Chronic fetal hypoglycemia inhibits the later steps of stimulus-secretion coupling in pancreatic β-cells

Paul J. Rozance,1 Sean W. Limesand,2 Gary O. Zerbe,3 and William W. Hay, Jr.1

1Perinatal Research Center, University of Colorado Health Sciences Center, Aurora, Colorado; 2Department of Animal Sciences, University of Arizona, Tucson, Arizona; and 3Department of Preventive Medicine and Biometrics, University of Colorado Health Sciences Center, Denver, Colorado

Submitted 1 June 2006; accepted in final form 4 January 2007

Rozance PJ, Limesand SW, Zerbe GO, Hay WW Jr. Chronic fetal hypoglycemia inhibits the later steps of stimulus-secretion coupling in pancreatic β-cells. Am J Physiol Endocrinol Metab 292: E1256–E1264, 2007. First published January 9, 2007; doi:10.1152/ajpendo.00265.2006.—We measured the impact of chronic late gestation hypoglycemia on pancreatic islet structure and function to determine the cause of decreased insulin secretion in this sheep model of fetal nutrient deprivation. Late gestation hypoglycemia did not decrease pancreas weight, insulin content, β-cell area, β-cell mass, or islet size. The pancreatic islet isolation procedure selected a group of islets that were larger and had an increased proportion of β-cells compared with islets measured in pancreatic sections, but there were no morphologic differences between islets isolated from control and hypoglycemic fetuses. The rates of glucose-stimulated pancreatic islet glucose utilization (126.2 ± 25.3 pmol glucose-islet−1·h−1), hypoglycemic, vs. 93.5 ± 5.5 pmol glucose-islet−1·h−1, control, P = 0.47) and oxidation (10.5 ± 1.7 pmol glucose-islet−1·h−1, hypoglycemic, vs. 10.6 ± 1.6 pmol glucose-islet−1·h−1, control) were not different in hypoglycemic fetuses compared with control fetuses. Chronic late gestation hypoglycemia decreased insulin secretion in isolated pancreatic islets by almost 70% in response to direct nonnutrient membrane depolarization and in response to increased extracellular calcium entry. β-Cell ultrastructure was abnormal with markedly distended rough endoplasmic reticulum in three of the seven hypoglycemic fetuses studied, but in vitro analysis of hypoglycemic control islets showed no evidence that these changes represented endoplasmic reticulum stress, as measured by transcription of glucose regulatory protein-78 and processing of X-box binding protein-1. In conclusion, these studies show that chronic hypoglycemia in late gestation decreases insulin secretion by inhibiting the later steps of stimulus-secretion coupling after glucose metabolism, membrane depolarization, and calcium entry.

insulin secretion; pancreatic islet; glucose; metabolism; unfolded protein response

INTRAUTERINE GROWTH RESTRICTION (IUGR) is associated with decreased glucose-stimulated pancreatic islet secretion (GSIS) in the human fetus (22). The mechanism linking IUGR and decreased fetal GSIS is currently unknown, but its identification is important, as infants born small for gestational age have decreased insulin secretion (5) and an increased risk of developing type 2 diabetes as adults (4, 14), the pathogenesis of which includes both insulin resistance and an insulin secretory defect. Decreased insulin secretion and pancreatic islet development are features in many animal models of IUGR (8, 11, 21, 30), especially those with placental insufficiency and decreased nutrient supply to the fetus. The ability to identify a critical nutrient that reduces pancreatic islet growth and function in these models has been limited, however, because none has involved experimental restriction of one specific nutrient.

To evaluate the role of glucose as a limiting nutrient for pancreatic islet development and function in IUGR, we have previously used late gestation hypoglycemic fetal sheep as a model of a selective nutrient deficiency that restricts fetal growth (10). Hypoglycemia is a feature of human IUGR (23), and we have previously shown that experimentally limiting glucose to the fetus for 2 wk in late gestation not only reduces fetal growth but also decreases in vivo and in vitro nutrient-stimulated insulin secretion while preserving islet insulin content. These results indicated an intrinsic islet defect in insulin secretion downstream from insulin biosynthesis or storage, but the nature of the defect was not determined. Arginine and lysine both stimulate insulin secretion by direct membrane depolarization (7, 28), and both are defective for stimulating in vivo insulin secretion in this IUGR model (20, 25). This suggests that the intrinsic islet defect is beyond membrane depolarization in the stimulus-secretion coupling pathway. However, leucine, which stimulates insulin secretion by increasing cellular metabolism (29), is not defective in stimulating in vivo insulin concentrations in this IUGR model (25). Further complicating the nature of the secretory defect, in vitro stimulation of insulin secretion by glucose, arginine, lysine, and leucine is completely abolished in islets isolated from chronically hypoglycemic fetal sheep, whereas islets from normal control sheep fetuses are responsive (25).

The goal of this study, therefore, was to determine whether decreased insulin secretion in chronically hypoglycemic fetal sheep is due to a defect in islet metabolism or in the later steps of stimulus-secretion coupling. To accomplish this, we quantified isolated pancreatic islet glucose utilization and oxidation rates as well as tested the ability of direct nonnutrient β-cell membrane depolarization and the ability of increased cytosolic calcium to stimulate insulin secretion. The impact of chronic late gestation hypoglycemia on isolated islet size, individual endocrine cell population, and β-cell ultrastructure also was determined. We also tested the impact of chronic fetal hypoglycemia on endocrine pancreas morphology to determine whether decreased β-cell mass is partly responsible for the decreased fetal insulin secretion under the hypoglycemic conditions. Our results show that the defect in GSIS occurs in the later steps of stimulus-secretion coupling after glucose metab-
olism, possibly in the regulation of insulin granule formation, trafficking, or exocytosis. Furthermore, decreased fetal insulin secretion is not due to decreased pancreatic insulin content or β-cell mass.

