Reductions in insulin concentrations and β-cell mass precede growth restriction in sheep fetuses with placental insufficiency


1Department of Animal Sciences, University of Arizona, Tucson, Arizona; and 2Section of Neonatology, Department of Pediatrics, University of Colorado-Denver, Aurora, Colorado

Submitted 24 August 2012; accepted in final form 31 December 2012

Limesand SW, Rozance PJ, Macko AR, Anderson MJ, Kelly AC, Hay WW, Jr. Reductions in insulin concentrations and β-cell mass precede growth restriction in sheep fetuses with placental insufficiency. Am J Physiol Endocrinol Metab 2013;204: E516–E523. First published December 31, 2012; doi:10.1152/ajpendo.00435.2012.—In pregnancy complicated by placental insufficiency (PI) and intrauterine growth restriction (IUGR), the fetus near term has reduced basal and glucose-stimulated insulin concentrations and reduced β-cell mass. To determine whether suppression of insulin concentrations and β-cell mass precedes reductions in fetal weight, which would implicate insulin deficiency as a cause of subsequent IUGR, we measured basal and glucose-stimulated insulin concentrations and pancreatic histology at 0.7 gestation in PI fetuses. Placental weights in the PI pregnancies were 40% lower than controls (265 ± 26 vs. 442 ± 41 g, P < 0.05), but fetal weights were not different. At basal conditions blood oxygen content, plasma glucose concentrations, and plasma insulin concentrations were lower in PI fetuses compared with controls (2.5 ± 0.3 vs. 3.5 ± 0.3 mmol/l oxygen, P < 0.05; 1.11 ± 0.09 vs. 1.44 ± 0.12 mmol/l glucose; 0.12 ± 0.01 vs. 0.27 ± 0.02 ng/ml insulin, P < 0.05). During a steady-state hyperglycemic clamp (~2.5 ± 0.1 mmol/l), glucose-stimulated insulin concentrations were lower in PI fetuses than controls (0.28 ± 0.02 vs. 0.55 ± 0.04 ng/ml, P < 0.01). Plasma norepinephrine concentrations were 3.3-fold higher (P < 0.05) in PI fetuses (635 ± 104 vs. 191 ± 91 pg/ml). Histological examination revealed less insulin area and lower β-cell mass and rates of mitosis. The pancreatic parenchyma was also less dense (P < 0.01) in PI fetuses, but no differences were found for pancreatic progenitor cells or other endocrine cell types. These findings show that hypoglycemia, hypoxia, and hypercatecholaminemia are present and potentially contribute to lower insulin concentrations and β-cell mass due to slower proliferation rates in early third-trimester PI fetuses before discernible reductions in fetal weight.

intrauterine growth restriction; pancreas; glucose; oxygen; norepinephrine

PLACENTAL INSUFFICIENCY (PI) is a common complication of human pregnancy that limits the supply of oxygen and nutrients to the developing fetus (11, 38). At later stages of pregnancy, fetuses with PI develop hypoglycemia, hypoxia, and intrauterine growth restriction (IUGR) (37). Fetuses with established IUGR also have lower plasma insulin concentrations, impaired β-cell responsiveness, and, in more severe cases, less β-cell mass (7, 32, 50). Impairments in β-cell function coincide with increased indexes for insulin sensitivity in small-for-gestational-age fetuses and newborns (14, 46). We have established an ovine model of chronic IUGR induced by PI beginning early in pregnancy that possesses all of the complications reported for near-term human fetuses with chronic IUGR (39, 51), including impaired fetal insulin secretion and reduced β-cell mass due to a slower rate of mitosis (8, 24, 26, 29). We have also demonstrated greater insulin sensitivity for the rate of glucose utilization in the IUGR fetus (28, 48). Together, these findings indicate that, at later stages of gestation, fetal β-cells are susceptible to oxygen and nutritional deficiencies created by PI. They also indicate that suppression of insulin secretion, which is a predominant anabolic hormone in the fetus, contributes to slower rates of fetal growth (16, 19, 22).

