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Pulsatile hyperglycemia increases insulin secretion but not pancreatic β-cell mass in intrauterine growth-restricted fetal sheep

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Original Article

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Abstract

Impaired β-cell development and insulin secretion are characteristic of intrauterine growth-restricted (IUGR) fetuses. In normally grown late gestation fetal sheep pancreatic β-cell numbers and insulin secretion are increased by 7–10 days of pulsatile hyperglycemia (PHG). Our objective was to determine if IUGR fetal sheep β-cell numbers and insulin secretion could also be increased by PHG or if IUGR fetal β-cells do not have the capacity to respond to PHG. Following chronic placental insufficiency producing IUGR in twin gestation pregnancies ($n = 7$), fetuses were administered a PHG infusion, consisting of 60 min, high rate, pulsed infusions of dextrose three times a day with an additional continuous, low-rate infusion of dextrose to prevent a decrease in glucose concentrations between the pulses or a control saline infusion. PHG fetuses were compared with their twin IUGR fetus, which received a saline infusion for 7 days. The pulsed glucose infusion increased fetal arterial glucose concentrations an average of 83% during the infusion. Following the 7-day infusion, a square-wave fetal hyperglycemic clamp was performed in both groups to measure insulin secretion. The rate of increase in fetal insulin concentrations during the first 20 min of a square-wave hyperglycemic clamp was 44% faster in the PHG fetuses compared with saline fetuses ($P < 0.05$). There were no differences in islet size, the insulin$^+$ area of the pancreas and of the islets, and β-cell mass between groups ($P > 0.23$). Chronic PHG increases early phase insulin secretion in response to acute hyperglycemia, indicating that IUGR fetal β-cells are functionally responsive to chronic PHG.

Introduction

Severely intrauterine growth-restricted (IUGR) human fetuses are characterized by decreased plasma glucose and insulin concentrations, impaired glucose-stimulated insulin secretion (GSIS) and reduced β-cell mass.[1–3] Lower β-cell mass may be one of the reasons why IUGR infants have an increased risk of developing type 2 diabetes as adults, a disease characterized by increased insulin resistance and an inability of the β-cells to secrete enough insulin to compensate for the insulin resistance.[4–6] In addition to fewer numbers of β-cells, the β-cells in IUGR fetuses also might develop a permanent defect in their ability to respond to increased insulin demand from insulin resistance and hyperglycemia due to a persistent functional defect.[3,7,8]

Late gestation pregnant sheep have been used to study the ability of fetal pancreatic β-cells to respond to changes in glucose supply.[9–11] In these previous studies, constant experimental fetal hyperglycemia for 7–10 days initially increased fetal insulin plasma concentrations, which returned to normal control values within 5 days, and reduced fetal GSIS.[9,10] In contrast, when experimental fetal hyperglycemia was provided in the form of 1-h pulsatile glucose infusion three times a day for 10 days, fetal GSIS increased.[9] Furthermore, after 1 week of experimental pulsatile hyperglycemia (PHG), the proportion of β-cells in the fetal pancreas was increased.[10] These studies show that at the end of gestation, fetal pancreatic β-cells respond robustly to pulses of increased fetal glucose supply.

In contrast to normal sheep fetuses, the ability of IUGR fetal sheep islets and β-cells to respond to chronic changes in glucose supply has been less well studied. Acute hyperglycemia fails to elicit normal insulin secretion in a fetal sheep model of severe and progressive placental insufficiency with IUGR.[12] Pancreatic islets in this model are smaller with less β-cells than control animals.[13] When a direct experimental glucose infusion into the IUGR sheep fetus was used to test the ability of the pancreatic islets and β-cells to respond to constantly and chronically increased fetal glucose concentrations, there was no increase in fetal insulin concentrations, GSIS or β-cell mass.[14] Whether fetal PHG for 7 days would increase GSIS and the pancreatic β-cell population in IUGR fetal sheep as it does in normal fetal sheep is unknown. Therefore, we tested the impact of late gestation PHG on insulin secretion,

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islet size and β-cell mass in late gestation IUGR fetal sheep following chronic placental insufficiency. We hypothesized that pulsatile hyperglycemic glucose infusions three times a day for 1 week would increase insulin secretion, islet size and β-cell mass in these IUGR fetal sheep, similar to normally grown fetal sheep.[9,10]

Materials and methods

Animal preparation

All experiments were conducted at the Perinatal Research Center, University of Colorado School of Medicine, Aurora, CO in compliance with the Institutional Animal Care and Use Committee. This Center is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Studies were conducted in pregnant Columbia-Rambouillet sheep ($n=7$) carrying twin fetuses. Beginning at 39 ± 1 days of gestation (dGA), pregnant sheep were exposed to elevated ambient temperatures (40°C for 12 h, 35°C for 12 h) for 72 ± 2 days to induce placental insufficiency.[14–16] Surgeries were performed to place indwelling catheters into the fetal abdominal aorta and femoral vein and maternal catheters into the femoral artery and vein on 121 ± 1 dGA, with appropriate anesthesia and antibiotics as previously described.[17] Animals were allowed at least 5 days for recovery before treatment assignment.