**MATERIALS AND METHODS**

*Experimental design and islet isolation.* Studies were conducted in pregnant Columbia-Rambouillet ewes during the last 20% of gestation (term of 147 days gestational age; dGA). 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 and approved by the University of Colorado Health Sciences Center Institutional Animal Care and Use Committee. Indwelling arterial and venous catheters were surgically placed into both the ewe and fetus. Animals were then randomly placed into either a euglycemic control (C) (n = 28) or chronically hypoglycemic treatment (T) (n = 26) group as previously described (25). After an average of 14.6 days of continuously infused maternal insulin (adjusted on average twice daily to decrease maternal and fetal plasma arterial glucose concentrations by 50%), the ewes were anesthetized with ketamine (4.4 mg/kg) and diazepam (0.11 mg/kg), a hysterec­tomy was performed, and the ewe was then killed by intravenous pentobarbital sodium (10 ml, Sleepaway; Fort Dodge Animal Health, Fort Dodge, IA). Control and the ewe was then killed by intravenous pentobarbital sodium (10 ml, Sleepaway; Fort Dodge Animal Health, Fort Dodge, IA). Control ewes were killed at a comparable gestational age. The fetus was removed, weighed, and dissected for islet isolation and organ weights. Maternal and fetal arterial plasma glucose concentrations were determined as previously described (25).

In seven animals in each group, the pancreas was removed, weighed, and divided into hepatic and splenic portions. The hepatic portion was snap-frozen and then stored at −80°C for subsequent measurement of insulin and glucagon content; the splenic portion was placed in 4% paraformaldehyde in phosphate-buffered saline (PBS; wt/vol) for fixation. For the other fetuses, islets were isolated and purity confirmed as previously described (21). Briefly, before removal of the pancreas, the 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), and then the pancreas was removed by blunt dissection, placed into 25 ml of collagenase solution, and incubated at 37°C for 20–30 min with gentle mixing every 5–10 min until complete digestion was observed. Islets and acinar clusters were washed three times, and islets were purified over a discontinuous gradient of a 2:1 solution of Histopaque (1.119 g/ml; Sigma) and KRB with 0.5% bovine serum albumin (BSA; wt/vol) and centrifuged at 800 g for 15 min. This was followed by hand selection under low magnification visualization. Islets recovered from the isolation procedure for 45–90 min in KRB with 0.5% BSA (wt/vol) at 37°C in 95% O2-5% CO2.

**Insulin and glucagon contents.** Insulin and glucagon contents were determined as previously described (19). Briefly, fetal pancreas tissue from the hepatic portion was pulverized in liquid nitrogen. Pancreatic hormones were acid-ethanol extracted from 35 mg of tissue in 1 ml of 1 M HCl, 70% ethanol, at −20°C. Insulin and glucagon contents were evaluated in three different samples per fetus to derive the average content for each fetus. The concentration of insulin was determined using ovine insulin ELISA (Alpco, Windham, NH), and the concentration of glucagon was measured with a Glucagon RIA kit (Linco Research, St. Charles, MO). Data are presented as micrograms of pancreatic insulin or glucagon per gram of pancreas.

**Histology of the fetal pancreas.** Five tissue sections of 5 μm were cut from a paraffin-embedded control and IUGR pancreases at 50-μm intervals for histological evaluation as previously described (19). Mature pancreatic endocrine hormones were identified in the fetal sheep pancreas with guinea pig anti-porcine insulin (Dako, Carpenteria, CA; 1:500), mouse anti-porcine glucagon (Sigma-Aldrich, St. Louis, MO; 1:500), rabbit anti-human somatostatin (Dako, 1:500), and rabbit anti-human pancreatic polypeptide (Dako, 1:500). Immuno­complexes were detected with affinity-purified secondary antisera: anti-rabbit IgG conjugated to Cy2, anti-mouse IgG conjugated to Texas Red, and anti-guinea pig IgG conjugated to 7-amino-4-methylcoumarin-3-acetic acid (AMCA; Jackson ImmunoResearch Laboratories, West Grove, PA) (all 1:500).

Fluorescent images were visualized on an Olympus BX51 microscope system and digitally captured with a Pixera 600CL camera. Morphometric analysis was performed with ImagePro 6.0 software (Media Cybernetics, Silver Spring, MD). β-Cell (insulin+ cells) and α-cell (glucagon+ cells) mass was determined by multiplying the pancreas weight by the percent total pancreas area positive for each respective hormone. Fetal pancreatic islet size was examined by triple immunofluorescence detection with insulin, glucagon, and somatostatin plus pancreatic polypeptide. Fetal islets were defined as endocrine cell clusters >500 μm2 containing at least two endocrine cell types.

**Histology of fetal isolated pancreatic islets.** Histology of isolated fetal pancreatic islets was determined for eight C and five T fetuses. Between 200 and 400 isolated islets were placed in tissue freeze media and sectioned. Islet sections were then fixed with 4% paraformaldehyde for 20 min at 22°C, washed three times with PBS, and then blocked with 1% BSA in PBS for 30 min. Pancreatic endocrine hormones were identified with the same antibodies used for evaluating the histology of the pancreas, as noted above, except anti-mouse IgG conjugated to Rhodamine Red (Jackson ImmunoResearch Laboratories, 1:500) was used in place of anti-mouse IgG conjugated to Texas Red. Fluorescent images were visualized and analyzed with the same system used for evaluating the histology of the pancreas. Isolated islets were again defined as endocrine cell clusters with a minimum area of 500 μm2 and containing at least two different endocrine cell types. Approximately 25 islets were examined from each animal (range 12–52), and each islet was evaluated for total area. The mean area was then determined for islets isolated from a single animal. These means were then averaged for each study group for comparison. The total area positive for insulin, glucagon, or the combination of somatostatin and pancreatic polypeptide was determined for every islet evaluated from an individual animal. The sum of the areas for each hormone in each individual animal was divided by the total islet area evaluated for that animal to determine the percentage of total islet area for each hormone. These percentages were then averaged for all animals in each study group for comparison.

