Effects of chronic hypoglycemia and euglycemic correction on lysine metabolism in fetal sheep

Sean W. Limesand,1* Paul J. Rozance,2* Laura D. Brown,2 and William W. Hay, Jr.2

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

Submitted 13 October 2008; accepted in final form 25 January 2009

Limesand SW, Rozance PJ, Brown LD, Hay WW, Jr. Effects of chronic hypoglycemia and euglycemic correction on lysine metabolism in fetal sheep. Am J Physiol Endocrinol Metab 296: E879–E887, 2009. First published February 3, 2009; doi:10.1152/ajpendo.90832.2008.—In this study, we determined rates of lysine metabolism in fetal sheep during chronic hypoglycemia and following euglycemic recovery and compared results with normal, age-matched euglycemic control fetuses to explain the adaptive response of protein metabolism to low glucose concentrations. Restriction of the maternal glucose supply to the fetus lowered the net rates of fetal (umbilical) glucose (42%) and lactate (36%) uptake, causing compensatory alterations in fetal lysine metabolism. The plasma lysine concentration was 1.9-fold greater in hypoglycemic compared with control fetuses, but the rate of fetal (umbilical) lysine uptake was not different. In the hypoglycemic fetuses, the lysine disposal rate also was higher than in control fetuses due to greater rates of lysine flux back into the placenta and into fetal tissue. The rate of CO2 excretion from lysine decarboxylation was 2.4-fold higher in hypoglycemic than control fetuses, indicating greater rates of lysine oxidative metabolism during chronic hypoglycemia. No differences were detected for rates of fetal protein accretion or synthesis between hypoglycemic and control groups, although there was a significant increase in the rate of protein breakdown (P < 0.05) in the hypoglycemic fetuses, indicating small changes in each rate. This was supported by elevated muscle specific ubiquitin ligases and greater concentrations of 4E-BP1. Euglycemic recovery after chronic hypoglycemia normalized all fluxes and actually lowered the rate of lysine decarboxylation compared with control fetuses (P < 0.05). These results indicate that chronic hypoglycemia increases net protein breakdown and lysine oxidative metabolism, both of which contribute to slower rates of fetal growth over time. Furthermore, euglycemic correction for 5 days returns lysine fluxes to normal and causes an overcorrection of lysine oxidation.

pregnancy; intrauterine growth restriction, glucose; amino acids

GLUCOSE, LACTATE, AND AMINO ACIDS are the primary substrates for energy production and tissue growth in the fetus. Both sufficient supply and appropriate balance of these nutrient substrates are necessary to promote optimal fetal energy metabolism and growth rates. Experiments among a variety of models of fetal undernutrition have shown characteristic fetal metabolic responses. Specifically for experimental conditions that limit the supply of glucose to the fetus and cause fetal hypoglycemia (e.g., maternal underfeeding, insulin-induced hypoglycemia, or partial placental ablation) (5, 6, 8, 13), two characteristic metabolic changes occur. First, fasting acutely reduces fetal glucose supply, leading to fetal hypoglycemia and increased amino acid oxidation, which maintains fetal oxidative metabolism (measured as the rate of fetal oxygen consumption) (30). Second, over longer periods of reduced glucose supply, fetal glucose uptake decreases 75%, but glucose utilization is reduced by only 20% and glucose oxidation is not reduced at all due the appearance of endogenous fetal glucone production (8). This indicates that, in contrast to acute glucose deprivation when amino acids produced by protein breakdown directly substitute for glucose for oxidation (30), eventually chronic glucose deprivation leads to fetal glucose production, allowing for maintained glucose oxidation and conservation of amino acids for protein turnover (6, 8). If the glucose deprivation in this case is singular, i.e., oxygen and amino acid supplies are normally abundant, oxidative metabolism can be maintained.

The metabolic adaptations to acute glucose deprivation appear to be correctable by simple reintroduction of glucose (9, 10). In contrast, there has been little investigation to determine whether the metabolic adaptations to chronic glucose deprivation are permanent (“programmed”) or remedial by reintroduction of glucose. In our previous study, in which we examined pancreatic β-cell function following 2 wk of persistent glucose deprivation and hypoglycemia that also produced an 18% reduction in fetal weight, we found that 5 days of euglycemic correction resulted in a correction of fetal weights (13). Factors responsible for this catchup growth did not include increased tissue water content, a greater male/female sex ratio, or differences in gestational age.

To confirm this rapid recovery in growth in the hypoglycemic euglycemic correction group and test whether adaptations in amino acid metabolism and accretion rates could explain these findings, we studied the rate and distribution of lysine disposal into fetal compartments in fetal sheep during chronic hypoglycemia and following euglycemic recovery and compared results with normal, age-matched euglycemic control fetuses. Lysine was selected because it is an essential amino acid that is abundant in body proteins and its deamination by 10.220.33.5 on October 14, 2017 http://ajpendo.physiology.org/ Downloaded from

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.
glycemic regulation of lysine metabolism

MATERIALS AND METHODS

Animal preparation. Studies were conducted during the final 20% of gestation in pregnant Columbia-Rambouillet ewes carrying singletons [term = 147 days gestational age (dGA)]. Indwelling catheters were surgically placed into the maternal and fetal vasculature at 115–125 dGA for blood sampling, and animals were maintained as described previously (6, 13, 14, 16). All animal procedures were in compliance with guidelines of the US 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 Health Sciences Center Institutional Animal Care and Use Committee.