Study design

Experimental protocol

For each pregnancy, one fetus was assigned to a pulsatile hyperglycemic infusion (PHG; $n=7$ fetuses) and the other to a saline infusion (SAL; $n=7$ fetuses). At surgery, fetal lower limb length was measured and fetuses with the longer limb were alternately assigned to each group. Infusions were initiated at 128 ± 1 dGA in both treatment groups. PHG was induced by administering a high rate, 60-min infusion of 33% dextrose [w/v dextrose in water (D33)] three times daily (9 am, 3 pm and 9 pm) that was adjusted to achieve an 80% increase in glucose concentrations. Throughout the infusion period, PHG fetuses also received a continuous, low-rate infusion of D33 adjusted to prevent a decrease in pre-pulsatile fetal arterial glucose concentrations associated with increased fetal insulin secretion during the pulsed infusion.[9] SAL fetuses were infused throughout the infusion period with 0.9% saline at a constant rate of 0.3 ml/h, which was similar to the average fluid infusion rate of the low-rate glucose infusion of the PHG group. Fetal infusions were maintained for 7 days. Fetal arterial blood samples were collected before experimental infusions and immediately before and after the daily dextrose pulses at 9 am and 3 pm. The fetal plasma glucose concentrations immediately after the dextrose pulses were used to adjust the rate of the subsequent dextrose pulse infusions, as noted above.

In vivo fetal insulin and glucagon secretion

On the final day of the chronic infusions, instead of a morning PHG infusion, a 105 min square-wave fetal hyperglycemic clamp followed by a 4-min fetal arginine infusion (0.375 mM/min) was used to measure fetal GSIS, glucose-potentiated arginine-stimulated insulin secretion (ASIS), and arginine-stimulated glucagon secretion in both groups.[9,14] The chronic dextrose and saline infusions continued through the insulin secretion studies and tissue collection.

Organ isolation

After completion of the insulin secretion studies, animals were sacrificed and the placenta and fetal organs were dissected and weighed as previously described.[17] The fetal pancreas was divided into hepatic and splenic portions. The hepatic portion was snap frozen in liquid nitrogen and stored at −80°C. The splenic portion was fixed in 4% (wt/vol) paraformaldehyde and stored for histological analysis in Optimal Cutting Temperature Freeze Media following graded equilibration with 30% sucrose (w/v) OTC.[18]

Biochemical analyses

Arterial blood was collected in heparinized syringes and immediately analyzed for pH, blood gases and hematocrit (ABL520; Radiometer America Inc., Brea, CA). Additional arterial blood samples were collected in EDTA coated syringes and centrifuged at 4°C for 3 min. Plasma concentrations of glucose and lactate were determined using a YSI 2700 biochemistry analyzer (Yellow Springs Instruments, Yellow Springs, OH). Additional plasma aliquots were stored at −80°C before analyses. Plasma concentrations of insulin, insulin-like growth factor-1 (IGF-I) and cortisol were measured by ELISA (ALPCO Immunoassays, Salem, NH). Intra- and inter-assay coefficient of variation (CV) was 5.6 and 4.7%, respectively, for insulin (sensitivity, 0.14 ng/ml); 3.1 and 5.6%, respectively, for IGF-I (sensitivity 0.09 ng/ml); and 3.1 and 5.6%, respectively, for cortisol (sensitivity 0.1 ng/ml). Glucagon and norepinephrine were measured in plasma by radioimmunoassay and HPLC, respectively, as previously described.[16,19] Intra- and inter-assay CV for glucagon were 4.8 and 11.7%, respectively (Millipore; sensitivity, 18.5 pg/ml) and 9.2 and 9.0%, respectively, for norepinephrine (sensitivity 170 pg/ml).

Pancreatic mRNA and protein analysis

RNA was extracted from the pulverized pancreas and reverse transcribed into cDNA.[20] Quantitative PCR assays for insulin (*INS*; for-TCA GCA AAC AGG TCC TCG CAA G, rev-GGG CCA GGT CTA GTT ACA GTA G), glucagon (*GCG*; for-TCA CTC TCT CTT CAC CTG CTC TGT, rev-GAC ACA CTT ACT TCC TGT CAG), pancreatic polypeptide (*PPY*; for-TGC TCC TTC TGT CCA CGT G, rev-ACC TGG GGA CTG CTG CTG AG), somatostatin (*SST*; for-TCT CCA TCG TCC TGG CTC TTG, rev-CTC CAG CCT CAT TTC CAT CCT G), glucokinase (*GCK*; for-TTT CCT GTG AGG CAC GAA GAC; rev-CGT GCT CAG GAT GTT GTA GA), *SLC2A2* [glucose transporter-2 (GLUT2); for-AGC TGG CTG TTG TCA CGG GC, rev-GGC TGG CAC AGC AGA CAA ACC A], *IGF1* [insulin-like growth factor-1 (IGF1); for-GAG ACC CTC TGC GGG GCT GA, rev-CTG CTC GAG CCG TAC CCC GT], *IGF2* (IGF-2; for-TGT GGG GAC CGC GGC TTC TA, rev-CAG GGC CAG GTC GCA GCT TC), *IGFBP2* (IGF-binding protein 2; for-ACC TTG GCC TGG AGG AGC CC, rev-TCC AGG GGA CCC CGC TCA TC) and pancreatic and duodenal homeobox-1 (*PDX1*; for-TTT CCC GTG GAT GAA GTC TAC, rev-CGG TGC GTG TCC GCT TGT TCT) mRNA were performed.[13,18,21] The samples were analyzed in triplicate and the standard curve method of relative quantification was utilized.[22] Target genes were normalized to the geometric mean of three reference genes [ribosomal protein s15 (*RPS15*); for-ATC ATT CTG CCC GAG ATG GTG, rev-CGG

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GCC GGC CAT GCT TTA CG), actin beta (*ACTB*; for-TGC AGA AAG AGA TCA CTG CC, rev-GAC AGC GAG GCA GGA TGG) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; for-TGG AGG GAC TTA TGA CCA CTG, rev-TAG AAG CAG GGA TGA TGT TCT). Results are presented as the relative fold change to the SAL group.

