Decreased nutrient-stimulated insulin secretion in chronically hypoglycemic late-gestation fetal sheep is due to an intrinsic islet defect

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

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

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Rozance, Paul J., Sean W. Limesand, and William W. Hay, Jr. Decreased nutrient-stimulated insulin secretion in chronically hypoglycemic late-gestation fetal sheep is due to an intrinsic islet defect. Am J Physiol Endocrinol Metab 291:E404–E411, 2006. First published March 28, 2006; doi:10.1152/ajpendo.00643.2005—We measured in vivo and in vitro nutrient-stimulated insulin secretion in late gestation fetal sheep to determine whether an intrinsic islet defect is responsible for decreased glucose-stimulated insulin secretion (GSIS) in response to chronic hypoglycemia. Control fetuses responded to both leucine and lysine infusions with increased arterial plasma insulin concentrations (average increase: 0.13 ± 0.05 ng/ml leucine; 0.99 ± 0.26 ng/ml lysine). In vivo lysine-stimulated insulin secretion was decreased by chronic (0.37 ± 0.18 ng/ml) and acute (0.27 ± 0.19 ng/ml) hypoglycemia. Leucine did not stimulate insulin secretion following acute hypoglycemia but was preserved with chronic hypoglycemia (0.12 ± 0.09 ng/ml). Isolated pancreatic islets from chronically hypoglycemic fetuses had normal insulin and DNA content but decreased fractional insulin release when stimulated with glucose, leucine, arginine, or lysine. Isolated islets from control fetuses responded to all nutrients. Therefore, chronic late gestation hypoglycemia causes defective in vitro nutrient-regulated insulin secretion that is at least partly responsible for diminished in vivo GSIS. Chronic hypoglycemia is a feature of human intrauterine growth restriction (IUGR) and might lead to an islet defect that is responsible for the decreased insulin secretion patterns seen in human IUGR fetuses and low-birth-weight human infants.

hypoglycemia; glucose

INSULIN IS SECRETED BY THE HUMAN FETUS in response to glucose infusion, and this is attenuated in fetuses with intrauterine growth restriction (IUGR) (25). Decreased insulin secretion is likely an adaptive response by the IUGR fetus to coordinate growth with nutrient supply. This adaptive response of the developing pancreatic islets to low nutrients may explain some of the decreased β-cell function in small-for-gestational-age infants, most of whom had IUGR (2). Decreased β-cell function and development is also present in many animal models of IUGR (7, 11, 19, 31). A main feature of human IUGR is hypoglycemia (26), which may be responsible for the decrease in pancreatic islet function in IUGR fetuses and infants. Previously, we (18) have shown that 2 wk of hypoglycemia during late gestation in fetal sheep impairs glucose-stimulated insulin secretion (GSIS) and arginine-stimulated insulin secretion. In the present study, reported herein we have used this same model to assess fetal pancreatic islet adaptation to chronic hypoglycemia to identify defects that would cause decreased insulin secretion.

To achieve this, we quantified the impact of chronic and acute hypoglycemia on leucine-stimulated insulin secretion (LeuSIS) and lysine-stimulated insulin secretion (LySIS). These amino acids stimulate pancreatic insulin secretion by different mechanisms: leucine stimulates insulin secretion by increasing β-cell metabolism and ATP production, whereas lysine stimulates insulin secretion mainly by direct β-cell membrane depolarization (5, 29, 30). Decreased LeuSIS indicates a defect in cellular metabolism, whereas decreased LySIS indicates a defect in the later regulatory steps of insulin secretion, such as calcium entry, insulin granule formation, insulin granule trafficking, or insulin exocytosis. We also determined whether in vivo changes in the stimulation of insulin secretion by glucose, leucine, arginine, and lysine persisted in pancreatic islets isolated from chronically hypoglycemic fetal sheep. The results demonstrate interactive effects between the duration of low glucose concentration and the supply of selected amino acids in regulating fetal insulin secretion and also show that the inhibitory effect of hypoglycemia on in vivo islet insulin secretion persists in isolated islets studied in vitro.

MATERIALS AND METHODS

Animal preparation. Studies were conducted in Columbia-Rambouillet sheep during the last 20% of gestation [term of 147 days gestational age, (dGA)]. Animals were randomly assigned to one of two study groups, normal euglycemic control (C; n = 20) or chronic hypoglycemic treatment (T; n = 22). Indwelling femoral arterial and venous catheters were surgically placed into the ewe and fetus by use of standard surgical and anesthetic procedures at ~118 dGA for T ewes and ~128 dGA for C ewes. Previously, we (1, 3) determined that the 10-day difference in gestational age at surgery does not change basal or stimulated insulin secretion. Animals were maintained as previously described (18). 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 Health Sciences Center Institutional Animal Care and Use Committee.

