Increased amino acid supply potentiates glucose-stimulated insulin secretion but does not increase β-cell mass in fetal sheep

Monika M. Gadhia,1 Anne M. Maliszewski,1 Meghan C. O’Meara,1 Stephanie R. Thorn,1
Jinny R. Lavezzi,1 Sean W. Limesand,2 William W. Hay, Jr.,1 Laura D. Brown,1,3 and Paul J. Rozance1,3

1Perinatal Research Center, Department of Pediatrics, University of Colorado Denver School of Medicine, Aurora, Colorado; 2Department of Animal Sciences, University of Arizona, Tucson, Arizona; and 3Center for Women’s Health Research, University of Colorado Denver School of Medicine, Aurora, Colorado

Submitted 27 July 2012; accepted in final form 29 November 2012

Increased amino acid supply potentiates glucose-stimulated insulin secretion but does not increase β-cell mass in fetal sheep. Am J Physiol Endocrinol Metab 304: E352–E362, 2013. First published December 4, 2012; doi:10.1152/ajpendo.00377.2012.—Amino acids and glucose acutely stimulate fetal insulin secretion. In isolated adult pancreatic islets, amino acids potentiates glucose-stimulated insulin secretion (GSIS), but whether amino acids have the same effect in the fetus is unknown. Furthermore, we tested the effects of increased fetal amino acid supply on GSIS and morphology of the pancreas. We hypothesized that increasing fetal amino acid supply would potentiate GSIS. Singleton fetal sheep received a direct intravenous infusion of an amino acid mixture (AA) or saline (CON) for 10–14 days during late gestation to target a 25–50% increase in fetal branched-chain amino acids (BCAA). Early-phase GSIS increased 150% in the AA group (P < 0.01), and this difference was sustained for the duration of the hyperglycemic clamp (105 min) (P < 0.05). Glucose-potentiated arginine-stimulated insulin secretion (ASIS), pancreatic insulin content, and pancreatic glucagon content were similar between groups. β-Cell mass and area were unchanged between groups. Baseline and arginine-stimulated glucagon concentrations were increased in the AA group (P < 0.05). Pancreatic α-cell mass and area were unchanged. Fetal and pancreatic weights were similar. We conclude that increasing fetal amino acid supply for 10 to 14 days during pathological growth restriction may potentiate fetal GSIS, but does not increase β-cell mass.


Address for correspondence: P. J. Rozance, Dept. of Pediatrics, Perinatal Research Center, 13243 E. 23rd Ave., MS F441, Aurora, CO 80045 (e-mail: Paul.rozance@ucdenver.edu).

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amino acid infusion would be present in response to an acute amino acid infusion.

MATERIALS AND METHODS

Animal Care and Surgical Procedure

Studies were conducted in pregnant Columbia-Rambouillet sheep, each carrying a singleton late-gestation fetus (term = 147 days gestation). Surgery was performed to place fetal catheters into the abdominal aorta and femoral veins and maternal catheters into the femoral artery and vein on 117 ± 3 days gestation, as described previously (38). This gestational age (and thus duration of amino acid infusion) was chosen to optimize fetal size for catheter placement and maintenance of catheter patency for infusions and fetal blood sampling. Animals were allowed to recover for ≥5 days prior to randomization into treatment groups described below. All animal procedures were in compliance with the 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 Institutional Animal Care and Use Committee.

Experimental Design

Study no. 1: chronic (10–14 days) amino acid-potentiated GSIS. Twenty-two animals were randomly assigned to an amino acid infusion group (AA) or control group (CON). The AA fetuses received a continuous intravenous infusion of Trophamine, an amino acid mixture that is relatively enriched with essential amino acids (University of Colorado Central Admixture Pharmacy, Aurora, CO). The rate of infusion was adjusted daily to achieve a 25–50% increase in fetal sodium delivery from Trophamine in the AA group. Prior to the start of the infusion (day 1), fetal arterial plasma was sampled for insulin, glucose, lactate, and amino acid concentrations, and fetal arterial whole blood was sampled for pH, partial pressure of carbon dioxide and oxygen (Paco2 and Paco3, respectively), blood hemoglobin-O2 saturation (SaO2), blood O2 content, and hematocrit. During the infusion period, fetal arterial plasma was sampled daily for glucose and lactate concentrations. On days 2, 5, 8, and 11 and the day of the GSIS study (days 10–14), fetal arterial blood was sampled for plasma amino acid concentrations, blood gas measurements, and plasma insulin concentrations. On the day of the GSIS study, fetal arterial plasma was also sampled for insulin-like growth factor-1 (IGF-1), norepinephrine, cortisol, and glucagon concentrations. Maternal arterial blood was sampled prior to the start of the infusion and on day 8 and the day of the GSIS study for blood gas measurements, plasma glucose and lactate concentrations, and plasma amino acid concentrations (Fig. 1A). Six fetuses (3 in the intervention group and 3 in the control group) did not survive to measurement of in vivo GSIS and were excluded from analysis, leaving eight animals in each group. There was one fetal demise in the AA group after measurement of insulin secretion, but it was prior to collection of the pancreas and other fetal organs. In one control fetus, the pancreas was not adequately dissected free of associated tissue. Therefore, seven pancreases from each group were analyzed.