**Islet glucose metabolism.** Glucose metabolism was determined in fetal islets from six C and seven T animals by methods based on those described by Ashcroft et al. (2, 3). Batches of 25 islets (n ≥ 4 incubations for each glucose concentration) were used to determine glucose utilization (1.1, 5.5, 11, and 16.7 mM glucose (18), by 3H2O production from D-[5-3H]glucose (6, 8, 9, and 10 μCi/ml, respectively; PerkinElmer, Boston, MA). Glucose oxidation was determined at 1.1, 5.5, 11, and 16.7 mM glucose by the formation of 14CO2 from 2-[1-14C]glucose (8, 12, 16, and 20 μCi/ml, respectively; PerkinElmer) (21).

**Insulin secretion.** Insulin secretion was determined for seven C and five T fetuses and was assayed by static islet incubations of 10 islets (n ≥ 3 per glucose concentration) for 1 h in KRB-BSA with 10 μmol/l forskolin (Sigma-Aldrich) as previously described (25). Incubations included islets under basal conditions with no additions to the KRB-BSA with 10 μmol/l forskolin and additions of 30 μmol/l potassium chloride (KCl) or 1 μmol/l ionicomycin (to increase extracellular calcium entry). Insulin concentrations were measured by an ovine insulin ELISA. Insulin secretion was quantified as the fraction of total islet insulin content released into the incubation media. The total islet insulin content was determined by adding the amount of insulin present in the incubation media with the amount of residual insulin remaining in the islets after the 60-min incubation.
Electron microscopy. β-Cells from five C and seven T fetuses were evaluated by electron microscopy. Morphometric analysis was performed with Image Pro 4.5 software (Media Cybernetics). Three or four micrographs with at least one β-cell in the image were analyzed for each fetus. β-Cells were identified by the characteristic appearance of the secretory granules, which contain contents of variable electron density with a surrounding halo (6, 9, 31, 33). In addition, >90% of the total islet area contained insulin-positive β-cells (see RESULTS), facilitating selection of β-cells for analysis. Values for each of the micrographs obtained from one fetus were averaged to determine the value for that fetus. These values were used to calculated group statistics for C and T fetuses. Secretory granule number per cytoplasm area, secretory granule diameter, total combined area of all secretory granules, and total combined area of all mitochondria per cytoplasm area were calculated.

Evaluation of potential endoplasmic reticulum stress response in fetal fibroblasts and pancreatic islets. Sheep fetal fibroblasts were isolated from an early gestation fetal skin biopsy. Fibroblasts were propagated in DMEM and supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin–streptomycin (PNS, Sigma-Aldrich). Thapsigargin (1 µmol/l; Upstate, Lake Placid, NY) was used as a positive control for the endoplasmic reticulum (ER) stress response. To test the response to low glucose, DMEM without glucose (Biosource) supplemented with 10% FBS was used as incubation media. Media glucose concentration was measured with the YSI 2700 Select Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH), and the addition of 10% FBS resulted in an average glucose concentration of 0.41 ± 0.03 mmol/l. Following incubations, cells were lysed and RNA extracted with RNeasy Mini kit (Qiagen, Valencia, CA) and then stored at −80°C for analysis by polymerase chain reaction (PCR). For each medium, three independent experiments were performed with sheep fetal fibroblast cells following the 4th–8th passage.

Fetal pancreatic islets for this experiment were isolated from normal late gestation fetal sheep as described above. Data from these fetuses are not used for any other experiments described in this manuscript. Pancreatic islets were incubated overnight in RPMI 1640 (Sigma-Aldrich) with 3 mmol/l glucose, 1% FBS, and 1% PNS equilibrated with 95% O2–5% CO2 at 37°C. Following this, islets were transferred to test medium: RPMI 1640 and 1% FBS plus 3 mmol/l glucose (control), 0.5 mmol/l glucose (low glucose), or 3 mmol/l glucose with 1 µmol/l thapsigargin, all equilibrated with 95% O2–5% CO2 at 37°C. After 150 min, islets were collected and frozen at −80°C. Total RNA was extracted from the islets using the RNeasy Micro kit (Qiagen) and stored at −80°C until PCR analysis.

Ovine glucose regulatory protein-78 (GRP78), X-box binding protein-1 (XBP1), and ribosomal protein s15 (S15) (GenBank accession nos.: DQ029323, EF107521, and AY949774, respectively) were cloned from ovine mRNA by reverse transcripase-PCR (reagents were obtained from Invitrogen, Carlsbad, CA). DNA products were amplified with the following oligonucleotide primer pairs: GRP78, sense 5’-TGACACAGGAGCATCAAGTTC-3’, antisense 5’-GTCAGGCRYTGTGTGCTACT-3’ (1,279 bp); XBP1, sense 5’-CGGAAGAAGAATCTGGAAGGAC-3’, antisense 5’-AGATGTTCTTGAGGAGGTTA-3’ (293 bp, spliced product 267 bp); or S15, sense 5’-TGCCGCAATTCTACATCACC-3’, antisense 5’-CGGCCGCGCTCATGCTTTACG-3’ (362 bp). Amplified PCR products were TA cloned using a TOPO PCR II kit and transformed into Escherichia coli chemically competent cells (Invitrogen). Plasmid DNA was purified using QIAprep Spin Miniprep kit (Qiagen), and the nucleotides were sequenced to confirm cloned DNA.

