Prolonged maternal amino acid infusion in late-gestation pregnant sheep increases fetal amino acid oxidation

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Rozance PJ, Crispo MM, Barry JS, O’Meara MC, Frost MS, Hansen KC, Hay WW Jr, Brown LD. Prolonged maternal amino acid infusion in late-gestation pregnant sheep increases fetal amino acid oxidation. Am J Physiol Endocrinol Metab 297: E638–E646, 2009. First published July 14, 2009; doi:10.1152/ajpendo.00192.2009.—Protein supplementation during human pregnancy does not improve fetal growth and may increase small-for-gestational-age birth rates and mortality. To define possible mechanisms, sheep with twin pregnancies were infused with amino acids (AA group, n = 7) or saline (C group, n = 4) for 4 days during late gestation. In the AA group, fetal plasma leucine, isoleucine, valine, and lysine concentrations were increased (P < 0.05), and threonine was decreased (P < 0.05). In the AA group, fetal arterial pH (7.365 ± 0.007 day 0 vs. 7.336 ± 0.012 day 4, P < 0.005), hemoglobin-oxygen saturation (46.2 ± 2.6 vs. 37.8 ± 3.6%, P < 0.005), and total oxygen content (3.17 ± 0.17 vs. 2.49 ± 0.20 mmol/l, P < 0.0001) were decreased on day 4 compared with day 0. Fetal leucine disposal did not change (9.22 ± 0.73 vs. 8.09 ± 0.63 μmol·min⁻¹·kg⁻¹, AA vs. C), but the rate of leucine oxidation increased 43% in the AA group (2.63 ± 0.16 vs. 1.84 ± 0.24 μmol·min⁻¹·kg⁻¹, P < 0.05). Fetal oxygen utilization tended to be increased in the AA group (327 ± 23 vs. 250 ± 29 μmol·min⁻¹·kg⁻¹, P = 0.06). Rates of leucine incorporation into fetal protein (5.19 ± 0.97 vs. 5.47 ± 0.89 μmol·min⁻¹·kg⁻¹, AA vs. C), release from protein breakdown (4.20 ± 0.95 vs. 4.62 ± 0.74 μmol·min⁻¹·kg⁻¹), and protein accretion (1.00 ± 0.30 vs. 0.85 ± 0.25 μmol·min⁻¹·kg⁻¹) did not change. Consistent with these data, there was no change in the fetal skeletal muscle ubiquitin figures MaFbX1 or MuRF1 in the protein synthesis regulators eEF2, eIF2α, and p70S6K. Decreased concentrations of certain essential amino acids, increased amino acid oxidation, fetal acidosis, and fetal hypoxia are possible mechanisms to explain toxicity during maternal amino acid supplementation.

Previous work has shown that maternal infusion of complete and balanced mixtures of intravenous amino acids into pregnant sheep for 2 and 12 h increases fetal concentrations of the branched-chain amino acids (BCAA; leucine, isoleucine, and valine), phenylalanine, and methionine but decreases threonine and serine concentrations. This was found to be due to the competitive inhibition of placental transport of certain essential amino acids to the fetus (14, 16, 33). The impact of this competitive inhibition on the fetus over a longer period of time has not been evaluated but is of critical importance because several large human clinical trials have shown that both high-protein and isocaloric-protein supplementation in normal pregnancies increases the risk for small-for-gestational-age (SGA) birth and might even increase perinatal mortality (26, 42). Despite the concerning data from human trials, maternal amino acid supplementation, especially when a pregnancy is affected by intrauterine growth restriction (IUGR), remains an intriguing option. Both human and animal studies have consistently shown a reduction of placental transport of essential amino acids such as leucine and threonine in IUGR pregnancies (6, 11, 12, 25, 32, 38). However, before studies in IUGR pregnancy can be considered, studies in normal pregnancies using appropriate animal models are required for defining how the fetus responds to amino acid supplementation at the physiological and molecular levels. Therefore, the purpose of this study was to determine the effect of prolonged, 4-day maternal amino acid infusion enriched with essential amino acids in normal sheep pregnancies during late gestation on fetal amino acid, protein, glucose, and oxygen metabolism as well as measurements of fetal well-being such as acid-base balance.

