Enhanced insulin secretion and insulin sensitivity in young lambs with placental insufficiency-induced intrauterine growth restriction

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Running Title: Enhanced Insulin Secretion and Sensitivity in IUGR Lambs

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Abstract

Intrauterine growth restriction (IUGR) is associated with persistent metabolic complications, but information is limited for IUGR infants. We determined glucose-stimulated insulin secretion (GSIS) and insulin sensitivity in young lambs with placental insufficiency-induced IUGR. Lambs with hyperthermia-induced IUGR (n=7) were compared to control lambs (n=8). GSIS was measured at 8±1 days of age, and at 15±1 days body weight-specific glucose utilization rates were measured with radiolabeled D-glucose during a hyperinsulinemic-euglycemic clamp (HEC). IUGR lambs weighed 23% less ($P<0.05$) than controls at birth. Fasting plasma glucose and insulin concentrations were not different between IUGR and controls for either study. First-phase insulin secretion was enhanced 2.3-fold in IUGR lambs compared to controls. However, second-phase insulin concentrations, glucose-potentiated arginine-stimulated insulin secretion, and β-cell mass were not different, indicating that IUGR β-cells have an intrinsic enhancement in acute GSIS. Compared to controls, IUGR lambs had higher body weight-specific glucose utilization rates and greater insulin sensitivity at fasting (1.6-fold) and hyperinsulinemic periods (2.4-fold). Improved insulin sensitivity for glucose utilization was not due to differences in skeletal muscle insulin receptor and glucose transporters 1 and 4 concentrations. Plasma lactate concentrations during HEC were elevated in IUGR lambs compared to controls, but no differences were found for glycogen content or citrate synthase activity in liver and muscle. Greater insulin sensitivity for glucose utilization and enhanced acute GSIS in young lambs are predicted from fetal studies, but may promote conditions that exaggerate glucose disposal and lead to episodes of hypoglycemia in IUGR infants.

Introduction

Studies in human and animal models demonstrate that low birth weight, a proxy for intrauterine growth restriction (IUGR), predisposes offspring to metabolic diseases, such as glucose intolerance and Type 2 Diabetes (21; 26; 45; 55). The causal link between IUGR and glucose intolerance in adulthood provides one example of the developmental origins of health and disease hypothesis, namely that adaptation to detrimental stimuli in development will shape the individual's health for life (3). In addition to birth weight as a predictor for increased risk of diabetes, neonatal events that impact growth trajectory have also been shown to influence metabolic outcomes (43). There is a paucity of information on the transition of IUGR infants into childhood because insulin action and secretion continue to develop and mature with age (23; 31; 46). To our knowledge, only a few studies in IUGR infants have investigated the early transition into neonatal life (24 to 72 hours). Even though these studies associate SGA with glucose dysregulation, the results are inconsistent across studies, which might reflect a dynamic interaction between insulin sensitivity and insulin secretion (5; 20; 44; 54). In sheep fetuses with placental insufficiency-induced IUGR, we have shown defects in pancreatic insulin secretion due to a reduced number of β-cells, but in vitro fetal sheep islets (and thus individual β-cells) have increased insulin secretion capacity. IUGR fetal sheep also have increased peripheral insulin sensitivity (35; 36; 47; 52). We also have shown that chronic exposures to high plasma catecholamine concentrations, which are characteristic of IUGR fetuses with placental insufficiency, inhibit insulin secretion in fetal sheep, but decreasing chronic adrenergic stimulation results in a compensatory hypersecretion of insulin that persists for several days (12; 13; 32; 41), likely accounting for at least some of the increased islet and β-cell insulin secretion capacity.

We have not, however, followed these placental insufficient IUGR fetuses postnatally, after normalization of plasma catecholamine, glucose, and insulin concentrations. This is important to do, because many human infants with IUGR have persistent evidence of increased glucose requirements to prevent hypoglycemia, indicative of increased insulin secretion and peripheral insulin sensitivity relative to current glucose concentrations. Therefore,

we tested the hypothesis that glucose-stimulated insulin secretion (GSIS) and insulin sensitivity for glucose disposal are enhanced in IUGR lambs during their first 2 weeks after birth, but the increase in GSIS is relatively greater than the increase in peripheral insulin sensitivity; both of these conditions would predispose IUGR lambs to excessive glucose utilization and hypoglycemia.

Materials and Methods

Animal Preparation

Animal experiments were approved by the Institutional Animal Care and Use Committee at The University of Arizona, Tucson, AZ, which is accredited by the American Association for Accreditation of Laboratory Animal Care. Time-mated, Columbia-Rambouillet cross-bred ewes were purchased from Nebeker Ranch (Lancaster, CA). Singleton pregnancies were confirmed by ultrasonography prior to treatment assignment and animal husbandry was provided as previously described (10). Briefly, ewes were assigned by simple randomization method to one of two experimental groups, control or IUGR, at receipt. Placental insufficiency-induced IUGR lambs (n = 7) were produced by exposing pregnant ewes to elevated ambient temperatures (40°C for 12 h; 35°C for 12 h; dew point 22°C) from 39 ± 1 day gestational age (dGA) to 85 ± 3 dGA (term 148 ± 1 dGA). Control lambs (n = 8) were from ewes maintained at 25 ± 2°C that were pair fed to the average feed intake of ewes in the IUGR group (term 149 ± 1 dGA). After delivery, lambs were removed from the ewe to eliminate confounding maternal variability and raised in individual pens adjacent to each other. Lambs received colostrum before being placed solely on milk replacer (Milk Specialties Co., Dundee, IL, USA) with *ad libitum* access. Weights were measured within 3 hour of birth, usually before the lamb suckled, and lambs were also weighed before each study. Crown rump length (poll to tail head), hindlimb length (hip to hoof), and head circumference were measured in lambs at birth.

Surgical preparation

At 3 ± 1 days of age, indwelling polyvinyl catheters were surgically placed in the femoral artery and vein for blood sampling and intravenous infusions. Prior to surgery lambs were fasted for 3-4 hours. Lambs were

anesthetized and maintained by inhalation of 1.5-4% isoflurane in oxygen. All catheters were filled with heparinized saline (30 units/ml, 0.9% wt/vol NaCl, Nova-Tech, Inc., Grand Island, NE). After placement, catheters were tunneled subcutaneously to the lamb's flank, exteriorized through a skin incision, and kept in a plastic mesh pouch sutured to the skin. Lambs were allowed to recover for at least 3 days before performing GSIS studies. Catheters were flushed daily with heparinized saline.

Insulin Secretion Responsiveness

Glucose-stimulated insulin concentration was determined with a square-wave hyperglycemic clamp at 8 ± 1 days of age. Lambs were fasted for 3 hours, placed into a custom-made Panepinto sling, and covered with a cloth drape. After approximately 20 minutes of acclimation, basal, fasting blood samples (1.5 ml) were collected in EDTA-lined syringes at −20, -10, and −1 minutes for plasma glucose and insulin measurements as described previously (11). All GSIS sample times are presented relative to the dextrose bolus (time = 0). The hyperglycemic clamp was initiated with an intravenous dextrose bolus (150 mg/kg) followed by a constant infusion of 33% (wt/vol) dextrose (D-glucose) solution that was adjusted (109 ± 9 μmol/min/kg) to maintain arterial plasma glucose concentrations at 15.0 ± 0.6 mmol/L, which was approximately twice the baseline arterial plasma glucose concentration. At the onset of the infusion, arterial blood samples (0.5 ml) were collected every 3–5 minutes for 20 minutes to establish the steady-state plasma glucose concentrations, after which three blood samples (1.5 ml) were collected between 30 and 60 minutes (10-15 minute intervals). Steady-state hyperglycemic conditions were confirmed during the sampling period when arterial plasma glucose concentrations varied less than ±10% of the mean. Acute, first-phase insulin concentration was calculated as AUC for the first 20 minutes of insulin secretion.

Glucose-potentiated arginine-stimulated insulin concentration (GPAIS) was determined with a follow-on arginine bolus (0.5 mmol/kg) to the GSIS study. Arginine was administered over 4 minutes after the final GSIS sample was collected at 60 minutes. Blood samples (0.5 ml) for plasma insulin concentrations were collected at 65, 75 and 90 minutes.