Diagnosis of IUGR usually occurs after 24 wk of gestation in women when anthropometry measurements by ultrasonography detect a slower fetal growth trajectory (15). Our fetal sheep model of PI-induced IUGR also possesses a similar decline in fetal growth after midgestation (2, 40). The widening disparity in body size between normal and PI-IUGR fetuses is expected, because placental transport capacity progressively fails to meet the oxygen and nutritional needs required for fetal metabolism and growth. One potential mechanism that slows the fetal growth trajectory is reduced lower insulin concentrations as a result of PI-induced hypoxemia and hypoglycemia. Increased fetal insulin secretion normally occurs in direct response to increasing nutrient supply and coordinates nutrient availability with fetal growth (1, 20, 25, 29, 32). Because the slower rate of fetal growth follows reduced oxygen and nutrient transport capacity of the placenta with transport insufficiency (natural, pathological, or experimentally induced), we hypothesized that low insulin concentrations and decreased β-cell mass are antecedents to fetal growth restriction, in keeping with experimentally reduced insulin secretion (e.g., from fetal pancreatectomy or fetal streptozotocin injections) that restricts fetal growth independently of nutrient supply (16, 22). To test this hypothesis, we measured fetal insulin concentrations during glucose and arginine stimulation in sheep fetuses from pregnancies complicated by PI at the beginning of the third trimester, when fetal nutrient and oxygen supplies are moderately decreased, but before measurable fetal growth restriction.

MATERIALS AND METHODS

Fetal sheep preparations. Sixteen Columbia-Rambouillet crossbreed ewes carrying singleton pregnancies were purchased from Colorado State University (Fort Collins, CO), and litter size was confirmed by ultrasonography. All animal care and use were conducted with institutional approval at the Perinatal Research Center at the University of Colorado Denver (Aurora, CO), which is accredited by the American Association for Accreditation of Laboratory Animal Care, the National Institutes of Health, and the United States Department of Agriculture. PI (n = 10) fetuses were created by exposing pregnant ewes to elevated ambient temperatures (40°C for 12 h; 35°C for 12 h) and moderate humidity (dew point at 22°C) from 39 ± 1

Address for reprint requests and other correspondence: S. W. Limesand, Dept. of Animal Sciences, Univ. of Arizona, 1650 E. Limberlost Dr., Tucson, AZ 85719 (e-mail: limesand@ag.arizona.edu).
days gestational age (dGA; mean ± SD) until 93 ± 1 dGA to produce PI as previously described (8, 27, 49). Control fetuses (n = 6) were from healthy pregnant ewes that were maintained at 25°C and pair-fed to the average feed intake of treated ewes. Ewes received alfalfa pellets, which had a dry matter composition of 19.5% crude protein, 32.6% acid detergent fiber, 42.6% neutral detergent fiber, 1.23 Mcal/kg net energy of maintenance, and 0.66 Mcal/kg net energy for reproductive processes (Dairy One Forage Testing Laboratory, Ithaca, NY). Six fetuses from each treatment group completed in vivo studies because four PI fetuses were lost during treatment exposure.

**Surgical preparation.** At 99 ± 1 dGA, indwelling catheters (Tygon Microbore Tubing formulation 5–54-HL; 1.1 mm outer diameter; Norton Performance Plastics, Akron, OH) were surgically placed in the fetus for blood sampling and infusion as described previously (25). Fetal catheters for blood sampling were placed in the abdominal aorta via the femoral arteries, and infusion catheters were placed in the femoral veins via the saphenous veins. Maternal catheters were placed in the femoral artery and vein for arterial sampling and venous infusions. All catheters were tunneled subcutaneously to the ewe’s flank, exteriorized through a skin incision, and kept in a plastic mesh pouch sutured to the ewe’s skin. Ewes were allowed to recover for 4 days before conducting the in vivo experiments.