Experimental design. Pregnant ewes were randomly assigned to one of three treatment groups: euglycemia control (n = 8), chronic hypoglycemia (n = 8), and chronic hypoglycemia with euglycemic recovery (n = 7) animals. The control ewes were age matched and maintained in the laboratory alongside the hypoglycemic and recovery ewes. The hypoglycemic ewes received a chronic intravenous insulin infusion (30–60 pmol·min⁻¹·kg⁻¹, Humulin R; Eli Lilly, Indianapolis, IN) in 0.5% bovine serum albumin (BSA; Sigma Chemicals, St. Louis, MO) and saline (0.9% NaCl; Abbott Laboratories) to lower maternal plasma glucose concentrations by 50%. Lysine metabolism was determined in hypoglycemic fetuses after 14 ± 0.3 days of chronic hypoglycemia treatment (range 13–16 days of treatment). The recovery ewes were made chronically hypoglycemic (1.9 ± 0.1 mmol/l, not different from hypoglycemic ewes) for 13 days (range 12–13 days), and then their glucose concentrations were corrected by slowly lowering the insulin infusion rate over 4 days until no exogenous insulin was being administrated. The recovery fetuses were studied on the 4th (n = 1) and 5th (n = 5) days of euglycemic recovery. Two fetuses were examined twice, first on the 2nd or 3rd day of euglycemic recovery and then again on the 5th day of recovery. Patency of the catheter in umbilical vein was lost in one recovery fetus prior to the metabolic study being performed, leaving six fetuses studied in the recovery group.

The metabolic study was designed to measure fetal lysine metabolism with the use of 1-[¹⁴C]lysine and ³H₂O as tracers (New England Nucleotides; PerkinElmer Life Sciences, Boston, MA). On the day of the study, time 0 samples were collected for background-specific radioactivities of lysine, ¹⁴CO₂, and ³H₂O. Each fetus was then infused with a solution of 1-[¹⁴C]lysine (5 µCi/ml) and ³H₂O (14 µCi/ml) into the venous catheter placed in the inferior vena cava. A bolus of 1-[¹⁴C]lysine (10 µCi) and ³H₂O (28 µCi) was given as a priming dose and was followed by a constant infusion of 1-[¹⁴C]lysine (0.17 µCi/min) and ³H₂O (0.47 µCi/min). After 2 h of tracer infusion, when the free lysine concentration had reached a plateau according to preliminary experiments, six blood samples were collected at 15- to 20-min intervals to determine blood flow rate, blood oximetry, plasma metabolites, and hormone concentration. Steady-state condition during the sampling period was confirmed during the sampling period when arterial plasma free 1-[¹⁴C]lysine (dpm/ml) concentrations varied less than ±10% of the mean and showed no systematic trend with time. After each blood collection, maternal blood was given to the fetus to avoid fetal anemia.

Biochemical analysis. Blood oxygen saturation and hemoglobin concentrations were measured with an ABL 520 blood gas analyzer (Radiometer, Copenhagen, Denmark). Oxygen content was determined as the product of oxygen saturation and oxygen capacity. The pH, PO₂, and hematocrit were determined at 39.2°C (mean laboratory core body temperature) (6). Whole blood collected in EDTA-coated syringes was centrifuged (14,000 g) for 3 min at 4°C. Plasma was aspirated from the pellet 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). Arterial amino acid concentrations were measured using a Dionex 3000 model 4500 amino acid analyzer (Dionex, Sunnyvale, CA) after deproteinization with sulfosalicylic acid. Plasma insulin concentrations were measured with an ovine insulin ELISA (Mercodia, Winsten-Salem, NC).

1-[¹⁴C]lysine incorporates into small plasma proteins that remained in the supernatant after deproteinization (19). To separate the free 1-[¹⁴C]lysine and unlabelled amino acids from these plasma proteins, we used cation exchange chromatography. The cation exchange resin AG 50W-X8 (Bio-Rad, Hercules, CA) was added to water to form a slurry, which was used to fill glass wool-plugged pasteur pipettes with a 2-cm packed volume. The columns were washed with distilled water (20 ml). In preparation for the columns, the fetal plasma (0.5 ml) pH was lowered to <2.0 with 200 µl of 1 M HCl, mixed, and then added to the AG 50W-X8 column. The resin was washed with 4 ml of distilled water, and the amino acids were eluted with 4 ml of 2 mol/l NH₄OH. The eluant pH was neutralized with concentrated HCl and dried. The amino acid fraction was resuspended in 0.01 mol/l HCl and divided for amino acid measurements (by HPLC) and scintillation counting in BioSafe II scintillation cocktail (Research Products International, Mount Prospect, IL). The recovery for each separation was determined, and all were >90%. We confirmed that the cation exchange chromatography separated the free 1-[¹⁴C]lysine from the incorporated 1-[¹⁴C]lysine by size exclusion chromatography with Sephadex G-75, as reported previously by Meier et al. (19). Correction for recovery efficiency and volume displacement was accounted for in our calculations for lysine arteriovenous-specific activity (dpm/mmol).