Insulin Sensitivity for Glucose Utilization

At 15 ± 1 days of age, insulin sensitivity of glucose utilization was measured in fasted conditions and during a hyperinsulinemic-euglycemic clamp (HEC)(19). Body weight-specific rates of glucose utilization (disposal) were determined by the net disappearance rate of [^{14}C(U)]-D-glucose (PerkinElmer Life Sciences, Boston, MA) in fasting and hyperinsulinemic steady state periods. Lambs were fasted 4 hours and placed into the Panepinto sling. A constant infusion (2ml/h) of radiolabeled glucose (37.2 μCi/ml) in saline was initiated with a 4 ml priming bolus. After 40 minutes, four arterial blood samples (1.2 mL each) were collected at 8-10 minutes intervals and basal (fasted) glucose utilization rates (μmol/min/kg), plasma concentrations of insulin, glucose, and lactate, and arterial blood gasses were measured. Hyperinsulinemia was initiated with a priming dose of insulin (175 mU/kg; HumulinR; Lilly; Indianapolis IN) followed by a constant infusion (2 mU/min/kg). Euglycemia was maintained with a 33% (wt/vol) dextrose infusion that was adjusted in response to the plasma glucose concentrations taken every 5-10 minutes until steady state was achieved, usually within an hour. Steady-state euglycemic conditions were confirmed during the sampling period when arterial plasma glucose concentrations varied less than ± 9% of the mean. Arterial blood samples (1.2mL each) were collected at 8-10 minutes intervals during the HEC.

Following an overnight recovery from the HEC study, lambs (16 ± 1 days of age) were euthanized with an intravenous overdose of sodium pentobarbital (86 mg kg−1) and phenytoin sodium (11 mg kg−1; Euthasol, Virbac Animal Health, Fort Worth, TX, USA). Organs were dissected, weighed, snap frozen in liquid nitrogen, and stored at −80°C. The splenic portion pancreas (body and tail from the pancreatic notch) was fixed in 4% paraformaldehyde overnight and then embedded in Tissue-Tek® O.C.T. Compound (Sakura® Finetek USA, Inc.) as described previously (14).

Biochemical Analysis and Calculations

Concentrations of plasma glucose and lactate were measured immediately with a YSI model 2700 Select Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH). Blood gasses and oxygen saturations

were measured in blood collected in heparin-lined syringes (Elkins-Sinn, Cherry Hill, NJ) using an ABL520 (Radiometer, Copenhagen, Denmark), and values were temperature corrected for the lambs core body temperature at the start of the study. Whole blood ^{14}C-glucose (0.5 ml) was determined in supernatants after being deproteinized by mixing whole blood with 0.3N Zinc Sulfate Heptahydrate and 0.3M Barium Hydroxide in a final volume of 5 ml. The deproteinized supernatant was aliquoted in triplicate, dried, and radioactivity was measured with a LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Fullerton, CA) in Hionic Fluor Liquid Scintillation Cocktail (PerkinElmer, Waltham, MA). Blood plasma was stored at −80°C until insulin concentrations were measured with an ovine insulin ELISA (ALPCO Diagnostics, Windham, NH; sensitivity 0.07 ng/mL; intra- and interassay coefficients of variation were 2.9% and 5.6%, respectively).

The rate of glucose disappearance (utilization) equals the rate of glucose appearance under steady-state conditions for plasma glucose concentrations. The body weight-specific net glucose utilization (disposal) rate was calculated as the ratio of the rate of ^{14}C-glucose (dpm/min) and the steady-state arterial plasma ^{14}C-glucose specific activity (dpm/μmol glucose). Endogenous (hepatic) glucose production was calculated as the difference between the net ^{14}C-glucose utilization rate and the exogenous net dextrose (D-glucose) infusion rate (μmol/min). Insulin sensitivity for glucose utilization rate (μmol/min/kg/μg/L) was calculated as the steady state net rate of glucose utilization (μmol/min/kg) divided by the arterial plasma insulin concentration (μg/L). All results were normalized to lamb body weight (kg).

Endocrine Pancreas Morphology

Tissue sections (6 μm) for histological evaluation were cut from the tail of the dissected pancreas. Procedures for fluorescent immunostaining on cryosections were performed to detect insulin, glucagon, and the combination of somatostatin and pancreatic polypeptide as reported previously (14; 32; 33). Fluorescent images were visualized with a Leica microscope, digitally captured, and analyzed with ImagePro software (Media Cybernetics, Silver Spring, MD), as described previously (32; 33). Positive areas were determined for at least 25 fields of view (0.39

mm^2) on two pancreas sections per animal and tissue sections were separated by ≥100 μm interval. Data are expressed as a percentage of total pancreas area, and cell mass was calculated by multiplying the pancreas weight by the percent positive area.

Glycogen Content

Glycogen content in liver and skeletal muscle (semitendinosus muscle) was determined as previously described with modifications (4; 35). Briefly, 200 mg of frozen tissue was pulverized and digested in 2 ml of 30% KOH at 95^{o}C for 30 minutes. The homogenate (150 μl) was placed on No. 1 Whatman filter paper and washed in 66.6% ethanol with constant stirring for 30 minutes. The filter paper was removed, dried, and cut into small pieces. Glycogen was converted to glucose with 41.2 mg/ml amyloglucosidase (Sigma-Aldrich, St. Louis, MO) in 0.2 M acetate buffer (pH 4.8, 0.5% glacial acetic acid, 0.12 M sodium acetate) at 37°C for 65 minutes. Glucose concentration of this solution was determined in triplicate using the YSI 2700 Select Biochemistry Analyzer. Results are expressed as milligrams glucose per grams tissue (wet weight).

Citrate Synthase Activity

Citrate synthase content in liver and skeletal muscle was determined using the citrate synthase assay kit (Sigma-Aldrich). Protein was extracted from snap-frozen liver and skeletal muscle (100 mg) into 2 ml of CelLytic MT reagent following the CelLytic MT mammalian tissues lysis procedure (Sigma-Aldrich; Catalog Number C3228). After protein extraction, total protein concentrations were determined by Pierce BCA Assay (Thermo Fisher, Rockford, IL). Citrate synthase activity of the supernatant was measured in a 96 well plate in triplicates. Each reaction contained 20 ug of protein. Reaction was initiated after adding oxaloacetic acid and absorbance of the reaction was measured for 1.5 minutes at a wavelength of 412 nm.

Immunoblotting

Protein lysates were prepared from semitendinosus muscle (30-40 milligrams) with CelLytic MT Cell Lysis Reagent (Sigma-Aldrich) and protease inhibitors (0.5mM PMSF, 2 μg/ml Aprotinin, 2.5 μg/ml Leupeptin). Lysates

were homogenized with the TissueLyser LT (Qiagen, Hilden, Germany) at 50 Hz for 5 minutes and were centrifuged for 10 minutes at 16,100 g. Protein concentrations of the supernatant were determined with Pierce BCA Assay (Thermo Fisher). Immunoblots were performed as previously described (35; 52). Primary antibodies against glucose transporter (GLUT)1 (1:250, Millipore, Temecula, CA), GLUT4 (1μ/ml, Sigma-Aldrich) and insulin receptor β-subunit (1:100, Santa Cruz) were detected with anti-rabbit immunoglobulin G horseradish peroxidase conjugated secondary antibody(1:15,000; Bio-Rad Laboratories, Hercules, California) and chemiluminescence (West Pico Chemiluminescent Substrate; Thermo Fisher). Protein levels were quantified using photographed images and densitometric analyses (Scion Image Software, Frederick, MD), to accommodate the number of samples, two blots were run simultaneously and contain 4 overlapping samples for internal controls. Data are presented as percent of control means.

Statistical Analysis

The statistical analysis was performed on IUGR (n = 7; 5 males and 2 females) and control (n = 8; 3 males and 5 females) lambs unless otherwise noted. For the HEC analysis, one IUGR lamb did not meet steady state criteria during the clamp and another lamb became ill and both were excluded from the analysis. Skeletal muscle and liver tissues were not collected for 1 control and 2 IUGR lambs. After surgery, lambs were assigned a new identification number that did not correspond with treatment or previous identification to facilitate blinding and eliminate bias in subsequent analyses. Lamb morphometric characteristics, pancreas histology, citrate synthase activity, glycogen content, and immunoblots were analyzed by one way ANOVA using general linear means procedure of SAS software (version 9.4; SAS Institute), and differences were determined with a post hoc least significant difference test. The GSIS and GPAIS studies were analyzed by ANOVA using the MIXED procedure of SAS. The model included experimental groups (control and IUGR), draw time, and their interaction. Acute, first-phase insulin concentration for the first 20 minutes was calculated as AUC with GraphPad Prism version 6 (GraphPad Software, La Jolla, California) of insulin secretion and analyzed by one-way ANOVA using general linear means procedure of

SAS. Insulin sensitivity study parameters were also analyzed by ANOVA using the MIXED procedure of SAS with lamb as the random effect. Main effects were experimental group (control and IUGR), period (basal and hyperglycemic), and their interaction. By experimental design insulin sensitivity was analyzed at basal and hyperglycemia independently with one way ANOVA using general linear means procedure of SAS and differences were determined with a post hoc least significant difference test. For all data, sex effect was removed from the model if $P > 0.30$. Means were separated using the PDIFF option of the LSMEANS statement of SAS and were considered significant when $P \leq 0.05$. In the absence of interactions ($P > 0.05$) for the repeated measures model, significant main effects are reported. Data are presented as the mean ± standard error mean.