**Glucose and glucose-potentiated arginine-stimulated insulin secretion.** A square wave hyperglycemic clamp was used to determine insulin secretion in response to glucose at 103 ± 1 dGA as previously reported (17, 42). Briefly, a continuous transfusion of maternal blood in the fetus (6 ml/h) was started 45 min before baseline sampling and maintained for the duration of the study to compensate for blood collection and to stabilize fetal hematocrit. All sample times are presented relative to the start of the fetal glucose bolus and continuous glucose infusion (time = 0). Baseline plasma glucose and insulin concentrations were determined at −30, −20, −10, and −5 min. Whole blood collected in syringes lined with EDTA (Sigma Chemicals) was centrifuged (13,000 g) for 2 min at 4°C. Plasma was aspirated from the cell pellet and stored at −80°C for hormone measurements. Blood gas and oxygen saturation were measured in blood collected in syringes lined with heparin (Elkins-Sinn, Cherry Hill, NJ). The hyperglycemic clamp was initiated with a dextrose bolus (controls, 397 ± 57 mg/kg; and PI fetuses, 461 ± 49 mg/kg; not different) directly in the fetal circulation followed by a constant infusion of 33% dextrose (controls, 14.0 ± 0.8 mg·min⁻¹·kg⁻¹; PI fetuses, 13.5 ± 1.1 mg·min⁻¹·kg⁻¹; not different) to maintain fetal arterial plasma glucose concentration at 2.5 mmol/l, which was shown to produce maximal insulin concentrations in fetal sheep (18). At the onset of the glucose infusion, fetal arterial plasma samples were collected every 5–10 min for the initial 30 min to establish steady-state hyperglycemia, after which fetal blood and plasma samples were collected at 45, 60, and 80 min during steady-state hyperglycemic conditions. During basal (time = −30 to 0 min) and hyperglycemic (45–80 min) steady-state periods fetal blood was collected for blood gas and oximetry measurements, and plasma was collected for glucose and insulin measurements. Following the final hyperglycemic sample, a glucose-potentiated arginine-stimulated insulin secretion test was conducted by injecting a bolus of arginine (0.5 mmol/kg estimated fetal weight mixed with 1 ml of 2 mol/l sodium acetate and 4 ml saline) over 4 min in the fetal circulation and collecting plasma samples at 5, 10, 20, and 30 min for subsequent measurement of insulin concentrations.

**Biochemical analysis.** Blood oxygen saturation and hemoglobin concentrations were measured with an ABL 520 with values temperature corrected at 39°C (Radiometer, Copenhagen, Denmark). Plasma glucose and lactate concentrations were measured immediately using an YSI model 2700 SELECT Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin concentrations were measured with an ovine insulin ELISA (intra- and interassay coefficients of variation: 5.6 and 2.9%, respectively; ALPICO Diagnostics, Windham, NH). Other hormones measured included glucagon with a glucagon radioimmunoassay (Linco Research, St. Charles, MI) and norepinephrine with a Noradrenaline ELISA (inter- and intrassay coefficients of variation: 20 and 22%, respectively; Labor Diagnostik Nord). Plasma norepinephrine concentrations were only determined for five controls and three PI fetuses because of limited plasma volumes. All other measurements were performed on all fetuses.

**Postmortem examination.** After completion of the in vivo studies, the pregnant ewe and her fetus were recovered to prestudy steady-state conditions and killed within 20 h with an intravenous overdose of pentobarbital sodium (86 mg/kg) and phenytoin sodium (11 mg/kg, Euthasol; Virbac Animal Health, Fort Worth, TX). The fetus was weighed and dissected. The entire fetal pancreas was collected for histology as reported previously (10).

**Immunofluorescent staining.** Pancreatic sections, 10 μm thick, were cut for histological evaluation at >100-μm intervals. Immunofluorescent procedures for endocrine pancreas analysis were described previously (10, 24, 26). Cells expressing mature pancreatic endocrine hormones were identified with guinea pig anti-porcine insulin (1:500; Dako, Carpinteria, CA), mouse anti-porcine glucagon (1:500; Sigma-Aldrich, St. Louis, MO), rabbit anti-human somatostatin (1:500; Dako), and rabbit anti-human pancreatic polypeptide (1:500; Dako). Immunoperoxidase detection for insulin, glucagon, and other endocrine cells (somatostatin/pancreatic polypeptide) was achieved with species-specific immunoglobulin affinity-purified secondary antisera conjugated to Cy2, Texas Red, or 7-amino-4-methylcoumarin-3-acetic acid (Jackson ImmunoResearch Laboratories, West Grove, PA). β-Cell proliferation was determined by dual immunostaining with mouse anti-sheep insulin C-peptide (1:500; see Ref. 26) and rabbit polyclonal anti-phospho-Histone H3 (pH3, 7.5 μg/ml; Upstate, Lake Placid, NY). Antigen retrieval procedures for this staining included an incubation in 0.2% Triton X-100 PBS for 15 min, a 10-min Proteinase K digestion (20 μg/ml in 10 mM Tris, pH 8.0), and a 10 mM citric acid, pH 6.0, microwave treatment before incubation with primary antibodies. Immunocomplexes were detected with affinity-purified anti-rabbit IgG conjugated to Cy2 and anti-mouse IgG conjugated to Texas Red. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and mounted in 50% glycerol and 10 mM Tris-HCl, pH 8. Newly formed β-cells were identified as those that coexpressed an epithelial cell marker, mouse anti-human cytokeratins, clone AE1/AE3 (1:200; Dako), and rabbit anti-mouse Pdx-1 (1:1,000; Millipore, Billerica, MA).

