instructions and exposed to Kodak X-ray film below film saturation. GLUT1 or GLUT4 immunocomplexes were removed with a 62.5 mmol/l Tris, pH 6.8, 2% SDS, and 100 mmol/l β-mercaptoethanol solution incubate for 30 min at 50°C. Immunodetection for GAPDH (Novus Biologicals, Littleton, CO) was performed and used to normalize for loading differences. Densitometry was analyzed with Scion Image software β-version 4.0.2 (Scion, Frederick, MD) and presented as means ± SE.

CREB immunoblots. Immunoblots for CREB and Ser133-phosphorylated CREB were performed as follows. Protein was extracted from...
pulverized hepatic tissue (200 mg) by the addition of 600 µl of ice-cold lysis buffer [150 mmol/l NaCl, pH 7.4, 20 mmol/l Tris, 1% (vol/vol) Nonidet P-40, 2 mmol/l EDTA, 2.5 mmol/l Na3P2O7, 10% (vol/vol) glycerol, 20 mmol/l β-glycerophosphate, 0.575 mmol/l PMSE, 2% (vol/vol) Sigma mammalian protease inhibitor cocktail, 0.5% (vol/vol) Sigma phosphatase inhibitor] followed by 30 min on an orbital rocker at 4°C. The samples were then sonicated for 30 s, agitated, and placed on an orbital rocker for another 30 min at 4°C. The protein was separated from cellular debris by centrifugation at 21,000 g for 20 min at 4°C. The supernatant was removed and the protein concentration quantified with the Bio-Rad DC protein assay. Protein samples (30 µg) were separated and blots prepared as described for the GLUT proteins. Immunoblot detection wasaccomplished with rabbit anti-Ser133-phosphorylated CREB (1:500; Cell Signaling Technology, Danvers, MA) in PBST with 5% bovine serum albumin, rabbit anti-CREB (1:1,000; Santa Cruz Biotechnology), and mouse anti-actin (1:40,000; Medimmune, Gaithersburg, MD) in PBST with 5% nonfat dried milk using anti-rabbit IgG or with anti-mouse IgG (1:20,000; Upstate) HRP-conjugated secondary antibodies. Immunocomplexes were visualized and analyzed as described above for the glucose transporters.

Glycogen content. Glycogen content in liver and skeletal muscle was determined as previously described (9, 15). Briefly, 100 mg of hepatic or skeletal muscle tissue was pulverized and digested in 2 ml of 30% KOH at 95°C for 30 min. The homogenate (150 µl) was placed on No. 1 Whatman filter paper and washed in 66% ethanol with constant stirring for 30 min. The filter paper was removed, dried, and cut into small pieces. Glycogen was converted to glucose with 31.1 U amylloglucosidase (Sigma Chemical) in 0.2 M acetate buffer (pH 4.8, 0.5% glacial acetic acid, 0.12 M sodium acetate) at 37°C for 60 min. Glycogen concentration of this solution was determined in triplicate on a Yellow Springs analyzer and compared with concurrently run standards of glycogen (Sigma Chemical). Results are expressed as milligrams glycogen per grams tissue (wet weight).

RNA extraction and quantitative real-time PCR. Total RNA from fetal liver was extracted with Tri Reagent (Molecular Research Center, Cincinnati, OH) and cleaned up using a Qiagen Mini RNeasy column (Qiagen, Valencia, CA). Prior to designing quantitative PCR primers against the sheep nucleotide sequences, ovine cDNA clones were generated using primers designed against orthologs and are available upon request. The PCR products were amplified by reverse transcription PCR from the polyadenylated RNA using Superscript II reverse transcriptase and Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA) as previously described (51). PCR products were TA cloned using TOPO PCR II kit and transformed into Mach1 T1 phage-resistant, chemically competent E. coli (Invitrogen Life Technologies). Plasmid DNA was purified using QIAprep Spin Miniprep kit (Qiagen, Valencia, CA), and nucleotide sequence was confirmed by sequencing both strands of the cDNA clone. The ovine nucleotide sequences were deposited into GenBank: phosphoenolpyruvate carboxykinase (PEPCK) accession no. EF062862, glucose-6-phosphate dehydrogenase (6-Pase) accession no. EF062861, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) accession no. 8 e95761, and ribosomal protein S15 accession no. AY949774. For each pair of oligonucleotide primers designed against the sheep nucleotide sequence, the specificity was determined by melting curve analysis, agarose gel electrophoresis, and nucleotide sequencing the PCR product after amplification with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). PCR efficiency was determined with gene-specific plasmid DNA that was linear over eight orders of magnitudes. Samples were run in triplicate, the results were normalized to the reference gene S15 that was analyzed by the comparative ∆CT method (CT gene of interest − CT reference gene), and fold change was determined by the Pfaffl method (56, 72). Standard curves for each gene product also were run concurrently to determine the absolute mass by linear regression analysis.

