Acute supplementation of amino acids increases net protein accretion in IUGR fetal sheep

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Acute supplementation of amino acids increases net protein accretion in IUGR fetal sheep. *Am J Physiol Endocrinol Metab* 303: E352–E364, 2012. First published May 29, 2012; doi:10.1152/ajpendo.00059.2012—Placental insufficiency decreases fetal amino acid uptake from the placenta, plasma insulin concentrations, and protein accretion, thus compromising normal fetal growth trajectory. We tested whether acute supplementation of amino acids or insulin into the fetus with intrauterine growth restriction (IUGR) would increase net fetal protein accretion rates. Late-gestation IUGR and control (CON) fetal sheep received acute, 3-h infusions of amino acids (with euninulinemia), insulin (with euglycemia and euaminoacidemia), or saline. Fetal leucine metabolism was measured under steady-state conditions followed by a fetal muscle biopsy to quantify insulin signaling. In CON, increasing amino acid delivery rates to the fetus by 100% increased leucine oxidation rates by 100%. In IUGR, amino acid infusion completely suppressed fetal protein breakdown rates but increased leucine oxidation rate by only 25%, resulting in increased protein accretion rates by 150%. Acute insulin infusion, however, had very little effect on amino acid delivery rates, fetal leucine disposal rates, or fetal protein accretion rates in CON or IUGR fetuses despite robust signaling of the fetal skeletal muscle insulin-signaling cascade. These results indicate that, when amino acids are given directly into the fetal circulation independently of changes in insulin concentrations, IUGR fetal sheep have suppressed protein breakdown rates, thus increasing net fetal protein accretion.

intrauterine growth restriction; insulin signaling; fetal muscle; protein synthesis; insulin; leucine

THE CAPACITY FOR REVERSING intrauterine growth restriction (IUGR) by restoration of nutrient supply and anabolic growth factors, particularly after the fetus has adapted to nutrient deprivation, remains undetermined. Testing for such capacity is important because, if not reversed, fetal IUGR from nutrient and anabolic hormone insufficiency predisposes to serious later-life diseases including insulin resistance and type 2 diabetes (54, 58). Underlying these diseases is the failure of muscle growth and function to return to normal, even postnatally. Epidemiological evidence, in fact, shows that deficits in muscle mass in the IUGR fetus persist into adulthood (24, 59). Long-term functional capacity of muscle also is compromised by IUGR. Thus, there are direct links between IUGR, decreased muscle mass, and insulin resistance in adulthood (23, 51, 52), with evidence that insulin signaling through its primary protein synthesis regulator mTOR is inhibited (22, 41). In cases of IUGR produced by chronic placental insufficiency, transplacental transport of amino acids and fetal insulin concentrations are at their lowest during the last third of gestation, when fetal skeletal muscle protein accretion and growth rates are maximal (8, 11, 53). Therefore, it is essential to determine the potential for restoring normal muscle growth after chronic reductions in nutrient supply have occurred in utero and to understand the mechanisms that might lead to peripheral insulin resistance.

Previous studies using normal fetal sheep have explored the effects of nutrient and/or growth factor stimulation on fetal growth and protein metabolism. Insulin given acutely to the normally growing sheep fetus during late gestation stimulated amino acid utilization and skeletal muscle fractional protein synthesis rates under conditions of amino acid sufficiency (5, 36, 49). However, chronically increasing insulin concentrations over 2–3 wk in the normally growing fetus during late gestation did not further accelerate fetal growth (14, 35). Normally growing and mildly growth-restricted fetal sheep responded to an acute amino acid infusion by increasing protein synthesis and accretion (10, 27). However, those studies allowed for concurrent amino acid-stimulated increases in plasma insulin concentrations, thereby limiting our knowledge of how amino acids themselves might stimulate fetal growth. On the basis of those previous studies, we tested whether acute stimulation with either amino acids or insulin would increase protein accretion in chronically nutrient-deprived IUGR fetal sheep. Furthermore, we tested whether restricted signaling cascades within IUGR fetal muscle might account for reduced growth potential in the IUGR fetus. We acutely supplemented both control (CON) and IUGR fetal sheep with either amino acids or insulin and measured fetal protein metabolic rates in each case using the stable isotope [1-13C]leucine. Under conditions of acute hyperinsulinemia or hyperaminoacidemia, we quantified the activity of insulin signaling in fetal skeletal muscle.

