Chronic pulsatile hyperglycemia reduces insulin secretion and increases accumulation of reactive oxygen species in fetal sheep islets

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Abstract

Children from diabetic pregnancies have a greater incidence of type 2 diabetes. Our objective was to determine if exposure to mild–moderate hyperglycemia, by modeling managed diabetic pregnancies, affects fetal β-cell function. In sheep fetuses, β-cell responsiveness was examined after 2 weeks of sustained hyperglycemia with 3 pulses/day, mimicking postprandial excursions, and compared to saline-infused controls (n = 10). Two pulsatile hyperglycemia (PHG) treatments were studied: mild (mPHG, n = 5) with +15% sustained and +55% pulse; and moderate (PHG, n = 10) with +20% sustained and +100% pulse. Fetal glucose-stimulated insulin secretion and glucose-potentiated arginine insulin secretion were lower (P < 0.05) in PHG (0.86 ± 0.13 and 2.91 ± 0.39 ng/mL plasma insulin) but not in mPHG fetuses (1.21 ± 0.08 and 4.25 ± 0.56 ng/mL) compared to controls (1.58 ± 0.25 and 4.51 ± 0.56 ng/mL). Islet insulin content was 35% lower in PHG and 35% higher in mPHG vs controls (P < 0.01). Insulin secretion and maximally stimulated insulin release were also reduced (P < 0.05) in PHG islets due to lower islet insulin content. Isolated PHG islets also had 63% greater (P < 0.01) reactive oxygen species (ROS) accumulation at 11.1 mmol/l glucose than controls (P < 0.01), but oxidative damage was not detected in islet proteins. PHG fetuses showed evidence of oxidative damage to skeletal muscle proteins (P < 0.05) but not insulin resistance. Our findings show that PHG induced dysregulation of islet ROS handling and decreased islet insulin content, but these outcomes are independent. The β-cell outcomes were dependent on the severity of hyperglycemia because mPHG fetuses had no distinguishable impairments in ROS handling or insulin secretion but greater insulin content.

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Introduction

Diabetic conditions during pregnancy have been associated with an increased incidence of impaired glucose tolerance, insulin resistance, and type 2 diabetes in the offspring (Pettitt et al. 1985, Silverman et al. 1995, Plagemann et al. 1997, Dabelea et al. 2000, Sobngwi et al. 2003). Current clinical management guidelines allow pregnant mothers with diabetes to be mildly hyperglycemic, with fasting glucose 20–40% above normal, and to have larger postprandial excursions that are approximately twice the normal fasting glucose concentrations (Gillmer et al. 1975, Parretti et al. 2001, American Diabetes Association 2004, Stegmund et al. 2008). Fetal phenotypes, such as macrosomia, are strongly associated with maternal blood glucose concentrations (Karlsson & Kjellmer 1972, Metzger et al. 2008), and postprandial glucose is more predictive of macrosomia than fasting glucose (Jovanovic-Peterson et al. 1991, Combs et al. 1992, de Veciana et al. 1995, Most & Langer 2007). Fetal glucose is dependent on maternal concentrations; therefore, fetal exposure to chronic mild hyperglycemia with postprandial pulses appears to drive the adverse outcomes in offspring of diabetic pregnancies. Although basal insulin is increased in human fetuses exposed to diabetic conditions (Silverman et al. 1995, Metzger et al. 2008), it is not known if β-cell responsiveness to glucose is affected. However, animal studies have shown that β-cell outcomes in offspring are dependent on the magnitude of hyperglycemic exposure (Aerts & van Asse 1977, Kervran et al. 1978, Carver et al. 1996).

Oxidative stress is one possible mechanism for hyperglycemia-induced β-cell dysfunction. Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H₂O₂) are formed as byproducts of multiple metabolic pathways that are increased with hyperglycemia (Takahashi et al. 2004, Kaneto et al. 2005, Nakayama et al. 2005, Tsuibouchi et al. 2005). In pregnant rats with severe hyperglycemia, oxidative stress increases embryological malformations and spontaneous abortions (Erikson & Borg 1993, Kinalska et al. 1999, Cederberg et al. 2001), and similar complications have been observed in women with severe pre-existing diabetes (Suhonen et al. 2000). Women with gestational diabetes also exhibit increased placental oxidative stress (Coughlan et al. 2004, Lappas et al. 2004); however, to our knowledge, fetal measures have not been obtained. Chronic oxidative stress in...
isolated islets and immortalized β-cell lines decreases glucose-stimulated insulin secretion (GSIS) and lowers insulin content (Maechler et al. 1999, Kaneto et al. 2001, Noda et al. 2002, Tanaka et al. 2002, Sakai et al. 2003, Takahashi et al. 2004, Tang et al. 2007). Compared to other cell types, adult β-cells have relatively low levels of antioxidant enzymes and a limited ability to upregulate these enzymes in response to oxidative stress (Lenzen et al. 1996, Tiedge et al. 1997), therefore making them more vulnerable to oxidative stress. It is not yet known whether fetal β-cells possess similar deficiencies and vulnerability to ROS accumulation.

Another potential mechanism linking hyperglycemia and fetal β-cell dysfunction is endoplasmic reticulum (ER) stress. Abnormal proinsulin processing and protein folding induced by hyperglycemia can result in ER stress, distended rough ER, and insulin secretion defects (Araki et al. 2003). Distension of the ER was observed in β-cells of fetal rats subjected to an experimental model of gestational diabetes (Aerts & van Assche 1977). ER stress would reflect possible defects caused by nitric oxide, calcium storage, or cytokines (Oyadomari & Mori 2004).