Materials and Methods

Animal Preparation

Studies were conducted during the final 20% of gestation in 11 pregnant Columbia-Rambouillet ewes carrying twins (term = 147 days gestational age; dGA). Indwelling catheters were surgically placed into the vasculature of the mother and both fetuses at 125.1 ± 0.2 dGA as previously described (3). All animal procedures were in compliance with guidelines of the United States Department of Agriculture, the National Institutes of Health, and the American Association for the Accreditation of Laboratory Animal Care. The animal care and use protocols were approved by the University of Colorado Denver Institutional Animal Care and Use Committee.

Experimental Design

Schematic representation of the study design is shown in Fig. 1. Pregnant sheep were randomly assigned to one of two groups, either an amino acid infusion group (AA group; n = 7 sheep) or a saline control group (C group; n = 4 sheep). Due to fetal catheter failure, complete studies were obtained on ten fetuses from seven sheep in the AA group and six fetuses from four sheep in the C group; in no case was there catheter failure in both twins from a single ewe. Animals were allowed to recover from surgery for 5 days prior to start of infusions. Beginning on 130.9 ± 0.3 dGA, infusions were started in both treatment groups. The AA group received 4 days of continuous, intravenous mixed amino acids (Trophanine; Central Admixture Pharmacy, Aurora, CO) via the maternal femoral vein, adjusted daily to achieve a 25–50% increase in maternal branched-chain amino acids (BCAA). Plasma BCAA concentrations were measured with a spectrophotometric assay described by Beckett et al. (2) using an Ultrospec 4300 Pro UV/VISIBLE spectrophotometer (GE Healthcare Bio-
the molar percent enrichment (MPE) of the L-[1-13C]leucine infusion pump rate by the concentration of leucine in the infusate and by concentrations were multiplied by umbilical plasma flow (Fick principle) venous-arterial differences in glucose, lactate, and amino acid concentrations and fetal arterial plasma IGF-I and glucagon were measured daily. Fetal and maternal arterial plasma insulin concentrations and fetal arterial plasma insulin concentrations and fetal arterial plasma IGF-I and glucagon were measured every other day.

At the end of the 4-day infusion (134.9 ± 0.4 dGA), a metabolic tracer study was performed to measure umbilical substrate uptakes and whole fetal leucine metabolism as previously described (5, 38). After samples were collected at time zero for naturally occurring isotopic enrichments, one twin fetus from a pregnancy was infused with a solution containing 3H2O (15 μCi/ml, starting bolus of 45 μCi followed by constant infusion at 0.5 μCi/min; PerkinElmer Life Sciences, Boston, MA), and the other twin was infused with ethanol (300 mg bolus followed by 12.8 mg/min; AAPER Alcohol and Chemical, Shelbyville, KY) to measure umbilical blood flow. Each fetus was infused with L-[1-13C]leucine (45.7 μmol/ml, starting bolus of 137 μmol followed by constant infusion at 1.5 μmol/min; Cambridge Isotope Laboratories, Woburn, MA) to measure fetal leucine metabolism. Tracers were infused for 180 min prior to obtaining four steady-state blood samples drawn simultaneously from the umbilical vein and fetal aorta. This blood was used to measure fetal oxygen content, concentrations of fetal plasma glucose, lactate, amino acids, α-ketoisocaproic acid (KIC), 3H2O, ethanol concentration, stable isotopic enrichments of plasma [1-13C]leucine and [1-13C]KIC, and whole blood 13CO2.

Calculations

Umbilical plasma flows were determined by the transplacental diffusion method, with 3H2O or ethanol as the flow indicator (27). Umbilical blood flows were then calculated by dividing umbilical plasma blood flow by (1 – fractional fetal hematocrit). Umbilical venous-arterial differences in glucose, lactate, and amino acid concentrations were multiplied by umbilical plasma flow (Fick principle) to calculate nutrient uptake across the fetus. Oxygen utilization was calculated by multiplying umbilical venous-arterial differences in O2 content by umbilical blood flow. [1-13C]leucine tracer fluxes were used to calculate fetal oxygen consumption, concentrations of fetal plasma glucose, lactate, amino acids, α-ketoisocaproic acid (KIC), 3H2O, ethanol concentration, stable isotopic enrichments of plasma [1-13C]leucine and [1-13C]KIC, and whole blood 13CO2.