Protein was extracted from pulverized pancreas, separated by gel electrophoresis and Western blotting performed as described previously.[19] Membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (v/v) and 5% non-fat dried milk (v/v) before incubation with primary antibodies; rabbit anti-glucokinase (1:1000; Abcam Inc., Cambridge, MA), rabbit anti-GLUT2 (1:1000; Abcam) and mouse anti-actin (1:10,000; MP Biomedicals, Salon, OH). Primary antibodies were diluted in Tris-buffered saline with 0.1% Tween-20 (v/v) with 5% bovine serum albumin (v/v), incubated with membranes overnight, and detected with goat anti-rabbit IRDye 800CW and goat anti-mouse IRDye 680RD (LI-COR Inc., Lincon, NE). Immunocomplexes were visualized and quantified using an Odyssey Fc imaging system (Image Studio; LI-COR Inc.). Densitometry for glucokinase and GLUT2 were normalized to actin and presented as the relative fold change to the SAL group.

Histology of the fetal pancreas

Histological evaluation of the fetal pancreas was modified slightly from our previous protocols.[13,23] In brief, four 5 µm thick sections were cut at 100 µm intervals from each pancreas. Frozen sections were adapted to room temperature for 30 min before three, 5 min washes in deionized water. Sections were transferred to a 10 mmol/l citric acid buffer (pH 6.0) and maintained in a 90°C water bath for 30 min. Sections were cooled for 20 min, washed three times in phosphate buffered saline (PBS) for 10 min. Sections were blocked for 30 min in 1.5% normal donkey serum in PBS. Endocrine hormones were identified with guinea pig anti-porcine insulin (1:250; 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). Immunocomplexes were detected with the following affinity-purified secondary antiserum (1:500): 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 (Jackson ImmunoResearch Laboratories, West Grove, PA).

Fluorescent images were visualized on an Olympus IX-83 microscope system (Olympus, Waltham, MA). Images were captured and morphometric analyses were performed using the cellSense software (Olympus). Insulin + cells and glucagon + cells were used to determine the β-cell and α-cell mass, respectively, by multiplying the pancreas weight by the percent total pancreas area positive for each hormone. The entire pancreatic section was used to determine β- and α-cell mass. Triple immunofluorescence with insulin, glucagon and somatostatin + pancreatic polypeptide was used to determine fetal pancreatic islet size in >20 fields of view (FOV = 0.07 mm^2) in each section ($n = 74 \pm 5$ islets per animal). Islets were defined as endocrine cell clusters containing at least two endocrine cell types that were >500 μm^2.[13] Percent fluorescent islet area for insulin, glucagon and somatostatin + pancreatic polypeptide is calculated relative to the total fluorescent islet area. Percent areas for each FOV and section were averaged to provide a percent area for each animal before comparative analyses.

Statistical analysis

All data are expressed as means ± S.E. To test the impact of the pulsatile infusions on PHG and SAL fetal plasma glucose, data from immediately before and after each pulsatile infusion for each animal were averaged for comparisons. Measurements made multiple times throughout the 7-day study or during the GSIS and ASIS were analyzed by repeated measures ANOVA (PROC MIXED; SAS Institute Inc., Cary, NC) with treatment groups (PHG *v.* SAL) and time as main effects and a term to account for pairing of fetuses from the same pregnancy. Fetal biochemical values for days 4, 5 and 6 are included in the analysis as one-time point due to variation in the day of sampling for individual animals. Area under the response curves during the hyperglycemic clamp was calculated using GraphPad Prism 5. The rate of increase of insulin concentrations from 0 to 20 min of the hyperglycemic clamp was calculated and differences between infusions were performed with Wilcoxon's non-parametric signed rank test for paired observations. Measurements made once in the experiment were analyzed by mixed model ANOVA with a term to account for pairing of fetuses from the same pregnancy. Norepinephrine values were log transformed before analysis. Baseline periods are the mean of measurements at −15, −10 and −5 min before initiation of the square-wave hyperglycemic clamp and homogeneity of variance for glucose and insulin concentrations during the hyperglycemic clamp were confirmed using the Levene's test. Individual means were compared using Fishers protected least squares difference.

Results

Fetal biochemical parameters during infusion

Daily fetal arterial pre-pulsatile glucose concentrations did not differ between PHG and SAL fetuses (Fig. 1). The initial pulsatile glucose infusion delivered 64 ± 3 µmol/min of glucose in PHG fetuses and glucose concentrations increased 60% ($P < 0.001$). For the remainder of the pulsatile glucose infusions, the rate was 117 ± 6 µmol/min, and the glucose concentrations increased 83% in the PHG fetuses (Fig. 2; $P < 0.001$). In the PHG fetuses the

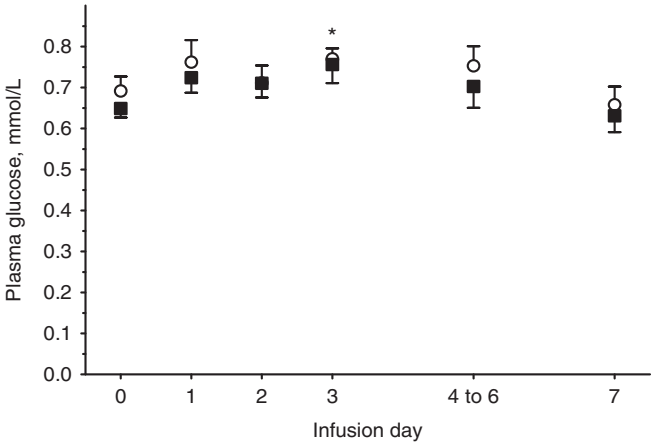

Fig. 1. Plasma glucose concentrations in intrauterine growth-restricted fetal sheep during seven days of pulsatile hyperglycemic (PHG) or saline (SAL) infusions. Fetal arterial glucose concentrations before the first daily pulsatile glucose infusion are presented in SAL (open circles, $n = 7$) and PHG (black squares, $n = 7$) fetuses. Data were analyzed by mixed model ANOVA with repeated measures and presented as mean ± S.E.M. Treatment, $P = 0.23$; day, $P \leqslant 0.05$; treatment × day, $P = 0.99$. *Day 3 was significantly different than day 0 ($P \leqslant 0.05$).