MATERIALS AND METHODS

Animal care and surgical procedures. Studies were performed in 42 late-gestation Columbia-Rambouillet mixed-breed sheep with singleton pregnancies at the University of Colorado Perinatal Research Center between 2004 and 2009, using protocols approved by the Institutional Animal Care and Use Committee. The Perinatal Research Center is accredited by the US Department of Agriculture, the National Institutes of Health, and the American Association for the Accreditation of Laboratory Animal Care. At 38.4 ± 0.3 days gestation (dGA), sheep were randomly allocated to environmental chambers with either elevated ambient temperatures to produce placental insufficiency and IUGR (40°C for 12 h; 35°C for 12 h; IUGR group, n = 28) or normal temperatures (20°C; CON group, n = 19) for 79 ± 0.4 days (term 148 dGA) (44). All sheep were housed in normothermic environments at least 5 d before surgery and for the remainder of their studies. All sheep were given ad libitum access to water. Maternal feed intake was matched between ewes in the CON and IUGR groups.
Maternal and fetal surgery was performed at 126.2 ± 0.3 dGA, each fetus was randomly assigned to one of three treatment groups: hyperaminoacidemia with euinsulinemia (independent amino acid effect: CON-AA, n = 8; IUGR-AA, n = 8), hyperinsulinemia with euaminoacidemia (independent insulin effect: CON-HI, n = 6; IUGR-HI, n = 10), or saline (CON-SAL, n = 5; IUGR-SAL, n = 5). All fetuses received a primed (5 μCi/kg) constant infusion of 3H2O (0.5 μCi/min; PerkinElmer, Waltham, MA) to measure umbilical blood flow by use of the transplacental diffusion method (34). A subset of fetuses (those studied from 2007 through 2009) also received a primed (30 μmol/kg) infusion with L-[1-13C]leucine (∼0.5 μmol·min−1·kg−1 estimated fetal weight; Cambridge Isotope Laboratories, Woburn, MA) to measure fetal leucine metabolism. After 180 min of tracer infusion equilibration, four fetal blood draws were obtained at baseline steady-state conditions for measurements of fetal arterial and umbilical venous plasma glucose, lactate, and amino acid concentrations including α-ketoisocaproic acid (KIC) and whole blood hemoglobin, pH, PaO2, PacO2, O2 saturation, blood O2 content, and 3H2O values. Fetal arterial plasma insulin, IGF-I, norepinephrine, and cortisol concentrations were measured. In those fetuses that were infused with L-[1-13C]leucine, isotopic enrichments of leucine, KIC, and 13CO2 were also measured under steady-state conditions (Fig. 1B).

After baseline sampling was complete, experimental treatment infusions were initiated as previously described (6). CON-AA and IUGR-AA fetuses received a mixed amino acid infusion (Trophamine; Central Admixture Pharmacy Services, Denver, CO) to increase fetal amino acid concentrations twofold greater than baseline. To suppress concurrent amino acid-stimulated fetal insulin secretion, somatostatin (Sigma Aldrich, St. Louis, MO) was prepared in 0.9% NaCl in water to provide a primed (0.2 mg/kg) constant infusion (4 μg·min−1·kg−1 estimated fetal weight). CON-HI and IUGR-HI fetuses received a primed (0.3 U/kg) constant infusion of recombinant insulin (Humulin R; Eli Lilly, Indianapolis, IN) prepared in 0.9% NaCl in water with 0.5% BSA at 3 mU·min−1·kg−1. Glucose (10% dextrose) and Trophamine were infused at variable rates based on measurements including umbilical venous plasma glucose, lactate, and cortisol concentrations twofold greater than baseline. To suppress plasma glucose, lactate, and amino acid concentrations were multiplied by their respective net fetal uptake rates and adding them together. Net fetal nitrogen uptake rate was determined by multiplying the number of nitrogen molecules in each amino acid by their respective net fetal uptake rates and adding them together. Net fetal nitrogen delivery rate included both the net fetal nitrogen uptake rate and the contribution of nitrogen molecules from Trophamine.

[1-13C]leucine tracer fluxes between the placenta and fetal plasma and between fetal plasma and fetal tissues were calculated per equations in Table 1 and as previously described (7). The leucine infusion rate from Trophamine was subtracted from the rate of protein degradation (Eq. 5, Table 1) and added to the equation for protein accretion rate (Eq. 7, Table 1).

Skeletal muscle protein analysis. Protein was extracted from pulverized skeletal muscle for Western blot analysis as previously described (6). Polyvinylidene difluoride membranes were incubated with L-[1-13C]leucine and [1-13C]KIC were determined using GC-MS as previously described (46). Isotopic enrichments of fetal and umbilical 13CO2/12CO2 were measured using continuous-flow isotope ratio mass spectrometry (5).

Calculations. Umbilical blood flow rates were calculated by dividing umbilical plasma flow by (1 − fractional fetal hematocrit). Umbilical venous-fetal arterial differences in plasma glucose, lactate, and amino acid concentrations were multiplied by the umbilical plasma flow rate (Fick principle) to calculate net umbilical (fetal) nutrient uptake rates. Net fetal O2 consumption rates were calculated by multiplying umbilical venous-fetal arterial differences in whole blood O2 content by umbilical blood flow rate. The glucose/oxygen quotient (the theoretical fraction of total fetal O2 consumption required to completely oxidize the net uptake rate of glucose by the fetus from the umbilical circulation) was calculated by dividing the umbilical venous-arterial difference in plasma glucose concentration by the umbilical venous-arterial difference in blood O2 content and multiplying that result by 6 (20). Individual amino acid delivery rates were calculated by adding the net fetal uptake rate to the exogenous infusion rate from Trophamine for each amino acid.

Protein was measured from pulsed fluorometry of skeletal muscle after tritium labeling with 3H2O (0.3 dGA for 50 ml per sampling period). At 177 ± 4 min of experimental infusion, the fetuses were delivered via maternal laparotomy and hysterotomy within 5 min of induction of maternal diazepam-ketamine anesthesia, and samples of the fetal biopsies femoris skeletal muscle were obtained under acute experimental conditions. The skeletal muscle tissue was immediately frozen with liquid nitrogen and stored at −70°C. Placental, fetal (whole body), and fetal organ weights were measured at necropsy.

Biochemical analysis. Fetal plasma glucose, lactate, amino acid, insulin, and cortisol concentrations and fetal blood gas, O2 content, and hematocrit values were measured as previously described (6, 31). IGF-I concentrations were measured by ELISA (ALPCO Immunoassays, Salem, NH, with an intra-assay CV of 7% and interassay CV of 7%). Fetal arterial plasma norepinephrine concentrations were measured as previously described (32). Isotopic enrichments of [1-13C]leucine and [1-13C]KIC were determined using GC-MS as previously described (46). Isotopic enrichments of fetal and umbilical 13CO2/12CO2 were measured using continuous-flow isotope ratio mass spectrometry (5).