Results

Weights

IUGR lambs were lighter at birth than control lambs (3.6 ± 0.3 kg vs. 4.7 ± 0.3 kg; $P < 0.05$). At the GSIS study IUGR lambs weighed less (4.8 ± 0.2 kg; $P \leq 0.01$) than control lambs (6.3 ± 0.4 kg). The body weights of the IUGR lambs were less (6.0 ± 0.4 kg; $P < 0.01$) compared to control lambs (7.4 ± 0.3 kg) at the HEC study. At necropsy the average body weights of IUGR lambs remained 30% lighter ($P < 0.05$) than control lambs.

Glucose-Stimulated Insulin Secretion

Basal plasma glucose and insulin concentrations were not different between IUGR and control lambs (Figure 1). Plasma glucose and insulin concentrations were increased ($P < 0.01$) with hyperglycemia in both groups. Glucose concentrations (Figure 1A) during hyperglycemia were similar between IUGR and control lambs, but average insulin concentrations were greater in IUGR (5.79±0.90 µg/L, $P = 0.05$) than in control lambs (3.14±0.84 µg/L). During the square-wave hyperglycemic clamp, first phase insulin secretion in response to the clamp-induced hyperglycemia was determined during the first 20 minutes of hyperglycemia, and the change in insulin concentration by time calculated by AUC was greater ($P \leq 0.05$) in IUGR lambs compared to controls (Figure 1B).

However, there were no differences between IUGR and control lambs during steady-state hyperglycemia, which represents second phase of insulin secretion.

Glucose-potentiated arginine-induced insulin secretion

Insulin concentrations increased further ($P < 0.01$) after an arginine bolus (Figure 2). Maximum insulin concentrations were reached at 5 minutes and return to pre-bolus insulin concentrations by 30 minutes. No experimental group by time interaction or experimental group effect was found for GPAIS.

Insulin Sensitivity for Net Body Weight-Specific Glucose Utilization Rate

During the HEC insulin concentrations increased from basal (fasting) period, as expected, but were not different between IUGR and control lambs in either period (Table 1). Glucose concentrations were not different between experimental groups or between study periods. Arterial blood pH, PaO_2, $PaCO_2$, O_2 content, and hematocrit were not different between IUGR and control lambs (Table 1). Although differences in period means were found, there was no experimental group by period interaction. Hyperinsulinemia decreased pH (7.400 ± 0.010 vs. 7.443 ± 0.001 at basal), O_2 content (5.8 ± 0.26 mM vs. 6.12 ± 0.25 mM at basal), and hematocrit (31.6 ± 1.3 % vs. 33.3 ± 1.3 % at basal) means, but increased PaO_2 (79.7 ± 2.2 mmHg vs. 74.6 ± 2.2 mmHg at basal) from basal levels. Plasma lactate concentrations were 21.8% higher in IUGR lambs compared to control lambs during the HEC.

Net body weight-specific glucose utilization rates (μmol/min/kg) were similar in IUGR and control lambs in the basal (fasting) period (Figure 3). However, steady state rates of glucose utilization were 1.7 fold greater ($P \leq 0.05$) in IUGR lambs than controls during the HEC. Glucose utilization rates were increased ($P \leq 0.05$) during the HEC compared to basal in both IUGR (1.8 fold change) and control (1.4 fold change) lambs.

Insulin sensitivity for glucose utilization (μmol/min/kg/μg/L; Figure 4) was 1.6 fold greater ($P \leq 0.05$) in IUGR lambs than in control lambs under basal conditions and 2.4 fold greater ($P \leq 0.05$) during the HEC. Hepatic glucose production was not different between IUGR (11.8 ± 2.9 mg/min/kg) lambs and controls (5.4 ± 2.5 mg/min/kg).

Endocrine Pancreas Morphology

Pancreas weights were not different between IUGR (9.87 ± 0.11 g) and control (12.25 ± 0.91 g) lambs. Similarly, the proportion of pancreas weight to body weight was not different between IUGR and control lambs. Insulin positive area was not different between IUGR (3.2 ± 0.8 %) and control (2.8 ± 0.5 %) lambs. β-cell mass also was not different between IUGR (302 ± 50.8 mg) and control (372 ± 113.6 mg) lambs. β-cell mass relative to lamb body weight was similar between IUGR (50.3 ± 10.7 mg/kg) and control (47.9 ± 12.1 mg/kg) lambs. Glucagon positive area (IUGR 1.1 ± 0.2 % vs. control 1.1 ± 0. 2%) and α-cell mass (IUGR 117 ± 20 mg vs. control 132 ± 28 mg) were similar between experimental groups. In addition, α-cell mass relative to lamb body weight was not different between IUGR (19.6 ± 4.3 mg/kg) and control (17.1 ± 2.6 mg/kg) lambs. Total endocrine area, a combination of insulin-, glucagon-, somatostatin-, and pancreatic polypeptide-positive areas, was similar between IUGR (6.2 ± 1.0%) and control (5.0 ± 0.9%) lambs. Total endocrine mass (IUGR 714 ± 83 mg vs. control 529 ± 164 mg) and total endocrine mass relative to lamb body weight (IUGR 88.0 ± 17.9 mg/kg vs. control 92.1 ± 15.6 mg/kg) were also not different between experimental groups.

Glycogen Content and Citrate Synthase Activity

Glycogen concentrations in muscle were similar between IUGR (17.2 ± 1.9 glucose mg/g) and control lambs (16.0 ± 1.6 glucose mg/g). Liver glycogen concentrations were not different between experimental groups (IUGR 32.3 ± 9.9 glucose mg/g vs. control 37.7 ± 7.5 mmol/kg). Similarly, citrate synthase activities were not different between groups in muscle (IUGR 0.52 ± 0.04 μmol/min/μg vs. control 0.47 ± 0.03 μmol/min/μg) and liver (IUGR 0.08 ± 0.01 μmol/min/μg vs. control 0.08± 0.01 μmol/min/μg).

Glucose Transporters and Insulin Receptor Levels

No differences were found in skeletal muscle for GLUT1 (control 100 ± 12% vs. IUGR 108 ± 13%), GLUT4 (control 100 ± 5% vs. IUGR 99 ± 9%), or insulin receptor β concentrations (control 100 ± 8% vs. IUGR 104 ± 13%) between groups (Figure 5).

Discussion

Although the association between IUGR and metabolic complications in adulthood is well known, information is scarce on the metabolic consequences of IUGR in such infants shortly after birth. Here we show enhanced insulin secretion and insulin action for glucose in young lambs born with IUGR caused by placental insufficiency. Amplification of the acute GSIS response in lambs occurs despite several earlier reports demonstrating impaired GSIS in the IUGR fetus, including studies in this ovine model of placental insufficiency-induced IUGR (25). Recent evidence indicates that inhibition of insulin secretion in the IUGR fetuses with placental insufficiency is due to elevated catecholamines that cause a hyper-secretory response of insulin following cessation of the adrenergic stimulation (12; 13; 32). IUGR lambs have improved insulin sensitivity for glucose, which parallels findings in IUGR fetuses, where body weight-specific glucose utilization rates are maintained despite lower insulin concentrations (35; 53; 53). The current results indicate that the increase in insulin sensitivity persists beyond birth without affecting glucose transporter or insulin receptor concentrations in skeletal muscle. These young IUGR lambs have inappropriately increased insulin concentrations for their degree of increased insulin sensitivity, which together could explain the transient relative hyperinsulinemic hypoglycemia observed in some IUGR infants (48; 51).