Experimental design. The T ewes received a continuous intravenous insulin infusion (30–60 pmol·min⁻¹·kg⁻¹ Humulin R; Eli Lilly, Indianapolis, IN) in 0.5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) and 0.9% NaCl adjusted on average twice daily to produce a 50% reduction in maternal plasma glucose concentration (from 60–70 to 30–35 mg/dl) for 2 wk. The C ewes were maintained for 1–2 wk in the same laboratory as the T animals but did
not receive an insulin infusion. Amino acid-stimulated insulin secretion (AASIS) was determined at comparable gestational ages (135.1 ± 2.7 dGA C, 134.7 ± 2.4 dGA T) and after an average of 13 ± 2 days of treatment with Humulin R in the T animals. Animals in which both in vivo and in vitro insulin secretion were tested were allowed to return to baseline study conditions for at least 2 days following AASIS determination prior to necropsy.

**In vivo insulin secretion.** Baseline fetal arterial glucose and insulin concentrations were measured 10 and 5 min prior to the amino acid infusion. Leucine (0.5 mmol/kg) was dissolved in normal saline (15 ml), and lysine (0.5 mmol/kg) was dissolved in 4 ml of normal saline and 1 ml of 2 N Na acetic acid. The amino acid solution (either leucine or lysine) was then infused into a fetal venous catheter over 4 min (start time = 0 min), and fetal arterial insulin and glucose samples were collected at 5, 10, 20, and 30 min after the bolus infusion was started. Fetal arterial plasma amino acid concentrations were determined 5 min before and 5 min after the amino acid infusion. The magnitude of insulin secretion was calculated as the difference between the maximal insulin concentration achieved after the amino acid infusion and the baseline insulin concentration (the average of the 2 samples taken before the lysine infusion).

To test the impact of chronic vs. acute hypoglycemia on AASIS, C animals were made acutely hypoglycemic (8–15 mg/dl), with a maternal infusion of Humulin R. In addition, T animals were made acutely normoglycemic (18–24 mg/dl), with a fetal infusion of 33% dextrose in saline to acutely raise the plasma glucose concentrations. Fetuses were maintained at the desired level of glycemia for at least 45 min to achieve a steady-state arterial plasma insulin concentration. LysSIS was determined in seven C fetuses and eight T fetuses. LeuSIS was determined in eight C fetuses and 12 T fetuses. In three C and five T fetuses LeuSIS was determined 2–3 days after LySIS was determined. There was no difference in the LeuSIS pattern in these fetuses vs. those studied without prior LySIS determination.

**Biochemical analysis.** Whole blood was collected in EDTA-coated syringes and immediately centrifuged (14,000 × g) for 3 min at 4°C. Plasma was removed and the glucose concentration immediately determined using the YSI model 2700 select biochemistry analyzer (Yellow Springs Instruments, Yellow Springs, OH). The remainder of the plasma was stored at −70°C for hormone and amino acid measurements. The arterial amino acid concentrations were measured using a Dionex 300 model 4500 amino acid analyzer (Dionex, Sunnyvale, CA) after deproteinization with sulfosalicyclic acid. Plasma insulin concentrations were measured by an ovine insulin ELISA (Alpco, Windham, NH). Blood oxygen saturation and hemoglobin concentrations were measured with an OSM III hemoximeter (Radiometer, Copenhagen, Denmark). Oxygen content was determined as the product of oxygen saturation and oxygen capacity. The pH, PO₂, and hematocrit were determined at 39.2°C (normal ovine fetal blood temperature) using an ABL 520 blood gas analyzer (Radiometer).

**Necropsy and islet isolation.** Necropsies were performed 2 days after the insulin secretion studies. To obtain fetal tissues under conditions closely approximating in vivo study conditions, the ewe and fetus were anesthetized with maternally administered intravenous ketamine (4.4 mg/kg) and diazepam (0.11 mg/kg). After a hysterectomy, the fetus was removed, weighed, and dissected for pancreatic islet isolation and organ weights. The ewe was then killed by administering intravenous concentrated pentobarbital sodium (Sleepaway, 10 ml; Fort Dodge Animal Health, Fort Dodge, IA). The fetus died under anesthesia following an intracardiac injection of pentobarbital sodium (1 ml).