Study no. 2: acute (3 h) amino acid-potentiated GSIS. To determine whether changes seen in fetal GSIS after a chronic amino acid infusion would be present in response to an acute amino acid infusion, a second in vivo study was conducted in a separate set of 10 pregnant Columbia-Rambouillet sheep, each carrying a singleton late-gestation fetus (Fig. 1B). The animals were randomly assigned to an AA group (n = 5) or CON group (n = 5). After a minimum 5-day recovery from surgery, the AA fetuses received a 190-min direct intravenous infusion of Trophamine, and the CON group received a fetal intravenous infusion of 0.9% NaCl at a rate adjusted to match the rate of sodium delivery from Trophamine in the AA group.

Insulin Secretion Studies

Study no. 1: chronic (10–14 days) amino acid-potentiated GSIS. A square-wave hyperglycemic clamp was used to determine fetal GSIS, as described previously (Fig. 1C) (29, 30). A continuous transfusion of maternal blood into the fetus was started 45 min prior to baseline sampling and maintained for the duration of the study to compensate for blood collection. All sample times were relative to the start of the fetal glucose infusion (time 0). Baseline plasma glucose and insulin concentrations were determined at −60, −45, −30, and −15 min. The hyperglycemic clamp was initiated with a 33% dextrose (wt/vol in saline) bolus (825 mg of glucose) into the fetus, followed by a variable infusion of 33% dextrose (wt/vol in saline) that was adjusted to maintain fetal arterial plasma concentration at 45 mg/dl, which elicits 90% maximal insulin concentrations in fetal sheep (15). The dextrose infusion was held constant beginning at minute 45. Fetal arterial plasma samples were collected at 5, 10, 15, 20, 30, 45, 60, 75, 90, and 105 min for glucose and insulin measurement. To measure glucose-potentiated arginine-stimulated insulin secretion (ASIS), a bolus of arginine (0.5 mmol/kg estimated fetal weight) in 5 ml of 0.4 mol/l sodium acetate and 0.9% NaCl was injected over 4 min into the fetus, followed by a variable infusion of 33% dextrose (wt/vol in saline) that was adjusted to maintain fetal arterial plasma concentration at 45 mg/dl, which elicits 90% maximal insulin concentrations in fetal sheep (15). The dextrose infusion was held constant beginning at minute 45. Fetal arterial plasma samples were collected at 5, 10, 15, 20, 30, 45, 60, 75, 90, and 105 min for glucose and insulin measurement. To measure glucose-potentiated arginine-stimulated insulin secretion (ASIS), a bolus of arginine (0.5 mmol/kg estimated fetal weight) in 5 ml of 0.4 mol/l sodium acetate and 0.9% NaCl was injected over 4 min into the fetal circulation beginning at 110 min. Fetal arterial plasma samples were collected 5, 10, 20, and 30 min after the start of the arginine infusion for measurement of glucose, insulin, and glucagon concentrations. Fetal arterial plasma glucagon concentrations were also measured at 90 and 105 min. The chronic amino acid and saline infusions were continued through the GSIS study and until euthanasia.
was administered immediately prior to autopsy and tissue collection. Steady-state hyperglycemic clamp insulin concentrations were defined as the average of insulin concentrations between minutes 60 and 105 of the hyperglycemic clamp.

Study no. 2: acute (3 h) amino acid-potentiated GSIS. A transfusion of maternal blood into the fetus was started 30 min prior to baseline sampling and maintained for the duration of the study to compensate for blood collection. Amino acid or saline infusions were initiated 100 min prior to the square-wave hyperglycemic clamp that was performed as described above. Fetal plasma arterial insulin and glucose were measured 15, 10, and 5 min prior to the initiation of the amino acid or saline infusion, as well as 15, 10, and 5 min prior to the initiation of the glucose clamp, and then at minutes 5, 10, 15, 20, 30, 45, 60, 75, and 90 after the start of the clamp. Amino acids also were measured during the basal and hyperaminoacidic periods.