We investigated insulin secretion responsiveness, islet function, islet ROS accumulation, and markers of ER stress in sheep fetuses exposed to chronic mild pulsatile hyperglycemia (mPHG). The sheep model was chosen for this study, because exogenous dextrose can be chronically infused into the pregnant ewe with precision in both magnitude and pattern (Carver et al. 1996). In vivo and ex vivo insulin responsiveness can be measured in the sheep fetus (Limesand et al. 2006, Green et al. 2011) and shares similarities with that in the human in during the progression of pancreas development (Green et al. 2010). Sheep fetuses were exposed to sustained mild-to-moderate hyperglycemia with three superimposed hyperglycemic pulses per day (mimicking postparturial excursions) for 2 weeks during late gestation. At the end of the treatment, GSIS was measured in the fetuses and their isolated pancreatic islets. Additionally, ROS accumulation in islets was measured in vitro, and markers of systemic oxidative stress and ER stress were assessed in fetal tissues. We found that PHG lowered islet insulin content and impaired fetal GSIS and ROS regulation, though these defects occurred independently and were dependent on the magnitude of PHG.

Materials and Methods

Animal preparation

Pregnant Columbia-Rambouillet ewes carrying singletons were purchased from Nebeker Ranch Inc. (Lancaster, CA, USA) and managed in compliance with the Institutional Animal Care and Use Committee of the University of Arizona. All animal experiments were conducted at the William J Parker Agricultural Research Complex, Tucson, AZ, USA, which is accredited by the National Institutes of Health, the United States Department of Agriculture, and the American Association for the Accreditation of Laboratory Animal Care. Animal rooms were maintained at 22 ± 1 °C with a 14 h light:10 h darkness cycle. Except as noted below, food and water were available ad libitum, and food intake was recorded daily. Food was withheld for 24 h and water for 18 h prior to surgery. At ~119 dGA, fetuses were surgically instrumented with indwelling polyvinyl catheters as described previously (Limesand & Hay 2003, Limesand et al. 2007).

Fetal catheters for blood sampling were placed in the abdominal aorta via hind limb pedal arteries and infusion catheters were placed in the femoral veins via the saphenous veins. Maternal catheters were placed in the femoral artery for arterial sampling and the femoral vein for infusions. All catheters were tunneled subcutaneously to the ewe’s flank, exteriorized through a skin incision, and kept in a plastic mesh pouch sutured to the ewe’s skin. Ewes were given 4–5 days to recover from surgery before treatment.

Study design

Animals were randomly assigned to one of the three treatment groups: control (n = 10), mPHG (n = 5), and PHG (n = 10). Treatments were initiated at 124 ± 0.5 dGA with maternal i.v. infusions of 50% dextrose (weight/volume in H2O) and were maintained for 14 days. In the mPHG and PHG animals, dextrose was infused to raise maternal plasma glucose concentrations 15 and 20% above euglycemia (determined in control fetuses) respectively. Arterial plasma glucose concentrations were measured at least twice daily, and dextrose infusion rates were adjusted to maintain the specified treatment conditions. In addition, mPHG and PHG ewes received 45 min boluses of dextrose at 0800, 1400, and 2000 h each day at a rate sufficient to achieve peak plasma glucose concentrations 55 and 100% above euglycemic values (compared to controls) respectively. The magnitude and pattern of the hyperglycemia treatments were specifically chosen to represent pregnant women with well-controlled diabetes (mPHG) and marginal glucose control (PHG), according to the current clinical guidelines (Carpenter & Coustan 1982, American Diabetes Association 2004, Gilmartin et al. 2008). Plasma glucose concentrations during boluses were checked at least every other day and bolus infusion rates adjusted when needed. Control ewes were randomly paired to PHG ewes and received saline infusions of equal volumes. Maternal and fetal arterial blood samples were collected daily between 0700 and 0800 h, and basal plasma glucose, lactate, and insulin, and blood gas and pH levels were measured. Seven maternal and fetal plasma samples (time 0–120 min) were collected for the 1400 h bolus on day 9 of treatment to quantify the fetal plasma insulin response to the boluses.

GSIS and glucose-potentiated arginine-stimulated insulin secretion studies

On day 13 or 14 of treatment, while continuing the chronic sustained maternal dextrose infusions and in place of the
arginine (0.5 mmol/kg estimated fetal weight mixed with studies (GPAIS) test was conducted by injecting a bolus of glucose-potentiated arginine-stimulated insulin secretion measured. Following the 61 min hyperglycemic sample, a plasma glucose, lactate, and insulin concentrations were hyperglycemic (45–61 min) periods, blood gas and pH and insulin was measured with an ovine insulin ELISA (ALPCO Diagnostics, Windham, NH, USA; intra-assay and interassay CV were 20 and 80% respectively). Within 24 h of the GSIS studies, while remaining under treatment conditions, ewes and fetuses were killed with an i.v. overdose of sodium pentobarbital (86 mg/kg) and phenytoin sodium (11 mg/kg, Euthanol; Virbac Animal Health, Fort Worth, TX, USA). The fetus was blotted, dried and weighed. The fetal pancreas was perfused and digested with Liberase Blendszyme III (0·175 mg/ml; Roche) in Krebs Ringer buffer (KRB; 118 mmol/l NaCl, 4·8 mmol/l KCl, 2·5 mmol/l CaCl₂, 1·2 mmol/l MgSO₄, 1·2 mmol/l KH₂PO₄, 25 mmol/l NaHCO₃, pH 7·3) and then individual islets were purified as described previously (Limesand et al. 2006, Rozance et al. 2006, Leos et al. 2010). After removal, the pancreas, brain, liver, skeletal muscle, spleen, kidney, lung, heart, and perirenal adipose tissues were dissected and weighed. Liver (left lobe) and semintidinosus muscle samples were snap frozen in liquid nitrogen and stored at −80 °C for RNA and protein extraction.