Islet isolation and confirmation of islet purity were performed as previously described (19). The fetal pancreatic ducts were perfused with a collagenase solution, 0.075 mg/ml Liberase Blendzyme III and 0.02% DNase I (Roche, Indianapolis, IN) in Krebs-Ringer buffer (KRB: 118 meq NaCl, 4.8 meq KCl, 25 meq NaHCO₃, 1.2 meq MgSO₄, 1.2 meq KH₂PO₄, 25 meq CaCl₂). This perfusion was accomplished by introducing a polyvinyl catheter (20 gauge) into the common hepatic duct and advancing it past the level of the common bile duct. The outlet of the common bile duct was then clamped at the duodenum. The collagenase solution was infused into the pancreas until it was fully distended (~18 ml). The pancreas was removed by blunt dissection, placed into 25 ml of collagenase solution, and incubated at 37°C for 30–40 min with gentle mixing every 5–10 min. Islets and acinar clusters were washed three times by sedimentation with KRB medium containing 0.5% BSA. Islets were partially purified with a discontinuous gradient of Histopaque (density 1.119 mg/ml; Sigma-Aldrich) and KRB-BSA (2:1). After being centrifuged at 750 g for 15–20 min, islets were removed from the interface and washed three times in KRB medium. Islets were recovered from the isolation procedure for 45–90 min in KRB-BSA at 37°C in 95% O₂-5% CO₂.

**In vitro insulin secretion and islet DNA content.** Insulin secretion by isolated islets was assayed by static incubations of 10 islets (n ≥ 3 incubations per medium condition) for 1 h in KRB-BSA. After recovery from isolation the islets were washed twice in KRB-BSA with 10 μM forskolin (Sigma-Aldrich). Following this, 10 islets were placed into an incubation vial. One milliliter of pregassed 95% O₂-5% CO₂ supplemented KRB-BSA with 10 μM forskolin (Sigma-Aldrich) was added to each incubation vial. Forskolin was added to the incubations to activate adenylate cyclase and augment insulin release. Supplements to KRB-BSA included 1.1 mM glucose, 11 mM glucos, and leucine, arginine, or lysine as indicated, as well as 11 mM glucose incubated on ice to control for icelet cell breakdown. In addition, incubations were performed in medium without nutrient additions. Islets were incubated at 37°C for 1 h and then pelleted at 2,300 g for 3 min. Medium was then removed and stored separately at −80°C. To determine insulin content of the islets after the 60 min, incubation acid ethanol extract (0.5 ml of 1 M HCl and 80% EtOH) was added to the islets, which were then sonicated for 1 min to disrupt cell membranes and release intracellular insulin. The resulting mixture was stored at −20°C overnight. Cellular debris was removed by centrifugation (20 min at 15,000 g), and the supernatant was stored at −80°C until insulin concentrations were measured with an ovine insulin ELISA (Alpco).

In vitro insulin secretion was quantified as the fraction of total islet insulin content that was released into the incubation medium. The total islet insulin content was determined by adding the amount of insulin present in the incubation medium with the amount of residual insulin remaining in the islets after the 60-min incubation. The results are expressed as the difference between fractional secretion of insulin under stimulated conditions and the basal fractional insulin release with no glucose or amino acids added.

Islet DNA content was determined for seven C and nine T fetuses. DNA content was determined for batches of 25 islets, (n ≥ 8 for each fetus). To lyse the islet cells 10 mM Tris·HCl, 1 mM EDTA, and 0.1% SDS were added to the islets, which were then sonicated for 1 min and frozen. Upon being thawed, the DNA concentration was determined using the PicoGreen dsDNA Quantitation kit (Molecular Probes, Eugene, OR).

**Statistical analysis.** Statistical analysis was performed using either SAS version 9.1 (28) or GraphPad Prism version 4.0 for Windows (12). Results are expressed as means ± SD. A repeated-measures mixed-effects ANOVA was performed with adjustment of the P value by Tukey’s multiple comparison test. Comparisons of in vivo insulin secretion were made using a repeated-measures mixed effects ANOVA including terms for
treatment, period, and treatment-by-period interaction with a random animal factor. \( P \) values were adjusted by Tukey’s method of multiple comparisons.

To compare insulin secretion between T and C islets for each particular medium condition, and between medium with increased nutrients added vs. low glucose only, a mixed-models ANOVA including terms for treatment, medium, and treatment-by-medium interactions with a random animal factor was used. The differences between mean secretion in T and C islets for a given medium and between nutrient-stimulated insulin secretion and secretion with low glucose were compared using Fisher’s protected least significant difference test. Islet insulin content, islet DNA content, basal islet insulin secretion, fetal and organ weights at autopsy, and baseline biochemical, hormonal, and hematological measurements were analyzed with the Mann-Whitney rank sum test or Student’s \( t \)-test when appropriate.