**Morphometric analysis.** Fluorescent images were visualized on a Leica DM5500 Microscope System and digitally captured with a Spot Pursuit 4 Megapixel CCD camera (Diagnostic Instruments, Sterling Heights, MI). Immunopositive areas were determined with Image Pro Plus 6.3 software (Media Cybernetics, Silver Spring, MD). Data are expressed as a percent of total pancreas area. The pancreatic parenchyma area was measured by autofluorescence. Insulin, glucagon, and other endocrine cell-positive areas were determined in ≥25 fields of view (≥0.31 mm²/section). Three pancreatic sections were evaluated per animal, and each section was separated by ≥100 μm. β-Cell (insulin⁺ cells) and α-cell (glucagon⁺ cells) mass was determined by multiplying the pancreas weight by the percent positive area. The proportion of β-cells undergoing mitosis (pH3/insulin⁺) was determined by evaluating >3,000 nuclei (DAPI⁺) of insulin-positive cells within each fetal pancreas on ≥3 pancreatic sections/fetus separated by 100 μm. Cytokeratin and insulin colocalization was determined in >300 insulin-positive cells/fetus.

**Statistical analysis.** All data are expressed as means ± SE. Period means for each animal were used for biochemical and hematological value comparisons. Statistical analyses for biochemical, hematological, and histological values were conducted by one-way ANOVA using the general linear means procedure in SAS Proc GLM, and differences were determined with a post hoc least-significant difference test (45). Insulin concentrations during the glucose-stimulated insulin secretion (GSIS) basal and hyperglycemic steady states were subjected to a repeated-measures ANOVA with fixed effects for
treatment group and period (draw time) and random effects for sheep (SAS Proc MIXED; see Ref. 45). Statistical analysis for arginine-stimulated insulin secretion was an ANOVA (SAS Proc GLM).

RESULTS

*Maternal parameters during treatment*. Within 7 days of exposure to elevated ambient temperatures, maternal core body temperatures increased from $39.2 \pm 0.2^\circ C$ to $39.6 \pm 0.1^\circ C$, reaching an average plateau of $39.7 \pm 0.1^\circ C$ for the 53 days of treatment, greater ($P < 0.01$) than the control ewes’ average of $39.1 \pm 0.2^\circ C$ during this same period. Ewes in the control group were pair-fed according to daily average feed intakes of ewes in the treatment group; therefore, feed intakes were not different between treatments: $1.4 \pm 0.1$ kg/day in PI ewes and $1.3 \pm 0.1$ kg/day in controls. Body weights of control ewes ($51.6 \pm 1.7$ kg) were not different from PI ewes ($53.1 \pm 1.9$ kg).

*Basal steady-state blood and plasma values*. In PI fetuses arterial blood oxygen content was 30% lower ($P < 0.05$) and arterial oxygen tension was 22% lower ($P < 0.05$) compared with control fetuses (Table 1). Basal fetal plasma glucose concentrations were lower ($P \leq 0.05$) in PI fetuses (Fig. 1A). Carbon dioxide tension, pH, bicarbonate, and hematocrit values were not different between treatment groups (data not shown). Plasma lactate concentrations were not different between treatments. Maternal arterial oxygen tension, oxygen content, glucose, and lactate were not different between treatments.