Biochemical analyses

Blood gases, pH, and oximetry parameters were measured in whole blood collected in heparin-lined syringes (Elkins-Sinn, Inc., Cherry Hill, NJ, USA) with an ABL 720 (Radiometer, Copenhagen, Denmark). Sample values were temperature corrected at 39·1 °C. Whole blood collected in EDTA-lined syringes (Sigma–Aldrich) was centrifuged (13 000 g) for 2 min at 4 °C, and the plasma was aspirated from the red blood cells. Plasma glucose and lactate concentrations were measured with a YSI Model 2700 SELECT Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA). The remaining plasma was stored at −80 °C until insulin was measured with an ovine insulin ELISA (ALPCO Diagnostics, Windham, NH, USA; intra-assay and interassay coefficients of variation (CV) were 5·6 and 2·9% respectively). In the control and PHG fetuses, remaining plasma from the −21 and −13 min samples were pooled, and nor-epinephrine concentrations were analyzed by Noradrenaline ELISA (Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany; intra-assay and interassay CV were 20 and 22% respectively).

Necropsy and islet isolation

Isolated islets were cultured overnight at 37 °C in 95% O₂/5% CO₂ in RPMI-1640 medium (Sigma–Aldrich) supplemented with 2% fetal bovine serum (FBS), 2·8 mmol/l glucose, and penicillin–streptomycin (50 U and 50 μg; Sigma–Aldrich). One day after isolation, in vitro experiments on islets were performed, and an aliquot of hand-picked islets was frozen and stored at −80 °C for RNA and protein extraction.

In seven control, five mPHG, and eight PHG fetuses, insulin secretion was measured in static islet incubations (Limesand et al. 2006). Fetal islets were washed twice in KRB with 0·5% BSA and a third time in KRB/BSA supplemented with 10 μmol/l Forskolin (Sigma–Aldrich) equilibrated to 37 °C and 95% O₂/5% CO₂. Ten islets were hand-picked (n = 3–4 replicates/condition) and incubated at 37 °C for 1 h in KRB/BSA/Forskolin media with the following conditions: no glucose, 1·1 mmol/l glucose (nonstimulatory concentration), 11·1 mmol/l glucose (maximal glucose stimulatory concentration for fetal sheep islets (Limesand et al. 2006)), or 1·1 mmol/l glucose plus 30 mmol/l KCl. Negative control islet incubations in 11·1 mmol/l glucose on ice were included to evaluate cellular integrity. Following the incubation, islets were pelleted by centrifugation (3 min at 800 g) at 4 °C. The media was removed and frozen, and islet insulin was extracted with acid–ethanol (1 mol/l HCl/70% ethanol). Insulin concentrations were measured with the ovine insulin ELISA. The data were analyzed as nanogram insulin release per islet.

Rates of glucose oxidation were measured by the formation of 14CO₂ as previously described (Limesand et al. 2006) for islets from five control and five PHG fetuses; mPHG islets were not assessed. Twenty-five islets were hand-picked into a 1 ml cryotube affixed inside a scintillation vial and sealed. The islets were incubated for 2 h at 37 °C in
concentrations were determined with the BCA protein Electrophoresis System (Bio-Rad Laboratories). Protein RNA integrity was confirmed with an Experion Automated ND-1000 Spectrophotometer, Wilmington, DE, USA), and measuring absorbance at 260 and 280 nm (NanoDrop Langerhans using the Allprep DNA/RNA/Protein Mini Kit for 10 min, and the supernatant was frozen and stored at −80 °C. Total RNA and protein were extracted from purified islets of Langerhans using the Allprep DNA/RNA/Protein Mini Kit (Qiagen). RNA concentrations were determined by measuring absorbance at 260 and 280 nm (NanoDrop ND-1000 Spectrophotometer, Wilmington, DE, USA), and RNA integrity was confirmed with an Experion Automated Electrophoresis System (Bio-Rad Laboratories). Protein concentrations were determined with the BCA protein assay (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

**Tissue protein oxidative damage**

Protein carbonyl incorporation was measured in skeletal muscle and liver from eight PHG and ten control fetuses and in islet protein lysates from six PHG and six control fetuses using the OxyBlot Protein Oxidation Detection Kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions except as noted here. Five micrograms of islet protein and 7.5 µg of liver and skeletal muscle protein lysates were derivatized to 2,4-dinitrophenylhydrazine (DNPH) by reaction with 2,4-dinitrophenylhydrazine or were treated with a negative solution for a negative control. Proteins were separated on 10% SDS-PAGE, transferred onto a polyvinylidene fluoride membrane (Bio-Rad), and blocked in the provided blocking/dilution buffer at room temperature for 1 h. Immunoblot detection was achieved by overnight incubation at room temperature with the provided rabbit anti-DNP antibody. The primary antibody was detected with anti-rabbit IgG HRP conjugated secondary antibody (1:10 000; Bio-Rad) for 1 h at room temperature and detected using SuperSignal West Pico (Thermo Fisher Scientific) exposed to Kodak X-ray film. Because the density of individual bands did not change independently, the sum of the density of all DNP bands in each lane was quantified with ImageJ Software version 1.41 (National Institutes of Health, Bethesda, MD, USA). Multiple exposure times were analyzed to confirm that the films quantified were not saturated. Protein from each fetus was analyzed in triplicate, and paired control and PHG fetuses were analyzed on the same gel. In addition, protein from one select control fetus was included in triplicate on every gel, and the band density for all other lanes was normalized to the mean for the control protein.

**PCR and quantitative real-time PCR (qPCR)**

Synthetic oligonucleotide primers were designed against sequences for genes of interest (GenBank accession numbers are listed in parentheses and are for ovine sequences unless otherwise noted): antioxidants superoxide dismutase 1 (SOD-1; JF546075), SOD-2 (GQ221055.1), GPx-1 (FJ728302), catalase (GQ421282) and uncoupling protein 2 (UCP2; bovine sequence NM_0010533611); insulin (U00659) and insulin transcription factors pancreatic and duodenal homeobox 1 (PDX-1; JF728303) and V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA; bovine sequence NM_001053637.1); ER stress response genes glucose regulatory protein-78 (GRP78; DQ029323), and DNA-damage inducible transcript-3 (DDIT-3; AY943948); and the reference gene ribosomal protein S15 (S15; AY949746), and DNA-damage inducible transcript-3 (DDIT-3; AY949748); and the reference gene ribosomal protein S15

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verified by confirming product size after separation in a 1% agarose DNA gel. PCR products were then inserted into the TOPO TA cloning expression vector pCR II (Invitrogen) and transformed into One Shot Mach T1 Phage-Resistant Chemically Competent Escherichia coli (Invitrogen). Plasmids were prepared for nucleotide sequencing with a QIAprep Spin Miniprep Kit (Qiagen) and sequenced at the University of Arizona DNA Sequencing Service.