Analysis of the biphasic insulin secretion response shows greater acute, first-phase GSIS in IUGR lambs that is due to enhanced pancreatic β-cell responsiveness. We based this conclusion on the observations that second-phase insulin secretion, GPAIS, and β-cell mass were not different between IUGR and control lambs. Furthermore, equivalent insulin concentrations during the HEC, when insulin doses are identical, indicate that the metabolic clearance rate of insulin is comparable between IUGR and control lambs. These findings indicate that the enhanced acute insulin concentration response is intrinsic to the β-cells in IUGR lambs.

Our previous work in the IUGR fetus indicates that chronic suppression of insulin secretion from elevated plasma catecholamines promotes the β-cell hyper-responsiveness in IUGR lambs. Placenta insufficiency causes fetal hypoglycemia and hypoxia, which elevate plasma catecholamines (34; 36). We have shown that plasma

catecholamine concentrations are chronically elevated in late gestation in such IUGR fetuses and continually inhibit their insulin secretion (40; 41). A pharmacological blockade of adrenergic receptors improves GSIS to a greater extent in IUGR fetuses than controls despite significantly less β-cell mass in the IUGR fetus (32; 40). Isolated islets from IUGR fetal sheep also have a greater fractional insulin secretion relative to their insulin content than control islets, which further supports β-cell hyper-responsiveness in the absence of adrenergic receptor activation (36). We have experimentally isolated the effects of hypercatecholaminemia in otherwise unperturbed sheep fetuses by continuously infusing norepinephrine for one week. Following termination of norepinephrine infusion, GSIS studies revealed β-cell hyper-responsiveness to glucose, which persisted for days in the fetus and more specifically their islets (12; 13).

We have previously shown in our ovine model that IUGR fetuses at 0.9 of gestation weighed 58% less than controls and had a similar reduction (59%) pancreas weight compared to control fetuses (33). In this study we see a modest, but significant, 23% reduction in birth weight compared to control lambs. However, pancreas weight and β-cell mass were not significantly different between IUGR and control lambs at 16 days of age when the IUGR lambs were 30% lighter in weight than control lambs. We attribute these differences to the shorter length of hyperthermia exposure in the current study, because it was demonstrated previously that the durations of environmental heat stress were associated with the severity of fetal growth restriction (22). To ensure that the lambs were viable without extensive measures to stabilize them after birth, we chose to expose the ewes to a shorter 6 week duration of environmental heat stress instead of the 80 days previously used for studies on the fetus (32; 33). Although the severity of fetal growth restriction was less in this cohort of IUGR lambs (23%) compared to fetal cohorts (40-58%), many similarities such as increased insulin secretion responsiveness (with adrenergic receptor antagonists) and increased insulin sensitivity were identified, suggesting that previously described impairments in the fetus occur and persist in these IUGR lambs (32; 35; 53). These data are consistent with the uterine carunclectomy model of placental restriction, which also shows a positive association between fetal weight

and absolute β-cell mass, and that this correlation is lost in 43 day old lambs (24). These data indicate that the severity of placental insufficiency and IUGR are important for the development of β-cell mass, but additional experiments are required to fully understand the postnatal relationships for molecular mechanisms that regulate cell proliferation, which were identified previously in islets from fetal sheep with placental insufficiency and IUGR (30).

Body-weight specific rates of glucose utilization increase with hyperinsulinemia, and in IUGR lambs insulin-stimulated glucose utilization rates are increased to a greater extent compared to control lambs. Rates of glucose utilization in relation to insulin concentrations demonstrate increased insulin sensitivity in IUGR lambs at fasting and hyperinsulinemic periods. This increase in insulin sensitivity in IUGR lambs may be a continuation of adaptations seen in utero, where, despite lower insulin concentrations, IUGR fetuses have relatively normal weight-specific glucose utilization rates (35; 53) and increased insulin receptor concentrations (52). In the IUGR fetus, however, the capacity for glucose oxidation is less than in normally grown fetuses, which has been proposed to promote lactate production in skeletal muscle to supply hepatic gluconeogenesis (i.e., via the Cori cycle) (7; 35; 57). While promoting mechanisms to spare glucose from oxidative metabolism is advantageous for the IUGR fetus and most likely dependent on endocrine responses to high catecholamines and low insulin, we also detect disparities between utilization and oxidation in the lambs, because lactate concentrations are greater in IUGR lambs and increased to a greater extent during the HEC. Additional evidence for glucose entry into glycolysis and not glycogenesis is that glycogen concentrations are similar between experimental groups in skeletal muscle and liver. Elevated lactate concentrations may indicate that, despite the maintained rates of glucose non-oxidative disposal, pyruvate entry into the tricarboxylic acid cycle is restricted, or at least not increased in response to increased glucose uptake and glycolysis. Limited oxidation rates of pyruvate do not appear to be dependent on differences in mitochondrial number because activity of citrate synthase, a mitochondrial matrix enzyme, was not different between experimental groups. Therefore, limitations in glucose oxidative capacity are dependent on

pyruvate metabolism, which is a proposed site of regulation because pyruvate dehydrogenase kinase 4 expression is increased in IUGR fetuses. Pyruvate dehydrogenase kinase 4 inhibits pyruvate dehydrogenase activity to reduce the flux into the tricarboxylic acid cycle, thereby promoting pyruvate conversion to lactate, which is catalyzed by lactate dehydrogenase (8). Additional studies are needed to fully characterize the metabolic flux and its dysregulation in skeletal muscle and liver, but these studies support the hypothesis that limitations in glucose oxidative rates in IUGR fetuses persist postnatally.

Like the β-cell hyper-responsiveness, the dysregulation of glucose metabolism in skeletal muscle may be produced by chronically elevated catecholamines, because sustained adrenergic stimulation increases insulin-stimulated non-oxidative glucose disposal in adult humans and rats (9; 28; 29; 39; 49). We have shown adrenergic receptor expression is down regulated in insulin-responsive tissues in IUGR fetuses and lambs (56; 58). Studies are ongoing to address the adrenergic-induced developmental programming and its role in glucose metabolism in IUGR fetuses and lambs.

Although the relationship between insulin secretion and sensitivity is enhanced early, this effect is most likely transient, based on studies carried out in adults showing a higher incidence of glucose intolerance. In a sheep model of placental restriction, IUGR lambs displayed increased insulin sensitivity to circulating free fatty acids at 6 weeks of age but had impaired insulin secretion to an intravenous glucose tolerance test (17). Normally, insulin sensitivity decreases with age. In sheep with placental restriction IUGR, increased whole-body insulin sensitivity for glucose is present at 4 weeks after birth, but glucose intolerance is present in adulthood (16; 23; 38; 45). To our knowledge, only a few studies have evaluated the early transition into neonatal life in humans. A human longitudinal study showed that SGA infants have increased insulin sensitivity for glucose at 48 hours (6; 44; 50). However, by 1 year of age SGA infants that showed catch up growth had higher fasting insulin concentrations compared to infants that did not gain weight and those born appropriate for gestational age (AGA) (44; 50), indicating the development of insulin resistance. Interestingly, by 3 years of age, glucose disposition index in SGA

infants was reduced even though they were the same weight as AGA infants (44). These studies in human SGA infants and sheep models of IUGR support early enhancement of the insulin disposition index that declines with age to a greater degree than what is observed for normal maturation. This indicates that there is a postnatal component contributing to the developmental origins for glucose intolerance, and although not shown in this study, may be associated with later catch-up growth, particularly of adipose tissue and total body fat (18).

Perspectives and Significance

Placenta insufficiency-induced IUGR causes hypoglycemia and hypoxia, which are present in human IUGR fetuses and in fetuses in our ovine model of IUGR (36). These fetal conditions associated with IUGR are expected to decrease insulin secretion and action, either directly or indirectly through hypercatecholaminemia and other endocrine factors(37). We have shown that catecholamines inhibit insulin secretion in IUGR sheep fetuses, and also suppress growth rates independent of circulating insulin concentrations, suggesting direct actions on other metabolic tissues (15; 41; 42). Persistent activation of adrenergic receptors in humans increases insulin-stimulated non-oxidative glucose disposal (49). This adaptation and also potential adaptations to low plasma glucose and insulin concentrations in IUGR fetuses may accentuate glucose extraction in skeletal muscle resulting in the appearance of increased insulin sensitivity for glucose utilization. In addition chronic adrenergic inhibition of insulin secretion due to hypercatecholaminemia leads to persistent hyper-secretion of insulin following the cessation of adrenergic stimulation (13; 32). In this study, we show that both enhanced GSIS and increased insulin sensitivity persist at least transiently (up to two weeks after birth) in lambs that were IUGR, indicating that the adaptive outcomes developed in utero. A common problem after birth for human IUGR newborns is transient hyperinsulinism and hypoglycemia (2; 5; 27; 51). Although the adaptations observed in IUGR lambs did not result in hypoglycemia, they resemble features that could explain this common problem in human IUGR newborns. Clearly, developmental impairments in insulin secretion, insulin action, and glucose homeostasis are not limited to

fetal adaptations in IUGR, but produce a mismatch between secretion and sensitivity of insulin in the neonate that perpetuates such impairments after birth and the consequent metabolic responses, including hypoglycemia.