Synthetic oligonucleotide primers to the sheep nucleotide sequences were developed for quantitative real-time PCR (RT-PCR). Primer sequences were as follows: GRP78, sense 5’-gaaatgtaggaggtct-3’, antisense 5’-gcagagaaattctagctc-3’ (191 bp); S15, sense 5’-ATCATTTGCCCCAGATGTGGT-3’, antisense 5’-TGCTTTTAGGGCATTGATTG-3’ (135 bp). For both primer–gene sets, RT-PCR efficiencies were between 90 and 110% over eight orders of magnitude. Cloning primers were used for XBP-1 PCR.

Total RNA (tRNA) from sheep fetal pancreatic islets and sheep fetal fibroblast cells was reverse transcribed to cDNA with Superscript III reverse transcriptase (Invitrogen). PowerSyberGreen (Applied Biosystems, Foster City, CA) RT-PCR mix was used to amplify the DNA, which was monitored with an Opticon II (Bio-Rad) RT-PCR machine. Specificity of the primers was confirmed by gel electrophoresis separation in an agarose gel, inspection of individual melting curves, and DNA sequencing. Expression of GRP78 was normalized to expression of S15 and for fibroblast experiments is presented relative to time zero. Activated XBP-1 mRNA is characterized by an intracytosolic splicing event that removes 26 bases (34). Standard PCR for XBP-1 was performed on all samples; PCR products were electrophoresed through a 2.5% agarose gel to resolve active vs. inactive XBP-1 mRNA products.

Statistical analysis. Statistical analysis was performed using either SAS version 9.1 (26) or GraphPad Prism version 4.0 for Windows (25). Results are expressed as mean ± SE unless otherwise noted. A repeated-measures mixed-effects ANOVA with a random animal factor was performed to determine when maternal and fetal plasma arterial glucose concentrations in the T group decreased significantly from baseline. The same procedure was used to determine when GRP78 expression increased significantly from baseline in fetal fibroblasts exposed to thapsigargin or low glucose conditions. GRP78 expression in pancreatic islets incubated in thapsigargin and low glucose media was compared with expression in islets incubated in control media with a one-way ANOVA. The plasma arterial glucose concentrations, fetal and organ weights, total islet area, percent insulin, glucagon, somatostatin or pancreatic polypeptide area, pancreatic insulin and glucagon content, β-cell and α-cell mass, and ultrastructural characteristics were compared using the Student’s t-test or Mann-Whitney test (for nonparametric analysis) when appropriate (12). To compare islet glucose metabolism and insulin secretion between T and C islets for each particular media condition, and between media conditions within each experimental treatment group, a mixed-models ANOVA including terms for treatment, media, and treatment-by-media interactions with a random animal factor was used. To describe the relationship between glucose concentration and pancreatic islet glucose metabolism, Michaelis-Menten curves were fitted using a hierarchical nonlinear stochastic parameter regression model.

RESULTS

Experimental condition. The hypoglycemic group received an insulin infusion for 14.6 ± 4 days that significantly decreased maternal and fetal arterial plasma glucose concentrations from baseline by day 1 (3.89 ± 0.08 to 2.74 ± 0.11 maternal and 1.14 ± 0.04 to 0.89 ± 0.04 mmol/l fetal, P < 0.05). Immediately before islet isolation, arterial plasma glucose concentrations in the fetus were 1.22 ± 0.08 mmol/l C and 0.65 ± 0.06 mmol/l T (P < 0.0001) and in the maternal ewe were 3.89 ± 0.30 mmol/l C and 2.17 ± 0.21 mmol/l T (P < 0.0001). Gestational age at necropsy (137.0 ± 0.4 vs. 137.4 ± 0.5 dGA in C and T, respectively) and the percentage of male fetuses were similar in both groups (37 vs. 44% in C vs. T, respectively). Fetal weight decreased by 16.8% in the group (3.37 ± 0.104 kg) compared with the C group (4.011 ± 0.128 kg). The proportion of organ to fetal weight was similar to our previously published percentages (25).

Pancreas characteristics. Table 1 provides characteristics of the pancreases from C and T fetuses. For the seven animals in each group for which the weight of the pancreas was obtained, there were no differences between C and T groups. The islet isolation procedure prevented accurate determination of pan-
creas weight from the remaining animals. No significant differences were found in any characteristic measured except for a greater ($P<0.05$) pancreatic glucagon content in T fetuses.  

**Histology of isolated fetal pancreatic islets.** The size and proportion of endocrine cell types of the isolated islets were measured to facilitate comparisons of islet glucose metabolism between T and C islets. The average sizes of the isolated islets were not different between the T and C groups (29,900 ± 10,600 μm² T vs. 20,700 ± 4,800 μm² C, $P=0.39$). The proportions of insulin (94.9 ± 1.3% T vs. 91.8 ± 1.4% C, $P=0.16$), glucagon (3.8 ± 1.3% T vs. 6.5 ± 1.1% C, $P=0.16$), and somatostatin- and pancreatic polypeptide (1.4 ± 0.4% T vs. 1.7 ± 0.5% C, $P=0.60$)-positive areas of the total isolated islet area also were not different between the T and C groups. In both T and C groups, most of the isolated islets were very large and consisted almost completely of insulin-positive cells (Fig. 1).