**RESULTS**

**Experimental condition and autopsy information.** The hypoglycemic (T) group received an insulin infusion for \( 15.5 \pm 1.2 \) days that significantly \(( P < 0.01)\) decreased both maternal and fetal arterial plasma glucose concentrations by day 1 (Fig. 1). This treatment significantly reduced fetal weight by 16.8% \((4.17 \pm 0.6 \) to \(3.47 \pm 0.5 \) kg, \( P < 0.01)\). Gestational age at autopsy \((137.8 \pm 2.1 \) vs. \(137.7 \pm 2.2 \) dGA in C and T, respectively) and the percentage of male fetuses \((37\% \) vs. \(43\% \) in C and T, respectively) were similar in both groups. Fetal weight and the proportion of organ weights to fetal weight are in Table 1. Baseline arterial blood gas and hematocrit values are shown in Table 2.

**In vivo insulin secretion.** There was no correlation between the change (peak minus basal) in plasma arterial insulin concentration and the peak plasma arterial lysine or leucine concentration or the change of plasma arterial leucine or lysine concentrations for all study periods (data not shown). Therefore, within the range of peak leucine and lysine concentrations achieved, insulin secretion was maximally stimulated.

**LeuSIS.** Baseline glucose and insulin concentrations were significantly lower \((P < 0.05)\) in the T group \((10.1 \pm 2.0 \) mg/dl glucose, \(0.10 \pm 0.04 \) ng/ml insulin) vs. the C group \((19.7 \pm 2.7 \) mg/dl glucose, \(0.32 \pm 0.12 \) ng/ml insulin). In acutely normoglycemic T fetuses, glucose \((21.3 \pm 1.6 \) mg/dl) and insulin \((0.27 \pm 0.18 \) ng/ml) concentrations increased to values not different from those of C fetuses. In acutely hypoglycemic C fetuses, glucose \((12.0 \pm 1.9 \) mg/dl) and insulin \((0.16 \pm 0.05 \) ng/ml) concentrations decreased and were not different from those of the T group. Baseline fetal arterial plasma leucine concentrations were significantly higher in the T group than in the C group \((239.1 \pm 36.8 \) nmol/ml T vs. \(178.9 \pm 42.2 \) nmol/ml C, \(P < 0.05\)\). Leucine concentrations in C fetuses made acutely hypoglycemic \((199.4 \pm 56.7 \) nmol/ml) and in T fetuses made acutely normoglycemic \((220.3 \pm 47.0 \) nmol/ml) were between baseline fetal leucine concentrations for the two groups but were not significantly different from either.

Baseline LeuSIS was determined in eight C fetuses and 12 T fetuses (Fig. 2). A significant increase in mean fetal arterial plasma insulin concentration occurred by 5 min in both groups but was not significantly different between the groups \((0.13 \pm 0.05 \) ng/ml C; \(0.12 \pm 0.09 \) ng/ml T). To determine whether LeuSIS would be affected similarly by acute and chronic periods of hypoglycemia C fetuses were made acutely hypoglycemic, and there was no insulin response to the leucine infusion (Fig. 2). We also increased the glucose concentrations in T fetuses to levels comparable with that of C fetuses, and postleucine administration insulin concentrations did not increase significantly until 30 min after the leucine infusion (Fig. 2). The change in insulin concentration \((0.19 \pm 0.13 \) ng/ml) was not significantly different from the baseline insulin secretion for T or C fetuses.

**LySIS.** Similar to the LeuSIS study, baseline glucose and insulin concentrations were significantly lower \((P < 0.05)\) in the T group \((10.4 \pm 1.8 \) mg/dl glucose, \(0.11 \pm 0.03 \) ng/ml insulin) vs. the C group \((20.0 \pm 3.5 \) mg/dl glucose, \(0.30 \pm 0.10 \) ng/ml insulin). In acutely normoglycemic T fetuses, glucose \((22.1 \pm 1.5 \) mg/dl) and insulin \((0.30 \pm 0.12 \) ng/ml) concentrations increased to values not different from those of C fetuses. In acutely hypoglycemic C fetuses, glucose \((11.8 \pm 1.3 \) mg/dl) insulin \((0.15 \pm 0.07 \) ng/ml) concentrations decreased and were not different from those of the T group. Differences in the fetal plasma lysine concentration between the T group \((156.8 \pm 60.1 \) nmol/ml) and the C group \((78.7 \pm 18.2 \) nmol/ml) did not reach statistical significance. Fetal plasma lysine concentrations were significantly higher \((P < 0.05)\) in the T group made acutely normoglycemic \((198.0 \pm 101.4 \) nmol/ml) vs. the C group at baseline, but acute hypoglycemia in the C group did not significantly change the lysine concentration \((136.0 \pm 43.9 \) nmol/ml).