One microgram of RNA extracted from islets from nine control and five PHG fetuses was used to generate cDNA with SuperScript III Reverse Transcriptase (Invitrogen). The relative mRNA expression for each gene of interest was determined by qPCR using SYBR Green (Qiagen) in an iQ5 Real-Time PCR Detection System (Bio-Rad) as reported previously (Chen et al. 2010). After initial denaturation at 95°C for 15 min, all reactions went through 40 cycles of 96°C (30 s), annealing temperature of 60–62°C (30 s), and 72°C (10 s) at which point the fluorescence was measured. Melt curve analysis was performed at the end of the amplification to confirm product homogeneity. PCR efficiency was determined with islet cDNA (80–100%) and was linear over six orders of magnitude. RNA samples were run in triplicate for each qPCR. The results were normalized to the reference gene S15 for each qPCR, and the average cycle threshold (ΔCt) was analyzed by the comparative ΔCt method (ΔCt gene of interest − ΔCt reference gene) (Schmittgen & Livak 2008).

Western immunoblot analyses
Western immunoblot analyses for SOD-1 and SOD-2, glutathione peroxidase 1/2 (GPx-1/2), insulin receptor-β (IRβ), β-tubulin, and ribosomal protein S6 were conducted as previously described (Chen et al. 2010) on liver and skeletal muscle protein preparations. Protein samples from each fetus were run in duplicate and 30 μg protein were loaded per lane. Paired control and PHG fetuses were analyzed on the same gel. Statistical analyses of feed and energy intake; the effect of body weight and the interaction was found between body weight and treatment. Protein carbonyl data and other western immunoblot data were analyzed by paired t-test to determine differences between protein expression in paired control and PHG fetuses separated in the same gel. Statistical analyses were completed in JMP 8 and SAS 9.2 (SAS, Cary, NC, USA).

Results

Maternal body weight and feed intake
Before treatment, maternal body weight was not different between control (44.2±1.7 kg), PHG (41.1±2.7 kg), and mPHG ewes (49.9±3.0 kg). During the treatment period, feed intake was lower (P<0.01) in PHG ewes (1.28±0.06 kg/day) than mPHG (1.78±0.08 kg/day) and control ewes (1.84±0.07 kg/day). However, total daily energy intake (feed+dextrose infusion) was not different between mPHG (2.88±0.13 Mcal/day) and PHG ewes (2.61±0.10 Mcal/day), and both were greater than that of control ewes (2.26±0.09 Mcal/day; P<0.01). In all treatments, energy intake was in excess of the requirements for maintenance and gestation (National Research Council 2007). Ewe body weight was included as a covariate in the analyses of feed and energy intake; the effect of body weight was significant for both parameters (P<0.01), but no interaction was found between body weight and treatment.

Fetal body and organ weights
At necropsy, average fetal body weights were similar between control (3.9±0.2 kg), mPHG (4.3±0.2 kg), and PHG fetuses (3.6±0.1 kg). Fetal body weight was included as a covariate for all analyses of fetal organ weights, and no treatment effect was found for individual fetal organ weights (data not shown).

Maternal and fetal plasma values during treatment
Prior to treatment, maternal and fetal plasma glucose and fetal plasma insulin concentrations were not different between treatment groups (Table 1). Per our experimental design, the
magnitude and pattern of hyperglycemia were tightly controlled during the treatment period (Table 1 and Fig. 1). The interanimal CV in control, mPHG, and PHG sheep respectively were 3.2, 4.0, and 3.3% for maternal plasma glucose and 20.7, 13.6, and 13.2 for fetal plasma glucose. The within-animal CV for daily samples did not differ between treatments for either maternal (mean 5.4±0.4%; P=0.50) or fetal plasma glucose concentrations (mean 8.7±0.7%; P=0.26). Basal maternal plasma glucose was 15±2% higher in mPHG ewes and 20±2% higher in PHG ewes than euglycemic controls (P<0.01; Table 1 and Fig. 1). Fetal plasma glucose was 16±7 and 21±5% higher in the mPHG and PHG fetuses respectively (P<0.05). Fetal insulin concentrations were not different between groups during the treatment period (Table 1).

At the peak of the boluses, plasma glucose concentrations were 1.56-fold higher than euglycemic control concentrations in mPHG ewes (5.72±0.09 mmol/l) and fetuses (1.67±0.10 mmol/l), 2.03-fold higher in PHG ewes (7.44±0.03 mmol/l), and 2.01-fold higher in PHG fetuses (2.16±0.10 mmol/l; P<0.01; Fig. 1C). Plasma insulin concentrations were increased 1.40-fold (0.74±0.11 ng/ml; P<0.05) in mPHG and 1.58-fold (1.60±0.07 ng/ml; P<0.01) in PHG compared to basal values (P<0.05). The peak bolus glucose concentrations had interanimal CV of 3.5 and 7.0% for maternal glucose and 13.9 and 12.5% for fetal glucose in mPHG and PHG animals respectively. The average within-animal CV (determined from at least six boluses on different days) were 6.4±0.5% for ewes and 8.7±0.7% for fetuses and were not different between treatments.