Biochemical Analysis

Insulin was measured by ELISA (Alpco; inter- and intra-assay CVs 2.9 and 5.6%, respectively) (39). Glucagon (Linco Research, St. Charles MO; inter- and intra-assay CVs 11.7 and 6.1%, respectively) and IGF-I (Diagnostics Systems Laboratories, Webster, TX; inter- and intra-assay CVs 4.5 and 2.6%, respectively) were measured by RIA (39). Plasma amino acids were measured by HPLC using a Dionex 300 model 4500 analyzer (Dionex, Sunnyvale, CA) (39). KIC concentrations were measured using methods described by Milley et al. (29). Maternal and fetal arterial pH, partial pressure of carbon dioxide (Pco2), partial pressure of oxygen (Po2), hemoglobin-oxygen saturation SaO2, total oxygen content CaO2, and hematocrit were determined using an ABL 520 analyzer (Radiometer, Copenhagen, Denmark) (39).

To determine umbilical blood flow using 3H2O, 0.1 ml of plasma was mixed with 0.5 ml of water and 15 ml of Biosafe II scintillation cocktail (Research Products International, Mount Prospect, IL). Counts were measured in a Packard Tri-Carb 460 C liquid scintillation counter (PerkinElmer, Boston, MA). Ethanol concentrations were determined in triplicate aliquots with a quantitative enzymatic UV determination method (cat. no. Alcohol 332-UV; Sigma, St. Louis, MO) (8). There were no systematic differences in umbilical blood or plasma flow as measured by 3H2O or ethanol.

Isotopic enrichments for [1-13C]leucine and [1-13C]KIC were determined using GC-MS (model 5975; Agilent Technologies, Wilmington, DE), equipped with an HP5-MS column (30 m × 0.25 mm × 0.25 μm) as previously described (5). Briefly, leucine in 0.5 ml of plasma was purified with 0.05 ml of the internal standard norleucine (10 μg/ml) and 0.15 ml of 50% acetic acid. Cation exchanger (AG-50, H+ form, 0.05 ml) was added, and the amino acid was eluted using 0.6 ml of 5 N ammonium hydroxide. The ammonium hydroxide solution was dried under reduced pressure, and plasma leucine isotopic enrichment was measured after conversion to t-BDMS derivative [0.05 ml of 50% N,O-bis(trimethylsilyl)trifluoroacetamide with 1% tri-methylchlorosilane in anhydrous acetonitrile incubated at 65°C for 1 h]. The leucine isotopic enrichment was determined using the selective ion monitoring (SIM) method at m/z 303/302. KIC was reduced to α-hydroxyisocaproic acid with sodium borohydride (0.025 ml, 10 mg/ml in water). The reaction was terminated by the addition of 0.025 ml of 4 N HCl. Ethyl acetate (1 ml) was added and washed with 0.5 ml of 0.04 M HCl, and the organic solvent was removed under reduced pressure. Plasma KIC isotopic enrichment was measured after conversion to α-hydroxyisocaproic acid t-BDMS derivative at 85°C for 20 min. The KIC isotopic enrichment was determined using the SIM method at m/z 304/303.4 for 3H2O.

For the measurement of fetal and umbilical 13CO2/12CO2 with continuous-flow IRMS (Delta V; Thermo Electron, Bremen, Germany), 1 ml of whole blood was transferred into a 20-ml Exetainer. Upon whole blood transfer, a constant flow of pure 0.30% CO2 (balance helium) over 5 min replaced all other gas in the Exetainer headspace above the whole blood samples. All CO2 was allowed to equilibrate between the sample and the pure headspace gas over 48 h and at a constant temperature (30°C). Upon equilibration, the headspace was sampled (10 μl) in duplicate for isotope ratio analyses. An average standard deviation for all samples was less than 0.0001 atom percent excess (AT%). Standard gas (high-purity CO2) was sampled five times prior to each sample being measured with a mean of 1.1022 AT% and standard deviation of <0.0001 AT%. Excess 13CO2 (AT% excess) was calculated relative to baseline CO2.

Organ Isolation

Organ isolation and measurements were performed at the completion of the physiological studies as previously described (22). Biopsies of the fetal biceps femoris were taken under steady-state experimental conditions and snap-frozen in liquid nitrogen followed by storage at −70°C until further analysis. The pancreas was removed, fixed overnight in 4% paraformaldehyde (wt/vol) in PBS and then transferred to 70% ethanol (vol/vol) until it was parafin embedded.
Tissue Analysis

For mRNA analysis, total RNA was extracted from pulverized skeletal muscle (200 mg) and reverse transcribed into complementary DNA (cDNA) as previously described (21). Ovine F-box only protein 32 (FBXO32, MaFBX1), ovine ribosomal protein s15 (GenBank accession nos. EU492872, EU525163, and AY949774, respectively) were measured by quantitative real-time PCR (qPCR) as previously described (20, 40). The standard-curve method of relative quantification was used to compare results (45). Ribosomal protein s15 was used as a housekeeping gene and was not different between treatment groups. Results are presented in arbitrary units as fold changes relative to C fetuses.