Postmortem exam. After completion of physiological studies, fetal tissues were collected under treatment conditions by anesthetizing the ewe and fetus with ketamine (4.4 mg/kg) and diazepam (0.11 mg/kg). The fetus was removed by hysterotomy and weighed. The fetus was killed by surgical dissection and removal of all internal organs to measure its weight. The ewe was killed with concentrated pentobarbital sodium (Sleepaway, Fort Dodge Animal Health) given intravenously immediately after removal of the fetus.

Calculations. The rate of umbilical blood flow (f; ml·min⁻¹·kg⁻¹) was calculated using the transplacental steady-state diffusion technique, with titrated water as the blood flow indicator, and normalized to fetal weight during the study (6, 15, 20). Net umbilical uptake rates (R₅₀₆; µmol·min⁻¹·kg⁻¹) for oxygen, glucose, and lysine by the fetus from the placenta were calculated as the product of the umbilical
blood (or plasma) flow and concentration difference between the umbilical vein (CV) and umbilical artery (CA) blood (O2) or plasma (glucose and lysine) concentrations. 

\[ R_{CV} = R_{CA} \] 

Net tracer fluxes (dpm/kg) of L-[U-14C]lysine secreted as 14CO2 via the umbilical circulation were calculated as the product of umbilical blood flow (mL/min x kg⁻¹ fetal weight) and umbilical arteriovenous concentration blood difference (dpm/ml).

Calculations for tracer fluxes between the placenta and the fetal plasma and between the fetal plasma and fetal tissues were adapted from Loy and colleagues (17, 18), Ross et al. (25), and Carver et al. (6) (Table 1 and Fig. 1).

**Skeletal muscle analysis.** Skeletal muscle from the biceps femoris was collected because it was easily accessible and contains a mixture of muscle fiber types. The tissues were snap-frozen in liquid nitrogen and stored at −70°C until further analysis. The tissue analysis was performed on 13 control fetuses, 15 hypoglycemic fetuses, and 12 recovery fetuses; selected data from some of these fetuses have been reported previously (13, 27). Skeletal muscle glycogen content was determined as described previously (1), and results are expressed as milligrams of glycogen per gram of skeletal muscle (wet weight).

For mRNA analysis, total RNA was extracted from pulverized skeletal muscle (200 mg) and reverse transcribed into complimentary DNA (cDNA), as described previously (15). Ovine F-box-only protein 32 (FBXO32; MAFbx-1) and ovine ring finger protein 28 (RFP28; MuRF1) (GenBank accession nos. EU492872 and EU525163, respectively) were cloned from ovine skeletal muscle cDNA by PCR (reagents obtained from Invitrogen, Carlsbad, CA). cDNA was amplified with the following oligonucleotide primer pairs: FBXO32 sense 5′-CTC ACA TTC CTG AGT GGC A-A′-3′ and FBXO32 antisense 5′-AAG TAC ATC ATC TTC CA A-3′ (493 bases); RFP28 sense 5′-TGT GCC AAC GAC ATC TTC CA A-3′ and RFP28 antisense 5′-GAT GAT GTT CTC CAC CAG CA-3′ (168 bases). Amplified PCR products were TA cloned using TOPO PCR II kit and transformed into Mach1 T1 phage-resistant, chemically competent E. coli (Invitrogen). Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and the nucleotides sequenced (Invitrogen). Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and the nucleotides sequenced (Invitrogen). Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and the nucleotides sequenced (Invitrogen). Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and the nucleotides sequenced (Invitrogen). Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and the nucleotides sequenced (Invitrogen).

For Western blot analysis, protein was extracted from pulverized skeletal muscle (200 mg) and the concentration quantified as described previously (26). Equal amounts of protein were separated by polyacrylamide gel electrophoresis under reduced conditions (5% β-mercaptoethanol, vol/vol). Proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad). All Western blots membranes were blocked for 1 h in phosphate-buffered saline with 0.1% Tween-20 (PBST; vol/vol) and 5% nonfat dried milk (NFDM; wt/vol) for 1 h at room temperature. The following primary antibodies were diluted in PBST with 5% NFDM: Thr177/385-phosphorylated 4E-BP1 (1:500), Thr189/Tyr204-phosphorylated p44/42 mitogen-activated protein kinase (MAPK, 1:500), Ser63-phosphorylated Akt (1:750), and β-actin (1:30,000). These primary antibodies were diluted in PBST with 5% bovine serum albumin (wt/vol); p44/42 MAPK (1:500), p70 S6 kinase (p70S6K, 1:1,000), and Akt (1:750). The following primary antibodies were diluted in PBST with 5% NFDM and 1% BSA: Thr223/227-phosphorylated p70S6K (1:250), 4E-BP1 (1:1,000), and the β-subunit of the insulin receptor (IR; 1:1,000). All antibodies except for β-actin (NP Biomedical, Solon, OH) and IR (Santa Cruz Biotechnology, Santa Cruz, CA) were obtained from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies were diluted in PBST with 5% NFDM and applied to membranes for 1 h at room temperature. Immunocomplexes were detected with enhanced chemiluminescence (Amersham ECL Plus, Piscataway, NJ), and densitometry was performed using Image J software.