**Table 1.** Ovine maternal plasma glucose and lactate and fetal plasma glucose, lactate, and insulin prior to and during the treatment period. Values are means±S.E.M. Pretreatment and treatment values are the means from samples taken daily prior to the 0800 h bolus infusion rates on day 14 of treatment were 3.6- and 2.4-fold higher than day 1 in the mPHG and PHG groups respectively, and the slopes of the infusion rates over time were positive for both treatments (P<0.01; Fig. 2). The bolus infusion rates also increased 1.3-fold (positive slope, P<0.01) and 1.1-fold (slope P=0.12) during the treatments, averaging 0.17±0.01 and 0.31±0.02 g/kg BW per h in the mPHG and PHG groups respectively.

![Image](https://via.placeholder.com/150)

**Hematological, lactate, and norepinephrine values during fetal GSIS**

Blood gases and blood pH were measured to evaluate physiological responses associated with hyperglycemia during the treatment and GSIS study. The fetal partial pressure of CO2 was greater in PHG fetuses (51.50±0.04 mmHg; P<0.01 for treatment effect) than controls (49.30±0.21 mmHg), and mPHG fetuses (49.99±0.04 mmol/l) were not different from either control or PHG treatments. The pCO2 increased in the hyperglycemic period (50.80±0.03 mmHg, P<0.01 for period) vs basal (49.72±0.17 mmHg), and there was no treatment by period interaction. Fetal blood oxygen content 

![Image](https://via.placeholder.com/150)

**Differences within treatment and period were found for fetal plasma lactate concentrations, but no treatment by period interaction was observed. Plasma lactate concentrations were greater (P<0.01) in PHG fetuses (2.63±0.03 mmol/l) than mPHG fetuses (2.22±0.04 mmol/l) and both were greater (P<0.01) than control fetuses (1.88±0.03 mmol/l). Plasma lactate concentrations increased (P<0.01) during the hyperglycemic clamp (2.48±0.03 mmol/l) from a basal period (1.99±0.03 mmol/l). Maternal plasma lactate concentrations were lower (P<0.05) in PHG ewes**
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Fetal GSIS and GPAIS

In the GSIS basal period fetal plasma glucose concentrations were not different from the treatment values reported above (Table 1 and Fig. 3). During the square-wave hyperglycemic clamp, glucose concentrations were increased to 2.3 ± 0.2 mmol/l, which was not different between treatments (Fig. 3A and B). Basal plasma insulin concentrations were not different between treatments and were 0.44 ± 0.08 ng/ml in control, 0.50 ± 0.06 ng/ml in mPHG, and 0.42 ± 0.07 ng/ml in PHG fetuses. At the hyperglycemic steady state, plasma insulin concentrations were lower \( (P < 0.05) \) in PHG fetuses \( (0.86 ± 0.13 \text{ ng/ml}) \) than control fetuses \( (1.58 ± 0.25 \text{ ng/ml}) \). Hyperglycemic insulin concentrations in the mPHG fetuses \( (1.21 ± 0.08 \text{ ng/ml}) \) were not different from the control \( (P < 0.08) \) or PHG \( (P < 0.09) \) treatments. GSIS responsiveness was calculated as the difference between hyperglycemic and basal steady state plasma insulin concentrations. PHG fetuses had lower \( (P < 0.05) \) GSIS responsiveness \( (0.45 ± 0.07 \text{ ng/ml}) \) compared to controls \( (1.14 ± 0.25 \text{ ng/ml}) \), while the mPHG fetuses were intermediate \( (0.71 ± 0.12 \text{ ng/ml}) \) and not different from either control or PHG fetuses (Fig. 3C and D). The fetal plasma insulin to glucose ratio did not differ between treatments during the basal period, but during the hyperglycemic clamp it was lower in PHG fetuses \( (0.35 ± 0.05; P < 0.05) \) than in controls \( (0.68 ± 0.11) \); mPHG fetuses \( (0.52 ± 0.03) \) were intermediate and not different from the other treatments (Fig. 3E).

Insulin concentrations following the arginine bolus reached maximum values after 5 min in all treatments (Fig. 4). Insulin concentrations during the GPAIS were lower in PHG fetuses than control and mPHG fetuses at both 5 min \( (P < 0.01) \) and 15 min \( (P < 0.05) \) following administration of arginine. The net incremental insulin area under the curve (AUC), when calculated from basal insulin concentrations, was lower in PHG fetuses \( (P \leq 0.05) \) than in control fetuses (Fig. 4B), reflecting impairment in the combined ability of glucose and arginine to enhance insulin secretion. In

\[(0.45 ± 0.01 \text{ mmol/l}) \text{ than mPHG ewes (0.49 ± 0.01 mmol/l) and were lower in both treatments than in controls (0.82 ± 0.01 mmol/l; } P < 0.01). \] The fetal hyperglycemic clamp did not alter maternal plasma lactate concentrations.

In the sheep fetus, norepinephrine in response to fetal stress has been shown to increase oxygen tension and lactate concentrations and decrease insulin secretion (Bassett & Hanson 2000, Jackson et al. 2000). To confirm that adrenergic stimulation was not responsible for the hematological and metabolic responses described above, we measured fetal plasma norepinephrine concentrations during the basal period of the GSIS study (Jackson et al. 2000). Fetal norepinephrine concentrations were not different between control \( (339 ± 78 \text{ pg/ml}) \) and PHG fetuses \( (402 ± 182 \text{ pg/ml}; P = 0.75) \).
contrast, when net incremental AUC was calculated from the hyperglycemic steady state insulin concentrations, the AUC values were not different between groups (\( P = 0.40 \); not shown).