### RESULTS

**Fetal and maternal hematological values.** Hematological variables for the pregnant ewes and fetuses during the basal control period are shown in Table 1. Fetal blood O2 content and the partial pressure of O2 were lower in the IUGR fetuses, but fetal pH, plasma bicarbonate concentration, and hematocrit were not different. Maternal hematological values for IUGR and control ewes were not different. A greater maternal-fetal arterial difference was found for blood oxygen content in the IUGR animals.

**Fetal plasma glucose, lactate, and insulin concentrations at steady states.** Maternal plasma glucose concentrations for IUGR (3.9 ± 0.1 mmol/l) and control (4.1 ± 0.3 mmol/l) ewes were not different. The fetal plasma glucose concentration was 52 ± 7% lower in the IUGR fetuses vs. controls during the basal period (Table 2). Maternal-fetal arterial glucose concentration gradients were greater (P < 0.05) in the IUGR group (3.5 ± 0.1) than in the control group (2.8 ± 0.1). The fetal plasma lactate concentrations were not significantly different between the groups at basal conditions but were 3.5 ± 1.4-fold greater in the IUGR fetuses during the hyperglycemic period. Consistent with experimental design, glucose concentrations increased significantly from the basal concentration during the hyperglycemic period (2.3 ± 0.2-fold for controls, 5.0 ± 1.1-fold for IUGR) to values that were different between treatment groups.

Plasma insulin concentrations were 69 ± 6 and 74 ± 11% lower in the IUGR fetus compared with control fetuses at basal and hyperglycemic steady states, respectively (P < 0.05; Table 2). The mean plasma insulin concentration increased 2.4 ± 0.2-fold in the control fetuses during the hyperglycemic period (P < 0.05), whereas only a marginal increase of 1.8 ± 0.5-fold in plasma insulin concentration was found in the IUGR fetuses.

**Fetal metabolic fluxes.** Net umbilical (fetal) glucose, oxygen, and lactate uptake rates were determined during basal and hyperglycemic steady-state periods (Table 3). During the basal steady-state period, umbilical blood and plasma flow rates were lower in IUGR compared with control fetuses (P < 0.05).

### Table 1. Fetal and maternal hematological values

<table>
<thead>
<tr>
<th>Treatment Condition</th>
<th>Control</th>
<th>IUGR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal arterial pH</td>
<td>7.35 ± 0.01</td>
<td>7.36 ± 0.01</td>
</tr>
<tr>
<td>Fetal arterial HCO3⁻</td>
<td>27.9 ± 0.5</td>
<td>29.0 ± 0.2</td>
</tr>
<tr>
<td>Fetal arterial P02</td>
<td>19.2 ± 1.2</td>
<td>11.7 ± 1.2*</td>
</tr>
<tr>
<td>Fetal O2 content, mmol/l</td>
<td>3.4 ± 0.2</td>
<td>1.4 ± 0.2*</td>
</tr>
<tr>
<td>Fetal hematocrit</td>
<td>37.5 ± 1.1</td>
<td>35.5 ± 3.3</td>
</tr>
<tr>
<td>Fetal arterial pH</td>
<td>7.47 ± 0.01</td>
<td>7.49 ± 0.01</td>
</tr>
<tr>
<td>Fetal arterial HCO3⁻</td>
<td>25.4 ± 0.7</td>
<td>26.5 ± 0.6</td>
</tr>
<tr>
<td>Fetal arterial P02</td>
<td>84.6 ± 1.8</td>
<td>82.2 ± 2.0</td>
</tr>
<tr>
<td>Maternal O2 content, mmol/l</td>
<td>6.8 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>Maternal hematocrit</td>
<td>35.8 ± 0.9</td>
<td>37.1 ± 0.7</td>
</tr>
<tr>
<td>Maternal-fetal O2 difference</td>
<td>3.4 ± 0.3</td>
<td>5.7 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE. IUGR, intrauterine growth restricted. *Significant difference, P < 0.05.
Table 2. Fetal arterial plasma glucose, lactate, and insulin concentrations during steady-state periods