Protein was extracted from pulverized skeletal muscle (200 mg) and prepared for Western blot analysis as previously described (4). The procedure for detection of Thr\(^{374/46}\)-phosphorylated eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), total 4E-BP1, Thr\(^{215/234}\)-phosphorylated p70 S6 kinase (p70S6K), total p70S6K, and β-actin have been previously described (4). Ser\(^{52}\)-phosphorylated eIF2α (1:3,000; Stressgen, Ann Arbor, MI) and eIF2α (1:1,500, Stressgen) were diluted in PBS-Tween 0.1% (vol/vol) with 5% nonfat dry milk (NFDM, wt/vol). Thr\(^{374/46}\)-phosphorylated eukaryotic elongation factor 2 (eEF2, 1:1,250; Cell Signaling Technology, Danvers, MA) and eEF2 (1:500, Cell Signaling Technology) were diluted in PBS-Tween 0.1% with 5% BSA (wt/vol). Horseradish peroxidase-conjugated secondary antibodies were diluted in PBS-Tween 0.1% with 5% NFDM and applied to membranes at room temperature for 1 h. Immunocomplexes were detected with enhanced chemiluminescence (Amersham ECL Plus, Piscataway, NJ), and densitometry was performed on every membrane to control for differences in transfer efficiency. Phosphorylated specific antibodies were normalized to the reference sample and persisted through day 1 of protein to the mother.

Statistical Analysis

Statistical analysis was performed using SAS v. 9.1 (SAS Institute). Results are expressed as means ± SE. A mixed-models ANOVA was performed to determine effects of treatment group (AA or C), day of infusion, and treatment by day of infusion interaction to determine whether changes over time were different between the two treatment groups. The experimental unit for comparisons between groups was the mother, and the analysis treated observations within twin pairs as nonindependent. Individual means were compared using Tukey’s test for multiple comparisons. Due to heterogeneity of variability, the fetal arterial plasma glucagon concentrations were log transformed.

RESULTS

Maternal Parameters During Treatment

The amino acid infusion rate required to achieve a 25–50% increase in maternal BCAAs concentrations provided 46.3 ± 7.1 g/day (0.9 ± 0.1 g·kg\(^{-1}·day^{-1}\)) of protein to the mother. Maternal feed and water intake did not vary between groups, and there were no changes in maternal arterial plasma lactate or insulin concentrations. Maternal arterial plasma glucose concentrations decreased on day 2 of the infusion but then increased back to baseline concentrations by day 4; this was similar in both groups. Glucose, lactate, and insulin concentrations on day 4 in the AA and C groups are shown in Table 1. By study design, the maternal BCAAs were increased by amino acid infusion in the AA group by day 1 and persisted through day 4 of infusion (Fig. 2). Several other essential and nonessential amino acid concentrations also were increased by amino acid infusion (Fig. 2). None of the amino acids were decreased by amino acid infusion, and no amino acids in the saline group changed. Maternal arterial pH, \(\text{PCO}_2\), \(\text{PO}_2\), \(\text{SaO}_2\), and \(\text{CaO}_2\) did not vary. Maternal arterial hematocrit decreased by 4% from baseline to day 4, but this was similar in both groups. Values on day 4 are shown in Table 1.

Fetal Parameters During Treatment

Arterial plasma glucose, lactate, and amino acid concentrations. Fetal arterial plasma glucose and lactate concentrations were not affected by treatment or day of infusion, and values on day 4 are shown in Table 2. We found significant changes in several fetal arterial plasma amino acid concentrations in the AA group, as shown in Fig. 3. Significant differences were found for almost all essential amino acids, with individual means comparisons showing that amino acid infusion in-