Biochemical analysis. Blood gases, pH, hematocrit, amino acids, glucose, lactate, insulin, cortisol, glucagon, IGFI, and norepinephrine concentrations were measured as described previously (30).

Tissue collection. Tissue was collected from the chronic (10–14 days) experimental infusion animals only and occurred 18–20 h after the insulin secretion studies. Maternal sheep and their fetuses were euthanized (intravenous pentobarbital sodium, 4,680 and 940 mg given to the mother and fetus, respectively) and tissues collected under in vivo study conditions. The fetus was removed, blotted dry, and weighed as described previously. The fetal pancreas was dissected free, weighed, and divided (9, 27). The hepatic portion of the pancreas was snap-frozen in liquid nitrogen and stored at −80°C for subsequent analysis. The splenic portion of the pancreas was fixed in 4% (wt/vol) paraformaldehyde in PBS overnight. One portion was embedded in paraffin, and a second portion was allowed to equilibrate with 30% sucrose (wt/vol) for 24 h. This portion was then placed in a 1:1 mixture of 30% sucrose and Optimal Cutting Temperature Freeze Media (OCT) for 24 h and then placed in a cryomold with OCT medium and stored at −80°C. Portions of the right lobe of the fetal liver and the fetal biceps femoris skeletal muscle were snap-frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

Analysis of the Fetal Pancreas

Measurement of pancreatic insulin and glucagon content. As described previously (41), frozen fetal pancreatic tissue was pulverized in liquid nitrogen. Pancreatic insulin and glucagon were acid ethanol extracted from ∼30 mg of tissue with 1 ml of 1 mol/l HCl in 70% ethanol at −20°C. Three independent samples per animal were analyzed and averaged to obtain a single value for insulin and glucagon protein content per fetus (41). The concentrations of insulin and glucagon were measured by ELISA and RIA, respectively.

Measurement of pancreatic mRNA. Total RNA was extracted from pulverized pancreas (100 mg) and reverse transcribed into complimentary DNA, as described previously (40). Real-time quantitative (q)PCR assays for PDX-1 (accession no. JF728303), GLUT2 (accession no. HQ585494), S15 (accession no. AY949774), and ß-actin (accession no. U39357) were performed as described previously (8).

Glucokinase and GAPDH (accession no. NM00190390) were identified as follows. PCR primers were designed for sheep sequences: glucokinase, forward TTT CCT GTG AGG CAC GAC GAC, reverse CTT GCT CAG GAT GGT GTA GA; GAPDH, forward TGG AGG AGC TTA TGA CCA CTG, reverse TAG AAG CAG GGA TGA TGT TCT. PCR products were cloned as described previously and sequenced to confirm identity (41).

Specificity of the primers for all genes was confirmed with agarose gel electrophoresis, melting curve analysis, and sequencing of qPCR products. Samples were analyzed in triplicate for each gene and the standard deviation method of relative quantification used (54). Genes of interest were normalized to an average of three housekeeping genes (S15, ß-actin, and GAPDH) that were not different between groups. Results are presented in arbitrary units as fold change relative to the CON group.

Histology of the Fetal Pancreas

Morphology of the fetal pancreas was analyzed and quantified by an individual masked to animal treatment group assignments, as described previously (27, 41). Briefly, tissue sections were cut from paraffin-embedded pancreases for histological and morphometric evaluation at a minimum of 70-µm intervals. Sections were dewaxed in xylene and then hydrated with a series of descending ethanol washes to water. Antigen retrieval was performed by microwaving sections in 10 mmol/l citric acid buffer (pH 6.0) to a temperature of 85–95°C for 20 min as well as a 10-min room temperature incubation in Triton X-100 (0.1%, vol/vol) in PBS. Sections were blocked with 1.5% normal donkey serum in PBS (vol/vol) for 30 min. Guinea pig anti-porcine insulin (1:250; Dako), mouse monoclonal anti-human glucagon (1:500; Sigma), rabbit anti-human somatostatin (1:500; Dako), and rabbit anti-human pancreatic polypeptide (1:500; Dako) were diluted in blocking buffer and sections incubated at 4°C overnight with these primary antibodies. The following day, immunocomplexes were detected with affinity-purified secondary antiserum (Jackson ImmunoResearch Laboratories): anti-guinea pig IgG conjugated to AMCA (blue), Alexa flour 594 goat anti-mouse IgG (red), and CY2 goat anti-rabbit IgG (green), all diluted 1:500 in blocking buffer for 60 min at room temperature.