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Hyperglycemic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>IUGR</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>1.05±0.09</td>
<td>0.50±0.10*</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>1.57±0.10</td>
<td>3.98±2.09</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.26±0.04</td>
<td>0.08±0.02*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significant difference (P < 0.05) between treatment groups (control and IUGR) during the study period (basal or hyperglycemic); †significant difference (P < 0.05) between study periods within a treatment group.

Umbilical (fetal) oxygen uptake and umbilical lactate uptake rates were lower in IUGR fetuses. The umbilical glucose uptake rate during the basal period was 33 ± 16% lower (P < 0.05) for the IUGR fetuses, but the fetal body weight-specific glucose utilization rate was not different between groups (Table 3). The difference between the fetal glucose utilization rate and total fetal glucose uptake rate, representing fetal glucose production rate, was positive at 41 ± 2% of fetal glucose utilization in the IUGR fetuses but not different from zero in the control fetuses. The GOx and glucose oxidation rate (GOxR) in IUGR fetuses were 32 ± 9 and 28 ± 6% less, respectively (each P < 0.05), compared with the control fetuses.

During the hyperglycemic steady-state period, umbilical blood and plasma flow rates were lower (P < 0.06 and P < 0.05, respectively) in the IUGR fetuses compared with control fetuses (Table 3). The net fetal lactate uptake rate was lower in the IUGR fetuses during acute hyperglycemia compared with basal rates and was lower (P < 0.05) than that in the control fetuses. Umbilical oxygen uptake tended (P < 0.06) to be less in the IUGR fetuses and was not different from the basal rates for either treatment group. No differences were found between groups for total net fetal glucose uptake rate (umbilical glucose uptake plus glucose infusion rates), glucose utilization rate (GUR), glucose production rate (GPR), GOx, or GOxR during the hyperglycemic steady-state period. Total net fetal glucose uptake rate, GUR, and GOxR increased from the basal rates in both IUGR and control fetuses. The administration of insulin decreased the fetal GPR in IUGR fetuses to undetectable levels that were not different from the control fetuses.