**Isolated islet insulin contents, insulin secretion, and glucose oxidation**

Islet insulin contents varied with treatment (\( P < 0.05 \); Fig. 5A); mPHG islets had greater insulin contents and PHG islets had lower insulin contents than control islets (\( P < 0.05 \)). Mean insulin release across the islet static incubations was reduced (\( P < 0.05 \)) in PHG islets (2.1 ± 0.3 ng/islet) compared to control (3.2 ± 0.4 ng/islet) and mPHG islets (3.9 ± 0.4 ng/islet), but control and mPHG means were not different. Among all treatments, insulin release was increased (\( P \leq 0.05 \)) in media conditions containing 11.1 mmol/l glucose (2.7 ± 0.4 ng/islet) and 30 mmol/l KCl (8.8 ± 0.4 ng/islet) compared to incubation conditions with 0 and 1.1 mmol/l glucose (1.2 ± 0.4 and 1.4 ± 0.4 ng/islet; Fig. 5B). The only difference between treatment groups within an incubation condition was found in the 30 mmol/l KCl condition; insulin release in PHG islets was less (\( P < 0.05 \)) than control and mPHG islets. Islets incubated on ice in 11.1 mmol/l glucose released equivalent

**Figure 3** Impaired GSIS in PHG fetuses. Graphs A and C show mean plasma glucose and insulin concentrations during basal (−21 to 0 min) and hyperglycemic (after time 0) periods. The bar graphs show the changes in (B) glucose and (D) insulin between the basal and hyperglycemic (45–61 min) steady state periods, which were calculated as the difference between hyperglycemia and basal mean concentrations. The elevation in glucose was similar between treatments, but PHG fetuses had a lower insulin response than control fetuses (\( P < 0.05 \)). Plasma insulin/glucose ratios (panel E) are shown for basal and hyperglycemic steady state periods. Bars or plots not sharing the same letter are different, \( P < 0.05 \).
amounts of insulin (1.4 ± 0.4 ng/islet) as islets in no glucose, demonstrating that insulin in the media was due to secretion and not cellular breakdown. Fractional insulin secretion, calculated as the percentage of total islet insulin released into the media, did not vary by treatment (data not shown).

The rate of glucose oxidation was measured in control and PHG islets. Stimulatory glucose concentrations (11.1 mmol/l) increased the rate of glucose oxidative phosphorylation in isolated islets twofold (8.1 ± 1.2 pmol/islet per h) compared to basal rates measured in 1.1 mmol/l glucose (4.1 ± 0.6 pmol/islet per h; P < 0.01). No differences between treatments were found.

**Islet ROS accumulation**

Fluorescence imaging of single isolated islets preloaded with CM-H$_2$DCFDA showed no difference between treatments in the rate of islet ROS accumulation at basal glucose concentrations (1·1 mmol/l); basal rates were 103 ± 17 a.u./min for control islets, 118 ± 37 a.u./min for mPHG islets, and 94 ± 33 a.u./min for PHG islets. Experimental islet variation due to CM-H$_2$DCFDA loading was normalized to the basal rate in each individual islet to compare rates of ROS accumulation in the presence of stimulatory glucose and H$_2$O$_2$. Elevation of glucose from 1·1 to 11·1 mmol/l led to a 63% increase in the ROS accumulation rate in PHG islets, but there were no changes in control or mPHG islets. The relative glucose stimulated (11·1 mmol/l) rate of ROS accumulation was greater in PHG islets than control and mPHG islets (P < 0·05; Fig. 6). The subsequent addition of 9 mmol/l H$_2$O$_2$ further increased the rate of ROS accumulation similarly in all treatments (Fig. 6), showing that the dynamic range of the probe was not exceeded during the experiment. These data indicate that isolated PHG islets have a greater rate of ROS accumulation under stimulatory glucose conditions.

**Islet gene expression for antioxidant enzymes, insulin expression, and ER stress**

In isolated islets, relative mRNA expression levels for SOD-1, SOD-2, catalase, and GPx-1 were similar between treatments (Fig. 7), indicating that the greater glucose-stimulated ROS accumulation in PHG islets was caused by increased production of ROS rather than a change in enzymatic ROS clearance. In addition, mRNA expression levels for insulin, PDX-1, MafA, UCP2, and ER stress response genes (GRP78 and DDIT-3) were not different between control and PHG islets (Fig. 7). Islets from mPHG fetuses were not evaluated.

**Tissue protein oxidative stress, antioxidant enzymes, and IR**

We evaluated whether chronic PHG caused islet or systemic oxidative stress by measuring carbonyl incorporation of proteins, which is an indicator of protein oxidative damage. Skeletal muscle from PHG fetuses had 46% greater intensities for carbonyl moieties compared to control fetuses (P < 0·05). However, there was no difference between PHG and control fetuses in carbonyl incorporation in liver or islet proteins. Protein concentrations of SOD1, SOD2, and GPx-1/2 in skeletal muscle and liver measured by western blot were not different between control and PHG treatments (Fig. 8A–D). Tissues from mPHG fetuses were not evaluated.

**Discussion**

The objective of this study was to determine if 2 weeks of PHG exposure, similar to conditions in managed diabetic
pregnancies, would impact fetal β-cell responsiveness. The major finding was that PHG treatment attenuated fetal insulin secretion in response to glucose and glucose-potentiated arginine. The GPAIS study showed that the readily releasable pool of insulin was reduced in PHG fetuses compared to controls. Islets isolated from PHG fetuses also had reduced insulin content and insulin release. Moreover, maximal insulin release induced by depolarizing the islets with KCl was lower in PHG islets. These data, together with the fetal GPAIS results, indicate that insulin content was a limiting factor for PHG islets because both measures are dependent on β-cell insulin content or β-cell mass (Seaquist & Robertson 1992, Robertson 2007). Fractional islet insulin release and islet glucose metabolism were not affected by PHG treatment, further supporting the hypothesis that reduced β-cell insulin content, rather than a defect in stimulus-secretion coupling, was the major factor explaining attenuated fetal GSIS. The second major finding of this study was that glucose-stimulated ROS accumulation was greater in the PHG islets, but there was no evidence for islet oxidative stress or ER stress. Therefore, oxidative stress does not appear to initiate the decline in insulin content; however, if persistent, it will cause islet oxidative damage and β-cell failure, as shown for adult models of hyperglycemia-induced diabetes (Kaneto et al. 1999, Tanaka et al. 1999, Tang et al. 2007). The major outcomes from PHG exposure were reduced fetal insulin secretion due to less insulin content and impaired islet ROS handling, but these two deficiencies do not appear to have a causal relationship.