**Islet glucose metabolism.** Islet glucose utilization and oxidation rates were measured and show that defective glucose metabolism is not responsible for decreased GSIS. Greater than 90% of the islet area in both C and T islets consists of β-cells (Fig. 1); therefore, the measured metabolic rates for isolated islets represent predominantly β-cell metabolic rates. In addition, because the percentage of β-cells was not different between T and C islets, any differences in glucose utilization or oxidation are due to changes in β-cell metabolism and not a change in β-cell number. To determine maximum rates of islet glucose metabolism, the relationship between the rate of glucose metabolism and glucose concentration was examined. Michaelis-Menten curves for islet glucose utilization and oxidation rates were generated. For glucose utilization, $V_{max}$ (the maximal islet glucose utilization rate) was 147.9 pmol/islet⁻¹·h⁻¹ (95% confidence interval: 97.6–198.3), and $K_m$ (the glucose concentration at which glucose utilization rate is one-half the maximum rate) was 4.69 mmol/l (95% confidence interval: 2.08–7.29). For islet glucose oxidation, $V_{max}$ was 12.4 pmol/islet⁻¹·h⁻¹ (95% confidence interval: 9.0–15.9), and $K_m$ was 2.60 mmol/l (95% confidence interval: 1.10–4.11). These results show that determining glucose metabolism for C and T islets at 1.1 and 11 mmol/l glucose tests baseline and maximally stimulated glucose metabolism, respectively. Comparison of islet glucose utilization and oxidation rates in C and T islets at 1.1 and 11 mmol/l glucose is shown in Fig. 2. Glucose utilization and oxidation rates increased significantly in 11 mmol/l glucose compared with 1.1 mmol/l glucose ($P<0.0001$) but similarly in both T and C islets. This demonstrates that decreased insulin secretion in response to chronic late gestation hypoglycemia is not due to decreased glucose metabolism.

**Islet insulin secretion.** Consistent with previously published results (25), total islet insulin content was maintained in the T group (11.8 ± 2.2 ng/islet T and 6.3 ± 1.6 ng/islet C, $P=0.07$). In vitro insulin secretion rates were measured to determine whether the defect in hypoglycemic fetal islet insulin secretion involves steps of stimulus-secretion coupling later than glucose metabolism, membrane depolarization, and calcium entry. Measurements were made under basal conditions and with 30 mmol/l KCl or 1 μmol/l ionomycin (Fig. 3). There

---

**Table 1. Characteristics of control and hypoglycemic treatment pancreases**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Treatment</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>3.60±0.29</td>
<td>3.10±0.23</td>
<td>0.20</td>
</tr>
<tr>
<td>Proportion of body weight, %</td>
<td>0.097±0.004</td>
<td>0.099±0.004</td>
<td>0.77</td>
</tr>
<tr>
<td>Insulin content, μg/g</td>
<td>73.8±11.4</td>
<td>97.3±9.1</td>
<td>0.14</td>
</tr>
<tr>
<td>%β-Cell area of pancreas</td>
<td>3.6±0.4</td>
<td>3.0±0.3</td>
<td>0.13</td>
</tr>
<tr>
<td>β-Cell mass, mg</td>
<td>138.6±26.0</td>
<td>91.3±13.5</td>
<td>0.07</td>
</tr>
<tr>
<td>Glucagon content, μg/g</td>
<td>7.81±1.20</td>
<td>12.72±1.42</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>%α-Cell area of pancreas</td>
<td>1.4±0.4</td>
<td>1.4±0.3</td>
<td>0.81</td>
</tr>
<tr>
<td>α-Cell mass, mg</td>
<td>55.1±19.4</td>
<td>43.2±10.2</td>
<td>0.50</td>
</tr>
<tr>
<td>Islet area, μm²</td>
<td>1,741±104</td>
<td>1,657±70</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Control and treatment values are means ± SE. Significant differences were determined by Student’s $t$-test.
lism, membrane depolarization, and calcium entry. Gestation hypoglycemic fetal sheep, beyond glucose metabolism. No differences were present between C and T β-cells for secretory granule number (1.93 ± 0.26 C, 1.81 ± 0.15 T, secretory granules/μm² cytoplasm), secretory granule radius (101 ± 7 C, 90 ± 1 T, nm), total combined area of all secretory granules (6.7 ± 1.5 C, 4.7 ± 0.4 T, %cytoplasm area), or total combined area of all mitochondria (7.6 ± 0.7 C, 8.4 ± 0.8 T, %cytoplasm area). There also was no difference in the location of the secretory granules between C and T β-cells. Representative micrographs are shown in Fig. 4. All β-cells photographed from three of seven T fetuses had distended rough ER (RER; Fig. 4B). This feature was not present in any β-cells evaluated from C animals (Fig. 4A).

**ER stress response in fetal sheep fibroblasts and pancreatic islets.** The ER stress response can decrease insulin secretion (17). Because of the distended RER noted in some of the hypoglycemic fetal β-cells, we determined whether this response could be activated by thapsigargin or hypoglycemia first in isolated fetal sheep fibroblast cells and then in isolated pancreatic islets. We quantified expression of GRP78 and demonstrated XBP-1 mRNA processing as markers for activation of the ER stress response (18, 34). In both sheep fetal fibroblast cells and isolated pancreatic islets, thapsigargin caused an ER stress response, but there was no evidence for ER stress caused by hypoglycemic culture conditions (Figs. 5–7).

**DISCUSSION**

In this study, we compared the structure and function of the pancreas and of isolated pancreatic islets from a fetal sheep model of IUGR produced by chronic hypoglycemia with the pancreas and isolated pancreatic islets from normally grown control fetal sheep to determine how in vivo hypoglycemia decreases fetal glucose-stimulated insulin secretion. The results show that chronic hypoglycemia in late gestation does not significantly decrease pancreatic islet size, pancreatic insulin content, or the pancreatic β-cell area. Hypoglycemia does, however, decrease insulin secretion at later steps of stimulus-secretion coupling. Glucose metabolism by the isolated islets is not abnormal and thus is not a cause of decreased glucose-stimulated insulin secretion. In addition, the defect in insulin secretion is not corrected by direct nonnutrient membrane depolarization with KCl and increased extracellular calcium entry with ionomycin significantly stimulated insulin release in C islets compared with basal incubation ($P < 0.05$). Neither KCl nor ionomycin was able to stimulate insulin release in T islets compared with basal conditions, and the fractional release of insulin in T islets under these conditions was significantly lower than in C islets ($P < 0.05$). These results demonstrate that a defect in the later steps of stimulus-secretion coupling limits GSIS in islets isolated from late gestation hypoglycemic fetal sheep, beyond glucose metabolism, membrane depolarization, and calcium entry.