β-Cell and α-cell area and mass. β-Cell and α-cell area and mass were quantified as described previously (27, 41). Fluorescent images were visualized on an Olympus BX51 systems microscope and captured digitally with a Pixera 600CL camera. Morphometric analysis was performed using Image Pro 4.5 software (Media Cybernetics, Silver Spring, MD). Four pancreatic sections were used for each animal. Insulin+ and glucagon+ areas were determined for 20 fields of view (FOV) on each pancreatic section. FOVs were selected randomly and represent all portions of the pancreatic section (1.1 × 0.75 cm). Insulin+ and glucagon+ areas were expressed as a percentage of total pancreas area evaluated within each FOV. The percent areas for all FOVs obtained from one section were averaged to provide the mean percent area for each section. Then, the percent areas for all sections from each animal were averaged to provide the mean percent area for each animal. This average was used for summary and comparative statistics. β-Cell and α-cell mass were calculated as the product of the relative β-cell and α-cell area, respectively, and the weight of the pancreas.

Pancreatic islet vessel density. Tissue sections were cut from cryo-preserved pancreases at a minimum of 150-µm intervals. Sections were then heated to 37°C for 30 min and rehydrated with water. Antigen retrieval was performed as described above, but without the Triton X-100 incubation. Sections were blocked with 0.5% NEN Block (Perkin-Elmer, Waltham, MA) for 60 min, followed by addition of the same primary antibodies noted above diluted in 1% BSA. Additionally, for quantification of pancreatic vascularity, FITC-conjugated Griffonia simplicifolia (GS-I) agglutinin (15 µg/ml; Vector Laboratories, Burlingame, CA) was added to the mixture of primary antibodies (green). After an overnight incubation at 4°C, samples were washed in PBS, and secondary antibodies, also suspended in 1% BSA, were applied for 1 h. Secondary antibodies were as described above with the exception of CY2 goat anti-rabbit IgG, which was replaced with Texas Red goat anti-rabbit (1:500; Jackson Immuno-Research Laboratories). Following this incubation, samples were washed in PBS three times and mounted with Fluormount (Sigma-Aldrich, St. Louis, MO). Fluorescent images were visualized and captured as described above. Morphometric analysis was performed using Image Pro 4.5 software. Pancreatic islets were defined as clusters of endocrine cells with an area of ≥500 µm2. Eight images per section for four sections were evaluated for an average of 22.9 ± 0.4 islets/animal.
Fetal Liver and Skeletal Muscle mRNA Total RNA was extracted from pulverized liver and skeletal muscle (100 mg) and reverse transcribed into complementary DNA, as described previously (49, 50). Real-time qPCR assays for PGC1α (peroxisome proliferator-activated receptor-γ coactivator-1), PFKL liver isoform (phosphofructokinase), FAS (fatty acid synthase), SREBP1C (sterol regulatory element-binding protein 1C), ERRα (estrogen-related receptor-α), CYTOC (cytochrome c), IR-A (insulin receptor A isoform), IR-B (insulin receptor B isoform), and IHS were performed as described previously (49, 50).

Real-time qPCR primers were developed for the following sheep sequences, as described previously (41): NRF1 (nuclear regulatory factor 1), forward ACGGAAAGTCCTCATGTGTT, reverse ATAGCTTGGCTGACACTGG; NRF2 (nuclear regulatory factor 2), forward GCATTGGCCAGACATTCG, reverse GCATTGAAGACTGGGCTCT; and PFKM muscle isoform, forward TGGACAGCAGGAAAGCAGG, reverse TGGACCCATTTAATGAGC. PCR products were sequenced to confirm identity. Genes of interest were normalized to the average of IHS and S15 for liver samples and S15, β-actin, and GAPDH for skeletal muscle samples, which were not different between groups. Results are presented in arbitrary units as fold change relative to expression in the CON group for each tissue.

Statistical Analysis

The statistical analysis was performed using SAS version 9.2 (SAS Institute). Results are expressed as means ± SE. For repeated measurements, a mixed-model ANOVA was performed to determine effects of treatment group (AA or CON), time, and treatment-time interactions. A term was included to account for repeat measurements made in the same animal. When the overall ANOVA was significant, posttest comparisons were made using Fisher’s least squares difference. Measurements made once were compared by Student’s t-test or Mann-Whitney U-test (for nonparametric data). P values < 0.05 were considered significant. The chronic and acute AA studies were analyzed separately.