Table 1. Fetal weight, and organ weights as a percentage of fetal weight, in control and hypoglycemic treatment fetuses

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>(n = 20)</td>
<td>(n = 22)</td>
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<tr>
<td>Total fetal weight, g</td>
<td>4.170±0.62</td>
<td>3.467±0.96*</td>
</tr>
<tr>
<td>Brain, %</td>
<td>75.9±1.0</td>
<td>76.3±1.2</td>
</tr>
<tr>
<td>Liver, %</td>
<td>1.1±0.2</td>
<td>1.5±0.2*</td>
</tr>
<tr>
<td>Lungs, %</td>
<td>2.6±0.3</td>
<td>2.6±0.3</td>
</tr>
<tr>
<td>Heart, %</td>
<td>3.6±0.6</td>
<td>3.5±0.7</td>
</tr>
<tr>
<td>Spleen, %</td>
<td>0.2±0.1</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>Kidney, %</td>
<td>0.6±0.1</td>
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Values are means ± SD. *Significant difference \((P < 0.05)\) by 2-sample \( t \)-test.

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**Fig. 1. Maternal and fetal arterial plasma glucose concentrations during the hypoglycemic/insulin treatment.** Concentrations are shown with SE bars and significantly declined from baseline by day 1; \( n = 22 \) (repeated-measures ANOVA).
Baseline LySIS was determined in seven C fetuses and eight T fetuses. Peak insulin concentration in both groups occurred at 5 min after the beginning of the lysine infusion and returned to baseline values by 30 min in all groups and periods. Unlike the situation for LeuSIS, T fetuses had significantly lower insulin secretion at baseline than C fetuses (Fig. 3). The change in plasma insulin concentrations in the T group was 0.37 ± 0.18 vs. 0.99 ± 0.26 ng/ml in the C group (P < 0.05). The effects of acute changes in glycemia were determined for T animals made normoglycemic and C fetuses made hypoglycemic (Fig. 3). The change in insulin secretion in acutely hypoglycemic C fetuses was 0.27 ± 0.19 ng/ml, which was significantly decreased from baseline LySIS in C fetuses (P < 0.05) but not different from the baseline LySIS for T fetuses. When T fetuses were made acutely normoglycemic the change in fetal insulin concentration was 0.84 ± 0.31 ng/ml, which was significantly increased from the baseline LySIS of these T fetuses (P < 0.05) but not different from the baseline LySIS for C fetuses.

**In vitro insulin secretion and islet DNA content.** The pancreatic islet insulin content was not different between the two groups (9.57 ± 8.99 ng/islet T vs. 5.91 ± 4.36 ng/islet C) and neither was islet DNA content (3.3 ± 2.0 ng/islet T vs. 3.5 ± 3.1 ng/islet C). The basal fractional release of insulin was also not different between the two groups (10.4 ± 7.6% T vs. 12.1 ± 10.2% C). Any negative insulin secretion values refer to a fractional insulin release for a supplemented medium condition that is less than the fractional insulin release when islets were incubated with unsupplemented KRB-BSA. In no case was there significantly less insulin released in supplemented conditions vs. basal conditions.

GSIS was significantly decreased in isolated islets from T fetuses vs. C fetuses, as shown in Fig. 4. In C fetal islets the fractional insulin release increased significantly in 11 vs. 1.1 mM glucose (P < 0.01). There was no increase in the fractional release of insulin from islets isolated from T fetuses incubated at 11 mM glucose vs. incubations with 1.1 mM glucose. GSIS for C islets at 11 mM glucose was significantly greater than GSIS in T islets incubated with 11 mM glucose (P < 0.01). The fractional release of insulin with incubation in 11 mM glucose on ice was not significantly different from incubation in 1.1 mM glucose in either T or C islets. In vitro LeuSIS was determined using 10 mM leucine and 1.1 mM glucose (Fig. 4). The addition of leucine to 1.1 mM glucose medium caused a significant increase in the fractional release of insulin compared with 1.1 mM glucose alone in C islets (P < 0.05). Leucine was unable to stimulate any increase in the fractional release of insulin from T islets compared with 1.1 mM glucose alone. Incubating C islets with 5 mM arginine plus 11 mM glucose increased the fractional insulin release vs. incubation with 1.1 mM glucose alone. Incubating T islets with 5 mM arginine plus 11 mM glucose did not increase insulin release compared with incubation with 1.1 mM glucose alone (the apparent trend toward increased secretion shown in Fig. 4 was not significant, P = 0.084).