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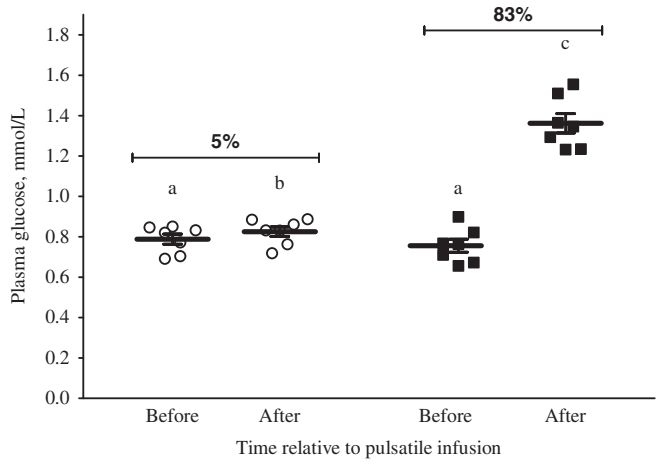

Fig. 2. Plasma glucose concentrations before and after pulsatile infusions in intrauterine growth-restricted (IUGR) pulsatile hyperglycemic (PHG) and IUGR saline-infused control fetal sheep. Fetal glucose concentrations immediately before or after pulsatile glucose infusions are presented in SAL (open circles, $n=7$) and PHG (black squares, $n=7$) fetuses. Each symbol represents the mean pre- or post-infusion concentrations on days 1–7 for a single animal. Data were analyzed by mixed model ANOVA with repeated measures and presented as means (black bars) ± S.E.M. Treatment, $P<0.001$; period, $P<0.001$; treatment × period, $P<0.001$. [a,b,c]Period means differ ($P\leqslant 0.05$).

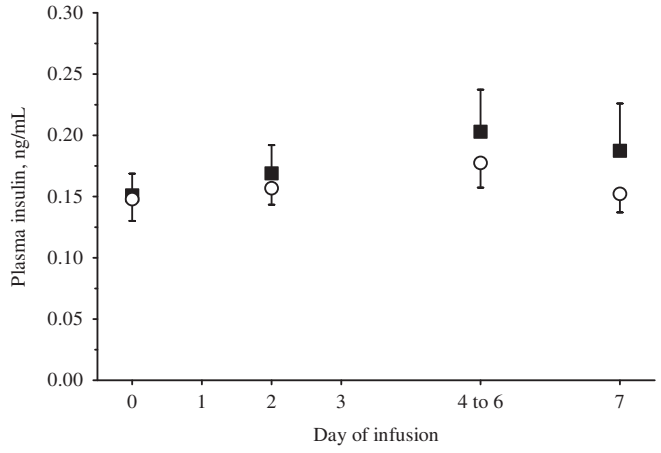

Fig. 3. Plasma insulin concentrations in intrauterine growth-restricted fetal sheep during 7 days of pulsatile hyperglycemic (PHG) or saline (SAL) infusions. Fetal arterial plasma insulin concentrations immediately before the first daily pulsatile glucose infusion are presented in SAL (open circles, $n=7$) and PHG (black squares, $n=7$) fetuses. Data were analyzed by mixed model ANOVA with repeated measures and presented as means ± S.E.M. Treatment, $P=0.23$; day, $P=0.40$; treatment × day, $P=0.91$.

constant low-rate glucose infusion delivered an average of 6.0 ± 0.1 μmol/min of glucose, which in previous studies, is about 10% of the fetal glucose utilization rate in this model of IUGR.[24] The basal fluid delivery rate was greater in SAL ($P<0.001$; 0.30 ± 0.01 ml/h) than in PHG fetuses (0.20 ± 0.01 ml/h).

PHG and SAL fetuses had similar arterial pre-pulsatile plasma concentrations of insulin throughout the 7-day study (Fig. 3). Pre-pulsatile fetal arterial pH, blood gases (pCO_2, pO_2, hemoglobin-O_2 saturation and O_2 content), hematocrit and arterial plasma lactate concentrations were similar in PHG and SAL fetuses on day 0 and after 7 days of PHG (Table 1). After 7 days of PHG, pre-pulsatile plasma arterial concentrations of IGF-I, cortisol, norepinephrine and glucagon did not differ between groups (Table 2).

Fetal insulin and glucagon secretion

Fetal insulin secretion was measured with a 105 min square-wave fetal hyperglycemic clamp after 7 days of experimental infusions. The hyperglycemic clamp increased arterial plasma glucose concentrations similarly in both groups to about 2.5 mmol/l, as designed (Fig. 4a). The rate of glucose infusion required to achieve the steady-state hyperglycemic clamp concentrations was not different between groups (83 ± 4, PHG and 87 ± 4 μmol/kg/min, SAL). Although PHG fetuses appeared to have an increased variation in plasma insulin concentrations during the hyperglycemic clamp, variance was statistically similar between groups ($P\geqslant 0.19$). Plasma concentrations of insulin increased faster in the PHG group compared to the SAL group throughout the first 20 min of the hyperglycemic clamp (slope: 0.036 ± 0.015, PHG and 0.025 ± 0.013 ng/min, SAL; $P<0.05$; Fig. 4b). Consistent with this, there was a trend toward a greater area under the insulin response curve in PHG fetuses (60 ± 17, PHG and 46 ± 14 ng × min/ml, SAL; $P=0.08$; Fig. 4b). A 4-min arginine infusion was started 110 min after initiation of the hyperglycemic clamp. ASIS was similar in both groups (Fig. 5). Arginine-stimulated glucagon secretion was lower in PHG fetuses compared with SAL fetuses (Fig. 6; $P\leqslant 0.05$).