<table>
<thead>
<tr>
<th>Essential amino acids</th>
<th>Non-essential amino acids</th>
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<tbody>
<tr>
<td>Glucose, mg/dl</td>
<td>C Group</td>
</tr>
<tr>
<td></td>
<td>AA Group</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>64.5 ± 5.7</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.71 ± 0.20</td>
</tr>
<tr>
<td>pH</td>
<td>7.45 ± 0.01</td>
</tr>
<tr>
<td>(\text{PCO}_2), mmHg</td>
<td>33.6 ± 0.3</td>
</tr>
<tr>
<td>(\text{PO}_2), mmHg</td>
<td>92.7 ± 4.2</td>
</tr>
<tr>
<td>(\text{SaO}_2), %</td>
<td>97.5 ± 1.2</td>
</tr>
<tr>
<td>(\text{CaO}_2), mmol/l</td>
<td>5.65 ± 0.27</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>29.9 ± 1.6</td>
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</tbody>
</table>

Values are means ± SE. C, saline control; AA, amino acid infusion.
creased leucine, isoleucine, valine, and lysine concentrations \((P < 0.05)\) and decreased threonine and methionine concentrations \((P < 0.05)\) by day 3. It should be noted, however, that methionine, serine, and phenylalanine were decreased by day 4 in the C group, and the decrease in phenylalanine concentrations in the C group was not observed in the AA group. There were no other significant individual means comparisons in the C group, nor were there any differences in fetal amino acid concentrations at baseline between the AA and C groups.

Several other nonessential amino acids were affected by amino acid infusion, as shown in Fig. 3.

**Insulin, IGF-I, and glucagon concentrations.** Fetal arterial insulin and IGF-I concentrations did not change. Values for both groups on day 4 are shown in Table 2. There was a significant increase in fetal arterial plasma glucagon concentrations in the AA group only \([36.9 \pm 5.8 \text{ day 0}, 51.2 \pm 9.6 \text{ day 2} (P < 0.05 \text{ vs. day 0}), 98.3 \pm 30.7 \text{ day 4} (P < 0.0005 \text{ vs. day 0}], \text{AA group}; 41.9 \pm 13.2 \text{ day 0}, 42.7 \pm 9.9 \text{ day 2}, 38.5 \pm 6.7 \text{ day 4}, \text{C group}; \text{pg/ml}]\).

**Arterial pH, blood gas, and hematocrit.** Daily values on days 0–4 of infusion are shown in Fig. 4. Fetal arterial pH decreased over time in the AA group but not in the C group \((P < 0.05)\). Fetal arterial \(\text{Pco}_2\) and \(\text{PO}_2\) did not vary between groups or by day of infusion. Fetal \(\text{SaO}_2\) decreased significantly over time in the AA group but not the C group \((P < 0.005)\). The fetal arterial blood \(\text{CaO}_2\) decreased in the AA group more than the C group \((P < 0.05)\), and the fetal hematocrit decreased more in the C group than the AA group \((P < 0.05)\).

**Fetal Metabolic Study.**

On day 4 of infusion, fetal substrate uptakes and fetal leucine flux rates were determined under steady-state conditions and compared between AA and C groups. Umbilical blood and plasma flows did not differ between groups, nor did glucose and lactate uptakes (Table 2). Fetal oxygen uptake tended to be higher in the AA group \((P = 0.06)\). No significant differences were found in umbilical uptake rates in either treatment group for any amino acids (Fig. 5). Calculated leucine flux rates to estimate protein metabolism are shown in Table 3. There was no difference in protein breakdown (Flux V), accretion (Flux VII), or synthesis (Flux VIII) between the AA and C groups; however, maternal amino acid infusion resulted in significantly higher leucine oxidation (Flux VI, \(P < 0.05)\).

**Placental and Fetal Measurements, Organ Weights, and Tissue Analysis.**

Placental and fetal measurements and organ weights did not change between the groups (Table 4). Due to the manner in which the pancreas was processed, accurate weights for this organ could not be obtained. There was not a significant difference in pancreatic \(\beta\)-cell area \((4.93 \pm 0.53 \text{ AA vs. } 4.58 \pm 0.66\% \text{ C})\) or \(\alpha\)-cell area \((2.27 \pm 0.34 \text{ AA vs. } 2.01 \pm 0.43\% \text{ C})\) between the two groups. The mRNA content of two muscle-specific ubiquitin ligases that mediate protein degradation, FBXO32 and RFP28, were not different in the AA group compared with the C group \((0.68 \pm 0.08 \text{ AA vs. } 1.00 \pm 0.22 \text{ C}, \text{FBXO32}, 1.92 \pm 0.74 \text{ AA vs. } 1.00 \pm 0.37 \text{ C, RFP28}; \text{arbitrary units})\). For four proteins involved in mRNA translation regulation, there were no differences in the ratio of phosphorylated to total amounts of each respective protein or in the total amount of each protein normalized to actin (Fig. 6).