Table 3. Fetal blood flows and nutrient flux rates

<table>
<thead>
<tr>
<th></th>
<th>Basal Period</th>
<th>Hyperglycemic Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>IUGR</td>
</tr>
<tr>
<td>Umbilical blood flow, ml·min⁻¹·kg⁻¹</td>
<td>164.0±7.3</td>
<td>126.0±22.6*</td>
</tr>
<tr>
<td>Umbilical plasma flow, ml·min⁻¹·kg⁻¹</td>
<td>103.8±4.2</td>
<td>74.6±16.1*</td>
</tr>
<tr>
<td>Net O₂ uptake from the placenta, μmol·min⁻¹·kg⁻¹</td>
<td>340±15</td>
<td>279±43*</td>
</tr>
<tr>
<td>Net lactate uptake rate from the placenta, μmol·min⁻¹·kg⁻¹</td>
<td>14.6±1.3</td>
<td>9.6±5.9*</td>
</tr>
<tr>
<td>Net glucose uptake rate from the placenta, μmol·min⁻¹·kg⁻¹</td>
<td>25.0±1.4</td>
<td>16.9±4.1*</td>
</tr>
<tr>
<td>Glucose utilization rate, μmol·min⁻¹·kg⁻¹</td>
<td>26.1±2.5</td>
<td>28.1±6.0</td>
</tr>
<tr>
<td>Glucose production rate, μmol·min⁻¹·kg⁻¹</td>
<td>1.1±1.8</td>
<td>11.2±1.8*</td>
</tr>
<tr>
<td>Glucose oxidation fraction, μmol·min⁻¹·kg⁻¹</td>
<td>0.66±0.05</td>
<td>0.45±0.06*</td>
</tr>
<tr>
<td>Glucose oxidation rate, μmol·min⁻¹·kg⁻¹</td>
<td>16.7±0.9</td>
<td>12.0±1.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significant differences (P < 0.05) between treatment groups (control and IUGR) within a study period (basal or hyperglycemic); †significant differences (P < 0.05) between the study period for that treatment group; ‡significantly different (P < 0.05) between treatments for the hyperglycemic period. Total glucose uptake is the umbilical glucose uptake during the basal period and the umbilical glucose uptake plus the glucose infusion rate during the hyperglycemic period.

Fetal and placental weights at necropsy. After completion of the in vivo study, the pregnant ewes and the fetuses were returned to basal conditions prior to being euthanized and necropsied between 128 and 136 dGA. There were three males and four females in the control group and four males and two females in the IUGR group. The IUGR fetuses and placentas weighed 59 ± 3 (P < 0.001) and 61 ± 3% less (P < 0.01), respectively, than controls.

GLUT concentrations in fetal tissue. GLUT concentrations were determined in the fetal brain, liver, and skeletal muscle (Fig. 1). GLUT1 concentrations were significantly higher in IUGR fetal brain tissue compared with controls (P < 0.05). Expression of GLUT1 was not different in fetal liver or skeletal muscle between IUGR and control fetuses. GLUT4 concentrations in skeletal muscle of the IUGR fetuses were not different between groups.

Fetal glycogen content. Glycogen content in skeletal muscle was 2.2 ± 0.2-fold greater (P < 0.01) in the IUGR fetuses compared with control fetuses (Fig. 2). The liver glycogen content was not different between the two groups.

Glucagon transcript expression. G-6-Pase expression in the IUGR fetal liver was 19.4-fold greater (P < 0.01) than in controls (Fig. 3). The PEPCK mRNA content was 9.7-fold greater (P < 0.05) in the IUGR liver compared with controls. The PGC-1α mRNA mean concentration (transcriptional coactivator for gluconeogenic enzymes) was 3.8-fold higher in the IUGR fetal liver, but this difference was not significant.

Transactivation of gluconeogenic enzymes occurs when protein kinase A activates CREB by phosphorylation at Ser²⁵³. Immunoblot analysis showed that the phosphorylated CREB protein concentration normalized to β-actin was 1.9 ± 0.3-fold higher (P < 0.05) in the IUGR fetal liver compared with control fetal liver. However, in the IUGR fetal liver the total CREB concentrations (CREB normalized to β-actin) were 51 ± 7% of the control fetal liver CREB content. Therefore, the fraction of phosphorylated CREB to total CREB was 4.2 ± 0.7-fold higher in IUGR liver vs. the control fetal liver (P < 0.05; Fig. 4).