*Table 2. Arterial blood gas and hematocrit values prior to AASIS determination*

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>PaCO₂, mmHg*</th>
<th>PaO₂, mmHg*</th>
<th>SåO₂, %*</th>
<th>O₂ Content, mM*</th>
<th>Hematocrit, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.38±0.02</td>
<td>49.0±2.1</td>
<td>19.5±3.7</td>
<td>48.6±12.7</td>
<td>3.3±0.7</td>
<td>36.0±4.9</td>
</tr>
<tr>
<td>Treatment</td>
<td>7.38±0.02</td>
<td>46.4±2.6</td>
<td>24.9±2.3</td>
<td>69.5±6.9</td>
<td>4.6±0.7</td>
<td>34.4±3.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 12 control, n = 16 treatment. AASIS, amino acid-stimulated insulin secretion. *Significant difference (P < 0.05) by 2-sample t-test.
Incubating T islets with 5 mM lysine + 11 mM glucose failed to increase fractional insulin release compared with 1.1 mM glucose alone, but in C islets 5 mM lysine + 11 mM glucose did increase the fractional insulin release compared with 1.1 mM glucose alone. However, as shown in Fig. 4, there was no increase in the fractional insulin release with 5 mM lysine + 11 mM glucose vs. 11 mM glucose alone in either group. Therefore, 5 mM lysine had no separate, discernable ability to stimulate insulin secretion in combination with 11 mM glucose. Because of this, we tested the response to 5 mM lysine + 11 mM glucose as well as the response to 10 mM lysine + 11 mM glucose in islets isolated from three C and three T fetuses (Fig. 5). Addition of 5 mM lysine to 1.1 mM glucose significantly increased fractional insulin release from C islets compared with 1.1 mM glucose alone (P < 0.05). Unlike C islets, islets isolated from T fetuses did not show any evidence for in vitro LySIS when 5 mM lysine was added to 1.1 mM glucose. Incubation in 10 mM lysine + 11 mM glucose did not significantly increase the fractional release of insulin from C or T islets vs. 11 mM glucose alone.

**DISCUSSION**

This study demonstrates an adaptive fetal islet response to chronic hypoglycemia that results in reduced in vitro insulin release, replicating the decreased in vivo GSIS seen in this model (18). These results, therefore, indicate that these islets develop a previously postulated (3, 18) intrinsic defect that is, at least in part, responsible for the in vivo defect. In addition, islets isolated from the hypoglycemic fetuses failed to secrete insulin in response to leucine or lysine (although there was a near significant trend toward a response to the combination of 11 mM glucose and 5 mM arginine), indicating defective insulin secretion in response to a variety of nutrients that stimulate insulin release by different mechanisms. This study also demonstrates the differential effects of hypoglycemia on in vivo fetal LeuSIS and LySIS; LeuSIS was decreased by acute hypoglycemia but not chronic hypoglycemia, whereas LySIS was significantly decreased by both acute and chronic hypoglycemia. This shows differential interactions between particular amino acids and the duration of hypoglycemia in regulating fetal insulin secretion.

Nutrient-stimulated insulin secretion is largely dependent on the ability of the nutrient to act as a fuel source for the pancreatic β-cell (21). For glucose, this occurs via metabolism by glycolysis and the tricarboxylic acid (TCA) cycle (14). Leucine stimulates insulin secretion by activating glutamate dehydrogenase (GDH). This enzyme converts glutamate to α-ketoglutarate, which is metabolized through the TCA cycle and generates signals for insulin secretion similar to glucose (22, 23, 30). Leucine may also stimulate insulin secretion via its own oxidative metabolism (20). Arginine and lysine stimulate insulin secretion by a different mechanism from leucine that primarily involves direct membrane depolarization independent of their metabolism. The positively charged amino acids are transported into the β-cell, depolarize the cell membrane, and thereby open voltage-dependent calcium channels, leading to an influx of calcium and insulin secretion (5).

Capitalizing on the different mechanisms of nutrient-stimulated insulin secretion we were able to distinguish a defect in the insulin secretion pathway. The absence of increased insulin secretion in response to lysine (which stimulates insulin secretion by direct membrane depolarization) indicates that, in our model, the intrinsic islet defect in nutrient-stimulated insulin secretion is downstream of membrane depolarization, possibly involving a defect in the regulation of calcium entry, insulin granule formation and transport, or the mechanisms of exocytosis. This conclusion is also supported by the absence of
insulin secretion in isolated islets in response to either glucose or leucine, two nutrients that stimulate insulin secretion by two different metabolic pathways. Normal islet insulin and DNA content suggests that the decreased insulin secretion from isolated hypoglycemic islets is not due to decreased cellularity or a decreased number of β-cells per islet, although this possibility deserves further investigation. Moreover, because the islet insulin content in the hypoglycemic fetuses is not decreased, there is an adequate ability to synthesize and store insulin. These islets simply are unable to release insulin in a normal, nutrient-regulated manner.