Characteristics of the fetal pancreas

Table 3 shows the relative expression of pancreatic mRNA and proteins known to regulate islet development and insulin secretion, and the histological characteristics of the fetal pancreases. Pancreases from PHG and SAL fetuses had similar percent insulin- and glucagon-positive areas and β- and α-cell masses. Islet size and the proportion of the islet staining for insulin and glucagon also were similar between groups. Pancreatic *INS* mRNA content tended to be 35% lower in PHG fetuses compared with SAL ($P=0.07$). Pancreatic content of *GCG*, *SST*, *PPY*, *SLC2A2*, *GCK*, *IGF1*, *IGF2*, *IGFBP2* and *PDX1* mRNA did not differ between groups. Pancreatic GLUT2 protein content was 33% lower in PHG fetuses compared with SAL ($P<0.05$). Pancreatic GCK protein content was similar between groups.

Necropsy measurements

Fetal body weights, organ weights, crown-rump length and hindlimb length were similar between PHG and SAL fetuses (Table 4).

Discussion

We aimed to determine whether chronic PHG would increase GSIS in the IUGR fetus and result in a higher β-cell mass within the pancreas. We showed that insulin concentrations increased faster during the first 20 min of a square-wave hyperglycemic clamp following 1 week of PHG in IUGR fetuses. This was despite lower pancreatic GLUT2 protein expression and a tendency towards lower pancreatic insulin mRNA expression and in the absence of changes in β-cell mass. This result is consistent with previous experiments with chronic PHG in normally grown fetuses demonstrating increased insulin secretion.[9] Although there was a trend toward a greater area under the insulin response curve during the square-wave hyperglycemic clamp in PHG fetuses, the variability was such that at no point during the clamp were mean insulin concentrations statistically significantly higher in the PHG group compared with the saline group.

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Table 1. Pre-pulsatile fetal arterial blood pH, gases, hematocrit and arterial plasma glucose and lactate before and after 7 days of PHG or saline infusions

	Day 0		Day 7		*P*-value		
Parameter	SAL	PHG	SAL	PHG	Trt	Time	Trt × time
Glucose (mmol/l)	0.69 ± 0.04	0.65 ± 0.02	0.72 ± 0.03	0.67 ± 0.04	0.32	0.82	0.83
pH	7.38 ± 0.01	7.39 ± 0.01	7.34 ± 0.01	7.35 ± 0.01	0.12	< 0.001	0.57
pCO_2 (mmHg)	53.7 ± 1.4	52.4 ± 0.7	53.8 ± 1.2	54.3 ± 0.9	0.71	0.46	0.37
pO_2 (mmHg)	14.2 ± 1.3	15.8 ± 1.0	13.6 ± 1.8	13.8 ± 1.5	0.70	0.58	0.76
Hematocrit (%)	38.1 ± 1.1	38.9 ± 1.4	39.2 ± 1.1	40.6 ± 1.2	0.37	0.12	0.72
Hemoglobin-O_2 saturation (%)	28.8 ± 5.1	34.7 ± 4.2	28.4 ± 6.6	28.6 ± 5.6	0.67	0.69	0.70
O_2 content (mmol/l)	2.3 ± 0.4	2.9 ± 0.2	2.2 ± 0.5	2.3 ± 0.4	0.56	0.61	0.69
Lactate (mmol/l)	2.94 ± 0.37	2.51 ± 0.40	4.35 ± 1.17	3.01 ± 0.64	0.23	0.20	0.53

Trt, treatment.
Mean ± S.E.M. from daily measurements made immediately before first infusion of saline (SAL; $n = 7$) and pulsatile glucose (PHG; $n = 7$) on study days zero and seven. Data were analyzed by mixed model ANOVA with repeated measures. Partial pressure CO_2 (pCO_2), partial pressure O_2 (pO_2).

Table 2. Pre-pulsatile plasma insulin-like growth factor-1 (IGF-I), cortisol, norepinephrine and glucagon concentrations after 7 days of pulsatile hyperglycemic or saline infusions

	SAL	PHG	*P*-value
IGF-I (ng/ml)	44.9 ± 7.5	49.0 ± 8.9	0.73
Cortisol (ng/ml)	20.2 ± 4.0	24.4 ± 6.2	0.58
Norepinephrine (pg/ml)[a]	2435 ± 633	1973 ± 520	0.56
Glucagon (pg/ml)	40.5 ± 4.5	33.0 ± 4.5	0.19

Means ± S.E.M. from measurements made after chronic infusion and immediately before final acute physiological studies are presented in saline-infused intrauterine growth-restricted (IUGR) (SAL; $n = 7$) and pulsatile hyperglycemic-infused IUGR fetuses (PHG; $n = 7$). Data were analyzed by mixed model ANOVA.
[a]Norepinephrine values were log transformed before analyses.