DISCUSSION

In this study we measured glucose metabolism in fetal sheep with placental insufficiency and intrauterine growth restriction...
control and IUGR fetuses. At baseline plasma glucose and insulin concentrations, fetal glucose utilization rates were predicted to be 30.1 $\mu$mol·min$^{-1}$·kg$^{-1}$ for control fetuses and 16.2 $\mu$mol·min$^{-1}$·kg$^{-1}$ for IUGR fetuses. Similarly, GUR predictions during the hyperglycemic period for control and IUGR fetuses were 46.8 and 38.1 $\mu$mol·min$^{-1}$·kg$^{-1}$, respectively. In both the basal and hyperglycemic periods the values of GUR for control fetuses predicted by this mathematical model were comparable to the observed values, whereas values for GUR predicted by the model were lower than observed values in the IUGR fetuses (Table 3). These comparisons between the observed data and the predicted values from the mathematical model indicate that fetuses with chronic placental insufficiency and IUGR exhibit increased insulin sensitivity because the biological response (GUR) was equivalent for basal and glucose-stimulated insulin concentrations that are established by the fetus in response to plasma glucose concentrations. Because our placental insufficiency model of intrauterine growth restriction produces asymmetric fetal growth restriction with reduced body content of skeletal muscle and relatively preserved adipose tissue mass (55), it is likely that the “normal” whole body weight-specific values of insulin action in the IUGR fetuses actually represent even greater values of increased glucose and/or insulin sensitivities in these individual tissues. Experiments to measure insulin action in the IUGR fetal hindlimb, which metabolically consists mostly of skeletal muscle, are underway to test this probability in muscle tissue directly.

We also show that the IUGR fetuses with placental insufficiency had a consistent and relatively large difference between the rate of net fetal glucose uptake from the placenta and their whole body rate of glucose utilization, demonstrating the presence of significant rate of fetal glucose production (Table 3). As shown many times before (39, 40), normal, well-nourished fetal sheep do not exhibit measurable rates of glucose production (Table 3). Fetal glucose production has been demonstrated in fetuses made chronically hypoglycemic by a maternal insulin infusion (23), including the induction of enzymes involved in gluconeogenesis (61). However, the induction of gluconeogenic enzymes in the chronically hypoglycemic fetuses by maternal insulin administration was not the
result of elevated fetal plasma glucagon or catecholamine concentrations (52). Together, our current data in the IUGR fetuses and the literature reports for hypoglycemic fetuses indicate that chronic hypoglycemia stimulates hepatic glucose production in the fetus, but the mechanism for induction differs between the two experimental models. The response in the hypoglycemic fetuses appears to result from increased fetal cortisol secretion and plasma concentrations (29) found in these fetuses (Rozance PJ, Limesand SW, and Hay WW Jr, unpublished results).

Increased plasma concentrations of the catabolic hormones glucagon and catecholamines, but not cortisol, have been found (55) in IUGR fetuses produced by placental insufficiency. Along with lower plasma insulin concentrations and relative hypoglycemia, the hormonal milieu in the IUGR fetuses in this study supports increased hepatic glucose production via gluconeogenesis (5, 21, 85). We also show enhanced mRNA expression of hepatic gluconeogenic enzymes PEPCK and G-6-Pase in the livers of the IUGR fetuses. Furthermore, the results indicate that the stimulation of these genes may be mediated through CREB, which transactivates PEPCK and G-6-Pase genes (7, 38, 87). Glucagon and catecholamines via their G protein-coupled receptors stimulate the adenylate cyclase pathway to phosphorylate CREB (7, 38). Surprisingly, only a marginal increase in PGC-1α was found in the IUGR fetal livers because PGC-1α expression also can be enhanced by phosphorylated CREB (43, 93). PGC-1α subsequently stimulates PEPCK and G-6-Pase transcription; however, PGC-1α is not required for PEPCK promoter induction but amplifies the basal and hormone-induced expression of these gluconeogenic enzymes (43, 44, 93). Therefore, the induction of PEPCK and G-6-Pase in the fetus might be less robust than the adult, but there is still a significant induction of gluconeogenic enzymes that is sufficient to promote markedly high rates of fetal glucose production.