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Fig. 1. Schema of experimental study design A: all measurements were performed at isotopic steady state, as demonstrated by molar percent enrichment (MPE) values for [1-13C]leucine (mean ± SE) in control (CON; ○) and intrauterine growth-restricted (IUGR; □) fetal arterial plasma (B).

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Table 1. Fetal leucine flux equations

<table>
<thead>
<tr>
<th>Flux</th>
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<tbody>
<tr>
<td>Equation</td>
</tr>
<tr>
<td>L-[1-13C] infusion rate (Inf)</td>
</tr>
<tr>
<td>Leu umb</td>
</tr>
<tr>
<td>Fetal plasma leucine disposal rate (DR)</td>
</tr>
<tr>
<td>Leucine flux into the placenta from fetal blood</td>
</tr>
<tr>
<td>Leucine flux into fetal tissues from fetal blood</td>
</tr>
<tr>
<td>Leucine flux into fetal blood from placenta</td>
</tr>
<tr>
<td>Leucine flux from fetal protein breakdown</td>
</tr>
<tr>
<td>Leucine oxidation rate</td>
</tr>
<tr>
<td>Net fetal protein accretion rate</td>
</tr>
<tr>
<td>Leucine flux into fetal protein synthesis</td>
</tr>
</tbody>
</table>

$[1-13\text{C}]\text{Leu}$, concentration of $[1-13\text{C}]$leucine in the isotopic infused; MPE, molar percent enrichment; $[1-13\text{C}]\text{Leu}_{\text{umb}}$, arterial-umbilical venous concentration difference $[1-13\text{C}]$leucine in fetal plasma; Leu umb, fraction of Inf taken up by the placenta; Leu Rf,p, net flux of leucine into the fetus from the placenta; Ex Leu Inf, exogenous leucine infusion rate; $[1\text{CO}_2]_{\text{a-v}}$, arterial-umbilical venous concentration difference $[1\text{CO}_2]_{\text{a-v}}$; UBF, umbilical blood flow; UF, umbilical plasma flow; KIC, $\alpha$-ketosuccinylacid.

Overnight at 4°C in antibodies to phosphorylated and total protein concentrations of protein kinase B (Akt, Ser175), mammalian target of rapamycin (mTOR, Ser2448), MAPK (Thr202/Tyr204), p70 S6 kinase (Thr421/Ser424), ribosomal protein S6 (rpS6, Ser235/236), eukaryotic initiation factor (eIF)4E-binding protein-1 (4E-BP1, Thr37/46), eu-


drather by 10% in CON fetuses and decreased by 30% in IUGR (Table 3). During the insulin infusion in IUGR fetuses, the change in fetal CO$_2$ accounted for $\sim 20\%$ of the change observed in fetal pH, indicating an increase in hydrogen ion concentration. Measurements of blood fetal O$_2$ values, including fetal arterial PaO$_2$, S$_2$ saturation, and blood O$_2$ content, were lower in IUGR fetuses at baseline (Table 3). Additionally, fetal whole blood O$_2$ content was 39% higher in males. O$_2$ saturation and content decreased in both CON and IUGR fetuses during the amino acid and insulin infusions, and they also decreased during the saline infusion in the IUGR fetuses. Umbilical venous PaO$_2$ and blood O$_2$ saturation also were lower in IUGR fetuses at baseline, regardless of group, further decreases in umbilical venous blood O$_2$ saturation were observed during the treatment infusions (Table 3). Change in hemoglobin-O$_2$ affinity from replacement of fetal hemoglobin with transfused maternal blood (associated with a shift in oxyhemoglobin dissociation curve to the right) contributed to reductions in fetal and umbilical blood O$_2$ values.

**Fetal substrate concentrations, uptake rates, and hormone concentrations.** Average maternal-to-fetal arterial plasma glucose concentration gradients across the placenta were 20% greater in IUGR fetuses than in CON, due to lower fetal glucose concentrations (Table 4). By study design, plasma glucose concentrations were similar to baseline values when insulin was infused into both CON and IUGR fetuses. During the amino acid infusion, however, fetal plasma glucose concentrations were not experimentally controlled and increased by 10% in CON fetuses and decreased by 30% in IUGR fetuses. Net fetal glucose uptake rates, although 15% lower in IUGR fetuses at baseline, demonstrated a nonsignificant 23% increase with amino acid infusion in IUGR fetuses and remained stable in CON fetuses (group $\times$ treatment interaction, $P = 0.06$). Similarly, the glucose/oxygen quotient increased by

Table 2. Fetal and placental weights

<table>
<thead>
<tr>
<th>Groups</th>
<th>CON</th>
<th>IUGR</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>Placenta weight, g</td>
<td>377 ± 17</td>
<td>171 ± 14*</td>
</tr>
<tr>
<td>Fetal weight, g</td>
<td>3,380 ± 99</td>
<td>1,932 ± 122*</td>
</tr>
<tr>
<td>Fetal/placental weight ratio</td>
<td>9.2 ± 0.4</td>
<td>12.0 ± 0.7*</td>
</tr>
<tr>
<td>Crown rump length, cm</td>
<td>48.5 ± 1.0</td>
<td>41.5 ± 1.0*</td>
</tr>
<tr>
<td>Lower extremity limb length, cm</td>
<td>36.7 ± 1.6</td>
<td>29.4 ± 0.9*</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>99.3 ± 3.7</td>
<td>52.2 ± 3.8*</td>
</tr>
<tr>
<td>Brain weight, g</td>
<td>46.5 ± 0.9</td>
<td>39.7 ± 1.0*</td>
</tr>
<tr>
<td>Brain/liver weight ratio</td>
<td>0.48 ± 0.02</td>
<td>0.84 ± 0.06*</td>
</tr>
<tr>
<td>Carcass weight, g</td>
<td>2,546 ± 65</td>
<td>1,329 ± 82*</td>
</tr>
<tr>
<td>Sex (%male)†</td>
<td>71</td>
<td>55</td>
</tr>
</tbody>
</table>