The stimulatory effect of PHG on GSIS in IUGR fetuses may have been limited by increased norepinephrine and/or hypoxemia suppression of β-cell function.[25–27] The hypoxemia and high norepinephrine concentrations that occur in IUGR fetuses both directly and indirectly inhibit GSIS.[25–27] Although differences in fetal blood oxygen values or plasma norepinephrine concentrations between IUGR groups were not observed, it is possible that these factor prevented higher GSIS in response to PHG in these IUGR fetuses compared with observations in normally grown fetuses.[9] It also is possible that lower GLUT2 protein expression in the PHG fetuses, similar to what has been observed in the chronically hyperglycemic rat,[28,29] inhibited β-cell glucose uptake. Decreased β-cell glucose uptake might have limited the stimulatory effect of PHG on GSIS in IUGR fetuses. Lower insulin mRNA expression also might have limited the stimulatory effects of PHG in GSIS in IUGR fetuses.

Although chronic PHG had a greater rate of increase in early phase insulin concentrations during the square-wave hyperglycemic clamp, chronic PHG did not impact any of the histological characteristics of the pancreases measured, including the pancreatic insulin$^+$ area (β-cell area), β-cell mass, islet area or the proportion of the islet that is comprised of β-cells. The results of our morphometric analysis of the fetal pancreas contrast with previous PHG experiments in normally grown fetal sheep. For example, the pancreatic insulin$^+$ area was 50% higher in pancreases from normal fetal sheep exposed to chronic PHG compared with normal saline-infused fetuses.[10] The lack of structural changes in the PHG IUGR fetal islets was consistent with equivalent mRNA expression levels for genes known to impact pancreatic islet growth and development such as PDX1, IGF-1, 2 and IGFBP2 between PHG IUGR and saline IUGR pancreases. These findings also are consistent with previous data showing that the pancreatic insulin$^+$ area and β-cell mass of IUGR fetuses was not impacted by experimentally increasing fetal glucose concentrations to match those of normal fetuses for 2 weeks in late gestation.[14] Together, these data show that the IUGR fetal islets and β-cells do not respond to chronically increased fetal glucose concentrations in terms of structural changes, whether the result of a chronic constant increase in fetal glucose concentrations, or periodic increases in the fetal glucose concentrations. We speculate that the developmental plasticity of the IUGR fetal pancreas to PHG is reduced by late gestation when effect of IUGR is more severe.

In the current study, body weight and hyperglycemic clamp insulin concentrations of IUGR fetuses are consistent with previous cohorts of IUGR fetuses in the same breed of sheep.[12,16] In these previous cohorts, fetal body weight and hyperglycemic clamp insulin concentrations were lower than normally grown fetuses.[12,16] The fetal body weights, hyperglycemic clamp insulin concentrations and β-cell mass reported in the current cohort of twin IUGR fetuses also were lower than previously reported in the normally grown twin fetuses in our previous studies,[30,31] further indicating that IUGR and pancreatic defects were achieved in the current study. It is important to note that not all studies investigating the impact of chronic PHG on insulin secretion in normally grown fetal sheep have found increased GSIS. Some of the differences among these studies may relate to the duration of the chronic exposure to PHG and to the magnitude of the PHG achieved by the pulsatile dextrose infusion.[9–11] We chose the current PHG protocol in order to match our most recent study in normally grown fetal sheep.[10] We cannot rule out the possibility, therefore, that GSIS would have been more consistently higher throughout the duration of the square-wave hyperglycemic clamp if the specific protocol producing PHG had been different. Another difference between the current study and previous experiments with chronic PHG in normal fetuses was the use of direct fetal dextrose infusion to produce PHG compared with the

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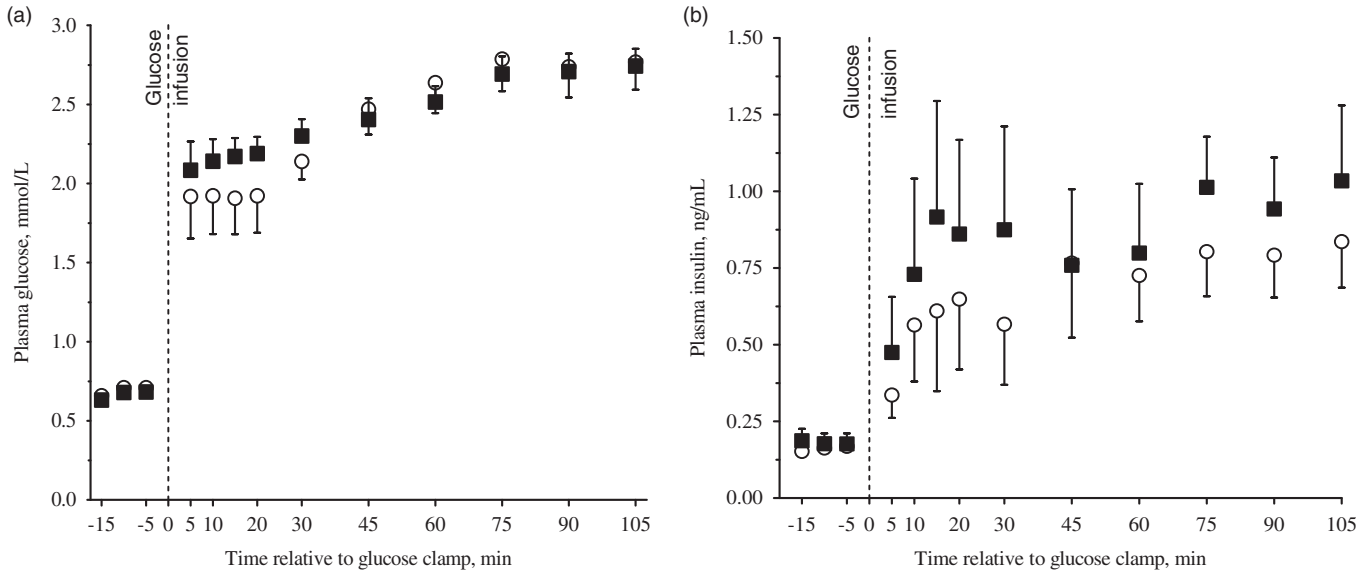