RESULTS

Maternal Parameters During 10–14 Days of Amino Acid Infusion

Maternal weight (CON: 56.0 ± 1.3 kg; AA: 52.7 ± 0.7 kg), feed intake (CON: 1.39 ± 0.03 kg/day; AA: 1.51 ± 0.04 kg/day), and water intake (CON: 4.79 ± 0.70 l/day; AA: 5.45 ± 0.16 l/day) were not different between AA and CON groups. There was no difference between groups for maternal glucose, lactate, pH, PaCO2, Pao2, blood O2 content, Sao2, or hematocrit (Table 1). Although several essential amino acids appeared to increase during the experimental infusion, the only statistically significant increase in AA concentrations during the experimental infusion (P < 0.05).
Fig. 3. Fetal arterial AA concentrations in the chronic AA study. Gray bars represent baseline CON (n = 8) AA concentrations, and black bars represent the CON average AA concentrations throughout the experimental infusion beginning on day 2. White striped bars represent baseline AA concentrations (n = 8), and open bars represent the average AA concentrations throughout the experimental infusion beginning on day 2. Values expressed as means ± SE. *Significant increase in AA concentrations during the experimental infusion (P < 0.05). Glutamine concentrations increased significantly (P < 0.05) on day 2 in the AA group and then returned to baseline.

significant differences between groups at the end of the infusion period were found for taurine and tryptophan (P < 0.05; Fig. 2).

Fetal Metabolites, Blood Gases, and Hormones During 10–14 Days of Amino Acid infusion

Durations of experimental infusions were the same in both groups (CON: 12.4 ± 0.5 days; AA: 12.6 ± 1.2 days). The amino acid infusion needed to maintain a 25–50% increase in branched-chain amino acid concentrations increased from an initial rate of 4.3 ± 0.2 to 10.6 ± 0.1 g/day at the end of the infusion in the AA group. Most essential and some nonessential amino acids were higher in the AA fetuses compared with CON (Fig. 3). For the amino acids that were significantly higher in the AA group, most were increased significantly by day 2 of the infusion and remained increased.

Fetal arterial plasma glucose concentrations decreased during the experimental infusion period in the AA group but not the CON group (P < 0.05; Fig. 4A). Fetal arterial pH, PaCO₂, PaO₂, Sao₂, blood O₂ content, and hematocrit were similar between groups throughout the experimental infusion (Table 1). Fetal arterial lactate concentrations were variably increased toward the middle of the experimental infusion period in the AA group (P < 0.05) but normalized by the end (Fig. 4B). Fetal arterial insulin concentrations during the experimental infusion period were similar in both groups (Fig. 4C). Fetal arterial plasma IGF-I, cortisol, and norepinephrine were measured at the end of the experimental infusion and were similar between groups. Glucagon concentrations at the end of the experimental infusion were significantly higher in the AA group (P < 0.05; Table 1).

In Vivo insulin Secretion Following 10–14 Days of Amino Acid infusion

During the fetal hyperglycemic clamp, early-phase insulin concentrations were >150% higher in AA vs. CON (P < 0.01), and this difference was sustained for the duration of the hyperglycemic clamp (Fig. 5). Glucose concentrations during the clamp were similar, as was the glucose infusion rate required to achieve these concentrations (CON: 12.8 ± 0.1 mg·min⁻¹·kg⁻¹; AA: 12.7 ± 0.1 AA mg·min⁻¹·kg⁻¹; Fig. 5). There were no differences in ASIS (maximum arterial plasma insulin concentrations: CON, 2.81 ± 1.89 ng/ml; AA, 2.96 ± 1.39 ng/ml), but glucagon concentrations following the arginine bolus were increased in the AA group (maximum concentrations: CON, 135.44 ± 16.84 pg/ml; AA, 279.20 ± 45.45 pg/ml) (P < 0.05).

One fetus in the AA group had a very high insulin response to the hyperglycemic clamp. Insulin concentrations were >10 times that of the other AA animals (a 10-min insulin concentration of 21.3 ng/ml). No other measured variables of this animal were unique. Because these insulin concentrations significantly increased the overall variability, they were excluded from the statistical analysis of GSIS.

![Fig. 4](http://ajpendo.physiology.org/)
Peripheral Insulin Sensitivity

To determine whether decreased insulin sensitivity in the AA fetuses might have contributed to increased insulin secretion, we plotted steady-state hyperglycemic clamp insulin concentrations vs. an estimate of fetal insulin sensitivity, the inverse of the product of basal insulin and glucose concentrations \([1/(\text{insulin}_{\text{basal}}) \times \text{[glucose]_{basal}}]\) [proportional to the HOMA-IS calculation (16, 46)] (Fig. 6). Comparison of these points with a plot of the mean and SE of historical data obtained in late-gestation fetal sheep \(n = 43\) (15) demonstrates that the AA group has higher hyperglycemic clamp insulin concentrations than the CON group independent of insulin sensitivity. We also quantified mRNA of genes encoding the insulin receptor and other insulin-sensitive genes from fetal skeletal muscle and liver. CYTOC expression was increased in AA fetal skeletal muscle. No differences were found in any of the other genes measured (Table 4). Finally, we quantified the protein concentrations of the insulin receptor and the phosphorylation of several insulin-responsive intracellular signaling protein kinases and their targets in fetal skeletal muscle and liver. The amount of insulin receptor and phosphorylation of proximal insulin signaling kinases Akt and MAPK were the same between groups (Fig. 7).