Values are means ± SE. CON, control; IUGR, intrauterine growth restriction. Group difference $*P < 0.005$. †Sex not recorded in 2 CON fetuses and 1 IUGR fetus.
### Table 3. Physiological measurements obtained at baseline and after experimental treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CON</th>
<th>IUGR</th>
<th>P&lt;0.001</th>
<th>G</th>
<th>T</th>
<th>G × T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>3.1</td>
<td>7.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HI</td>
<td></td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAL</td>
<td></td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HI</td>
<td></td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td></td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G × T</td>
<td></td>
<td>0.6</td>
<td></td>
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</tbody>
</table>

Values are means ± SE. E355PROTEIN ACCRETION IN IUGR FETAL SHEEP

20% during the amino acid infusion in IUGR fetuses but did not change in CON fetuses. However, the glucose/oxygen quotient also increased during the saline infusion in IUGR fetuses. Fetal plasma lactate concentrations were 120% higher in IUGR fetuses than in CON at baseline. Fetal lactate concentrations increased to similar degrees (by 30%) in CON and IUGR fetuses during the amino acid infusion and increased by 80% in IUGR fetuses infused with insulin. Net fetal lactate uptake rates were 43% lower in the IUGR group than in the CON group at baseline. Umbilical blood and plasma flow rates and net fetal O₂ consumption rates were 15 and 25% lower, respectively, in the IUGR group than in the CON group and were unaffected by treatment infusion.

Fetal plasma insulin concentrations were 55% lower under baseline conditions in IUGR fetuses compared with CON fetuses. By design, fetal plasma insulin concentrations were similar to baseline values during the amino acid infusion when somatostatin was concurrently infused. Insulin concentrations were increased to a pharmacological level during the fetal insulin infusion in both CON and IUGR fetuses. Fetal plasma IGF-I concentrations also were 50% lower in IUGR fetuses at baseline compared with CON fetuses. IGF-I concentrations increased during saline, amino acid, and insulin infusions in IUGR animals but not in CON animals. Fetal plasma cortisol and norepinephrine concentrations at baseline were 80 and 200% higher, respectively, in IUGR than in CON fetuses. Cortisol concentrations were 71% lower in males. Cortisol concentrations increased similarly during the amino acid infusion by ~80% in CON and IUGR fetuses but increased by only 40% during insulin infusion in IUGR fetuses. Norepinephrine concentrations increased by 75% during the amino acid infusion in IUGR fetuses only.

**Fetal protein metabolism.** IUGR fetuses compared with CON fetuses at baseline had 20% lower leucine disposal rates (CON 9.2 ± 0.5 vs. IUGR 7.1 ± 0.5 μmol·kg⁻¹·min⁻¹), 20% lower entry rates of leucine into the fetus from the placenta (CON 5.5 ± 0.4 vs. IUGR 4.4 ± 0.3 μmol·kg⁻¹·min⁻¹), and 20% lower leucine flux rates into fetal tissues (CON 7.5 ± 0.2 vs. IUGR 5.7 ± 0.5 μmol·kg⁻¹·min⁻¹) (group effects vs. P < 0.01). Flux rates of leucine back into the placenta at baseline were similar between CON and IUGR fetuses (CON 1.7 ± 0.2 vs. IUGR 1.5 ± 0.2 μmol·kg⁻¹·min⁻¹). Net fetal leucine disposal rates increased during amino acid infusion in CON and IUGR fetuses compared with baseline values (CON-AA 13.4 ± 1.0 vs. IUGR-AA 7.9 ± 1.6 μmol·kg⁻¹·min⁻¹, treatment effect P < 0.0001). Leucine disposal rates were similar to baseline values during the insulin infusion (CON-HI 8.8 ± 0.7 vs. IUGR-HI 7.4 ± 0.8 μmol·kg⁻¹·min⁻¹).

Leucine flux rates were used to estimate fetal protein metabolic rates, including net fetal protein breakdown, oxidation, accretion, and synthesis rates, during the treatment infusions (Fig. 2). Fetal protein breakdown rates decreased by 90% during amino acid infusion in IUGR fetuses only (Fig. 2A). Fetal leucine oxidation increased by 100% in CON fetuses and 20% in IUGR fetuses during amino acid infusion (Fig. 2B). Thus, protein accretion rates significantly increased by 150% in IUGR fetuses during the amino acid infusion (Fig. 2C). Protein metabolic rates during insulin infusion were similar to baseline metabolic rates in both CON and IUGR groups.