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Declaration of Interest

The authors have no conflict of interest.

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References

1. **Andersen G, Dalgaard LT, Justesen JM, Anthonsen S, Nielsen T, Thorner LW, Witte D, Jorgensen T, Clausen JO, Lauritzen T, Holmkvist J, Hansen T and Pedersen O**. The frequent UCP2 -866G>A polymorphism protects against insulin resistance and is associated with obesity: a study of obesity and related metabolic traits among 17 636 Danes. *Int J Obes (Lond)* 37: 175-181, 2013.
2. **Arya VB, Flanagan SE, Kumaran A, Shield JP, Ellard S, Hussain K and Kapoor RR**. Clinical and molecular characterisation of hyperinsulinaemic hypoglycaemia in infants born small-for-gestational age. *Archives of Disease in Childhood-Fetal and Neonatal Edition* fetalneonatal-2012, 2013.
3. **Barker DJP**. Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition* 13: 807-813, 1997.
4. **Barry JS, Davidsen ML, Limesand SW, Galan HL, Friedman JE, Regnault TR and Hay WW, Jr.** Developmental changes in ovine myocardial glucose transporters and insulin signaling following hyperthermia-induced intrauterine fetal growth restriction. *Exp Biol Med (Maywood)* 231: 566-575, 2006.
5. **Bazaes RA, Salazar TE, Pittaluga E, Pena V, Alegria A, Iniguez G, Ong KK, Dunger DB and Mericq MV**. Glucose and lipid metabolism in small for gestational age infants at 48 hours of age. *Pediatrics* 111: 804-809, 2003.
6. **Bazaes RA, Salazar TE, Pittaluga E, Pena V, Alegria A, Iniguez G, Ong KK, Dunger DB and Mericq MV**. Glucose and lipid metabolism in small for gestational age infants at 48 hours of age. *Pediatrics* 111: 804-809, 2003.
7. **Brown LD, Rozance PJ, Bruce JL, Friedman JE, Hay WW and Wesolowski SR**. Limited capacity for glucose oxidation in fetal sheep with intrauterine growth restriction. *Am J Physiol Regul Integr Comp Physiol* 309: R920, 2015.

8. **Brown LD, Rozance PJ, Bruce JL, Friedman JE, Hay WW and Wesolowski SR**. Limited capacity for glucose oxidation in fetal sheep with intrauterine growth restriction. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 309: R920, 2015.
9. **Budohoski L, Challiss RA, Dubaniewicz A, Kaciuba-Uscilko H, Leighton B, Lozeman FJ, Nazar K, Newsholme EA and Porta S**. Effects of prolonged elevation of plasma adrenaline concentration in vivo on insulin-sensitivity in soleus muscle of the rat. *Biochem J* 244: 655-660, 1987.
10. **Chen X, Fahy AL, Green AS, Anderson MJ, Rhoads RP and Limesand SW**. beta2-Adrenergic receptor desensitization in perirenal adipose tissue in fetuses and lambs with placental insufficiency-induced intrauterine growth restriction. *J Physiol* 588: 3539-3549, 2010.
11. **Chen X, Fahy AL, Green AS, Anderson MJ, Rhoads RP and Limesand SW**. beta2-Adrenergic receptor desensitization in perirenal adipose tissue in fetuses and lambs with placental insufficiency-induced intrauterine growth restriction. *J Physiol* 588: 3539-3549, 2010.
12. **Chen X, Green AS, Macko AR, Yates DT, Kelly AC and Limesand SW**. Enhanced insulin secretion responsiveness and islet adrenergic desensitization after chronic norepinephrine suppression is discontinued in fetal sheep. *Am J Physiol Endocrinol Metab* 306: E58-E64, 2014.
13. **Chen X, Kelly AC, Yates DT, Macko AR, Lynch RM and Limesand SW**. Islet adaptations in fetal sheep persist following chronic exposure to high norepinephrine. *Journal of Endocrinology* 2016.
14. **Cole L, Anderson MJ, Antin PB and Limesand SW**. One process for pancreatic β-cell coalescence into islets involves an epithelial-mesenchymal transition. *J Endocrinol* 203: 19-31, 2009.
15. **Davis MA, Macko AR, Steyn LV, Anderson MJ and Limesand SW**. Fetal Adrenal Demedullation Lowers Circulating Norepinephrine and Attenuates Growth Restriction but not Reduction of Endocrine Cell Mass in an Ovine Model of Intrauterine Growth Restriction. *Nutrients* 7: 500-516, 2015.

16. **De Blasio MJ, Gatford KL, Harland ML, Robinson JS and Owens JA**. Placental restriction reduces insulin sensitivity and expression of insulin signaling and glucose transporter genes in skeletal muscle, but not liver, in young sheep. *Endocrinology* 153: 2142-2151, 2012.
17. **De Blasio MJ, Gatford KL, McMillen IC, Robinson JS and Owens JA**. Placental restriction of fetal growth increases insulin action, growth, and adiposity in the young lamb. *Endocrinology* 148: 1350-1358, 2007.
18. **De Blasio MJ, Gatford KL, Robinson JS and Owens JA**. Placental restriction of fetal growth reduces size at birth and alters postnatal growth, feeding activity, and adiposity in the young lamb. *Am J Physiol Regul Integr Comp Physiol* 292: R875-R886, 2007.
19. **DeFronzo RA, Tobin JD and Andres R**. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 237: E214-E223, 1979.
20. **Falorni A, Fracassini F, Massi-Benedetti F and Maffei S**. Glucose Metabolism and Insulin Secretion in the Newborn Infant: Comparisons Between the Responses Observed the First and Seventh Day of Life to Intravenous and Oral Glucose Tolerance Tests. *Diabetes* 23: 172-178, 1974.
21. **Fernandez-Twinn DS and Ozanne SE**. Mechanisms by which poor early growth programs type-2 diabetes, obesity and the metabolic syndrome. *Physiol Behav* 88: 234-243, 2006.
22. **Galan HL, Hussey MJ, Barbera A, Ferrazzi E, Chung M, Hobbins JC and Battaglia FC**. Relationship of fetal growth to duration of heat stress in an ovine model of placental insufficiency. *Am J Obstet Gynecol* 180: 1278-1282, 1999.
23. **Gatford KL, De Blasio MJ, Thavaneswaran P, Robinson JS, McMillen IC and Owens JA**. Postnatal ontogeny of glucose homeostasis and insulin action in sheep. *Am J Physiol Endocrinol Metab* 286: E1050-E1059, 2004.

24. **Gatford KL, Mohammad SN, Harland ML, De Blasio MJ, Fowden AL, Robinson JS and Owens JA**. Impaired {beta}-cell function and inadequate compensatory increases in {beta}-cell mass following intrauterine growth restriction in sheep. *Endocrinology* 149: 5118-5127, 2008.
25. **Green AS, Rozance PJ and Limesand SW**. Consequences of a compromised intrauterine environment on islet function. *J Endocrinol* 205: 211-224, 2010.
26. **Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C and Winter PD**. Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ* 303: 1019-1022, 1991.
27. **Hoe FM, Thornton PS, Wanner LA, Steinkrauss L, Simmons RA and Stanley CA**. Clinical features and insulin regulation in infants with a syndrome of prolonged neonatal hyperinsulinism. *J Pediatr* 148: 207-212, 2006.
28. **Jacob S, Fogt DL, Dietze GJ and Henriksen EJ**. The beta2-adrenergic modulator celiprolol reduces insulin resistance in obese Zucker rats. *Life Sci* 64: 2071-2079, 1999.
29. **Jensen J, Jebens E, Brennesvik EO, Ruzzin J, Soos MA, Engebretsen EM, O'Rahilly S and Whitehead JP**. Muscle glycogen inharmoniously regulates glycogen synthase activity, glucose uptake, and proximal insulin signaling. *Am J Physiol Endocrinol Metab* 290: E154-E162, 2006.
30. **Kelly AC, Bidwell CA, McCarty FM, Taska DJ, Anderson AJ, Camacho LE and Limesand SW**. RNA sequencing exposes novel adaptive and immune responses to intrauterine growth restriction in fetal sheep islets. *Endocrinology* doi.org/10.1210/en.2016-1901 : 2017.
31. **Kervran A and Randon J**. Development of insulin release by fetal rat pancreas in vitro: effects of glucose, amino acids, and theophylline. *Diabetes* 29: 673-678, 1980.
32. **Leos RA, Anderson MJ, Chen X, Pugmire J, Anderson KA and Limesand SW**. Chronic exposure to elevated norepinephrine suppresses insulin secretion in fetal sheep with placental insufficiency and intrauterine growth restriction. *Am J Physiol Endocrinol Metab* 298: E770-E778, 2010.