**Electron microscopy.** β-Cell ultrastructure was analyzed to identify specific cellular features that might explain decreased insulin secretion despite maintenance of normal insulin content and glucose metabolism. No differences were present between C and T β-cells for secretory granule number (1.93 ± 0.26 C, 1.81 ± 0.15 T, secretory granules/μm² cytoplasm), secretory granule radius (101 ± 7 C, 90 ± 1 T, nm), total combined area of all secretory granules (6.7 ± 1.5 C, 4.7 ± 0.4 T, %cytoplasm area), or total combined area of all mitochondria (7.6 ± 0.7 C, 8.4 ± 0.8 T, %cytoplasm area). There also was no difference in the location of the secretory granules between C and T β-cells. Representative micrographs are shown in Fig. 4. All β-cells photographed from three of seven T fetuses had distended rough ER (RER; Fig. 4B). This feature was not present in any β-cells evaluated from C animals (Fig. 4A).

**ER stress response in fetal sheep fibroblasts and pancreatic islets.** The ER stress response can decrease insulin secretion (17). Because of the distended RER noted in some of the hypoglycemic fetal β-cells, we determined whether this response could be activated by thapsigargin or hypoglycemia first in isolated fetal sheep fibroblast cells and then in isolated pancreatic islets. We quantified expression of GRP78 and demonstrated XBP-1 mRNA processing as markers for activation of the ER stress response (18, 34). In both sheep fetal fibroblast cells and isolated pancreatic islets, thapsigargin caused an ER stress response, but there was no evidence for ER stress caused by hypoglycemic culture conditions (Figs. 5–7).

**DISCUSSION**

In this study, we compared the structure and function of the pancreas and of isolated pancreatic islets from a fetal sheep model of IUGR produced by chronic hypoglycemia with the pancreas and isolated pancreatic islets from normally grown control fetal sheep to determine how in vivo hypoglycemia decreases fetal glucose-stimulated insulin secretion. The results show that chronic hypoglycemia in late gestation does not significantly decrease pancreatic islet size, pancreatic insulin content, or the pancreatic β-cell area. Hypoglycemia does, however, decrease insulin secretion at later steps of stimulus-secretion coupling. Glucose metabolism by the isolated islets is not abnormal and thus is not a cause of decreased glucose-stimulated insulin secretion. In addition, the defect in insulin secretion is not corrected by direct nonnutrient membrane depolarization with KCl and increased extracellular calcium entry with ionomycin significantly stimulated insulin release in C islets compared with basal incubation ($P < 0.05$). Neither KCl nor ionomycin was able to stimulate insulin release in T islets compared with basal conditions, and the fractional release of insulin in T islets under these conditions was significantly lower than in C islets ($P < 0.05$). These results demonstrate that a defect in the later steps of stimulus-secretion coupling limits GSIS in islets isolated from late gestation hypoglycemic fetal sheep, beyond glucose metabolism, membrane depolarization, and calcium entry.

**Electron microscopy.** β-Cell ultrastructure was analyzed to identify specific cellular features that might explain decreased insulin secretion despite maintenance of normal insulin content and glucose metabolism. No differences were present between C and T β-cells for secretory granule number (1.93 ± 0.26 C, 1.81 ± 0.15 T, secretory granules/μm² cytoplasm), secretory granule radius (101 ± 7 C, 90 ± 1 T, nm), total combined area of all secretory granules (6.7 ± 1.5 C, 4.7 ± 0.4 T, %cytoplasm area), or total combined area of all mitochondria (7.6 ± 0.7 C, 8.4 ± 0.8 T, %cytoplasm area). There also was no difference in the location of the secretory granules between C and T β-cells. Representative micrographs are shown in Fig. 4. All β-cells photographed from three of seven T fetuses had distended rough ER (RER; Fig. 4B). This feature was not present in any β-cells evaluated from C animals (Fig. 4A).

**ER stress response in fetal sheep fibroblasts and pancreatic islets.** The ER stress response can decrease insulin secretion (17). Because of the distended RER noted in some of the hypoglycemic fetal β-cells, we determined whether this response could be activated by thapsigargin or hypoglycemia first in isolated fetal sheep fibroblast cells and then in isolated pancreatic islets. We quantified expression of GRP78 and demonstrated XBP-1 mRNA processing as markers for activation of the ER stress response (18, 34). In both sheep fetal fibroblast cells and isolated pancreatic islets, thapsigargin caused an ER stress response, but there was no evidence for ER stress caused by hypoglycemic culture conditions (Figs. 5–7).
depolarization or by increasing extracellular calcium entry into the β-cells. Although there was a trend toward decreased pancreatic β-cell mass in the hypoglycemic fetuses, this did not reach statistical significance. Given the normal pancreatic insulin content in these fetuses, we conclude that the defect in insulin secretion in the hypoglycemic fetuses is due to defective β-cell function and not disruption of pancreatic structure.