**Fetal skeletal muscle insulin signaling.** Total protein concentrations of the individual proteins measured were similar.
Table 4. Fetal substrate, hormone, and uptake rates

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Baseline</th>
<th>SAL</th>
<th>AA</th>
<th>HI</th>
<th>Baseline</th>
<th>SAL</th>
<th>AA</th>
<th>HI</th>
<th>Baseline</th>
<th>SAL</th>
<th>AA</th>
<th>HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>72.6 ± 2.7</td>
<td>72.0 ± 2.3</td>
<td>70.7 ± 2.5</td>
<td>68.6 ± 2.1</td>
<td>73.9 ± 2.5</td>
<td>73.4 ± 2.1</td>
<td>70.5 ± 2.3</td>
<td>68.1 ± 2.1</td>
<td>73.4 ± 2.5</td>
<td>73.1 ± 2.1</td>
<td>71.6 ± 2.3</td>
<td>69.5 ± 2.1</td>
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<td>n</td>
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<td>23</td>
</tr>
<tr>
<td>Umbilical blood flow, ml·min⁻¹·kg⁻¹</td>
<td>1872 ± 10.4</td>
<td>1885 ± 7.4</td>
<td>1924 ± 10.2</td>
<td>1866 ± 4.6</td>
<td>1882 ± 8.3</td>
<td>1869 ± 5.5</td>
<td>1844 ± 10.2</td>
<td>1851 ± 7.4</td>
<td>1832 ± 8.3</td>
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<td>1844 ± 10.2</td>
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<td>23</td>
<td>6</td>
<td>5</td>
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<td>23</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>7.3 ± 0.3</td>
<td>7.4 ± 0.3</td>
<td>7.3 ± 0.3</td>
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<td>7.3 ± 0.3</td>
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<tr>
<td>Fetal lactate, mmol/l</td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.2</td>
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</table>
| Values are means ± SE. Posttest comparisons (p < 0.05) differ from baseline within group; differ between groups within treatment. D, differs from control baseline value by Mann-Whitney test.

between treatments and groups. The phosphorylation state of several proteins within the insulin-signaling pathway increased during the amino acid infusion and to a greater extent during the insulin infusion (Fig. 3). During the insulin infusion, phosphorylation of Akt and mTOR increased in both CON and IUGR fetuses compared with SAL-infused fetuses. Phosphorylated p70 S6 kinase and rpS6 increased in CON and IUGR fetuses with insulin infusion. Two proteins known to be activated during periods of cellular stress and nutrient deprivation, AMPK and eIF2α, were also increased during the insulin infusion in both groups. During the amino acid infusion, phosphorylation of mTOR increased in CON fetuses. Phosphorylated eIF2α increased during the amino acid infusion in IUGR fetuses. Phosphorylation of eEF2, 4E-BP1, and MAPK were not different among groups and treatments (data not shown).

Fetal amino acid concentrations, net fetal uptake rates, and amino acid delivery rates. Fetal plasma amino acid concentrations were similar between CON and IUGR fetuses at baseline, with the exception of the following changes in IUGR fetuses compared with CON (Fig. 4): lower arginine (by 35%) and methionine (by 22%), and higher taurine (by 100%), asparagine (by 40%), proline (by 50%), alanine (by 35%), lysine (by 50%), and histidine (by 15%). Sex was a significant factor for glutamate (17% lower in males) and tyrosine (34% higher in males) concentrations. Amino acid infusion rates per kilogram fetal weight from Trophamine were similar between CON and IUGR fetuses (0.22 ± 0.01 CON-AA vs. 0.22 ± 0.02 g·kg⁻¹·h⁻¹ IUGR-AA). In both CON and IUGR fetuses infused with amino acids, all fetal amino acid concentrations were increased from baseline with amino acid treatment (treatment effects P < 0.0001; Fig. 4). IUGR fetuses infused with amino acids had higher plasma concentrations of leucine (by 25%), methionine (by 20%), lysine (by 55%), histidine (by 20%), glycine (by 30%), alanine (by 60%), proline (by 60%), ornithine (by 30%), and taurine (by 120%) compared with CON fetuses infused with amino acids, although CON fetuses had higher concentrations of arginine (by 58%) (group by treatment interactions P < 0.05; Fig. 4). There were minor changes (<20%) in amino acid concentrations in CON and IUGR fetuses infused with insulin (Fig. 4) despite similar infusion rates of amino acids into the fetus (0.07 ± 0.01 CON-HI vs. 0.08 ± 0.02 g·kg⁻¹·h⁻¹ IUGR-HI).

Net fetal nitrogen uptake rates were 20% lower in the IUGR group under baseline conditions compared with CON (55.7 ± 3.2 CON vs. 44.8 ± 2.8 μmol·kg⁻¹·min⁻¹ IUGR, group effect P < 0.05). Individual net fetal amino acid uptake rates at baseline also were lower in the IUGR group than in CON for isoleucine (by 30%), leucine (by 25%), phenylalanine (by 30%), tryptophan (by 40%), methionine (by 25%), and tyrosine (by 35%), and net fetal output of glutamate was reduced by 40% (group effects P < 0.05; Fig. 5). During insulin and amino acid infusions, net fetal nitrogen uptake rates from the placenta were similar to baseline nitrogen uptake rates (CON-AA 48.0 ± 6.9 vs. IUGR-AA 44.1 ± 10.6; CON-HI 45.3 ± 5.5 vs. IUGR-HI 37.9 ± 3.7 μmol·kg⁻¹·min⁻¹). Thus, individual net fetal amino acid delivery rates, calculated by adding the net fetal amino acid uptake rate to the exogenous infusion rate for each amino acid, were increased during the amino acid infusion for almost all amino acids in both CON and IUGR fetuses and to a similar extent (treatment effects P < 0.05).
0.05), whereas amino acid delivery rates were stable with insulin infusion (Fig. 5). Similarly, net nitrogen delivery rates were increased during the amino acid infusion in CON-AA and IUGR-AA fetuses compared with baseline net nitrogen uptake (treatment effect $P<0.0001$) and were unchanged from baseline with insulin infusion (CON-AA 91.3 ± 7.2 vs. IUGR-AA 88.0 ± 14.0; CON-HI 58.2 ± 4.4 vs. IUGR-HI 54.3 ± 6.6 μmol·kg$^{-1}$·min$^{-1}$).