Fig. 4. Plasma glucose (*a*) and insulin (*b*) in pulsatile hyperglycemic (PHG) and saline-infused intrauterine growth-restricted fetal sheep during a 105 min square-wave hyperglycemic clamp. Basal and hyperglycemic fetal arterial plasma glucose and insulin are presented in PHG (black squares, $n = 7$) and SAL (open circles, $n = 7$) fetuses. The slope of the insulin response curve determined during the first 20 min of the hyperglycemic clamp in PHG and SAL fetuses. Area under the insulin response curve was determined throughout the hyperglycemic clamp. Glucose and insulin response curves and insulin AUC were analyzed by mixed model ANOVA with repeated measures and presented as mean ± S.E.M. Plasma insulin: treatment, $P = 0.50$; time, $P < 0.001$; treatment × time, $P = 0.90$. Plasma glucose: treatment, $P = 0.51$; time, $P < 0.001$; treatment × time, $P = 0.79$.

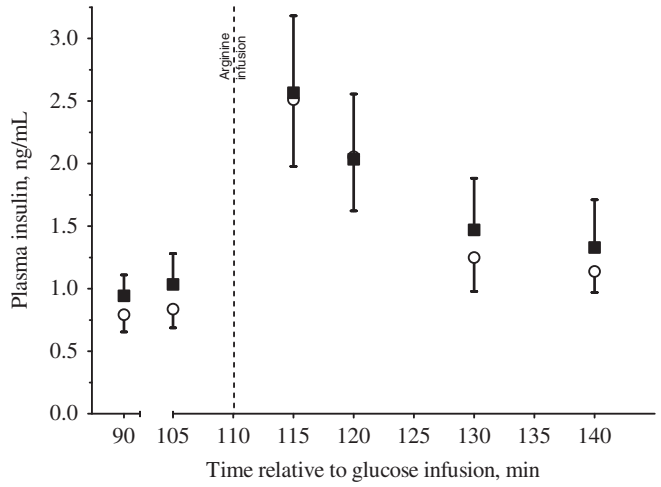

Fig. 5. Plasma insulin concentrations in pulsatile hyperglycemic (PHG) or saline-infused (SAL) intrauterine growth-restricted fetal sheep before and after a 4 min arginine infusion. Arginine-stimulated insulin secretion is presented in PHG (black squares, $n = 7$) and SAL (open circles, $n = 7$) fetuses. Data were analyzed by mixed model ANOVA with repeated measures and presented as mean ± S.E.M. Treatment, $P = 0.79$; time, $P < 0.001$; treatment × time, $P = 0.97$.

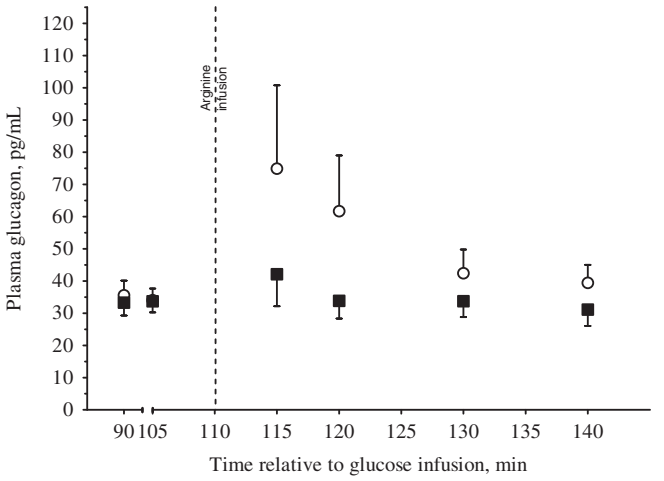

Fig. 6. Plasma glucagon concentrations in pulsatile hyperglycemic (PHG) or saline-infused (SAL) intrauterine growth-restricted fetal sheep before and after a 4-min arginine infusion arginine-stimulated insulin secretion is presented in PHG (black squares, $n = 7$) and SAL (open circles, $n = 7$) fetuses. Data were analyzed by mixed model ANOVA with repeated measures and presented as means ± S.E.M. Treatment, $P = 0.05$; time, $P = 0.11$; treatment × time, $P = 0.49$.

use of maternal dextrose infusions.[9] We opted to use direct fetal dextrose infusions in the current study in order to bypass the decreased transplacental glucose transport capacity of the IUGR pregnancy.[32] Had we not done so, maternal glucose concentrations required to achieve our target fetal glucose concentrations would likely have been much higher than those needed with normal pregnancies.[32] Our approach allowed for more precise experimental control of the fetal glucose concentrations in the IUGR pregnancies.

Comparing our results to those obtained with 10–12 days of constant amino acid supplementation in growth-restricted fetuses further demonstrates the capacity of the IUGR pancreas to increase insulin secretion in response to chronic changes in nutrient concentrations. Chronic constant supplementation of exogenous amino acids for 10–12 days resulted in higher GSIS compared with saline-infused normal and IUGR fetuses.[16,18] This was independent of any increase or decrease in fetal glucose concentrations.[16,18] Chronic constant infusion of amino acids into the IUGR fetus also increased pancreatic insulin$^+$ area and β-cell mass compared with saline-infused IUGR fetuses.[16] Therefore, while IUGR fetal islets and β-cells are responsive to both chronic amino acid supplementation and chronic PHG with respect to GSIS, they are only responsive to chronic amino acid supplementation and not to chronic hyperglycemia, pulsatile or constant, with respect to islet size, the proportion of β-cells within the pancreas, and β-cell mass.