The results of this study show that islet dysfunction is dependent on the magnitude of PHG. Strikingly, GSIS responsiveness was intermediate in mPHG fetuses, and the mPHG response to the GPAIS study was not different from controls but greater than PHG fetuses. The mPHG islet insulin content was also greater than controls and, coupled with normal fetal GSIS, indicates insulin stimulus-secretion may be reduced. This is also supported in the islet experiments, because no enhancement of islet insulin secretion was observed even with elevated insulin content. Together, these findings indicate that within a narrow range of exposure to mild or moderate PHG, fetal β-cells exhibit impaired insulin stimulus-secretion with a compensatory increase in insulin contents (mPHG) or lower insulin contents (PHG), depending on the magnitude of hyperglycemia.

Insulin secretion responsiveness varies in human neonates born to diabetic mothers, and this variation is probably a result of differences in the duration and magnitude of exposure to hyperglycemia, as in our study and other animal studies described below. One human study showed no difference in infants’ insulin responses to a glucose infusion (King et al. 1989). Others showed higher insulin concentrations in infants from diabetic mothers (Pildes et al. 1969, Obenshain et al. 1970, Pribylova & Kozlova 1979), similar to the findings from our mPHG group, which had increased islet insulin content. Another study that directly measured insulin responsiveness to an i.v. glucose infusion in 2-h old infants of normal and diabetic mothers found that infants of diabetic mothers had a higher first phase but lower second phase insulin response (Isles et al. 1968), indicating β-cell dysfunction. Regardless, children of diabetic pregnancies exhibit an increased incidence of glucose intolerance (Pettitt et al. 1985, Silverman et al. 1995), and by the time these offspring reach their early twenties, they have compromised acute or early insulin secretion compared to control subjects (Gautier et al. 2001, 2007).
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Sobngwi et al. 2003). Together, these data support the notion that insulin secretion is programmed in utero, and our findings suggest that there is a narrow range of hyperglycemia within which either insulin secretion or production can be impaired. It should also be noted that basal glucose concentrations were not significantly different between the mPHG and PHG treatments in our study, but the pulsatile excursions were greater in the PHG treatment. Thus, differences in outcomes between the mPHG and PHG fetuses appear to be driven by the pulsatile excursions rather than chronic sustained hyperglycemia, an observation that has also been made in human studies (Jovanovic-Peterson et al. 1991, Combs et al. 1992, de Veciana et al. 1995, Most & Langer 2007).

Other studies in pregnant sheep have evaluated effects of hyperglycemia on fetal insulin secretion. Carver et al. (1996) tested 10 days of chronic sustained hyperglycemia (+35%) and a pulsatile treatment (+17% sustained, +60% pulses 3X/day) similar to our mPHG group. They found that the magnitude and pattern of hyperglycemia affected β-cell function differently, because insulin secretion was suppressed with chronic constant hyperglycemia and enhanced in the PHG treatment (Carver et al. 1995, 1996). In our study, insulin secretion was suppressed after 14 days of PHG treatment (+20% sustained, +100% pulses 3X/day) but not mPHG treatment. We do not believe that this difference was due to duration of treatment, at least for the PHG fetuses, because comparison of the insulin: glucose ratio during one of the boluses on day 9 to the hyperglycemic clamp on day 14, which reached similar glucose concentrations, showed no difference between days 9 (0.32 ± 0.03) and 14 (0.31 ± 0.03). This indicates that the lower insulin secretion responsiveness in PHG fetuses had occurred by 9 days. Therefore, PHG might exceed a threshold of severity of hyperglycemia, resulting in lower insulin secretion, as found in fetuses with 35% chronic sustained hyperglycemia (Carver et al. 1996). We found greater insulin content in islets isolated from mPHG fetuses compared to control fetuses, while PHG fetuses had less islet insulin content (Fig. 5). These data also reflect a β-cell response that is dependent on the magnitude of hyperglycemic exposure. In the milder PHG treatments by Carver et al., duration could still be a factor and the increased insulin production or β-cell mass, which appears to occur in mPHG islets, could occur initially in PHG islets. However, with continued hyperglycemic exposure, the β-cells become exhausted and insulin content decreases.

Rodent models of diabetic pregnancies generated by streptozotocin injections or direct glucose infusions have outcomes in offspring that are also dependent upon the severity of hyperglycemia. Severe hyperglycemia (usually 3-4X normal) in rat fetuses leads to β-cell degranulation and exhaustion, hypoinsulinemia, and impaired insulin secretion (Aerts & van Assche 1977, Kervran et al. 1978). Postnatally, offspring have hypertrophy of the endocrine pancreas with an excess of small islets (Aerts et al. 1997) and are hypoglycemic and insulin resistant (Aerts & Van Assche 1981, Holumans et al. 1991). In contrast, rat fetuses exposed to moderate hyperglycemia (1-3-2X normal) have β-cell hyperplasia, increased insulin synthesis, and enhanced GSIS (Kervran et al. 1978, Aerts et al. 1997). However, withdrawal of

Figure 6 Greater glucose-stimulated ROS accumulation in PHG islets. In isolated fetal sheep islets, the fluorescence intensities from oxidation of the CM-H2DCFDA probe were determined in 1.1 mmol/l glucose, 11.1 mmol/l glucose, and 9 mmol/l H2O2 every minute for 15 min in each condition. Rates of ROS accumulation in stimulatory glucose and H2O2 were normalized to the basal rate (1.1 mmol/l glucose) for each islet, and the bars represent the mean ± S.E.M. for two to five islets from each of eight fetuses. The asterisk indicates a significant difference (P<0.05) between control and PHG treatments in ROS accumulation at 11.1 mmol/l glucose.