**DISCUSSION**

In this study, we determined whether acute supplementation of amino acids or insulin to normal and growth-restricted ovine fetuses would independently promote net fetal protein accretion rates and concurrently upregulate intracellular signaling involved in mRNA translation initiation in skeletal muscle. Under baseline conditions, the IUGR fetal sheep demonstrated decreased leucine disposal rates and decreased leucine flux into fetal tissues compared with CON. Direct infusion of amino acids into the IUGR fetal circulation, independent of changes in insulin concentrations, increased fetal amino acid and nitrogen delivery, suppressed protein breakdown rates, and only minimally increased leucine oxidation, thus yielding consistent increases in fetal protein accretion rates. CON fetuses, however, oxidized leucine to a greater degree than IUGR fetuses in response to increased amino acid delivery, which produced lower (and more variable) increases in fetal protein accretion rates. Amino acid infusion had relatively little effect on the pathway(s) for protein synthesis in CON or IUGR fetal skeletal muscle, further indicating that upregulation in protein accretion rate seen in IUGR fetuses occurred by suppression of protein breakdown rate and not by an increase of protein synthesis. Surprisingly, acute, pharmacological insulin infusions into both CON and IUGR fetuses under euglycemic-euaminoacidic conditions markedly upregulated signaling through mTOR in skeletal muscle but had little effect on whole fetal protein metabolic rates. Further analysis of insulin signaling, including AMPK and eIF2α, however, indicates the potential for stimulating cellular proliferation and growth under the proper conditions as discussed below.

**Potential for improving fetal growth in IUGR.** Nutrient and anabolic hormonal manipulation of the IUGR fetus to challenge programmed changes in growth pathways has been attempted previously. The most promising results have come from chronic, low-dose IGF-I infusions into the fetus in terms of improving fetal organ growth (13). Acute amino acid infusion with a concurrent increase in fetal plasma insulin concentrations increased protein accretion rates in the IUGR fetus (10); however, chronic glucose supplementation to the IUGR fetus resulted in hypoxia and acidosis (47). These investigations are important, because IUGR is a significant cause of increased fetal and neonatal morbidity and mortality, with few therapeutic options available aside from close fetal surveillance.
Furthermore, epidemiological evidence shows that, during childhood, impaired lean mass growth continues into childhood among infants born small for gestational age (19). The development of prenatal strategies to promote fetal growth and muscle development could improve the potential for postnatal lean mass growth, thereby minimizing the risk of an IUGR infant developing long-term insulin resistance and reductions in muscle mass (23, 51, 52). The results of the present study support further investigation into the use of supplementing amino acids to the IUGR fetus, as in the acute setting, amino acid supplementation suppressed fetal protein breakdown rates and promoted fetal protein accretion rates. The results also demonstrate the need to evaluate concurrent activation of pathways that sense hypoxemia or deprivation of other nutrients, such as AMPK signaling and/or secretion of counterregulatory hormones, that might occur as a result of such infusions.

**Increased capacity for nutrient utilization in IUGR.** In these studies, we used a model of progressive and severe placental insufficiency to replicate the fetal metabolic patterns and characteristics of human IUGR pregnancy (4). The IUGR group of fetal sheep demonstrated a severe phenotype, with marked hypoglycemia, hypoinsulinemia, hypoxemia, higher lactate concentrations, and reduced umbilical blood flow. They were, however, metabolically compensated for such adverse conditions, as they had a normal pH under baseline conditions. Placental, fetal (whole body), and fetal organ weights were reduced by 40%, with length and brain weights that were relatively spared. In fact, placental weight was more restricted than fetal weight. This finding indicates either increased placental substrate transport capacity or increased fetal capacity for nutrient utilization for growth. It is generally accepted that placental insufficiency results in decreased placental substrate transport. Previous human and animal studies in IUGR show reduced placental size and surface area with decreased glucose, amino acid, and oxygen transport capacity (12, 29, 44). As expected, the IUGR group of fetuses in the present study demonstrated lower net fetal glucose uptake, lower net fetal amino acid/nitrogen uptake, and lower unidirectional entry rate of leucine into the fetus from the placenta, all findings shown.
Fig. 4. Fetal arterial plasma amino acid concentrations. Fetal arterial plasma amino acid concentrations are shown at baseline (open bars), during infusion with saline (hatched bars), during infusion with amino acids (gray bars), and during infusion with insulin (filled bars) in CON and IUGR groups. Essential amino acids (A), nonessential amino acids in high concentrations (B), and nonessential amino acids in low concentrations (C) are shown. All data are expressed as means ± SE. Posttest comparisons (P < 0.05): *concentration differs from baseline within CON or IUGR group; †concentration differs between CON and IUGR groups within treatment. CON: baseline n = 19, SAL n = 5, AA n = 8, HI n = 6; IUGR: baseline n = 21, SAL n = 5, AA n = 7, HI n = 9.
Fig. 5. Net fetal amino acid uptake and delivery rates. Net fetal amino acid uptakes are shown at baseline (open bars), during SAL (hatched bars), AA (gray bars), and HI (filled bars). Cross-hatched bars demonstrate exogenous amino acid delivery rates in AA and HI groups, such that the total amino acid delivery rate for each amino acid is the sum of its net fetal uptake rate and exogenous delivery rate (with the exception of aspartate, serine, and glutamate, which demonstrate net fetal output to the placenta). Essential amino acids (A) and nonessential amino acids (B and C) are shown. All data are expressed as means ± SE. Posttest comparisons (P < 0.05): *uptake rate differs from baseline within CON or IUGR group; †uptake rate differs between CON and IUGR group within treatment; #delivery rate differs from baseline within CON or IUGR group; ‡uptake and delivery rates both differ between CON and IUGR groups within treatment. CON: baseline n = 19, SAL n = 5, AA n = 8, HI n = 6; IUGR: baseline n = 21, SAL n = 5, AA n = 7, HI n = 9.
previously in IUGR pregnancies in both animal models and humans (21, 53, 55).