Glucagon probably has little effect alone, because pharmacological concentrations of glucagon are required to induce glucose production in fetal sheep (21, 85). Effects of increased plasma norepinephrine concentrations are mixed, as norepinephrine increases plasma glucose concentrations in fetal sheep, which in turn reduces transplacental transport of glucose as well as inhibits insulin secretion and lowers plasma insulin concentrations (27, 46, 47, 82). However, induction of hepatic glycogenolysis occurs with norepinephrine in adult animals (16, 83), but hepatic tissue is less responsive to norepinephrine than epinephrine (17), which could partially contribute to the maintained liver glycogen content in the IUGR fetuses despite the lower glucose and insulin concentrations (Fig. 2). Additionally, norepinephrine acts on extrahepatic tissues to provide gluconeogenic substrates from hepatic glucose production, thereby potentially inducing the Cori cycle in the IUGR fetuses. Such changes in fetal metabolism from increased norepinephrine or epinephrine were reversed by infusing insulin into sheep fetuses (10), indicating that the primary role for catecholamines is to inhibit insulin release. Glucose production from gluconeogenesis rather than glycogenolysis is expected in the IUGR fetuses because glycogen contents remain unchanged or even increase (Fig. 2). Together, our results and such observations reported in the literature from other studies show that the combination of chronic effects of hypoglycemia, low insulin concentrations, and increased glucagon and norepinephrine concentrations lead to the induction of gluconeogenesis, but not glycogenolysis, in IUGR sheep fetuses with placental insufficiency.
Increased insulin sensitivity to glucose as shown in these IUGR fetuses might promote glucose storage as glycogen. We observed selective regulation for hepatic gluconeogenesis pathways that preserved glycogen storage but did not augment it, although we show that fetal insulin sensitivity is increased (Fig. 2). An explanation for the discordant tissue glycogen content in these IUGR fetuses was shown in another data set of IUGR fetuses (75) where glycogen synthase kinase-3β (GSK-3β) was suppressed in the liver. Phosphorylation of GSK-3β by insulin leads to its subsequent phosphorylation and inactivation of glycogen synthase. Lowering GSK-3β, therefore, will favor glycogen synthesis in the fetal liver and consequently maintain glycogen stores. In contrast, the skeletal muscle in the IUGR fetuses showed enhanced insulin sensitivity through proximal signaling by increased insulin receptor protein coupled with decreased levels of insulin signal transduction inhibitors, such as the p85α regulatory subunit of phosphatidylinositol 3-kinase, which would have the effect of enhancing insulin action to promote glycogen deposition (75).

It has recently been shown (14, 48) that adrenalin synergizes with insulin to activate protein kinase B (Akt), which subsequently inactivates GSK-3β, thereby promoting glycogen synthase and further explaining the increased skeletal muscle glycogen content. Indeed, studies in the ovine IUGR fetal myocardium (9) also document increased weight-specific glycogen concentrations in IUGR fetuses along with greater GLUT4 protein and higher insulin receptor concentrations.

Previous studies of maternal insulin infusions leading to fetal hypoglycemia (19) reported a decline in brain GLUT3, an increase in brain GLUT1, and a subsequent decline in liver GLUT1 but no significant reduction in insulin-sensitive myocardial, skeletal muscle, and adipose tissue GLUT1 or GLUT4 concentrations compared with gestational age-matched sham controls. Similar patterns for GLUT1 and GLUT4 were found in fetuses with placental insufficiency and intrauterine growth restriction in the present study (Fig. 1), where GLUT1 was upregulated in an insulin-independent tissue (brain) and did not differ from controls in insulin-responsive tissues (liver and skeletal muscle). Preliminary data from these tissues (75) suggest that insulin sensitivity is increased through the proximal insulin-signaling cascade. Regardless of mechanisms, such adaptive responses allow the fetus to preserve essential metabolic functions (i.e., oxidative metabolism) at the expense of its growth, which in this paradigm progressively slows during the final one-third of gestation as placental size and total nutrient supply become limiting (89–91).