These results contrast with a placental insufficiency model of IUGR in fetal sheep. The placental insufficiency IUGR model also has decreased fetal glucose and insulin concentrations and impaired in vivo GSIS. This reduction in insulin secretion, however, is due at least in part to significantly lower pancreatic islet insulin content, which, despite normal-to-increased fractional insulin secretion, limits their capacity to respond as well as control fetuses to acute in vivo hyperglycemia. Islets from the placental insufficiency IUGR pancreases also are significantly smaller, and there is significantly less β-cell area and mass. Another difference between the primary hypoglycemic model and the placental insufficiency model of IUGR is that primary hypoglycemia significantly increases pancreatic glucagon content without increasing pancreatic α-cell area or α-cell mass. In the placental insufficiency model of IUGR, pancreatic glucagon content, α-cell area, and α-cell mass all were not different from controls. In addition, islet glucose oxidation is significantly decreased in the placental insufficiency model of IUGR, which may contribute to its decreased islet insulin content and β-cell mass (19, 21). On the basis of these differences from the hypoglycemia model in this report, it is clear that the timing, duration, degree, and type of nutrient restriction involved in producing fetal IUGR are important for determining the specific type of pancreatic β-cell/islet adaptation that leads to decreased GSIS.

Fig. 4. Ultrastructural analysis of control and chronically hypoglycemic fetal sheep β-cells. An ultrastructural analysis of hypoglycemic (treatment) β-cells was performed. In both micrographs, the nucleus (N) and mitochondria (M) are marked. A: representative micrograph of a control fetal sheep β-cell, with arrows indicating rough endoplasmic reticulum (RER). B: representative micrograph of a β-cell with distended RER (indicated by large arrowheads). Distended RER was observed in all β-cells photographed from 3 of 7 hypoglycemic fetal sheep but was not seen in control fetal sheep (n = 5) β-cells.

Fig. 5. Glucose regulatory protein-78 (GRP78) expression is not increased by low glucose in fetal sheep fibroblasts. A: thapsigargin (1 μmol/l) increases GRP78 expression relative to S15 (reference gene) in sheep fetal fibroblast cells. B: low glucose (0.41 ± 0.03 mmol/l glucose) fails to increase GRP78 expression relative to S15. *Significant difference from time 0 by repeated-measures ANOVA (P < 0.05).
The mechanism of GSIS is well described, and other secretagogues use the same pathways. GSIS occurs via the metabolism of glucose, which produces several intermediates involved in insulin secretion. The best characterized is the generation of ATP, which increases the ATP-to-ADP ratio, thereby closing ATP-sensitive K⁺ channels on the β-cell membrane, leading to depolarization. This depolarization opens membrane voltage-dependent calcium channels, leading to increased entry of calcium into the β-cells and elevation of cytosolic calcium concentrations, triggering insulin granule exocytosis and insulin release (16). A defect in GSIS could occur at any point along this pathway.

Previously, we showed that chronic late gestation hypoglycemia decreased insulin secretion in response to several nutrients (20, 25). In this study, we have more mechanistically extended the earlier studies by demonstrating the inability of direct membrane depolarization to restore insulin release in our hypoglycemic fetal islets by incubating the islets with KCl, a nonnutrient direct membrane depolarizer. We also show that increasing extracellular calcium entry into the β-cells by incubating the islets with the calcium ionophore thapsigargin failed to restore insulin release from the hypoglycemic fetal islets.

In the present study, we also tested islet glucose metabolism to determine whether a decrease in the capacity for the islets to metabolize glucose is partly responsible for the defective GSIS. The results for our control islets were nearly identical to the results previously reported in another cohort of sheep fetuses (21). We observed the expected three- to fourfold increase in both glucose oxidation and utilization rates when the glucose concentration in the incubation media was increased from 1.1 mmol/l (normal physiological concentration for our fetal sheep) to 11 mmol/l. However, this increase was observed in both normal control fetal islets and islets isolated from the hypoglycemic fetuses. Therefore, chronic late gestation hypoglycemia had no impact on rates of glucose utilization or oxidation in isolated islets, leading to the conclusion that the defect in GSIS in islets in our hypoglycemic model of IUGR cannot be due to differences in glucose metabolism.

Previously, we reported that isolated islets from this chronically hypoglycemic model of IUGR did not have a reduction in total islet insulin content (25), and this was confirmed in the present study. Consistent with this data are the findings that chronic late gestation hypoglycemia does not decrease islet size, the percentage of β-cells within the pancreas and the isolated islets, or the pancreatic insulin content. In addition, we previously demonstrated that chronic late gestation hypoglycemia does not change isolated pancreatic islet DNA content (25). These data lead to the conclusion that the insulin content per β-cell is normal in our hypoglycemic fetuses. Although this conclusion was not tested directly with pure β-cell isolation and evaluation of β-cell insulin content, we did not find differences in secretory granule number or size in our β-cell ultrastructural analysis.

![Fig. 6. GRP78 expression is not increased by low glucose in fetal sheep pancreatic islets. Isolated pancreatic islets from 12 (n = 4 for each media type) late-gestation normal fetal sheep were incubated in RPMI 1640 with 1% FBS and 3 mmol/l glucose (control), 0.5 mmol/l glucose (low glucose), or 3 mmol/l glucose and 1 μmol/l thapsigargin for 150 min. *Significant increase in GRP78 expression relative to S15 (reference gene) in thapsigargin compared with control media by ANOVA (P < 0.05).](image1)