33. **Limesand SW, Jensen J, Hutton JC and Hay WW, Jr.** Diminished β-cell replication contributes to reduced β-cell mass in fetal sheep with intrauterine growth restriction. *Am J Physiol Regul Integr Comp Physiol* 288: R1297-R1305, 2005.
34. **Limesand SW, Rozance PJ, Macko AR, Anderson MJ, Kelly AC and Hay WW, Jr.** Reductions in Insulin Concentrations and beta-Cell Mass Precede Growth Restriction in Sheep Fetuses with Placental Insufficiency. *Am J Physiol Endocrinol Metab* 304: E516-E523, 2013.
35. **Limesand SW, Rozance PJ, Smith D and Hay Jr WW**. Increased Insulin Sensitivity and Maintenance of Glucose Utilization Rates in Fetal Sheep with Placental Insufficiency and Intrauterine Growth Restriction. *Am J Physiol Endocrinol Metab* 293: E1716-E1725, 2007.
36. **Limesand SW, Rozance PJ, Zerbe GO, Hutton JC and Hay WW, Jr.** Attenuated Insulin Release and Storage in Fetal Sheep Pancreatic Islets with Intrauterine Growth Restriction. *Endocrinology* 147: 1488-1497, 2006.
37. **Limesand SW and Rozance PJ**. Fetal adaptations in insulin secretion result from high catecholamines during placental insufficiency. *The Journal of Physiology* 2017.
38. **Liu H, Schultz CG, De Blasio MJ, Peura AM, Heinemann GK, Harryanto H, Hunter DS, Wooldridge AL, Kind KL, Giles LC, Simmons RA, Owens JA and Gatford KL**. Effect of placental restriction and neonatal exendin-4 treatment on postnatal growth, adult body composition, and in vivo glucose metabolism in the sheep. *American Journal of Physiology - Endocrinology and Metabolism* 309: E589, 2015.
39. **Liu X, Perusse F and Bukowiecki LJ**. Mechanisms of the antidiabetic effects of the beta 3-adrenergic agonist CL-316243 in obese Zucker-ZDF rats. *Am J Physiol* 274: R1212-R1219, 1998.
40. **Macko AR, Yates DT, Chen X, Green AS, Kelly AC, Brown LD and Limesand SW**. Elevated plasma norepinephrine inhibits insulin secretion, but adrenergic blockade reveals enhanced β-cell responsiveness in

an ovine model of placental insufficiency at 0.7 of gestation. *Journal of Developmental Origins of Health and Disease* doi: 10.1017/S2040174413000093: 1-9, 2013.

41. **Macko AR, Yates DT, Chen X, Shelton LA, Kelly AC, Davis MA, Camacho LE, Anderson MJ and Limesand SW**. Adrenal Demedullation and Oxygen Supplementation Independently Increase Glucose-Stimulated Insulin Concentrations in Fetal Sheep With Intrauterine Growth Restriction. *Endocrinology* 157: 2104-2115, 2016.
42. **Macko AR, Yates DT, Chen X, Shelton LA, Kelly AC, Davis MA, Camacho LE, Anderson MJ and Limesand SW**. Adrenal demedullation and oxygen supplementation independently increase glucose-stimulated insulin concentrations in fetal sheep with intrauterine growth restriction. *Endocrinology* 157: 2104-2115, 2016.
43. **Martin A, Connelly A, Bland RM and Reilly JJ**. Health impact of catch-up growth in low-birth weight infants: systematic review, evidence appraisal, and meta-analysis. *Maternal & Child Nutrition* n/a, 2016.
44. **Mericq V, Ong KK, Bazaes R, Pena V, Avila A, Salazar T, Soto N, Iniguez G and Dunger DB**. Longitudinal changes in insulin sensitivity and secretion from birth to age three years in small- and appropriate-for-gestational-age children. *Diabetologia* 48: 2609-2614, 2005.
45. **Owens JA, Thavaneswaran P, De Blasio MJ, McMillen IC, Robinson JS and Gatford KL**. Sex-specific effects of placental restriction on components of the metabolic syndrome in young adult sheep. *Am J Physiol Endocrinol Metab* .: 2007.
46. **Philipps AF, Dubin JW and Raye JR**. Maturation of early-phase insulin release in the neonatal lamb. *Biol Neonate* 39: 225-231, 1981.
47. **Rozance PJ, Anderson M, Martinez M, Fahy A, Macko AR, Kailey J, Seedorf GJ, Abman SH, Hay WW and Limesand SW**. Placental Insufficiency Decreases Pancreatic Vascularity and Disrupts Hepatocyte Growth Factor Signaling in the Pancreatic Islet Endothelial Cell in Fetal Sheep. *Diabetes* 64: 555-564, 2015.

48. **Rozance PJ and Hay WW**. New approaches to management of neonatal hypoglycemia. *Maternal health, neonatology and perinatology* 2: 1, 2016.

49. **Scheidegger K, Robbins DC and Danforth E Jr**. Effects of chronic beta receptor stimulation on glucose metabolism. *Diabetes* 33: 1144-1149, 1984.

50. **Soto N, Bazaes RA, Pena V, Salazar T, Avila A, Iniguez G, Ong KK, Dunger DB and Mericq MV**. Insulin sensitivity and secretion are related to catch-up growth in small-for-gestational-age infants at age 1 year: results from a prospective cohort. *J Clin Endocrinol Metab* 88: 3645-3650, 2003.

51. **Stanley CA, Rozance PJ, Thornton PS, De Leon DD, Harris D, Haymond MW, Hussain K, Levitsky LL, Murad MH and Simmons RA**. Re-evaluating transitional neonatal hypoglycemia: mechanism and implications for management. *The Journal of pediatrics* 166: 1520, 2015.

52. **Thorn SR, Regnault TR, Brown LD, Rozance PJ, Keng J, Roper M, Wilkening RB, Hay WW, Jr. and Friedman JE**. Intrauterine growth restriction increases fetal hepatic gluconeogenic capacity and reduces messenger ribonucleic acid translation initiation and nutrient sensing in fetal liver and skeletal muscle. *Endocrinology* 150: 3021-3030, 2009.

53. **Thorn SR, Brown LD, Rozance PJ, Hay WW and Friedman JE**. Increased Hepatic Glucose Production in Fetal Sheep With Intrauterine Growth Restriction Is Not Suppressed by Insulin. *Diabetes* 62: 65, 2013.

54. **Wang X, Cui Y, Tong X, Ye H and Li S**. Glucose and lipid metabolism in small-for-gestational-age infants at 72 hours of age. *J Clin Endocrinol Metab* 92: 681-684, 2007.

55. **Whincup PH, Kaye SJ, Owen CG, Huxley R, Cook DG, Anazawa S, Barrett-Connor E, Bhargava SK, Birgisdottir BE, Carlsson S, de Rooij SR, Dyck RF, Eriksson JG, Falkner B, Fall C, Forsen T, Grill V, Gudnason V, Hulman S, Hypponen E, Jeffreys M, Lawlor DA, Leon DA, Minami J, Mishra G, Osmond C, Power C, Rich-Edwards JW, Roseboom TJ, Sachdev HS, Syddall H, Thorsdottir I, Vanhala M,**

Wadsworth M and Yarbrough DE. Birth weight and risk of type 2 diabetes: a systematic review. *JAMA* 300: 2886-2897, 2008.

56. Yates, D. T., Fowden, A. L., Macko, A. R., Chen, X., Green, A. S., and Limesand, S. W. Adrenal demedullation abolishes hypoxemia-induced catecholamine supression of glucose stimulated insulin secretion in fetal sheep. J Pediatr Res 68, 179. 2010.