**DISCUSSION.**

Previous clinical studies have indicated that high-protein supplementations during pregnancy paradoxically decrease birthweight, increase the risk of SGA birth, and might even increase perinatal mortality \((18, 26, 42)\). These studies provided 35–40 g/day protein to the pregnant mother, which is \(-0.8 \text{ g kg}^{-1} \text{ day}^{-1}\) \((18, 42)\), comparable to our supplementation of \(46.3 \pm 7.1 \text{ g/day} (0.9 \pm 0.1 \text{ g kg}^{-1} \text{ day}^{-1})\). The data presented in this study are the first to combine in vivo and in vitro approaches in fetal sheep to help define the responsible mechanisms. Our data show that prolonged, intravenous maternal amino acid infusion enriched with essential amino acids increased fetal plasma leucine, isoleucine, valine, and lysine concentrations as well as fetal amino acid oxidation rates. The increase in amino acid oxidation might indicate preferential amino acid utilization for energy production rather than protein....

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**Table 2. Fetal values on day 4 of infusion**

<table>
<thead>
<tr>
<th></th>
<th>C Group</th>
<th>AA Group</th>
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<tbody>
<tr>
<td>Glucose, mg/dl</td>
<td>14.7 ± 0.9</td>
<td>16.3 ± 0.7</td>
</tr>
<tr>
<td>Glucose uptake, mg·kg(^{-1})·min(^{-1})</td>
<td>3.6 ± 0.6</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>2.81 ± 0.39</td>
<td>3.36 ± 0.68</td>
</tr>
<tr>
<td>Lactate uptake, μmol·kg(^{-1})·min(^{-1})</td>
<td>13.3 ± 2.4</td>
<td>19.0 ± 2.2</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.12 ± 0.01</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>IGF-I, ng/ml</td>
<td>18.9 ± 4.3</td>
<td>22.7 ± 2.5</td>
</tr>
<tr>
<td>Oxygen uptake, μmol·kg(^{-1})·min(^{-1})</td>
<td>249.6 ± 29.2</td>
<td>326.7 ± 23.5†</td>
</tr>
<tr>
<td>Umbilical blood flow, ml·kg(^{-1})·min(^{-1})</td>
<td>142.7 ± 26.9</td>
<td>151.6 ± 16.8</td>
</tr>
<tr>
<td>Umbilical plasma flow, ml·kg(^{-1})·min(^{-1})</td>
<td>105.1 ± 21.0</td>
<td>106.7 ± 12.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. †: Day effect; ‡: Treatment effect; §: Treatment × day interaction \((P < 0.05)\).
accretion. There also was a decrease in fetal threonine concentrations and mild fetal acidosis and hypoxia, which are possible etiologies for poor fetal outcomes as a result of high-protein supplementation during human pregnancy.

The present study has added to the data obtained from previously performed maternal amino acid infusion experiments in sheep (14–16). First, we used more prolonged supplementation by infusing amino acids for 4 days and increased maternal amino acid concentrations within a physiological range (25–50%). Second, we added a saline control group to account for naturally occurring changes in maternal and fetal amino acid concentrations that might occur as gestation progresses. Third, we infused Trophamine, a parenteral amino acid mixture designed for use in the human neonate, and which is relatively enriched in essential amino acids compared with standard parenteral amino acid preparations, to preferentially increase maternal and fetal essential amino acid concentrations. Finally, we measured additional fetal metabolic parameters and molecular markers to help define how mechanistically maternal high-protein supplementation might detrimentally affect fetal growth.

Our study showed that, even though all maternal plasma amino acid concentrations were increased or remained unchanged after 4 days of supplementation, certain fetal amino acid concentrations were decreased by the amino acid infusion (threonine, glycine, tyrosine, cysteine, and aspartate). Our data are consistent with previous studies that acutely infused various mixtures of amino acids into pregnant sheep and demonstrated competitive inhibition across the placenta of coinfused amino acids. Balanced mixtures of essential and nonessential amino acids infused into pregnant sheep for 2, 3.5, and 12 h similarly decreased concentrations of the fetal essential amino acid threonine (14–16). These data are also consistent with human studies in which acute maternal amino acid infusions administered just prior to delivery failed to increase fetal threonine concentrations (36, 37). Only maternal infusion of threonine alone increases fetal threonine concentrations (33). Persistently lower fetal arterial plasma concentrations of the essential amino acid threonine might ultimately prevent increased fetal growth in amino acid-supplemented pregnancies. Any future maternal amino acid supplementation studies during pregnancy will require the use of an amino acid mixture...
that simultaneously increases fetal concentrations of all essential amino acids, particularly including increased concentrations of threonine relative to the concentration of the BCAAs (15). Studies are currently planned to test this with longer-term supplementation that includes groups that receive threonine-enriched amino acid mixtures as well as direct fetal amino acid infusions of specific and mixed amino acids.