![Fig. 7. X-box binding protein-1 (XBP-1) mRNA is not spliced in response to low glucose in fetal sheep fibroblasts or pancreatic islets. A and B: the smaller spliced mRNA indicates activation of the ER stress response in fibroblasts incubated with 1 μmol/l thapsigargin (Tg) but not with low glucose (0.41 ± 0.03 mmol/l). B: Tg denotes a positive control: sheep fetal fibroblast cells incubated with 1 μmol/l Tg for 4 h. C: Tg (1 μmol/l) results in XBP-1 mRNA splicing in isolated pancreatic islets, but low glucose (0.5 mmol/l) does not. The three columns at right indicate sheep fetal fibroblast cells incubated with 1 μmol/l Tg for 0, 30, and 240 min, respectively.](image2)
Our average isolated islet area reported (20,720 ± 13,500 μm²) is more than 10-fold greater than that determined for islets in pancreatic sections. The large discrepancy is most likely due to selection from the isolation technique or processing for morphometric analysis. This combination of islet isolation and processing naturally favors selection of the largest islets. These types of islets with increased numbers of β-cells have been described previously in both late gestation fetal and neonatal sheep pancreas (19, 31) and in fetal bovine pancreas (6). Overselection of these large islets with a predominance of β-cells by our isolation technique is also likely to be responsible for the increased percent insulin-positive area in our islets compared with previously published values (19). One benefit to overrepresentation of large, predominantly insulin-expressing islets, however, is that >90% of the islet is comprised of β-cells. This makes our determination of islet glucose metabolism more likely to represent β-cell metabolism and also facilitates selection of β-cells for ultrastructural analysis by electron microscopy.

Ultrastructural analysis did not reveal cellular features responsible for impaired glucose-stimulated insulin secretion in all of the hypoglycemic β-cells. Some of the hypoglycemic treatment fetal β-cells, however, did have distended RER, but this did not decrease the size, number, or location of secretory granules present. Our measurement of secretory granule diameter is consistent with previously published values for 1-day-old lambs (31) but is smaller than published diameters for the human fetus (33) or neonate (9). There are no published values for secretory granule number, combined area of all secretory granules, or combined area of all mitochondria for fetal sheep β-cells for comparison.

Distended RER also has been described in β-cells of fetal rats subjected to an experimental model of gestational diabetes (1). In our hypoglycemic model, it is possible that stress from glucose deprivation disrupts the normal folding and processing of insulin, leading to the accumulation of insulin or proinsulin (both of which are measured by our insulin assay) in the pancreatic β-cell and an insulin secretory defect. Genetic manipulations that cause abnormal proinsulin processing and folding or a decreased capacity to attenuate protein translation in response to ER stress also result in distended RER. In the Akita mouse model of diabetes, a heterozygous mutation in the insulin 2 gene results in replacement of a cysteine residue with a tyrosine residue. This disrupts the normal folding and processing of proinsulin within the ER and leads to an insulin secretory defect, dilated RER, and activation of the ER stress response (17, 24, 32). Distended RER and low serum insulin concentrations also have been observed in the β-cells of mice deficient for double-stranded RNA-dependent protein kinase-like ER kinase (PERK, a resident ER kinase)-mediated phosphorylation of eukaryotic initiation factor-2α (eIF2α) and therefore deficient in the ability to decrease protein translation in response to ER stress (15, 27).

To determine whether primary hypoglycemia could be responsible for ER dysfunction, we conducted in vitro experiments using fetal sheep fibroblasts and isolated pancreatic islets. We measured GRP78 expression and XBP-1 mRNA processing as markers of the ER stress response. GRP78 is an ER chaperone protein that assists in correct protein folding, and XBP-1 is a transcription activator for many ER stress response genes (18, 34). Our results show that, while thapsigargin elicits the ER stress response in both fetal sheep fibroblasts and pancreatic islets, low glucose concentrations (0.4–0.5 mmol/l) do not. These results indicate that the distended RER may not be primarily due to defective proinsulin processing and RER stress as a direct result of hypoglycemia but instead may represent a defect in the coordination of insulin synthesis with insulin release and degradation.

In conclusion, 2 wk of marked hypoglycemia in late gestation fetal sheep do not decrease pancreatic islet size, insulin content, β-cell area, β-cell mass, or islet glucose metabolism. Hypoglycemia does, however, decrease insulin secretion by inhibiting later steps of stimulus-secretion coupling, as shown by normal pancreatic islet glucose metabolism and the inability of nonnutrient direct membrane depolarization with KCl to restore insulin secretion. This supports our earlier findings of decreased in vitro nutrient-stimulated insulin secretion with arginine or lysine, which also stimulate insulin secretion by direct membrane depolarization (25). Our conclusions also are supported by the persistent insulin secretory defect following a direct increase in extracellular calcium entry into the β-cell with the calcium ionophore ionomycin. Unlike a placent al insufficiency model of fetal sheep growth restriction, there is no difference in the size of the pancreatic islets. Our findings in the hypoglycemic islets may be explained by retention of proinsulin or insulin in the RER of the β-cells due to an inability to coordinate insulin synthesis with release and degradation. Further studies will be needed to confirm or refute these possible mechanisms.

ACKNOWLEDGMENTS

We thank Dr. John C. Hutton, Barbara Davis Center for Childhood Diabetes, University of Colorado School of Medicine, Aurora, CO; Kathryn E. Howell, Cell and Developmental Biology, University of Colorado School of Medicine, Aurora, CO, for assistance with electron microscopy; Dan LoTurco for technical assistance with RT-PCR; and Rafael Leos for technical assistance with histology of the fetal pancreas.

GRANTS

This work was supported by National Institutes of Health (NIH) Grants HD-42815 [W. W. Hay, Jr., Primary Investigator (PI)], DK-52138 [W. W. Hay, Jr., PI], HD-28794 [W. W. Hay, Jr., PI], HD-07166 [W. W. Hay, Jr., PI], and RR-00060 [W. W. Hay, Jr., Associate Director] and NIH-Colorado Clinical Nutrition Unit Pilot and Feasibility Project 2 P30 DK-048520-11 (P. J. Rozance, PI) and NIH-Loan Repayment Program 2 L40 CA-117814 (P. J. Rozance, PI). Histological core services were provided by the Barbara Davis Center for Childhood Diabetes, University of Colorado School of Medicine, which is supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant P30-DK-57516 (J. C. Hutton, PI).

REFERENCES