It is also important to note that many models of intrauterine growth restriction have been developed using vastly different methods to produce placental insufficiency (26, 30, 33, 49, 50, 60, 71, 78, 81, 84). In human pregnancies, intrauterine growth restriction is a frequent and serious complication. It has several known causes, but placental dysfunction is a major contributor leading to fetal nutrient deficiencies and a slower rate of growth (18, 34, 45, 63). In addition to fetal nutrient deficits, complications from placental dysfunction in human IUGR pregnancies include lower placental mass, lower rates of oxygen, amino acid, and glucose uptake by the fetus, and lower rates of umbilical venous blood flow (25, 41, 57, 68–70, 77). Clinical severity of human IUGR fetuses is determined by abnormalities in umbilical artery Doppler velocimetry (69), which is associated with an increased incidence of fetal hypoglycemia and hypoxia (57, 70). Very few animal models recapitulate all of the complications observed in human pregnancies with placental insufficiency and intrauterine growth restriction. However, our ovine model of placental insufficiency and intrauterine growth restriction, established by exposing pregnant ewes to a warm environment, as occurs naturally in warmer microclimates in all of the equatorial regions around the world, replicates all of the complications found in human pregnancies with moderate to even severe fetal growth restriction (2, 13, 31, 32, 74, 79, 88).

Importantly, at least two other experimental IUGR models in sheep with placental insufficiency have been developed. The uterine carunculectomy model (1) produces smaller placentas by surgically ablating the endometrial placental implantation sites (uterine caruncles) prior to pregnancy, thereby reducing the endometrial surface area into which the fetal trophoblast can invade. In addition, the overfed adolescent pregnant ewe model has a smaller placenta but no specific nutrient transport defects (90, 92). This model is produced by overfeeding adolescent ewes that are very early in their adolescent growth phase and have hormonally induced estrus and embryo transfer to initiate and establish, respectively, their pregnancies. Importantly, both of these models, which are markedly different from each other and from our placental insufficiency model, produce offspring (the fetus in the adolescent pregnant ewe model and lambs in the uterine carunculectomy model) that have evidence of increased insulin sensitivity and glucose uptake capacity as in our model (20, 90) in which placental size and transport capacity for glucose and selected amino acids are reduced. Therefore, the IUGR sheep fetus with placental insufficiency appears to have a common phenotype, regardless of how the smaller placenta is experimentally produced or whether or not the placenta is just smaller or also has selective nutrient transport defects, and thus provides a valuable model to evaluate fetal adaptations to nutrient restriction and related postnatal outcomes. As such, it is also an excellent model for studying in utero metabolic programming in mammals.

In terms of programming, it appears that IUGR fetuses produced by placental insufficiency and nutrient deprivation adapt to the hypoglycemic environment in utero by developing mechanisms that maintain or promote tissue glucose uptake and utilization. Teleologically, such adaptive mechanisms to maintain fetal energy stores help ensure fetal survival and, in terms of increased glycogen and fat stores, might help with postnatal survival as well. However, such mechanisms, if they persist, also might affect later developmental and adaptive conditions, and these adaptations might not always be beneficial in the long term. Thus, if the maintained or upregulated mechanisms of insulin sensitivity persist, IUGR fetuses might be predisposed to increased fat deposition when exposed to high-sugar and high-fat diets later in life. For example, the carunculectomy model of IUGR in sheep (20) develops increased insulin action that promotes utilization of both glucose and free fatty acids and increases visceral adiposity in young lambs at 1 mo of age. In addition, human IUGR infants demonstrate increased insulin-induced glucose disposal as early as 48 h after birth (12, 58). Therefore, the common phenotype in fetal sheep, in which IUGR is produced by very different methods, and human IUGR infants provides reasonable support for the hypothesis that placental nutrient insufficiency leads to common metabolic adaptations that will, if not
treated properly, lead to later-life pathology. These observations strengthen the case that postnatal nutrition must be matched to the infant’s growth rate if a leaner and thus potentially healthier development is to occur.

GRANTS
This work was supported by grants from the National Institutes of Health (HD-42815, W. W. Hay, Jr., principal investigator (PI)); S. W. Lensesand (PI) was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant KO1-DK-067393. P. J. Rozance and D. Smith were supported by National Institute of Child Health and Human Development Institutional Training Grant HD-07186 (W. W. Hay, Jr., PI).

REFERENCES
INSULIN SENSITIVITY IN IUGR FETAL SHEEP


## References

ajas:00000025

# Downloaded from http://ajpendo.physiology.org/ by 10.220.33.1 on June 15, 2017