Acute (3 h) Amino Acid-Potentiated insulin Secretion Study

Given the lack of changes in the structure of the pancreas or in key genes regulating GSIS, we next determined whether GSIS would be potentiated following an acute amino acid infusion. In a separate cohort of singleton fetuses, we measured GSIS following a 100-min direct fetal amino acid infusion, which raised fetal arterial plasma amino acid concentrations to values similar to those seen at the end of the chronic infusion.

Table 3. Fetal pancreatic characteristics

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin content, μg/g</td>
<td>23.28 ± 1.89</td>
<td>21.52 ± 1.32</td>
</tr>
<tr>
<td>Glucagon content, μg/g</td>
<td>2.09 ± 0.15</td>
<td>2.50 ± 0.13</td>
</tr>
<tr>
<td>β-Cell mass, g</td>
<td>0.15 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>β-Cell area, %</td>
<td>4.27 ± 0.30</td>
<td>4.49 ± 0.25</td>
</tr>
<tr>
<td>α-Cell mass, g</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>α-Cell area, %</td>
<td>2.24 ± 0.39</td>
<td>2.58 ± 0.26</td>
</tr>
<tr>
<td>Glucokinase mRNA (ratio)</td>
<td>1.00 ± 0.21</td>
<td>0.56 ± 0.60</td>
</tr>
<tr>
<td>GLUT2 mRNA (ratio)</td>
<td>1.00 ± 0.12</td>
<td>1.21 ± 0.07</td>
</tr>
<tr>
<td>PDX-1 mRNA (ratio)</td>
<td>1.00 ± 0.06</td>
<td>0.84 ± 0.04</td>
</tr>
<tr>
<td>Islet vascular density (%)</td>
<td>10.86 ± 0.01</td>
<td>9.52 ± 0.01</td>
</tr>
<tr>
<td>Islet area, μm²</td>
<td>1,800.50 ± 171.00</td>
<td>1,602.40 ± 134.20</td>
</tr>
</tbody>
</table>

Values are means ± SE; CON (\(n = 7\)) and AA (\(n = 7\)).
DISCUSSION

Amino acids acutely (hours) stimulate fetal insulin secretion (7, 11, 14, 26). Furthermore, in isolated adult pancreatic islets and insulin-secreting β-cell lines, glucose and amino acids act synergistically to increase insulin secretion (13, 31). We used a direct fetal amino acid infusion for both 10–14 days (chronic) and 3 h (acute) in late-gestation pregnant sheep to test the hypothesis that glucose and amino acids would act synergistically to increase in vivo fetal insulin secretion. The novel finding of this study is that a direct amino acid infusion into a fetus (which already receives a continuous supply of amino acids from the placenta), when administered both acutely (3 h) and chronically (10–14 days), potentiated GSIS. Amino acids might potentiate glucose-stimulated fetal insulin secretion by a variety of mechanisms, including direct effects on the pancreatic islet or by indirect effects (34, 51). We did not find associated changes in pancreatic insulin content, β-cell mass, pancreatic morphology, including changes in islet vascularity, or α-cell mass as a result of chronic amino acid supplementation or evidence of peripheral insulin resistance.

Our goal was to test whether amino acids and glucose would act synergistically in vivo in the fetus to increase insulin secretion. We also sought to determine whether changes in pancreatic morphology (increased β-cell mass and islet vascularity) and increases in glucagon secretion and α-cell mass might contribute to amino acid-potentiated GSIS. The most compelling evidence for a causative role of amino acid supply regulating fetal β-cell mass and function comes from pregnant rats fed a low-protein diet whose fetuses have decreased islet insulin secretion, vascularity, and β-cell mass (4, 5, 10, 48). However, fetal amino acid delivery has not been measured directly in the low-protein diet model of IUGR, and there have been no studies in normal pregnancies with normally growing fetuses of chronic increases in fetal amino acid supply on fetal GSIS, islet vascularity, or β-cell mass. However, such studies are important to perform because the human trial that found a detrimental effect of protein supplementation to prevent IUGR was performed in women who, for the most part, had normal pregnancies and no IUGR (they were selected for inclusion in the study because they were at risk of IUGR) (42). Therefore, we used normally growing fetuses in normal sheep pregnancies to quantify the effects of controlled amounts of fetal amino acid delivery on fetal pancreatic function and morphology. Although our results demonstrate a role for increased amino acid supply in increasing fetal GSIS, we did not find evidence for a role in regulating fetal pancreatic β-cell or α-cell mass or islet vascularity. However, we did find increased fetal glucagon secretion, which might contribute to the potentiation of fetal GSIS by amino acids.