We previously demonstrated that restoring fetal glucose concentrations to normal for 5 days following 2 wk of late-gestation hypoglycemia partially restores fetal GSIS (18). This contrasts with our in vitro findings of a complete lack of GSIS in response to a 1-h hyperglycemic (11 mM) incubation. However, in the fetuses with restored glucose concentrations the β-cell responsiveness remained decreased, as evidenced by a persistent increase in the time needed to reach half-maximal plasma arterial insulin concentrations during a hyperglycemic clamp. In addition, arginine-stimulated insulin secretion remained defective in these fetuses as well (18). Clearly, β-cell defects persisted after the 5 days of normoglycemic correction, and these may be responsible for the decreased in vitro insulin secretion demonstrated in the present study. One difference between the present study and this previous study is that fetal brain weight was increased in this group of chronically hypoglycemic fetuses but not in the previously reported group (18). We are not prepared to speculate on the cause or significance of this discrepancy.

The results from our present study are similar to findings in a rat model of fetal nutrient deprivation. Fetal pancreatic islets from pregnant dams fed a low-protein diet also have normal islet insulin content but reduced fractional release of insulin when stimulated with glucose, leucine, or arginine (6). A similar phenotype was characterized in Rab3A-null mice, which shows the effects of a specific defect in one regulatory protein involved in the mechanism of insulin granule exocytosis, Rab3A. Similar to the hypoglycemic fetal sheep, these mice have decreased in vivo and in vitro GSIS and no decrement in total insulin content per islet (32). Our results contrast with a placental insufficiency model of IUGR in fetal sheep in which the fractional release of insulin in response to glucose is not decreased, but the number of β-cells in the islets and the content, storage, and thus secretion of insulin by the pancreatic islet is severely limited. Comparison of these models shows that the timing and type of nutrient restriction are important determinants of the resulting pancreatic islet adaptations (19).

Chronic hypoglycemia also had a negative impact on fetal insulin secretion in vivo, shown by decreased LySIS. Diminished LySIS was, however, fully restored with acute normoglycemic correction of the glucose deficiency. In addition, the LySIS defect seen in the chronic hypoglycemic fetuses was reproduced with acute hypoglycemia in control fetuses. These results indicate that the decreased LySIS in our chronically hypoglycemic fetuses was a function of the fetal glycemic state, not the duration of the hypoglycemic treatment, and that it was fully reversible with correction to normoglycemia. The dependence of LySIS on the glycemic state is in agreement with previous in vitro experiments showing increased LySIS in the presence of high glucose (29) and is the reason why we chose to test in vitro LySIS at a high glucose concentration initially. The dependence of LySIS on the fetal glycemic state at the time of the lysine infusion is also consistent with previous results in a group of normal fetal sheep, where the similar amino acid arginine was used (13).

In contrast to the results for LySIS, the effects of hypoglycemia on LeuSIS are significantly dependent on the duration of glucose deprivation. Acute hypoglycemia decreases LeuSIS, but chronic hypoglycemia restores it to control values, implying some adaptation to chronic hypoglycemia that allows the fetus to have a normal insulin response to leucine that develops over time. When chronically hypoglycemic fetuses were made acutely normoglycemic, the magnitude of LeuSIS was not decreased, but the time to significantly increase plasma arterial insulin concentrations was delayed from 5 to 30 min following the leucine infusion. There are no other studies characterizing the effects of leucine given as a single amino acid on fetal insulin secretion. One study of a maternal infusion of all three branched-chain amino acids found no impact on steady-state fetal sheep insulin concentrations despite a 3.6-fold increase in fetal arterial plasma concentrations of leucine. However, plasma insulin concentrations in this study were determined only 2 h after the amino acid infusion was begun (15). Another study (8) showed no change in insulin concentrations during a 2-h infusion of leucine into neonatal pigs sufficient to raise plasma concentrations of leucine almost 3.5-fold. In our study, we achieved a 5.4-fold increase in plasma arterial leucine concentrations averaged for all study periods. The difference in the fold increase in leucine concentration may explain why we observed in vivo LeuSIS, whereas these other two studies did not.