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Table 3. Characteristics of the fetal pancreas

	SAL	PHG	*P*-value
Histology			
Insulin$^+$ area (%)	1.94 ± 0.37	2.21 ± 0.30	0.64
Glucagon$^+$ area (%)	0.57 ± 0.06	0.65 ± 0.08	0.52
β-cell mass (mg)	40.60 ± 7.13	47.14 ± 7.44	0.60
α-cell mass (mg)	12.07 ± 1.41	13.85 ± 1.90	0.53
Islet area (μm^2)	1648 ± 239	1757 ± 263	0.23
Islet β-cell area (%)	30.1 ± 4.3	26.3 ± 3.7	0.46
Islet α-cell area (%)	11.3 ± 2.5	9.0 ± 0.1	0.58
mRNA			
INS (ratio)	1.00 ± 0.18	0.65 ± 0.10	0.07
GCG (ratio)	1.00 ± 0.28	0.85 ± 0.17	0.77
SST (ratio)	1.00 ± 0.15	1.05 ± 0.05	0.91
PPY (ratio)	1.00 ± 0.32	1.02 ± 0.20	0.99
IGF1 (ratio)	1.00 ± 0.16	0.75 ± 0.14	0.26
IGF2 (ratio)	1.00 ± 0.07	0.85 ± 0.06	0.12
IGFBP2 (ratio)	1.00 ± 0.13	0.93 ± 0.13	0.28
SLC2A2 (ratio)	1.00 ± 0.16	1.24 ± 0.26	0.44
GCK (ratio)	1.00 ± 0.24	0.81 ± 0.21	0.35
PDX1 (ratio)	1.00 ± 0.10	0.94 ± 0.08	0.61
Protein			
GLUT2 (ratio)	1.00 ± 0.12	0.65 ± 0.11	0.04
Glucokinase (ratio)	1.00 ± 0.20	0.71 ± 0.12	0.62

Mean ± S.E.M. from measurements of the fetal pancreas are presented in saline-infused intrauterine growth-restricted (IUGR) (SAL; $n = 7$) and pulsatile hyperglycemic-infused IUGR fetuses (PHG; $n = 7$). Pancreatic mRNA and protein expression are presented relative to saline-infused control fetuses (SAL). Data were analyzed by mixed model ANOVA and means were compared using Fisher's LSD. Insulin-like growth factor binding protein 2 (*IGFBP2*), glucose transporter-2 (*GLUT2*), pancreatic and duodenal homeobox-1 (*PDX1*).

We also observed lower arginine-stimulated glucagon secretion in PHG IUGR fetuses compared with saline-infused IUGR fetuses. This was despite having similar pancreatic and islet glucagon$^+$ positive areas, α-cell mass and pancreatic glucagon mRNA expression. In contrast, both IUGR and normal fetuses supplemented with exogenous amino acids for 10–12 days have been shown to have higher plasma glucagon concentrations and arginine-stimulated glucagon secretion.[16,18] The increased glucagon secretion with following chronic constant amino acid supplementation occurred despite similar pancreatic glucagon$^+$ area supplemented and saline-infused IUGR and normal fetuses.[16,18]

In conclusion, these studies provide important evidence that chronic PHG increases in the IUGR sheep fetus. This contributes to a growing body of literature demonstrating that IUGR fetal insulin secretion is responsive to a variety of factors, including glucose, amino acids, oxygen and norepinephrine.[16,25,26] However, chronic glucose supplementation, either constant or pulsatile, does not result in higher β-cell mass or a larger insulin$^+$ area in IUGR fetal sheep pancreases or islets. This limits the usefulness of glucose supplementation alone to correct impaired islet growth development in the late gestation IUGR fetus. We speculate that successful therapeutic interventions for impaired fetal islet growth and β-cell mass in IUGR may include glucose, but will most likely require supplementation of several key nutrients such as amino acids and oxygen, as well as the modulation of certain endocrine factors, to improve fetal insulin secretion, islet development and long-term growth and metabolic outcomes.

Table 4. Fetal body weight, measurements and organ weights in sheep fetuses after 7 days of pulsatile hyperglycemic or saline infusions

	SAL	PHG	*P*-value
Fetal weight (g)	2178.3 ± 162.2	2060.9 ± 148.1	0.60
Crown-rump length (cm)	42.8 ± 1.2	41.7 ± 1.4	0.59
Hindlimb length (cm)	30.5 ± 1.3	30.9 ± 1.3	0.84
Pancreas (g)	2.2 ± 0.2	2.2 ± 0.2	0.99
Liver (g)	58.5 ± 5.1	56.3 ± 3.9	0.73
Heart (g)	18.4 ± 0.9	19.2 ± 1.3	0.54
Lungs (g)	82.4 ± 5.54	77.7 ± 5.0	0.62
Kidneys (g)	13.8 ± 0.9	14.1 ± 0.8	0.80
Spleen (g)	4.0 ± 1.0	3.5 ± 0.8	0.47
Brain (g)	6.5 ± 1.8	7.2 ± 2.1	0.53

Gestational age of pair-matched saline-infused intrauterine growth-restricted (IUGR) (SAL; $n = 7$) and pulsatile hyperglycemic-infused IUGR (PHG; $n = 7$) fetal twins averaged 134 ± 1 days. Data were analyzed by mixed model ANOVA and presented as mean ± S.E.M.

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Conflicts of Interest. The authors have no conflicts of interest.

Ethical Standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the Association for Assessment and Accreditation of Laboratory Animal Care for the care and use of laboratory animals in compliance with the Institutional Animal Care and Use Committee at the University of Colorado School of Medicine, Aurora, Colorado.

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