    Ref Type: Abstract

57. **Yates DT, Green AS and Limesand SW**. Catecholamines Mediate Multiple Fetal Adaptations during Placental Insufficiency That Contribute to Intrauterine Growth Restriction: Lessons from Hyperthermic Sheep. *J Pregnancy* 2011:740408: 2011.

58. **Yates DT, Macko AR, Nearing M, Chen X, Rhoads RP and Limesand SW**. Developmental Programming in Response to Intrauterine Growth Restriction Impairs Myoblast Function and Skeletal Muscle Metabolism. *J Pregnancy* 2012:631038.: 2012.

Figure Legends

Figure 1. Glucose Stimulated Insulin Concentration. Square-wave hyperglycemic clamps were performed in control lambs (n=8) and in lambs with placental insufficiency-induced IUGR (n=7) at 8 days of age. A) Plasma glucose concentrations (mmol/L) are presented for each sample time (y-axis, minutes), which is relative to the start of the glucose bolus (time = 0). B) Plasma insulin concentrations (μg/L) are presented for each sampling point. The insulin area under the curve (AUC, μg.min/L) was calculated for the first 20 minutes of hyperglycemia, which represents acute, first phase insulin secretion, and the AUC mean ± SEM is graphed in the insert. P values from the statistical analysis are reported in the upper left corner of each graph.

Figure 2. Glucose-potentiated Arginine-induced Insulin Concentration. Insulin concentrations are presented for the GPAIS studies in control (n=8) and IUGR lambs (n=7). The arginine bolus is administered at 60 minutes, therefore values obtained immediately prior during the hyperglycemic clamp are baseline samples for this analysis. Results from the statistical analysis are presented in the upper left corner.

Figure 3. Glucose Utilization Rate. Rates of glucose utilization were measured with a radiotracer for D-glucose. Mean values are presented for control (n=8) and IUGR (n=5) lambs at basal and hyperinsulinemic steady states. There was an experimental group by period interaction and the letters denote significant difference ($P<0.05$).

Figure 4. Insulin Sensitivity for Glucose. Mean ± SEM values of insulin sensitivity are presented for control (n=8) and IUGR (n=7) lambs at basal (A) and hyperglycemic (B) steady state periods. The asterisks denotes significant difference ($P<0.05$) between IUGR and control groups.

Figure 5. Glucose Transporters and Insulin Receptor Levels. The protein expression of insulin receptor β, GLUT1, and GLUT4 was measured in control and IUGR lamb semitendinosus muscle samples by Western blot. Results were quantified and analyzed for control (n = 5) and PI-IUGR (n = 4) semitendinosus muscle. Representative image is shown for β-Tubulin which was run as internal control for each Western blot.

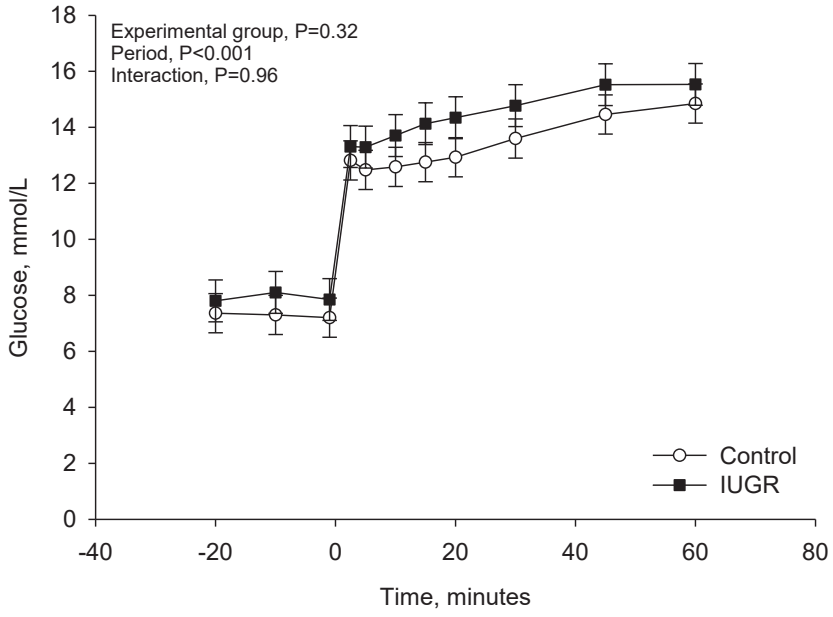
Table 1. Arterial blood gases, lactate, glucose and insulin concentrations during HEC study

Parameter	Fasting (Basal)		Hyperinsulinemia		*P*-value Group	*P*-value Period	*P*-value Interaction
	Control (8)	IUGR (5)	Control (8)	IUGR (5)			
pH	7.46 ± 0.01	7.43 ± 0.02	7.42 ± 0.01	7.38 ± 0.02	0.12	<0.01	0.85
PaO_2 (mmHg)	76.4 ± 2.8	72.8 ± 3.3	84.9 ± 2.8	74.6 ± 3.5	0.10	0.02	0.11
$PaCO_2$ (mmHg)	39.8 ± 1.5	42.4 ± 1.7	41.2 ± 1.5	44.1 ± 1.8	0.23	0.06	0.76
O_2 Content (mM)	6.1 ± 0.3	6.1 ± 0.4	6.0 ± 0.3	5.7 ± 0.4	0.74	0.03	0.22
Hematocrit (%)	32.4 ± 1.7	34.1 ± 1.8	31.1 ± 1.7	32.4 ± 1.9	0.59	<0.01	0.45
Lactate (mM)	1.01 ± 0.08^{ab}	1.09 ± 0.09^{ab}	0.84 ± 0.08^{b}	1.27 ± 0.10^{a}	0.02	0.95	0.05
Glucose (mmol/L)	8.54 ± 0.52	8.02 ± 0.60	8.27 ± 0.52	8.51 ± 0.63	0.87	0.66	0.09
Insulin (µg/L)	0.93 ± 0.37	0.75 ± 0.42	4.33 ± 0.37	3.54 ± 0.51	0.29	<0.01	0.46

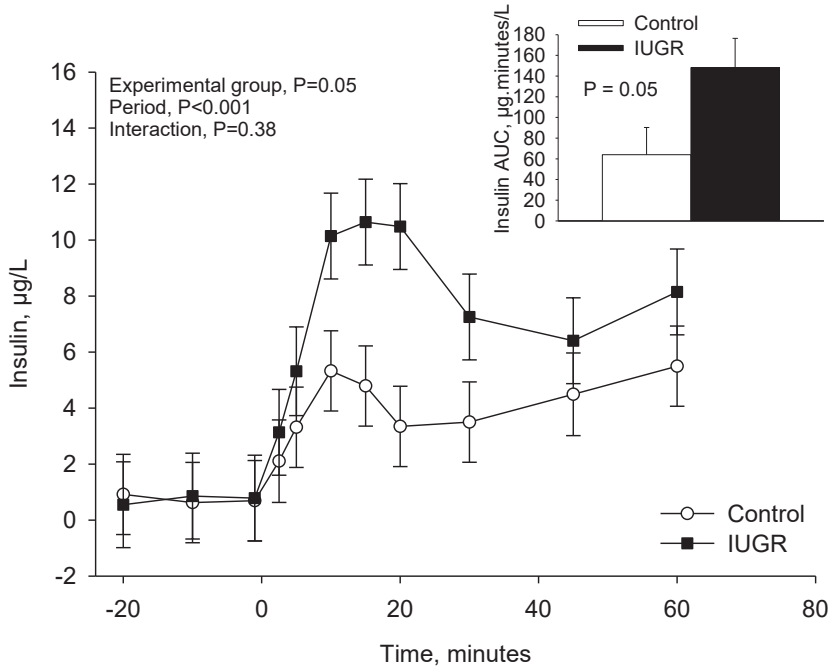
Superscript letters denote differences for significant interactions between experimental group and period.