**Table 1. Calculations for lysine fluxes**

<table>
<thead>
<tr>
<th>Description</th>
<th>Product Symbol</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction of lysine tracer infusion rate taken up by the placenta</td>
<td>LysNp</td>
<td>= LysRf,p/If,0</td>
</tr>
<tr>
<td>Fraction of metabolized L-[U-14C]lysine secreted as 14CO2 via the umbilical circulation</td>
<td>co2Namb</td>
<td>= co2Ramb/If,0</td>
</tr>
<tr>
<td>DR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Back flux of lysine from umbilical circulation to the placenta</td>
<td>Flux I</td>
<td>= I x (Sf - Samb)</td>
</tr>
<tr>
<td>Lysine flux into fetal tissues</td>
<td>Flux II</td>
<td>= DR x (LysNamb)</td>
</tr>
<tr>
<td>Net umbilical uptake rate</td>
<td>Flux III</td>
<td>= LysRf,p + flux II</td>
</tr>
<tr>
<td>Lysine flux into plasma from fetal tissues</td>
<td>Flux IV</td>
<td>= DR - flux IV</td>
</tr>
<tr>
<td>CO2 production from fetal lysine</td>
<td>Flux V</td>
<td>= DR x co2Ramb</td>
</tr>
<tr>
<td>Lysine for protein accretion</td>
<td>Flux VI</td>
<td>= LysRf,p - flux VI</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Flux VII</td>
<td>= flux VII + flux V</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Flux VIII</td>
<td></td>
</tr>
</tbody>
</table>

LysRD, L-[U-14C]lysine fetal infusion rate (dpm/min); Samb, specific activity (dpm/μmol); DR, disposal rate (μmol/min); LysRD, net flux of lysine from the fetus to the placenta; LysRD, net flux of lysine into the placenta from the fetus.

**Fig. 1. Model of lysine fluxes between placenta (p), fetal plasma (f), and fetus (t) estimated by mass balance of L-[U-14C]lysine (tracer) and L-lysine (tracee).**
Scion Image software (Scion, Frederick, MD). All results were normalized to β-actin to control for loading differences, and a reference sample was analyzed on every membrane to control for differences in transfer efficiency. Phosphorylated proteins were normalized to the total amount of each protein. Antibodies were stripped from the membranes with Restore Western stripping buffer (Pierce, Rockford, IL). Results of densitometry are presented in arbitrary units and as fold changes compared with control fetuses.

Statistical analysis. Calculations were based on the mean values for measured parameters collected during steady-state sampling period for the tracer lysine. Statistical analysis of differences for the flux rate calculations and biochemical, hematological, hormone, and fetal weight measurements were analyzed by general linear means ANOVA [Proc GLM (29)], and treatment means were separated by the least significant difference test for multiple comparisons. Differences for in vitro tissue analysis on the skeletal muscle were determined using an ANOVA with a Tukey’s test for multiple comparisons. Due to heterogeneity of variability, the data for FBXO32 and RFP28 mRNA were log transformed.

RESULTS

Maternal experimental parameters. Maternal plasma glucose concentrations in the insulin-infused hypoglycemic ewes averaged 2.0 ± 0.2 mmol/l, ~45% lower than the mean value of 3.7 ± 0.1 mmol/l in the control ewes during the same period. These values were not different from those of the recovery group: 3.5 ± 0.2 mmol/l prior to hypoglycemia, 1.9 ± 0.1 mmol/l during the hypoglycemic period, and 3.9 ± 0.2 mmol/l during the euglycemic recovery period.

Fetal weights. Fetal body and organ weights measured at necropsy are presented in Table 2. Gestational ages were not different between the treatment groups at the time of study. Fetal body weights were 16 and 19% lower (P < 0.05) in the hypoglycemic and recovery fetuses, respectively, than in the control fetuses. The brain weights of hypoglycemic and recovery fetuses were not different from control fetuses, but brain weights in recovery fetuses were less than in hypoglycemic fetuses. The hypoglycemic and recovery mean liver weights were less than those in control fetuses. There was no difference in crown-rump length among the groups.

Fetal blood flows and lysine uptake. Fetal studies were performed at 137 ± 1 dGA in control and hypoglycemic groups and at 138 ± 2 dGA in the recovery group. The umbilical blood and plasma flow rates per kilogram of fetal weight did not differ between treatment groups. The umbilical venous and arterial lysine concentrations were significantly higher in the hypoglycemic group compared with control fetuses. However, lysine concentrations of the recovery fetuses did not differ from the control fetuses. Net fetal (umbilical) lysine uptake rates were not different among all three groups (Table 3).