Interestingly, net fetal amino acid uptake rates in the AA group were not different from saline controls after a 4-day infusion, even though fetal amino acid concentrations remained different. This finding differs from previous 2- and 3.5-h maternal infusion studies, which showed that fetal plasma amino acids were accompanied by similar changes in umbilical uptakes (15, 16). However, longer amino acid infusion resulted in persistently increased fetal amino acid concentrations despite amino acid uptakes that were unchanged at 12 h (14). Chronic increases in maternal amino acid concentrations vs. acute changes might result in adaptation in both placental transport and fetal amino acid disposal. Alternatively, measurements of fetal amino acid metabolic rates and umbilical uptake rates might not be precise enough to detect true differences in umbilical amino acid flux between the groups.

Stable isotope methodology using L-[1-13C]leucine to estimate fetal protein metabolism demonstrated that amino acid oxidation was increased. However, an increase in most fetal essential amino acids, including leucine, did not stimulate protein synthesis as has been shown in postnatal and adult animal models and humans (1, 9, 10, 23, 24). There are several reasons that might explain this finding. First, a decrease in fetal threonine concentrations as mentioned above could negatively influence protein synthesis rates. Studies in piglets fed a threonine-deficient diet for 2 wk showed less weight gain and reduced protein synthesis rates in skeletal muscle and liver (44). Second, duration and route of the amino acid infusion also affect protein synthesis rates. Direct infusion of amino acids into the fetus will avoid competitive inhibition at the placenta among amino acids with similar properties. For example, when balanced mixtures of amino acids have been infused directly into the late-gestation sheep fetus for 2 and 4 h, fetal plasma amino acid concentrations, including threonine, protein synthesis rates, and accretion rates increased (7, 19). Importantly, insulin concentrations also were increased as a result of such direct amino acid infusions, unlike in the present study (7). Third, fetal insulin and IGF-I concentrations did not change as a result of supplementation in our study, and previous work suggests that protein synthesis rates are maximal only when amino acids are present with a concurrent increase in insulin or IGF-I concentrations (30, 31, 43). Consistent with these data, we recently demonstrated that in the late-gestation sheep fetus acute fetal amino acid infusion without an increase in fetal insulin concentrations did not upregulate translation initiation factors known to stimulate mRNA translation, such as 4E-BP1 and p70S6k (4). In the present study, we measured those proteins in addition to eIF2α and eEF2, all of which have been shown to be regulated by amino acids (13, 34). Consistent with our measurement of whole fetal protein synthesis, we did not find any changes in these regulatory proteins as a result of the 4-day maternal amino acid infusion. Finally, there are differences in the effects of hormone and nutrient stimulation on protein synthesis among the fetus, neonate, and adult (17, 35, 46, 47). These developmental differences might explain the lack of amino acid effect on protein synthesis observed in the present study compared with other studies performed during later stages in development. It should also be noted that our study was underpowered in order to detect a statistically significant increase in protein accretion of 18%.

There was evidence of fetal intolerance to amino acids in the AA group with the development of mild fetal acidosis and hypoxia. However, if amino acid infusions were given over several weeks during gestation, the acidosis and hypoxia might worsen and explain poor perinatal outcomes observed when high-protein supplementation was used in human pregnancies. One possible cause of these findings is the significant increase in fetal leucine oxidation that occurred. Increases in plasma amino acid concentrations such as leucine and phenylalanine