Table 4. mRNA concentrations of insulin-sensitive genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>CON</th>
<th>AA</th>
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<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR-A</td>
<td>1.00 ± 0.06</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>IR-B</td>
<td>1.00 ± 0.07</td>
<td>1.14 ± 0.10</td>
</tr>
<tr>
<td>PGC1A</td>
<td>1.00 ± 0.17</td>
<td>1.25 ± 0.18</td>
</tr>
<tr>
<td>PFK (liver isoform)</td>
<td>1.00 ± 0.11</td>
<td>1.24 ± 0.21</td>
</tr>
<tr>
<td>CYTOC</td>
<td>1.00 ± 0.04</td>
<td>1.12 ± 0.07</td>
</tr>
<tr>
<td>FAS</td>
<td>1.00 ± 0.07</td>
<td>0.98 ± 0.10</td>
</tr>
<tr>
<td>SREBP1C</td>
<td>1.00 ± 0.13</td>
<td>1.80 ± 0.51</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IR-A</td>
<td>1.00 ± 0.08</td>
<td>0.90 ± 0.10</td>
</tr>
<tr>
<td>IR-B</td>
<td>1.00 ± 0.07</td>
<td>0.87 ± 0.12</td>
</tr>
<tr>
<td>PGC1A</td>
<td>1.00 ± 0.11</td>
<td>0.96 ± 0.10</td>
</tr>
<tr>
<td>PFK (muscle isoform)</td>
<td>1.00 ± 0.08</td>
<td>0.95 ± 0.09</td>
</tr>
</tbody>
</table>
| CYTOC      | 1.00 ± 0.08| 1.30 ± 0.11+
| NRF1       | 1.00 ± 0.09| 0.86 ± 0.10|
| NRF2       | 1.00 ± 0.04| 1.10 ± 0.09|
| ERRB       | 1.00 ± 0.09| 1.11 ± 0.14|

Values are means ± SE; CON (n = 8) and AA (n = 7). IR-A, insulin receptor isoform A; IR-B, insulin receptor isoform B; PGC1A, PPARY coactivator-1α; PFK, phosphofructokinase; CYTOC, cytochrome c; FAS, fatty acid synthase; SREBP1C, sterol response element-binding protein-1; NRF1, nuclear regulatory factor 1; NRF2, nuclear regulatory factor 2; ERRA, estrogen-related receptor-α. mRNA concentrations are relative to the average of 3 reference genes for skeletal muscle samples (β-actin, GAPDH, and S15) and 2 reference genes for liver samples (S15 and IBS), which were not different between groups. Results are presented relative to expression in the CON group. *P < 0.05 between CON and AA.
Fig. 7. Insulin receptor and insulin-responsive proteins in fetal skeletal muscle and liver. A: Western blot analysis of protein expression of the insulin receptor and the total and phosphorylated (phospho) mitogen-activated protein kinase (MAPK), Akt, p70 S6 protein kinase (p70 S6K), ribosomal protein S6 (rp S6), and actin from CON (n = 8) and AA (n = 7) fetal skeletal muscle and liver. B: quantification of the Western blot analysis relative to actin. Values are means ± SE and are expressed relative to CON. *P < 0.05.
concentrations may partly explain increased GSIS following the chronic (10–14 days) amino acid infusion, they do not explain acute (3 h) amino acid-potentiated GSIS, as acute amino acid infusions do not increase fetal glucagon concentrations (22, 39).

We also measured pancreatic islet vascularity. Endothelial cells promote β-cell function through a complex set of mechanisms that include secreted growth factors as well as shared basement membrane proteins (20, 21, 35). Furthermore, there is decreased fetal pancreatic islet vascularity in the low-protein diet model of IUGR (4). Despite the increased GSIS in our AA group, we did not find any evidence for direct regulation of pancreatic islet vascularity by the fetal amino acid supply.