Insulin, glucose, lactate, and oxygen concentrations and umbilical uptakes. The mean arterial plasma insulin, glucose, and lactate concentrations and blood oxygen contents for each treatment group are shown in Table 4. Mean fetal plasma insulin concentration in the hypoglycemic group was 33% lower than in the control group (P < 0.01). Similarly, mean fetal plasma glucose concentration in the hypoglycemic group was 46% lower than in the control group (P < 0.05); mean fetal plasma insulin and glucose concentrations in the control and recovery groups were not different. Mean fetal plasma lactate concentration was 33% lower in the hypoglycemic fetuses compared with control fetuses (P < 0.05), but the mean control group value was not different from that in the recovery group. The mean blood oxygen content in the hypoglycemic group was 37% greater than in the control group (P < 0.05); there was no difference between mean oxygen contents in the control and recovery groups.

Mean net fetal (umbilical) glucose uptake rate was significantly lower in hypoglycemic than control fetuses (P < 0.05) and was not different between control and recovery groups. Net fetal (umbilical) uptake rates of lactate were marginally lower (P = 0.054) in the hypoglycemic group than the control group and not different between control and recovery groups. Net fetal (umbilical) uptake rates of oxygen were not different among all three groups.

Fetal lysine flux rates. The lysine fluxes between placental and fetal compartments were calculated for each treatment group, and results are shown in Table 5. The mean disposal rate for lysine (flux I) was 1.4 ± 0.1-fold greater (P < 0.05) in the hypoglycemic fetuses compared with the control group due to greater rates of lysine transfer into the placental (flux II, 1.8 ± 0.3-fold greater) than in the control group.
GLYCEMIC REGULATION OF LYSINE METABOLISM

Table 5. Lysine flux rates estimated by combining tracer and tracee data and using plasma lysine \( S_a \)

<table>
<thead>
<tr>
<th>Flux No.</th>
<th>Control</th>
<th>Hypoglycemic</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>I: fetal plasma lysine disposal rate</td>
<td>2.33±0.25b</td>
<td>3.16±0.21a</td>
<td>2.06±0.14a</td>
</tr>
<tr>
<td>II: lysine flux into the placenta from fetal blood</td>
<td>0.42±0.08b</td>
<td>0.76±0.12a</td>
<td>0.37±0.06b</td>
</tr>
<tr>
<td>III: lysine flux into fetal tissues from fetal blood</td>
<td>1.91±0.2b</td>
<td>2.40±0.11a</td>
<td>1.69±0.15b</td>
</tr>
<tr>
<td>IV: lysine flux into fetal blood from placenta</td>
<td>2.17±0.20</td>
<td>2.62±0.24</td>
<td>2.02±0.15</td>
</tr>
<tr>
<td>V: lysine flux into fetal blood from fetal proteins</td>
<td>0.16±0.1b</td>
<td>0.54±0.09a</td>
<td>0.04±0.1b</td>
</tr>
<tr>
<td>VI: CO₂ produced by fetus from fetal plasma (-[U-^{14}C])lysine</td>
<td>0.16±0.02b</td>
<td>0.38±0.07a</td>
<td>0.07±0.01a</td>
</tr>
<tr>
<td>VII: lysine flux into fetal protein accretion from fetal blood</td>
<td>1.59±0.16</td>
<td>1.49±0.15</td>
<td>1.58±0.16</td>
</tr>
<tr>
<td>VIII: lysine flux into fetal protein synthesis</td>
<td>1.75±0.19</td>
<td>2.02±0.12</td>
<td>1.62±0.15</td>
</tr>
</tbody>
</table>

Values are means ± SE. Different superscripts indicate differences where \( P \leq 0.05 \).

0.4-fold, \( P < 0.05 \) and fetal tissues (flux III, 1.3 ± 0.1-fold, \( P < 0.05 \)). The rate of lysine coming from the placenta into fetal plasma (flux IV, \( \text{LyS}_{p,f} \)) was not different between control and hypoglycemic fetuses, but the flux of lysine from the fetal tissues into the fetal plasma (fetal protein breakdown, flux V, \( \text{LyS}_{f,p} \)) was 3.4 ± 0.6-fold greater (\( P < 0.05 \)) in the hypoglycemic than in the control fetuses. No differences were found for rates of protein accretion (flux VII) or synthesis (flux VIII) using lysine tracer data between control and hypoglycemic fetuses. The rate of fetal CO₂ production from lysine (flux VI) was 2.4 ± 0.4-fold higher (\( P < 0.05 \)) in the hypoglycemic fetuses compared with the control fetuses.

Euglycemia correction following 2 wk of chronic hypoglycemia in recovery fetuses normalized all differences found for the lysine disposal rate and fetal-placental compartment flux rates II–V in the hypoglycemic fetuses. Evaluation of fetal lysine disposal rates on days 2, 3, 4, and 5 of euglycemic recovery showed no systematic trends, indicating that fetal adaptations were not progressively augmented but an immediate and persistent response during the 5 days of euglycemic recovery. In the recovery fetuses, the rate of CO₂ production from fetal lysine (flux VI) was 42 ± 7% (\( P < 0.05 \)) of control fetuses. No differences were found for protein accretion (flux VII) or protein synthesis (flux VIII) rates in the recovery fetuses compared with the hypoglycemic and control fetuses.