Decreased LeuSIS in the presence of increased glucose is a well-described phenomenon in vitro and is why we chose to test LeuSIS from our isolated islets in incubation medium containing 1.1 mM glucose, which is the normoglycemic concentration for fetal sheep from most breeds (including the one we studied) and not in medium with a higher glucose concentration. A proposed mechanism explaining increased LeuSIS in low vs. high glucose incubation conditions is that the ability of GDH to be activated by leucine is dependent on a low cellular energy state (9, 10, 16). LeuSIS was preserved in our chronically hypoglycemic fetal sheep but was decreased in acute hypoglycemia. The mechanism preserving LeuSIS in chronic hypoglycemia vs. acute hypoglycemia is unclear, but an initial secretory defect during acute hypoglycemia may be overcome with chronic glucose deprivation by a mechanism involving GDH. In vitro, the duration of hypoglycemia influences the magnitude of leucine stimulated insulin secretion (16) but not the number of GDH transcripts (20). The long-term in vivo effects of glucose deprivation on islet GDH have not been studied.

In vivo LeuSIS is preserved following 2 wk of late-gestation hypoglycemia, whereas in vitro LeuSIS is completely absent in the islets isolated from the hypoglycemic fetuses. Fetal insulin secretion in response to chronic hypoglycemia was tested with a fetal glucose of 10.1 mg/dl (0.56 mM). Although the absolute difference in maximal and basal insulin concentrations achieved was not different when hypoglycemic fetuses were made acutely normoglycemic to 21.3 mg/dl (1.18 mM), the time required to reach a significant increase from baseline was prolonged from 5 to 30 min. On the basis of these data, there...
is defective LeuSIS in chronically hypoglycemic fetuses when tested with the higher glucose concentration of 1.1 mM, an observation consistent with the effects of increased cellular glucose on GDH. We tested in vitro LeuSIS with 1.1 mM glucose, which may explain why we observed decreased in vitro LeuSIS. On the basis of the above data it is possible that LeuSIS in islets isolated from hypoglycemic fetal sheep could have been demonstrated in incubation medium with a lower glucose concentration; we did not test this. The acute and chronic effects of glucose on GDH and LeuSIS in the fetus deserve further investigation.

Another interesting result in these studies is the increased plasma arterial concentration of leucine and the trend toward an increase in the concentration of lysine in our chronically hypoglycemic fetuses. These results are consistent with our previous studies (18) demonstrating increased lysine concentrations using this model. The lack of a significant difference in lysine concentrations in the present study is due to increased variability in the hypoglycemic fetuses compared with our previous study and not to a smaller difference in mean lysine concentrations. However, other studies evaluating the effect of fetal hypoglycemia in sheep, either acute (24) or for 8 wk (4), did not find an increase in fetal leucine concentrations. The difference may be related to the duration of hypoglycemia. Our results are more consistent with a 5-day maternal-fasting model of fetal hypoglycemia in which fetal leucine concentrations were increased significantly (17). Preliminary data from our group indicate that lysine oxidation is increased following 2 wk of hypoglycemia (unpublished data), a result consistent with increased leucine oxidation in fetuses following a 5-day maternal fast (17). Leucine oxidation is not, however, increased following fetal hypoglycemia for either 3 h (24) or 8 wk (4). Taken together, these data indicate that one adaptation to fetal hypoglycemia is increased amino acid oxidation and that elevated lysine and leucine concentrations might provide an increased supply of non-glucose nutrients for oxidation by the fetus. This response requires more than 3 h to develop and disappears by 8 wk of hypoglycemia. Also consistent with this theory are the findings from our chronic placental insufficiency model of IUGR, in which fetal plasma arterial leucine concentrations are decreased but the rate of fetal leucine oxidation is not changed (27).

In conclusion, normal in vitro GSIS by fetal pancreatic islets is abolished by prior chronic in vivo hypoglycemia in the near-term fetal sheep, showing that hypoglycemia produces a persistent defect that inhibits GSIS. In the in vitro condition, this defect of GSIS is not completely restored by incubation of the islets with leucine, arginine, or lysine, indicating that the defect may involve later steps in the regulation of insulin secretion, such as granule synthesis, transport, or exocytosis. In contrast, pancreatic islet insulin secretion in vivo that is suppressed by hypoglycemia can be overcome, in part, by acute stimulation by selected amino acids. For example, in vivo lysine stimulation of insulin secretion is decreased by chronic hypoglycemia but is dependent on the glycemic state of the fetus at the time of the lysine infusion and not on the duration (chronic vs. acute) of the hypoglycemia. LeuSIS, however, is not affected by chronic hypoglycemia but is inhibited with acute hypoglycemia. The differential effect of hypoglycemia on LeuSIS and LySIS is likely caused by the difference in mechanisms of stimulated insulin secretion between these two amino acids. These results demonstrate the interactions between the duration of hypoglycemia and the supply of selected amino acids in regulating fetal insulin secretion. They also demonstrate that the inhibitory effect of hypoglycemia on in vivo islet insulin secretion persists in isolated islets studied in vitro.

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