Increased fetal capacity for nutrient utilization for growth and metabolism, however, is a feasible explanation for the increased fetal-to-placental ratio found in the IUGR group. Several different ovine IUGR models have found evidence of increased insulin sensitivity for glucose metabolism (30, 57). Studies that have examined the capacity for insulin or amino acids to independently stimulate amino acid metabolism in the IUGR fetus are more limited (10). When concurrent insulin secretion is blocked by somatostatin, our results show the ability of acute amino acid infusion to suppress fetal protein breakdown rates, yielding consistent increases in fetal protein accretion rates among all IUGR animals studied. Furthermore, as fetal oxygen consumption did not change, we speculate that glucose oxidation increased to maintain energy balance. This is based on the observation that, in IUGR fetuses infused with amino acids, the glucose/oxygen quotient increased. However, saline infusion into the IUGR fetus resulted in similar changes in the glucose/oxygen quotient, perhaps demonstrating a more limited capacity to tolerate manipulation and/or exogenous infusions. Interestingly, the CON group preferentially used excess amino acids for oxidative purposes with minimal change in glucose metabolism, consistent with previous observations (46). Thus, protein accretion rates were more variable, such that two CON fetuses demonstrated increased protein accretion rates and two demonstrated decreased protein accretion rates. Therefore, the present study demonstrates the ability of amino acid-infused IUGR fetuses to respond to exogenously administered amino acids to promote protein accretion, with further evidence for increased glucose use to provide energy for this process. These results support the hypothesis of increased capacity for nutrient utilization for growth as well as metabolism, including both amino acids and glucose, in the IUGR fetus.

Potential mechanisms for differences in protein metabolic rates in IUGR vs. CON fetuses. Differences in protein metabolic rates in response to an acute amino acid infusion between CON and IUGR fetuses allow for speculation regarding the potential mechanisms. In IUGR fetuses, plasma IGF-I, cortisol, and norepinephrine concentrations increased in response to amino acid infusion, whereas in CON fetuses only cortisol was increased. The capacity for IGF-I to promote fetal protein accretion and growth, especially in muscle, has been well documented by both Igf1 gene knockout models (43) and direct infusion experiments (1, 49). Notably, both norepinephrine and IGF-I suppress protein breakdown in the normal ovine fetus during late gestation (28, 38). These antiproteolytic effects could have contributed to the marked suppression in protein breakdown rates that we observed in IUGR fetuses in response to amino acid infusion. Furthermore, cortisol has been shown to increase IGF-I production in the fetal liver (26). Cortisol, however, has also been shown to increase proteolysis (37). It should be noted that IGF-I concentrations also increased in response to saline infusion (with maternal blood transfusion to maintain euvolemma) in IUGR fetuses without concurrent changes in plasma cortisol and norepinephrine. Further investigation is needed to understand the complex interactions between cortisol and the somatotropic axis and their effects on protein accretion and growth, especially in the context of IUGR.

Alternatively, IUGR fetuses may have limited capacity to oxidize excess amino acids. Amino acid-infused IUGR fetuses demonstrated suppression of protein breakdown rates with only minimally increased fetal leucine oxidation rates compared with CON fetuses similarly infused with amino acids. Muscle is the primary site for branched-chain amino acid oxidation due to the high concentration of branched-chain α-keto acid dehydrogenase (BCKAD) (17). Under normal conditions in humans, branched-chain amino acids (leucine, isoleucine, and valine) are more rapidly oxidized when protein is present in excess, due to increased BCKAD activity (18). During experimentally induced hypoxia in fetal sheep, however, leucine oxidation was suppressed (39), potentially due to decreased BCKAD enzyme activity and net efflux of amino acids from muscle (56). In the hypoxemic IUGR fetus, we speculate that BCKAD activity might also be suppressed. Thus, in response to supplemental amino acid treatment, there would be less oxidation of leucine in IUGR fetal muscle compared with CON. It also is possible that downregulation of amino acid transporters occurs in IUGR muscle to limit active uptake of excess amino acids by muscle for oxidative metabolism. Either process would enable the preferential use of supplemental amino acids for suppression of proteosomic activity and efflux of amino acids from muscle, as has been shown previously (15).

Lack of insulin effect on fetal protein metabolism. Unlike amino acid infusion, insulin had very little effect on amino acid delivery rates, fetal leucine disposal, or fetal protein accretion in either CON or IUGR fetuses despite robust signaling of the skeletal muscle insulin-signaling cascade. This was a surprising finding given that other investigations, including our own, have shown that insulin promotes amino acid utilization and fetal protein accretion in normally grown fetuses when amino acid concentrations are maintained by simultaneous infusions (5, 36, 49). There are several possible mechanisms to account for this observation. First, fetal insulin concentrations as a result of the insulin infusion were much higher than those achieved in previous studies, and, by study design, the infusion period was for a shorter duration to capture acute upregulation of signal transduction in muscle (2). Moreover, the marked increase in fetal insulin concentrations achieved by the insulin clamp, while stimulating protein translation capacity in skeletal muscle, may not promote net protein turnover along the same time frame in the whole animal. Finally, fetal cortisol concentrations increased during the acute insulin infusion, and fetal blood oxygen levels were lower, which could have suppressed fetal protein accretion (37, 39). A more physiological supplementation of insulin, as well as for a longer duration, might have produced a more anabolic effect on both CON and IUGR fetuses.