**Skeletal muscle analysis.** The mean glycogen content of the skeletal muscle was not different between the hypoglycemic and control fetuses but was significantly increased in the recovery fetuses compared with E fetuses (\( P < 0.01 \); Fig. 2). We also measured the mRNA content of two muscle-specific ubiquitin ligases. There was a higher amount of FBXO32 mRNA in hypoglycemic fetuses compared with control fetuses (\( P < 0.05 \); a similar trend was found for RFP28 mRNA (\( P = 0.08 \). The FBXO32 and RFP28 mRNA content in recovery fetuses was close to the values in the control fetuses, although they were not different from either control or hypoglycemic fetuses (Fig. 3).

We also examined the insulin-signaling pathways that regulate protein synthesis. The proportion of phosphorylated 4E-BP1 (Thr\(^{37/46}\)) to total 4E-BP1 was not different in hypoglycemic or recovery fetuses compared with control fetuses, although hypoglycemic fetuses tended to have a lower ratio than control fetuses (\( P = 0.09 \); Fig. 4). There was a significantly lower phosphorylated/total 4E-BP1 ratio in recovery compared with hypoglycemic fetuses (\( P < 0.005 \)). The total amount of 4E-BP1 relative to \( \beta \)-actin was higher in the skeletal muscle of the hypoglycemic fetuses compared with the control and recovery fetuses (\( P < 0.05 \)). No differences were found among the three groups for skeletal muscle content of IR (control 1.00 ± 0.09, hypoglycemic 0.89 ± 0.09, recovery 1.13 ± 0.18), Akt (control 1.00 ± 0.14, hypoglycemic 1.08 ± 0.29, recovery 1.19 ± 0.4), p44/42 MAPK (control 1.00 ± 0.12, hypoglycemic 0.99 ± 0.18, recovery 0.62 ± 0.10), or p70S6K (control 1.00 ± 0.11, hypoglycemic 0.81 ± 0.13, recovery 0.72 ± 0.13). Similarly, no differences were found among the three groups for the ratio of Ser\(^{24}\)-phosphorylated Akt (control 1.00 ± 0.16, hypoglycemic 1.79 ± 0.47, recovery 1.69 ± 0.32), Thr\(^{202/204}\)/Yyr\(^{363}\)-phosphorylated p44/42 MAPK (control 1.00 ± 0.21, hypoglycemic 1.59 ± 0.24, recovery 1.51 ± 0.22), or Thr\(^{24}\)/Ser\(^{24}\)-phosphorylated p70S6K (control 1.00 ± 0.20, hypoglycemic 1.47 ± 0.26, recovery 0.87 ± 0.23) to the total amount of the respective protein present.

**DISCUSSION**

In this study, we determined the rate and distribution of lysine disposal into fetal compartments in fetal sheep during chronic hypoglycemia and following euglycemic recovery and compared results with normal, age-matched euglycemic control fetuses. Our goal was to define the amino acid/protein metabolic adaptations to chronic glucose deficiency and to determine whether reintroduction of glucose could normalize amino acid and protein metabolism. In response to 2 wk of sustained glucose deficiency, the mean fetal plasma lysine concentration was 1.9-fold greater in hypoglycemic compared with control fetuses, and there was a higher rate of lysine disposal into the placental and fetal tissue compartments without a change in the rate of net fetal (umbilical) lysine uptake. The oxidative metabolism of lysine (the rate of CO₂ production...
from lysine decarboxylation) was 2.4-fold greater in hypoglycemic fetuses. Although there was no difference in fetal tissue protein synthesis or accretion, there was an increase in fetal tissue protein breakdown (the flux of lysine from fetal tissues into the plasma). Euglycemic recovery normalized all fluxes tissue protein breakdown (the flux of lysine from fetal tissues protein synthesis or accretion, there was an increase in fetal from lysine decarboxylation) was 2.4-fold greater in hypoglycemic fetuses. After 8 wk of chronic hypoglycemia produced no difference in the rate of protein synthesis (6). A major and novel finding of the present study is that reintroduction of glucose following a chronic, 2 wk restriction in glucose supply leads to resumption of normal amino acid and protein metabolism. Clearly, a reduced rate of protein accretion as a response to chronic hypoglycemia may decrease fetal growth over time by increasing ubiquitin ligase-mediated protein degradation rates. Of major importance, the chronic metabolic adaptations of lysine and protein metabolism to glucose deficiency were remedial, indicating that after at least 2 wk of glucose deprivation, the fetal sheep can shift back to normal glucose utilization. However, lysine oxidation rates were reduced compared with normal fetuses. Coupled with normalization of the rate of protein breakdown, this might allow for resumption of normal to marginally greater rates over time of fetal growth once the hypoglycemia is corrected.