Figure 7 Islet gene expression for antioxidant enzymes, insulin, and ER stress. The mRNA expression normalized to ribosomal protein s15 is shown for enzymatic antioxidant defense (SOD-1, SOD-2, catalase, and GPx-1); insulin transcription (insulin, PDX-1, and MafA); and ER stress (GRP78 and DDIT3) in islets isolated from control and PHG fetuses. Values shown are calculated by the comparative ΔΔCt method (Ct gene of interest × Ct reference gene). Gene expression was not different between control and PHG islets.
hyperglycemia after birth results in impaired GSIS with normal pancreatic endocrine mass and cell distribution at weaning (Bihoreau et al. 1986, Aerts et al. 1990, 1997, Gauguier et al. 1991, Van Asche et al. 2001, Boloker et al. 2002, Han et al. 2007). The work in rodent models further highlights differential outcomes resulting from moderate or severe hyperglycemia, similar to those found in our mPHG and PHG treatments, and it exemplifies the need for fetal evaluation of relevant hyperglycemic paradigms.

PHG islets demonstrated increased glucose-stimulated ROS accumulation compared to control or mPHG islets. Antioxidant enzyme expression was not different between control and PHG islets, and it exemplifies the need for fetal evaluation of relevant hyperglycemic paradigms.

A direct link between decreased islet insulin content and increased islet ROS accumulation is not apparent. No differences in oxidative damage to islet proteins or UCP2 expression (Brand et al. 2010) were detected between control and PHG groups. However, we did find an increase in carbonyl incorporation in PHG skeletal muscle, which indicates higher systemic oxidative stress. Hyperglycemia and oxidative stress have been associated with vascular dysfunction in islets and other cell types (Koukkou et al. 1998, Teixeira & Andrade 1999, Homo-Delarche et al. 2006, Segar et al. 2009), and defects in islet vasculature have been known to decrease insulin secretion (Eberhard et al. 2010, Richards et al. 2010). In the GK/Par spontaneous type 2 diabetes model, there was a threefold increase in GPx-1 expression. Thus, culture may be masking treatment effects on gene expression, but ROS accumulation was greater in PHG than control islets even after overnight culture and the presumed increased expression of antioxidant enzymes.

Figure 8 Antioxidant enzyme and insulin receptor concentrations in liver and skeletal muscle. Representative western immunoblots show protein concentrations of SOD-1, SOD-2, GPx-1/2, and the reference protein ribosomal protein S6, in (A and B) skeletal muscle and (C and D) liver and of insulin receptor β and the reference protein β-tubulin in (E and F) skeletal muscle (SM) and liver (E and F). Bar graphs show quantification of band density by analysis in ImageJ. Antioxidant enzyme and insulin receptor β protein concentrations were not different between control and PHG treatments. In panel E, C refers to control.
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diabetes rat model, oxidative stress markers are concentrated in the peri-islet vascular and inflammatory compartments (Lacraz et al. 2009). Defects in islet vasculature might explain the impairment of GSIS found in the fetus but not in isolated islets, as the isolation procedure would eliminate hemodynamic effects and might also prevent the detection of oxidative damage to peri-islet vasculature.

Although a direct mechanism linking ROS accumulation to impairment of insulin secretion in vivo has not been identified, increased ROS accumulation could represent an islet defect, which, if persistent, will cause oxidative damage and contribute to islet dysfunction in adulthood (Kaneto et al. 1999, Tanaka et al. 1999, Tang et al. 2007). In the GK/Par rat model, islets have greater ROS accumulation prior to the spontaneous onset of diabetes; after diabetes is evident, protective antioxidant enzymes are upregulated and oxidative damage is found in the pancreas (Lacraz et al. 2009). Chronic oxidative stress undermines the signaling potential of ROS and thus blunts insulin secretion function (Robertson & Harmon 2006, Pi et al. 2007). Therefore, we predict that the observed increase in glucose-stimulated ROS accumulation in PHG islets foreshadows overt oxidative stress and the deterioration of islet insulin secretion.

The dextrose infusion rate increased during the 2-week PHG treatment in order to maintain chronic hyperglycemia in the pregnant ewe (Fig. 2), indicating improved maternal glucose disposal. If the PHG fetuses also have improved insulin sensitivity, they would have greater insulin clearance and glucose disposal. If the PHG fetuses also have improved insulin clearance or insulin sensitivity due to an upregulation of IRs (Flier et al. 1982, Mittelman et al. 2000). Although insulin clearance or insulin sensitivity was not directly measured in the current study, multiple lines of evidence indicate that insulin sensitivity is not a major factor for reduced insulin concentrations in PHG fetuses. No differences were found in insulin to glucose ratios during basal treatment conditions (Fig. 3E). Insulin clearance following the arginine bolus was not different between treatments, though it tended (P=0.075) to be lower in PHG (−0.65 ± 0.01 ng/ml) than control (−0.107 ± 0.02 ng/ml) or mPHG (−0.108 ± 0.02) fetuses. Finally, in liver and skeletal muscle, IR concentrations were not different between control and PHG fetuses (Fig. 8). These findings indicate that lower plasma insulin concentrations following the GSIS or GPAIS challenge were due to decreased insulin secretion and not increased clearance, which is also supported by isolated islet experiments (Fig. 5).

In the current study, fetal insulin secretion was impaired following a 2-week regimen of PHG during late gestation. The PHG treatment was designed to replicate the magnitude and pattern of glycemic control found in pregnant women with diabetes managed under the current clinical guidelines. However, many women do not attain this degree of glycemic control during their pregnancies, and in these cases of fetal exposure to more severe hyperglycemia, the outcomes are likely worse. Islet ROS accumulation appears to occur independently of the impairment in insulin secretion, but if this defect persists, we expect it to cause further damage to islet function. Because of the clinical relevance of the PHG treatment regimen and the resulting diabetic phenotype in fetal offspring, additional work with this model will be useful in investigating the mechanisms for the association of fetal exposure to hyperglycemia and diabetes risk later in life.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

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