During the basal period plasma insulin concentrations were 66% lower in PI fetuses than controls ($0.12 \pm 0.01$ vs. $0.27 \pm 0.02$ ng/ml, $P < 0.05$; Fig. 1B). Plasma norepinephrine concentrations were 3.3-fold higher in PI fetuses ($635 \pm 104$ vs. $191 \pm 91$ pg/ml, $P < 0.05$). Plasma glucagon concentrations were lower ($P < 0.01$) in PI fetuses ($55.5 \pm 3.1$ pg/ml) compared with control fetuses ($78.6 \pm 2.6$ pg/ml).

Indexes demonstrate greater insulin sensitivity in PI fetuses at 0.7 gestation at basal conditions. We calculated the glucose-to-insulin ratio (G/I) and quantitative insulin sensitivity check index (QUICKI), the inverse of the product of basal insulin and glucose concentrations ($1/([\text{insulin}_{\text{basal}}(\mu \text{U/ml}) \times [\text{glucose}_{\text{basal}}(\text{mg/dl})])$), which have been previously used to establish fetuses and newborn insulin sensitivity in humans (14, 21, 46). The G/I (mmol·l$^{-1}$·μg$^{-1}$·l$^{-1}$) ratio in PI fetuses was $10.4 \pm 1.3$ and was greater ($P < 0.05$) than the control mean of $5.9 \pm 0.9$. The QUICKI calculation also shows greater insulin sensitivity in PI

<table>
<thead>
<tr>
<th>Table 1. Fetal arterial blood and plasma values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td><strong>Fetal</strong></td>
</tr>
<tr>
<td>$P_{O_2}$, mmHg</td>
</tr>
<tr>
<td>$O_2$ content, mmol/l</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
</tr>
<tr>
<td><strong>Maternal</strong></td>
</tr>
<tr>
<td>$P_{O_2}$, mmHg</td>
</tr>
<tr>
<td>$O_2$ content, mmol/l</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
</tr>
</tbody>
</table>

Values are means ± SE; PI, placental insufficiency. *Differences ($P \leq 0.05$) between control and PI fetuses.

Fig. 1. Lower insulin concentrations in fetuses with placental insufficiency (PI). Fetal plasma glucose (A) and insulin (B) concentrations determined for the in vivo glucose-stimulated insulin secretion (GSIS) study are presented as means ± SE for control ($n = 6$; open diamonds) and PI ($n = 6$; filled circles) fetuses. Administration of the glucose bolus and infusion were started at 0 min. Statistical analyses are presented in RESULTS for basal and hyperglycemic steady-state period averages.

Glucose-stimulated insulin concentrations. Following the basal steady-state period, a square-wave hyperglycemic clamp was established to raise fetal plasma glucose concentrations to levels determined to be maximally stimulatory for insulin secretion (18), and these glucose concentrations were not different between treatment groups (Fig. 1A). In response to this hyperglycemia, plasma insulin concentrations increased 2.1-fold to $0.55 \pm 0.04$ ng/ml in control fetuses and 2.3-fold in PI fetuses to $0.28 \pm 0.02$ ng/ml ($P < 0.01$; Fig. 1B). The maximal plasma insulin concentrations were lower ($P < 0.01$) in PI vs. control fetuses. In addition to monitoring absolute differences in circulating insulin concentrations, we also calculated the GSIS responsiveness (difference in insulin concentrations between hyperglycemia and basal periods). The change in
glucose-induced insulin concentrations was 50% lower in PI than control fetuses (P < 0.01; Fig. 2).

Glucose-potentiated arginine-stimulated insulin secretion. Insulin concentrations following the administration of arginine reached maximum values after 5 min in all fetuses in both treatment groups. The peak insulin concentrations were not different between controls (1.08 ± 0.26 ng/ml) and PI fetuses (1.11 ± 0.24 ng/ml). Administration of arginine also stimulated plasma glucagon concentrations, which peaked at 5 min, but the maximal concentrations were not different between control (108.1 ± 10.7 pg/ml) and PI (102.4 ± 12.6 pg/ml) fetuses.