Similar to the current study, previous measurements in our laboratory of leucine metabolism in fetal sheep that were chronically hypoglycemic for 8 wk revealed increased rates of leucine release from protein breakdown. In contrast, 8 wk of hypoglycemia resulted in reduced rates of overall protein accretion. Clearly, a reduced rate of protein accretion as a response to chronic hypoglycemia is an adaptation that progressively increases over time. Similar to our current study, however, 8 wk of chronic hypoglycemia produced no difference in the rate of protein synthesis (6). A major and novel finding of the present study is that reintroduction of glucose following a chronic, 2 wk restriction in glucose supply leads to resumption of normal amino acid and protein metabolism.

Following 2 wk of chronic hypoglycemia the rate of protein breakdown is increased, but protein accretion is maintained. This must be due to a nonsignificant, 15% increase in the mean rates of protein synthesis in the hypoglycemic fetuses. Analysis of fetal skeletal muscle corroborated our in vivo findings, except for our 4E-BP1 results. The total amount of 4E-BP1 increased in the hypoglycemic fetuses and then returned to normal following restoration of a normal fetal glucose supply. 4E-BP1 is a binding protein that normally binds to eIF4E and acts to inhibit translation initiation and protein synthesis. In the acute setting, 4E-BP1 is regulated by phosphorylation events that act to inhibit its eIF4E-binding function, thereby allowing for increased translation initiation (23). In the hypoglycemic fetuses, the proportion of phosphorylated 4E-BP1 compared with the total amount was not different. This may be one reason why protein synthesis is maintained despite increased amounts of the inhibitory 4E-BP1. Additionally, p70S6k and its phosphorylation were maintained, as were other proteins in the insulin-signaling pathways that regulate protein synthesis; however, the variation in these measurements increased during hypoglycemia, which might have limited our ability to identify the small but significant increases in insulin action. As for the increased release of amino acids from fetal tissues, FBXO32 and RFP28, also known as MAFbx-1 and MuRF1, have been identified as two muscle-specific ubiquitin ligases that mediate protein degradation due to a variety of experimental conditions (3). They appear to be part of a common final pathway that mediates skeletal muscle atrophy (3). Our results show that chronic fetal glucose deprivation increases these ligases, which in turn allows for greater amino acid release from fetal tissue into fetal plasma and for increased rates of lysine oxidation, as observed in hypoglycemic fetuses. Again, this is a reversible process since the expression of these ligases returns to normal following restoration of a normal glucose supply. To our knowledge, this is the first experimental demonstration of these ligases regulating fetal protein breakdown in situations of nutrient deprivation.

It is instructive to compare our results following 2 wk of hypoglycemia with those obtained using leucine kinetics to measure amino acid metabolism following 8 wk of hypoglycemia. After 8 wk there was not an increase in leucine oxidation, although we found a significant increase in lysine oxidation following 2 wk of hypoglycemia. One explanation for this discrepancy is that the chronically hypoglycemic fetus develops a progressive increase in fetal glucose production, presumably due to gluconeogenesis (8, 22, 26). This endogenously
produced glucose might progressively replace amino acid oxidation during chronic selective fetal hypoglycemia such that amino acid oxidation is increased toward the beginning of the hypoglycemia but then decreases somewhere between 2 and 8 wk. However, in very acute maternal insulin-induced fetal hypoglycemia (3 h), no increase in leucine oxidation was demonstrated (21). Therefore, the capacity of the fetus to oxidize amino acids in place of glucose during hypoglycemia appears to require more than 3 h to develop and then is downregulated somewhere between 2 and 8 wk. This time course also is consistent with leucine oxidation rates in a model of intrauterine growth restriction following chronic (>8 wk) placental insufficiency, which are normal (25) and have increased leucine oxidation rates obtained following 5 days of maternal fasting (12). An additional confounding variable includes the gestational age of onset of the nutrient restriction, which may also influence the fetal response. Of course, differences between fetal leucine and lysine utilization patterns cannot be excluded. These important differences between fetal leucine and lysine metabolism include the proportion of the disposal rate that represents fetal protein accretion and amino acid oxidation (2). Normally, most of the lysine that enters the fetal tissues from the fetal plasma is committed to protein accretion, with only a small percentage destined for oxidation and energy production (6). For leucine, a much higher percentage of the flux into fetal tissues is oxidized (~35%) (25). Further studies would be required to determine whether these differences are important and to better define the time course of amino acid oxidation as a substitute for glucose.

Another interesting comparison is the difference between protein accretion, synthesis, and breakdown over the various lengths of fetal hypoglycemia. Acute, 3-h, fetal hypoglycemia significantly decreased fetal protein accretion due to decreased protein synthesis and did not change rates of fetal protein breakdown (21). By 2 wk of hypoglycemia we found increased fetal protein breakdown with normal protein accretion and synthesis. At 8 wk of hypoglycemia there was a significant decrease in protein accretion due to a persistent increase in the rate of protein breakdown; rates of protein synthesis were not different. An early adaptation to hypoglycemia is decreased protein accretion due to decreased protein synthesis and not to increased protein breakdown. This is followed by a return of protein synthetic rates to normal and increased protein breakdown that eventually results in persistently lower rates of protein accretion by 8 wk. The implications for improvement of fetal growth during fetal growth restriction and chronic nutrient restriction are that interventions designed to reduce protein breakdown might be more effective than those designed to increase rates of protein synthesis. Studies are currently underway to evaluate these potential interventions and their effects on fetal protein breakdown.