Insulin effect on cell-signaling pathways in fetal muscle. Despite the lack of increase in fetal protein synthesis, we have confirmed previous observations of insulin’s significant, independent effect on activating the insulin signal transduction pathway through Akt and mTOR, leading to substantial upregulation of p70 S6 kinase and rpS6 in fetal skeletal muscle (6, 48). Acute insulin infusion has a preferential anabolic effect on muscle, as has been shown in fetal and neonatal cell culture and animal models (9, 50). Protein metabolic rates were measured across the umbilical circulation, reflecting changes in the whole fetus and not skeletal muscle specifically. Therefore, we
might not have appreciated subtle changes in muscle-specific protein metabolism as others have observed (40, 49). Other negative feedback pathways were activated as a result of the high-dose insulin infusion that might have counteracted mTOR mediated activation of net protein synthesis. For example, AMPK is activated by metabolic stresses that deplete or interfere with the generation of ATP, such as hypoxia, and activate catabolic pathways (16). Increased phosphorylation of AMPK at site Thr\(^{172}\) occurred during the insulin infusion, more significantly in IUGR than CON muscle. Measures of fetal oxygenation and fetal pH decreased during the insulin infusion, and more so in IUGR fetuses, which could have stimulated AMPK activation. In fact, insulin and amino acid infusion also resulted in the phosphorylation of eIF2\(\alpha\), a marker of endoplasmic reticular stress that downregulates protein synthesis (25). We speculate that the high-dose insulin infusion activated intracellular signals that both promote and inhibit protein synthetic pathways, resulting in no net gain in protein accretion. Further investigation of these highly complex interactions when introducing nutrient and growth factor supplementation to the fetus is required.

**Fetal amino acid profiles.** We have presented detailed analysis of complete amino acid profiles in these animals both at baseline and in response to the amino acid and insulin infusions. Under baseline conditions, plasma amino acid concentrations were maintained in IUGR fetuses, with increased plasma concentrations for taurine, asparagine, proline, alanine, lysine, and histidine compared with CON fetuses; only arginine and methionine were decreased. The severity of placental insufficiency likely predicts whether fetal plasma amino acid concentrations are higher or lower than normal, with contributions from placental transport and/or clearance capacity, degree of fetal protein breakdown, and degree of fetal hypoxia (33, 45). Acute amino acid infusion resulted in increased amino acid and nitrogen delivery rates, strikingly similar for CON and IUGR fetuses, with minimal suppression of fetal amino acid and nitrogen uptake from the placenta. However, it is interesting to note that, despite equal delivery rates of amino acids on a body weight-specific basis, several plasma amino acid concentrations were higher in IUGR fetuses than in CON fetuses. These findings provide additional evidence for reduced placental clearance capacity of amino acids in IUGR pregnancy (12). Finally, alanine is usually in equilibrium with lactate, and both can be produced by skeletal muscle when oxygen is limiting, allowing their availability for hepatic gluconeogenesis. It is possible that the addition of amino acids to the fetal circulation of a hypoxic fetus generated the production of lactate and alanine in fetal muscle, thus fueling the production of glucose (in addition to increasing glucose uptake from the placenta) to maintain glucose supply for vital organs (30, 53).

**Study limitations.** Although the study population of CON and IUGR fetal sheep was large, these fetuses were allocated among six different groups; thus, the power to appreciate more subtle differences between treatment groups was limited. Furthermore, as has been discussed, different doses and durations of fetal infusions can lead to different outcomes. We chose a dose of insulin that had previously been shown to increase amino acid utilization (5). However, fetal plasma insulin concentrations as a result of this dose of insulin were higher than expected. With treatment infusions, fetal cortisol and norepinephrine increased, and fetal blood oxygen values decreased. In addition to expected shifts in the oxyhemoglobin dissociation curve from transfused maternal blood, independent effects of insulin and amino acid infusion at the doses used in these studies likely contributed to decreases in fetal blood oxygen values. Additionally, AMPK and eIF2\(\alpha\) were activated during the infusions, both sensors of metabolic stress. The use of more physiological rates of insulin and amino acid infusions might minimize hypoxemia, cortisol, and norepinephrine secretion and maximize anabolic growth potential. We provided supplemental nutrients acutely; it is not known whether anabolic effects of supplemental nutrients can be sustained when administered chronically to an IUGR fetus with limited oxygen availability. Finally, studying a group of fetuses in which insulin was allowed to concurrently increase within a physiological range with amino acid infusion also would be valuable to determine potential synergistic effects of insulin and amino acids (40).

**Conclusion.** CON and IUGR fetuses from progressive and severe placental insufficiency responded differently to acute supplementation of amino acids and insulin. In response to acute amino acid infusion, the CON fetus preferentially oxidized the amino acids in contrast to more limited increases in leucine oxidation rates in the IUGR fetus. Thus, amino acids infused into IUGR fetuses preferentially suppressed protein breakdown rates, which specifically contributed to increased protein accretion rates. Insulin infusion upregulated insulin-signaling proteins in both CON and IUGR fetuses but did not affect whole fetal protein synthesis or accretion rates in either group. On the basis of the unique responses in the IUGR fetuses, we speculate that amino acids might be used to promote fetal growth in IUGR more effectively than hormonal strategies when metabolic responsiveness to insulin is limited. However, changes in fetal blood oxygen values, acid-base balance, production of anabolic and catabolic hormones, and activation of stress pathways must be evaluated with future prenatal interventional strategies. These investigations will allow for a better understanding of mechanistic adaptations that occur in the fetus to chronic nutrient deprivation when one is attempting to improve fetal growth.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

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