Fetal body and organ weights. At necropsy gestational age was not different between control (104 ± 2 days) and PI (104 ± 2 days) fetuses. All of the PI fetuses were males compared with 67% males in the control group. The mean weight of the conceptus (placenta and fetus) in the PI fetuses was 29% less than controls (Table 2). Mean weights of the uterus, placenta, and placental membranes were 24, 40, and 38% lower in PI than control fetuses, respectively. No differences were found between mean PI and control fetal weights or crown-rump lengths (34.9 ± 1.3 cm control vs. 33.4 ± 0.9 cm PI); mean brain, liver, and pancreas weights also were not different between treatment groups (Table 2). However, the brain-to-liver ratio was greater (P < 0.05) in PI fetuses, as was the proportion of brain mass to fetal mass (2.4 ± 0.1% PI vs. 1.9 ± 0.1% control, P < 0.05). The weight of the spleen was less in PI fetuses (1.9 ± 0.3 g PI vs. 3.1 ± 0.3 g control, P < 0.05); kidney weights also tended to be less (9.2 ± 0.5 g PI vs. 11.9 ± 1.2 g control, P < 0.07).

Pancreas histology. Immunofluorescent positive insulin area was 28% lower in PI fetuses compared with control fetuses (Table 3 and Fig. 3). β-Cell mass was 41% lower (P < 0.05) in PI fetuses compared with control fetuses (Fig. 4A). The rate of β-cell mitosis was lower (P < 0.05) in PI than control fetuses (Fig. 4B). Individual β-cell area was not different between control (108.8 ± 4.8 μm²) and PI (112.7 ± 5.6 μm²) fetuses. The rate of β-cell differentiation, determined by insulin and cytokeratin colocalization, was not different between PI and control fetuses. No differences were identified for immunofluorescent-positive areas for glucagon (α-cell) or somatostatin plus pancreatic polypeptide (δ- and F-cells). Pancreatic parenchyma area was lower (P < 0.01) in PI than control fetuses (Table 3). However, cytokeratin-positive area was not different between PI and control fetuses, 2.8 ± 0.3 and 3.1 ± 0.4%, respectively. A majority (90.2 ± 2.5%) of the cytokeratin-positive cells were positive for Pdx-1, which is similar to previous findings at younger ages (10).

DISCUSSION

Previous studies in fetal sheep have demonstrated that reduced insulin secretion and plasma insulin concentrations, independent of oxygen and nutrient supplies, reduce fetal growth rates (5, 16, 22). Such observations formed the hypothesis that reduced fetal growth due to PI occurred only after a reduction in fetal insulin and β-cell responsiveness. To test this possibility, we compared β-cell function and insulin concentrations in normal (control) fetuses and fetuses from an established model of chronic PI that consistently produces placental and fetal IUGR in later pregnancy (3, 6). Fetal plasma insulin concentrations were lower in PI fetuses at 0.7 gestation during basal and hyperglycemic steady-state conditions, before definitive fetal IUGR, and there was a diminished fetal insulin secretion response to glucose, but not arginine. Interestingly, and in support of the possibility that PI induces β-cell dysfunction, followed by fetal IUGR, lower β-cell mass due to reduced rates of mitosis preceded overt reductions in fetal weight, although at least one other early sign of asymmetric growth, a reduced liver-to-brain weight ratio, was present. These findings uniquely demonstrate that reduced β-cell mass and suppression of insulin concentrations in response to reduced nutrient supply by PI are precursors to fetal growth restriction.

PI was confirmed in our studies with measurement of smaller placental mass and moderate fetal hypoxemia and hypoglycemia.

Table 2. Postmortem measurements

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conceptus, kg</td>
<td>2.91 ± 0.29</td>
<td>2.06 ± 0.10*</td>
</tr>
<tr>
<td>Uterus, g</td>
<td>337 ± 21</td>
<td>258 ± 13*</td>
</tr>
<tr>
<td>Placenta, g</td>
<td>442 ± 41</td>
<td>265 ± 26*</td>
</tr>
<tr>
<td>Membranes, g</td>
<td>155 ± 14</td>
<td>96 ± 9*</td>
</tr>
<tr>
<td>Fetus, kg</td>
<td>1.23 ± 0.13</td>
<td>1.05 ± 0.07</td>
</tr>
<tr>
<td>Brain, g</td>
<td>23.4 ± 1.8</td>
<td>24.7 ± 0.9</td>
</tr>
<tr>
<td>Liver, g</td>
<td>67.5 ± 6.9</td>
<td>52.9 ± 4.6</td>
</tr>
<tr>
<td>Pancreas, g</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Brain-to-liver ratio</td>
<td>0.36 ± 0.03</td>
<td>0.48 ± 0.04*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Differences (P = 0.05) between control and PI fetuses.