Amino acids can cause insulin resistance for glucose utilization in adults (34, 51), which is important to consider in this study because of the hyperbolic relationship between insulin secretion and sensitivity that maintains normal glucose concentrations (1, 23). Despite increased glucose-stimulated insulin concentrations in the AA fetuses, the glucose infusion rate required to achieve comparable steady-state hyperglycemic clamp glucose concentrations was the same following chronic (10–14 days) experimental infusions, suggesting insulin resistance. However, maximal fetal glucose utilization rates are 11–15 mg·kg⁻¹·min⁻¹ and are reached at fetal glucose concentrations of ~45 mg/dl, which was the target glucose concentration of the square-wave hyperglycemic clamp used in this study (17). Further increases in fetal insulin concentrations do not increase glucose utilization rates beyond this maximum (17), which is essentially identical to the sum of the glucose infusion rate in the current study (12.8 mg·kg⁻¹·min⁻¹) and the expected glucose uptake rate from the placenta during a hyperglycemic clamp (~0–2 mg·kg⁻¹·min⁻¹) (17, 33). Therefore, these differences between AA and CON fetal hyperglycemic clamp insulin concentrations and equivalent glucose concentrations and glucose infusion rates likely represent achieving maximal rates of glucose utilization in both groups with increased fetal insulin secretion responsiveness for glucose in the AA group.

To show this, glucose-stimulated insulin concentrations from normal late-gestation fetal sheep in our laboratory were plotted as a function of an estimate of insulin sensitivity, the inverse of the product of basal glucose and insulin concentrations [proportional to the HOMA-IS calculation (46)] (Fig. 6). When plotted against these normative data, it is clear that most of the AA fetuses have increased insulin secretion compared with normal fetuses independent of insulin sensitivity (Fig. 6).

To further explore the possibility of insulin resistance in the AA fetuses, we measured mRNA, protein, and phosphorylated protein concentrations of the insulin receptor and several insulin-sensitive genes, protein kinases, and their targets in fetal skeletal muscle and liver. Although phosphorylation of P70 S6K is increased by amino acids, as expected (3, 47), we did not find evidence for inhibition of proximal insulin signaling through Akt or MAPK. Moreover, we did not find any changes in the insulin receptor or insulin-sensitive gene expression.
Fetal arterial plasma glucose concentrations decreased slightly over time in the AA group. This may be related to decreased fetal glucose uptake from the placenta, which we demonstrated previously following chronic fetal amino acid infusions (30). Lower fetal glucose concentrations lead to decreased fetal insulin secretion (28). Fetal arterial plasma cortisol and norepinephrine were nearly doubled in the AA group, although none of these failed to reach statistical significance. These hormones normally act to inhibit insulin secretion (25, 55). Therefore, the chronic infusion of amino acids into the fetus potentiated fetal GSIS despite the increase in norepinephrine and cortisol concentrations and decrease in glucose concentrations. We found significantly higher maternal taurine and tryptophan concentrations during the experimental infusions. Several other essential amino acids also appeared to increase with time in the AA group, but none of these reached statistical significance. It is possible, though, that the previously identified small and nonsignificant decreases in the uptake rates of these essential amino acids led to a decrease in maternal amino acid utilization rates and increased concentrations (30). It is not surprising that the concentrations of more essential amino acids increased compared with nonessential amino acids considering that the infused amino acid mixture was enriched in the essential amino acids. These maternal amino acid changes in the AA group were not associated with any changes in maternal glucose, lactate, pH, or blood gasses.

In conclusion, this is the first study to show that increasing fetal amino acid delivery increases fetal GSIS without a change in β-cell mass or area, pancreatic islet size, vascularity, or insulin content. We also found increased fetal glucagon concentrations and α-cell responsiveness following increased amino acid delivery. Because we found no change in pancreatic morphology, insulin, GLUT2, or glucokinase, we speculate that amino acids upregulate the generation of secondary messengers in the β-cell, leading to increased GSIS. Future studies will directly measure glucose metabolism and the generation of secondary messengers in isolated islets to determine their relative contributions to amino acid-potentiated GSIS.

ACKNOWLEDGMENTS

We thank Karen Trembler, David Caprio, Alex Cheung, Dan LoTurco, Jenai Kailey, Nicole Isenberg, and Gates Roe for their technical support.

GRANTS

This work was supported by a Pilot and Feasibility Award from the Diabetes and Endocrinology Research Center, University of Colorado [National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant P30-DK-57516], as well as National Institutes of Health (NIH) Grants R01-DK-088139 and K08-DK-060688 (P. J. Rozance). L. D. Brown was supported by NIH Building Interdisciplinary Careers in Women’s Health Scholar Award K12 HD-057022 and the Children’s Hospital Colorado Research Institute. S. R. Thorn was supported by K01-DK-090199.

DISCLOSURES

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIDDK or National Institute of Child Health and Human Development. No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