Although we did not measure glucose metabolism in this study, previously we used this model to investigate glucose metabolism with glucose tracers following 2 wk of hypoglycemia. The data demonstrated maintained fetal glucose oxidation rates relative to a significant reduction in fetal insulin and glucose concentrations (8). Furthermore, glucose transporter concentrations (GLUT1 and -4) in insulin-responsive tissues are unaffected by chronic glucose deprivation, indicating that avidity for glucose uptake is not the reason for maintained glucose oxidation (7). Consistent with this, we found skeletal muscle glycogen (1 product of glucose utilization) to be maintained in the hypoglycemic fetuses, similar to previously documented glycogen contents in the livers of these hypoglycemic fetuses (26). This suggests maintained or even increased insulin and/or glucose sensitivity in the hypoglycemic fetuses, at least for the pathways regulating fetal glucose utilization, oxidation, and glycogen content. Although we did not test this directly by measuring fetal glucose utilization rates during a hyperinsulinemic euglycemic and/or hyperglycemic euglycemic clamp, we did measure the relative phosphorylation of the proximal insulin-signaling proteins Akt and MAPK and found no difference in baseline phosphorylation in the fetuses. Maintained phosphorylation relative to the total amounts of these proteins despite a significant reduction in fetal insulin concentrations cannot be explained by an increase in skeletal muscle insulin receptor because this was likewise unchanged. Two phenomena in the recovery group suggest that there may be a residual increase in insulin and/or glucose sensitivity. One
is the rebound increase in fetal skeletal muscle glycogen. The other is that a significant reduction in lysine oxidation in this group compared with control fetuses might indicate increased glucose oxidation in the recovery fetuses, thus sparing lysine for nonoxidative pathways. A more detailed explanation of the mechanisms responsible for maintained or increased insulin and/or glucose sensitivity requires further studies, including measuring glucose utilization rates during hyperinsulinemic euglycemic and hyperglycemic euglycemic clamps followed by tissue collection under these experimental conditions.

In our previous study with euglycemic correction following 2 wk of persistent fetal hypoglycemia, we found that 5 days of euglycemic recovery resulted in a normalization of fetal weights (13). A primary reason for repeating this treatment design was to understand potential effects of amino acid metabolism that might be responsible for the catchup growth observed in the previous study. In the current data set, however, no correction in fetal weight was observed (Table 2), although all other in vivo results between the two cohorts of fetuses were similar. We did not measure fetal protein synthesis or net protein balance in the first study. In the present study, during the recovery period there was evidence for conservation of lysine into fetal protein (reduced release of lysine from protein and reduced oxidation of lysine), but protein synthesis and accretion from lysine were not different from controls, perhaps due to the unexplained lower fetal plasma lysine concentration. Thus, although this new data provides insight into the mechanisms involved in regulation of protein metabolism in response to nutrient deprivation and glucose replacement, it is possible that undetermined differences in study design and animal variability contributed to differences in fetal weight gain between the two studies. Clearly, the interaction between glucose and amino acid supply and the regulation of fetal protein metabolism and weight change require more thorough investigation, particularly if therapy of intrauterine fetal growth restriction should become a target for future experimental trials.

In conclusion, we have shown that fetal lysine oxidation and fetal protein breakdown increase as metabolic adaptations to 2 wk of fetal hypoglycemia. These results are consistent with data from fetuses made hypoglycemic for 8 wk (6) but add to our knowledge by indicating that these changes in amino acid and protein metabolism are progressive over time and are reversible, at least after the 2 wk of hypoglycemia that we studied, upon restoration of normal fetal glucose concentrations. Future studies are clearly needed to better understand the molecular events responsible for changes in glucose, insulin, and amino acid metabolism in this model, such as measurement of glucose and/or amino acid metabolism under various clamp conditions with varying levels of glucose, amino acids, and insulin.

ACKNOWLEDGMENTS

We extend our gratitude to C. Teng, K. Trembler, and A. Cheung for their valuable technical contributions.

GRANTS

This work was supported by grants from the National Institutes of Health (RO1-HD-42815 [W. W. Hay, Jr., principal investigator (PI)], KO1-DK-067393 [S. W. Limesand, PI], and Institutional Training Grant T32-HD-07186 [W. W. Hay, Jr., PI; P. J. Rozance, trainee]), The Children’s Hospital Research Institute, Research Scholar Awards (P. J. Rozance, PI; L. D. Brown, PI), and Pilot Project Awards from the Colorado Clinical Nutrition Unit (National Institute of Diabetes and Digestive and Kidney Diseases: 2-P30-DK-048520-11, J. Hill, PI; P. J. Rozance and L. D. Brown, awardees).

REFERENCES