Table 3. Pancreas histological characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control, %</th>
<th>PI, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Cell area</td>
<td>2.1 ± 0.2</td>
<td>1.5 ± 0.2*</td>
</tr>
<tr>
<td>α-Cell area</td>
<td>1.1 ± 0.3</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>δ/F-cell area</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Parenchyma area</td>
<td>42.8 ± 2.1</td>
<td>36.1 ± 1.1*</td>
</tr>
<tr>
<td>Differentiation rate</td>
<td>7.2 ± 1.1</td>
<td>9.4 ± 1.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Difference (P < 0.05).
The reduced size of the placenta alone in our model of chronic PI-IUGR imparts reduced oxygen and nutrient supply to the fetus. Additionally, specific placental molecular defects (e.g., reduced amino acid and glucose transporters) further contribute to impaired glucose and amino acid transport capacity (12, 49). Reduced glucose supply to the fetus and lower plasma glucose concentrations limit insulin production and secretion in this model (29).

The severity of hypoglycemia and hypoxemia in fetuses with PI-IUGR worsens as gestation progresses. In the present study, we found that as early as 0.7 gestation, PI fetuses have 29% lower blood oxygen content and 23% lower plasma glucose concentrations (Table 1) compared with 50% reduction in both parameters by 0.9 gestation compared with age-matched controls (27, 29). At 0.7 gestation, fetal weight was nearly normal in the PI fetuses in our study, including particularly brain weight, indicating that increasing fetal demand for oxygen due to increasing fetal mass likely contributed significantly to the progressive hypoxemia, in addition to the declining placental transport capacity (31, 39, 41, 49). Importantly, our findings at 0.7 gestation indicate that PI fetuses are chronically exposed to hypoglycemia and hypoxemia over the last third of gestation in this model of PI, even though the magnitude of such conditions increases as gestation advances. Chronic exposure to these adverse metabolic conditions or the endocrine...
responses caused by such conditions have been shown to initiate adaptive responses in the fetal β-cells that reduce insulin production and secretion, representing an appropriate physiological mechanism that matches nutrient and oxygen supplies with anabolic hormone production (1, 24, 25).

In PI-IUGR fetuses near-term β-cell mass is 78% lower than controls, which was associated with slower rates of β-cell mitosis but no changes in apoptosis (26). Interestingly, the current findings show deficiencies in β-cell mass and replication rates before fetal growth restriction (Fig. 4), and these deficiencies progressively worsen as gestational age advances in the context of PI (8, 26). In addition to nutritional status and circulating insulin, pancreatic expression of fibroblast growth factors and insulin-like growth factors is lower in near-term PI-IUGR fetuses (8). These factors may contribute to the observed limitation of β-cell and pancreatic parenchyma expansion, as shown in the present studies. The pancreatic progenitor cells, which were immunopositive for cytokeratin and Pdx-1, make up similar proportions of pancreas area between treatments. These findings also support no differences in rates of β-cells differentiation in the current study and previous findings at older gestational ages (8, 26).

In addition to reductions in β-cell mass we also demonstrate lower insulin concentrations and identified specific features of the 0.7 gestation PI fetus that might impair β-cell responsiveness to glucose, including low arterial oxygen content and increased plasma norepinephrine concentrations. Although low oxygen concentrations can directly inhibit insulin secretion (36), we have demonstrated in previous studies that chronically low oxygen in late-gestation PI-IUGR fetuses indirectly inhibits β-cell function via increased circulating norepinephrine (36). Acute fetal hypoxia also increases plasma norepinephrine concentrations, which inhibit insulin secretion (9, 13, 30). The chronic suppression of insulin in fetal sheep by norepinephrine in the absence of hypoxemia results in fetal growth restriction, which was alleviated with the administration of insulin (4, 5, 52).