<table>
<thead>
<tr>
<th>Flux No.</th>
<th>Flux Description</th>
<th>Equation</th>
<th>C Group</th>
<th>AA Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fetal plasma leucine disposal rate</td>
<td>[100 × ln(leucine MPE)–Inf]</td>
<td>8.09±0.63</td>
<td>9.22±0.73</td>
</tr>
<tr>
<td>II</td>
<td>Leucine flux into the placenta from fetal blood</td>
<td>DR × (Leuumb)</td>
<td>1.20±0.17</td>
<td>1.92±0.59</td>
</tr>
<tr>
<td>III</td>
<td>Leucine flux into fetal tissues from fetal blood</td>
<td>DR × (1–Leuumb)</td>
<td>6.89±0.76</td>
<td>7.30±0.93</td>
</tr>
<tr>
<td>IV</td>
<td>Leucine flux into fetal blood from placenta</td>
<td>Lο–Rf,p + flux II</td>
<td>3.47±0.25</td>
<td>5.02±0.74</td>
</tr>
<tr>
<td>V</td>
<td>Leucine flux from fetal protein breakdown</td>
<td>DR–flux IV</td>
<td>4.62±0.74</td>
<td>4.20±0.95</td>
</tr>
<tr>
<td>VI</td>
<td>Leucine oxidation rate</td>
<td>DR–flux IV</td>
<td>4.62±0.74</td>
<td>4.20±0.95</td>
</tr>
<tr>
<td>VII</td>
<td>Net fetal protein accretion rate</td>
<td>[13CO2]g–e × UBF/MPE KICRf,p–flux VI</td>
<td>1.84±0.24</td>
<td>2.63±0.16*</td>
</tr>
<tr>
<td>VIII</td>
<td>Net leucine umbilical uptake rate</td>
<td>flux VII + flux V</td>
<td>5.47±0.89</td>
<td>5.19±0.97</td>
</tr>
</tbody>
</table>

Results are given as μmol·min–1·kg–1 ± SE. Inf, [1–13C]leucine infusion rate; MPE, molar percent enrichment; DR, disposal rate; Leuumb, fraction of leucine tracer infusion rate taken up by the placenta; 13C0–Rf,p, net flux of leucine into the fetus from the placenta; [13CO2]g–e, umbilical venous-arterial differences in whole blood 13CO2; UBF, umbilical blood flow; KIC, α-ketoisocaproyl acid. *P < 0.05.
will promote their oxidation (19). A decrease in SaO2 and CaO2 with a trend toward increased fetal oxygen consumption suggests that overall fetal substrate oxidation rates were increased to handle the increase in substrate load. Systemic fetal hypoxia might inhibit fetal protein synthesis (28). Whether due to low threonine, insulin, or IGF-I concentrations or a developmental insensitivity of the fetus, it is possible that without fetal utilization of leucine for protein synthesis the excess leucine is shunted into oxidation. Conversely, leucine oxidation might represent the preferred metabolic pathway for exogenously increased fetal amino acids relative to protein synthesis during fetal life. The extent of placental metabolism of amino acids, lactate, oxygen, carbon dioxide, and glucose under these conditions and the effect of such metabolism on the fetus warrant further investigation.

In the present study, we found a progressive increase in fetal arterial glucagon concentrations in the AA group. Previously a 3.5-h maternal amino acid infusion did not affect fetal glucagon concentrations (16). These data are consistent with our data in that we observed an increase in fetal glucagon concentrations that was progressive; concentrations on day 2 were not as high as on day 4. To extend these findings we undertook a morphological analysis of the fetal pancreas, but we did not see an increase in the amount of pancreatic α-cells, which secrete glucagon; nor did we find a change in the amount of pancreatic β-cells, which was not surprising given the lack of change in fetal insulin concentrations. Interestingly, short-term administration of amino acids, either into the pregnant mother or directly into the fetus, acutely increases fetal insulin concentrations (4, 7, 16); however, our data indicate that this response is not sustained. A detailed understanding of the effects of a prolonged amino acid infusion on fetal insulin and glucagon requires further study.

In summary, prolonged maternal amino acid infusion during late gestation in pregnant sheep resulted in decreased fetal plasma threonine concentrations, acidosis, and hypoxia. Furthermore, this infusion increased fetal leucine oxidation. All of these findings are possible etiologies for the previously observed poor fetal outcomes following high-protein supplementation during pregnancy. Future studies are warranted to test whether an even longer maternal amino acid infusion to the pregnant ewe, or a prolonged and direct amino acid infusion to the fetus, might further increase fetal oxygen consumption to support increased rates of amino acid oxidation. It is also important to investigate the complex interactions between amino acids and insulin or amino acids and other nutrients and hormones and their role in promoting fetal growth when maternal pregnancy supplementations are considered. Concurrently, continued attempts at designing an optimal maternal amino acid supplement to promote more uniform increases in fetal plasma amino acids are worthwhile, especially to find ways to improve fetal growth in IUGR pregnancies.

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