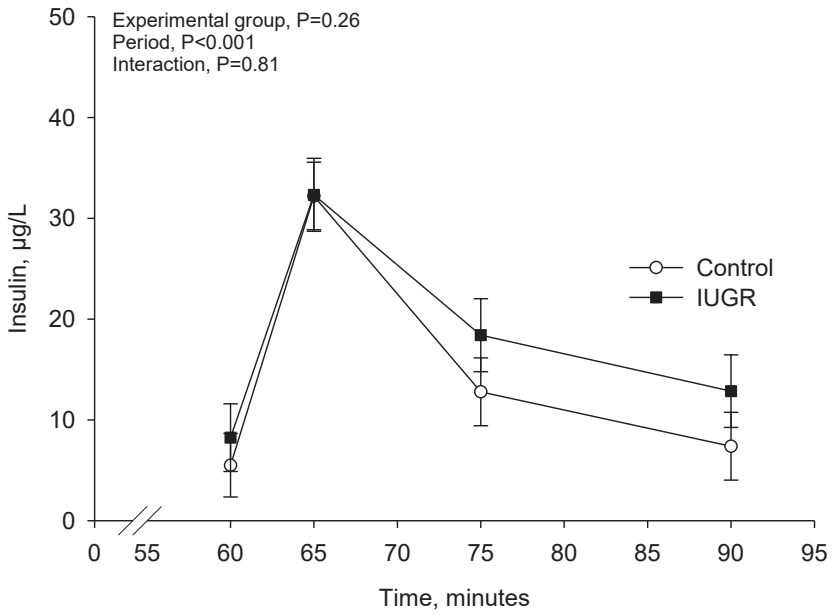
Values are means ± SEM.

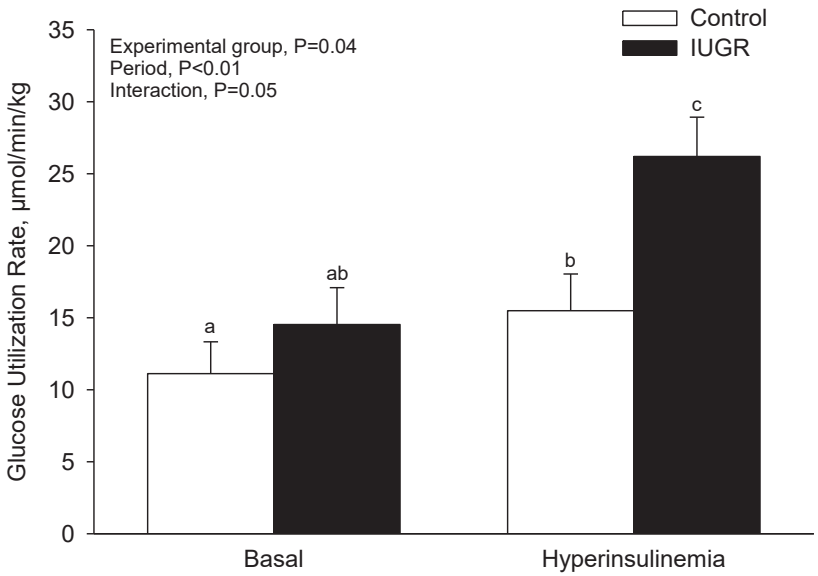
A


Experimental group, P=0.32
Period, P<0.001
Interaction, P=0.96
Glucose, mmol/L
Time, minutes
Control
IUGR

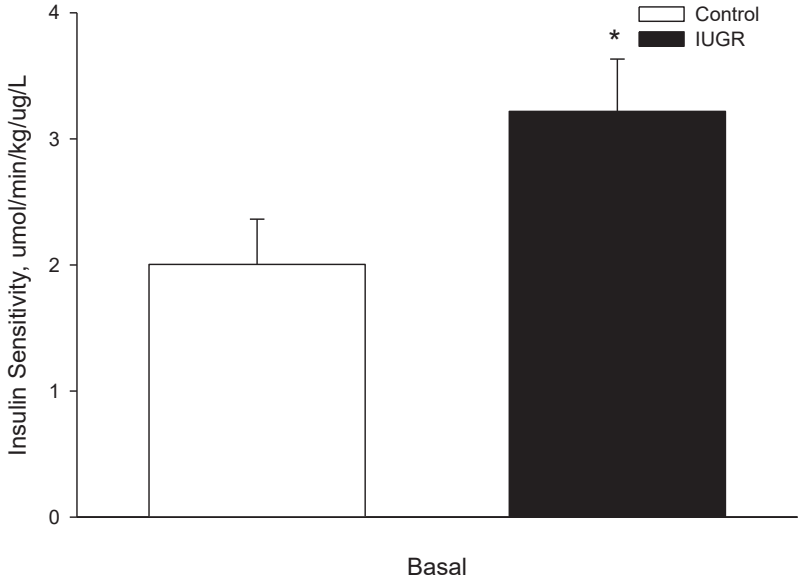
B


Experimental group, P=0.05
Period, P<0.001
Interaction, P=0.38
Insulin, µg/L
Time, minutes
Control
IUGR
Insulin AUC, µg.minutes/L
P = 0.05

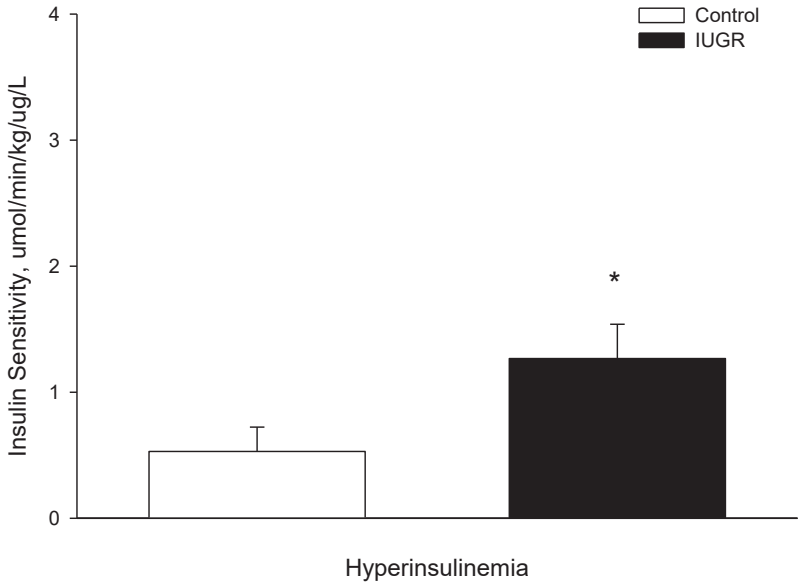

Experimental group, P=0.26
Period, P<0.001
Interaction, P=0.81
Insulin, µg/L
Time, minutes
Control
IUGR
0
10
20
30
40
50
0
55
60
65
70
75
80
85
90
95

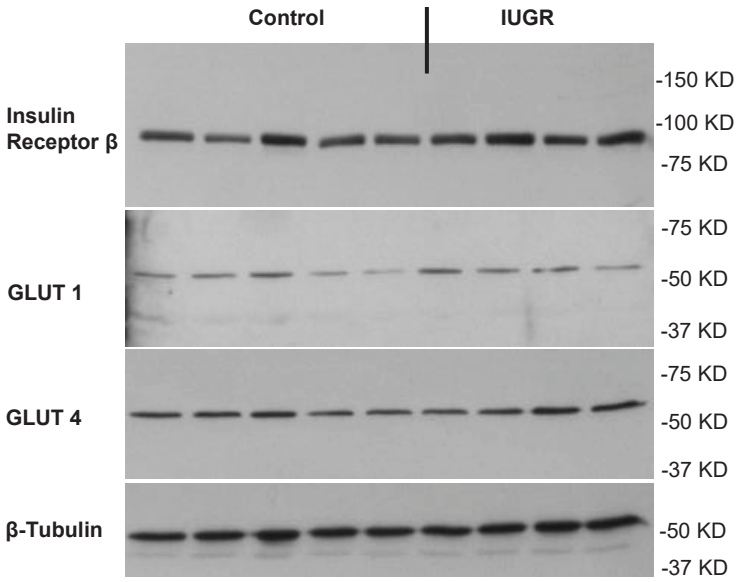

Control
IUGR
Experimental group, P=0.04
Period, P<0.01
Interaction, P=0.05
Glucose Utilization Rate, µmol/min/kg
35
30
25
20
15
10
5
0
a
ab
b
c
Basal
Hyperinsulinemia

A


Control
IUGR
*
Insulin Sensitivity, umol/min/kg/ug/L
0
1
2
3
4
Basal

B


Control
IUGR
*
Insulin Sensitivity, umol/min/kg/ug/L
0
1
2
3
4
Hyperinsulinemia


Control
IUGR
Insulin Receptor β
-150 KD
-100 KD
-75 KD
GLUT 1
-75 KD
-50 KD
-37 KD
GLUT 4
-75 KD
-50 KD
-37 KD
β-Tubulin
-50 KD
-37 KD