Another noteworthy feature is greater insulin sensitivity, which could be either a result or cause of lower insulin secretion in this model. Although not directly measured in this set of studies, indexes indicate that whole body insulin sensitivity for glucose utilization is greater, which has been demonstrated in the same model of fetal IUGR at 0.9 gestation (28, 47, 48). This is a consistent observation among all studies to date in IUGR fetal sheep, regardless of the model, including impaired β-cell function (33, 34). Together these data support increases in insulin sensitivity for glucose utilization in fetal sheep models of IUGR and that the increases are concurrent with impaired β-cell function, which is also shown in the current study at quite early stages of IUGR development.

Norepinephrine responsiveness has been examined in late-gestation fetal sheep by measuring changes in glucose and free fatty acid metabolism, which identified a threshold concentration for physiological action of 2,000 pg/ml (35). In PI-IUGR fetuses at 0.9 gestation norepinephrine concentrations exceeded this threshold (24, 29). However, the 3.3-fold greater norepinephrine concentrations in the PI fetuses at 0.7 gestation in the present study achieved concentrations below the 2,000 pg/ml threshold and were comparable to the concentrations of control fetuses at 0.9 gestation (9, 35). This raises questions about whether norepinephrine sensitivity is higher at earlier gestational ages or whether the higher concentrations identified in the current set of PI fetuses contribute to the lower fetal plasma insulin concentrations we identified. Experiments to test these two possibilities are ongoing.

We also identified lower fetal arterial plasma glucose concentrations in PI fetuses at 0.7 gestation. In previous studies 2 wk of experimentally induced hypoglycemia blunted fetal insulin secretion despite the presence of normal β-cell mass (25, 43). In these fetuses β-cell dysfunction was not associated with decreased arterial oxygen content or increased arterial plasma norepinephrine concentrations, indicating that glucose restriction alone is sufficient to lower insulin secretion responsiveness (25, 43). Therefore, the fetal hypoglycemia identified in the 0.7 gestation PI fetuses in the present study might be responsible for the lower fetal insulin concentrations. However, we have not directly tested the impact of selectively and chronically restricting fetal glucose supply, for example, with maternal insulin infusions, on β-cell mass and function at 0.7 gestation.

Another characteristic of the PI fetus at 0.7 gestation that might have contributed to reduced insulin secretion responsiveness was the 30% lower mean plasma glucagon concentration. Glucagon can potentiate glucose-induced insulin secretion (23, 44).

Together, these observations at 0.7 gestation in fetuses with PI indicate that multiple causes, decreased β-cell mass, increased norepinephrine, increased insulin sensitivity, lower glucose, lower oxygen, and lower glucagon, could be involved to produce the reductions in plasma insulin concentrations in PI fetuses that then would contribute to producing growth restriction and fetal IUGR.

In conclusion, the present study demonstrates that mild hypoxemia and hypoglycemia, as well as hypoinsulinemia and reduced β-cell mass, are present at the onset of the third trimester in fetal sheep with chronic PI. Moreover, these complications in PI fetuses at 0.7 gestation precede reductions in fetal weight. The findings indicate that fetal endocrine derangements (primarily decreased insulin, but also increased norepinephrine and reduced glucagon), as well as metabolic derangements (hypoglycemia, hypoxemia), that develop in response to nutrient and oxygen deficiencies from chronic PI precede, and likely contribute to, subsequent fetal growth restriction.

ACKNOWLEDGMENTS

We thank David Caprio for his technical assistance. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

GRANTS

This work was supported by National Institutes of Health (NIH) Grants R01-HD-42815 (W. W. Hay, Jr.) and R01-DK-084842 (S. W. Limesand). P. J. Rozance was supported by NIH Grants R01-DK-088139 and K08-HD-060688. A. R. Macko was supported by NIH Grant T32 HL-7249. The research also was supported by NIH Institutional Training Grant HD-07186 (W. W. Hay, Jr.) and the NIH-Colorado Clinical Nutrition Research Unit (P90-DK-048520-11, J. Hill).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

REFERENCES