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Islet Adaptations in Fetal Sheep Persist Following Chronic Exposure to High Norepinephrine

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Abstract

Complications in pregnancy elevate fetal norepinephrine (NE) concentrations. Previous studies in NE-infused sheep fetuses revealed that sustained exposure to high NE resulted in lower expression of α_2-adrenergic receptors in islets and increased insulin secretion responsiveness after acutely terminating the NE infusion. In this study, we determined if the compensatory increase in insulin secretion following chronic elevation of NE is independent of hyperglycemia in sheep fetuses and whether it is persistent in conjunction with islet desensitization to NE. Following an initial assessment of glucose-stimulated insulin secretion (GSIS) at 129±1 days of gestation, fetuses were continuously infused for seven days with NE and maintained at euglycemia with a maternal insulin infusion. Fetal GSIS studies were again performed on days 8 and 12. Adrenergic sensitivity was determined in pancreatic islets collected at day 12. NE infusion increased (P<0.01) fetal plasma NE concentrations and lowered (P<0.01) basal insulin concentrations compared to vehicle-infused controls. GSIS was 1.8-fold greater (P<0.05) in NE-infused fetuses compared to controls at both one and five days after discontinuing the infusion. Glucose-potentiated arginine-induced insulin secretion was also enhanced (P<0.01) in NE-infused fetuses. Maximum GSIS in islets isolated from NE-infused fetuses was 1.6-fold greater (P<0.05) than controls, but islet insulin content and intracellular calcium signaling were not different between treatments. The half-maximal inhibitory concentration for NE was 2.6-fold greater (P<0.05) in NE-infused islets compared to controls. These findings show that chronic NE exposure and not hyperglycemia produce persistent adaptations in pancreatic islets that augment β-cell responsiveness in part through decreased adrenergic sensitivity.

Keywords

β-cell; adrenergic receptor; islets of Langerhans; catecholamine; fetal stress

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Declaration of Interest

The authors have no conflict of interest.

Introduction

Fetal stress imposed by complications such as ischemic placental disease leads to prolonged increases in fetal circulating catecholamines (Okamura *et al.* 1990; Greenough *et al.* 1990; Ananth & Vintzileos 2006; Ananth & Friedman 2014). Elevated epinephrine and norepinephrine (NE) concentrations in the fetus are primary determinants for adaptive changes in metabolism and reduced growth rates (Apatu & Barnes 1991; Milley 1997; Bassett & Hanson 1998; Davis *et al.* 2015). Catecholamines suppress insulin release via α_2-adrenergic receptors (α_2-ARs) on pancreatic β-cells (Sperling *et al.* 1984; Jackson *et al.* 2000; Yates *et al.* 2012a). In an ovine model of placental insufficiency-induced intrauterine growth restriction (PI-IUGR), glucose-stimulated insulin secretion (GSIS) was attenuated, in part, by elevated catecholamine concentrations (Limesand *et al.* 2006; Macko *et al.* 2016). Pharmacological adrenergic blockade in IUGR sheep fetuses ameliorated the deficiency in GSIS, even though β-cell mass was substantially lower (Leos *et al.* 2010; Macko *et al.* 2013; Limesand *et al.* 2013). To define the islet response to chronic adrenergic stimulation during gestation, we infused NE into normal sheep fetuses for seven days and showed that continuous exposure to high NE concentrations suppressed fetal insulin secretion and raised fetal glucose concentrations. Moreover, three hours after terminating the chronic NE infusion, we observed an enhancement in insulin secretion responsiveness to glucose and arginine challenges (Chen *et al.* 2014). Expression levels of α_2-AR and $G\alpha_{i-2}$ protein were also reduced in islets from this cohort of NE fetuses (Chen *et al.* 2014). Our findings from these two distinct models of chronic hypercatecholaminemia, PI-IUGR and NE infusion, indicate that sustained adrenergic stimulation produces a compensatory augmentation in β-cell responsiveness. Therefore, we propose that NE-induced adaptations in fetal islets may produce a discord between insulin secretion and insulin action during the transition from fetal to postnatal life, and predispose offspring to metabolic complications.

In this study we test the hypothesis that the compensatory increase in insulin secretion responsiveness and adrenergic receptor desensitization in islets persist in sheep fetuses after a period of chronic hypercatecholaminemia and are not dependent upon fetal hyperglycemia. In our previous work, glucose concentrations were incongruent between IUGR and NE-infused fetuses and both hypo- and hyperglycemia negatively affect β-cell responsiveness (Carver *et al.* 1995; Green *et al.* 2012; Chen *et al.* 2014; Macko *et al.* 2016). To avoid confounding results from glucose, we maintained euglycemic conditions in the fetus during the NE infusion with a simultaneous maternal infusion of insulin. We show that the compensatory increase in insulin secretion responsiveness to glucose and arginine stimulation was present five days after discontinuing the NE treatment. Furthermore, isolated islets from the NE fetuses exhibited greater GSIS and NE desensitization.

Materials and Methods

Animal preparation

Studies were conducted in 15 pregnant Columbia-Rambouillet crossbred ewes carrying a singleton fetus (Nebeker Ranch, Lancaster, CA). Ewes were fed Standard-Bread Alfalfa Pellets (Sacate Pellet Mills, AZ) and provided water and salt *ad libitum*. Indwelling vascular catheters were surgically placed into ewes and fetuses for arterial blood sampling and

intravenous infusions at 123 ± 1 days gestational age (dGA). Animal husbandry was performed as previously reported (Chen *et al.* 2014; Macko *et al.* 2016). All animal procedures were approved by the Institutional Animal Care and Use Committee and performed at the University of Arizona, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Experimental treatments

Sheep fetuses were randomly assigned to control (n = 7) or NE (n = 8) groups at 129 ± 1 dGA. Control fetuses received an infusion of diluent (0.3% ascorbic acid in 0.9% saline), and ewes received an infusion of diluent (0.9% saline with 0.5% BSA). NE fetuses received an infusion of NE (Norepinephrine Bitartrate, Bedford Laboratories, OH; 1–4 μg/min; IV) for 7 days at preset rates for each day of treatment (day 1 at 1 μg/min, days 2–4 at 2 μg/min, and days 5–7 at 4μg/min; (Chen *et al.* 2014)). Insulin (Humulin R; Eli Lilly, Indianapolis, IN; 30–60 pmol/min/kg) was intravenously infused into ewes to maintain euglycemic conditions in fetuses during NE infusion (Limesand & Hay, Jr. 2003; Rozance *et al.* 2006). The rates of NE infusion were previously shown to recapitulate physiological concentrations observed in response to fetal hypoxemia in human and sheep (Greenough *et al.* 1990; Yates *et al.* 2012a; Chen *et al.* 2014). All infusions were terminated on day 7 of treatment, 24 hours prior to the second GSIS study.

Experimental design and insulin secretion studies

Three GSIS studies were performed before (GSIS-pre), 1 day after (GSIS-1d), and 5 days after (GSIS-5d) the chronic infusion in all but one control fetus, where the final GSIS study was lost due to loss of catheter patency. The GSIS studies were conducted as described previously (Limesand *et al.* 2006; Leos *et al.* 2010). Briefly, a continuous transfusion of maternal arterial blood into the fetus (10 mL/h) was started 30 min prior to sampling and maintained for the duration of the study to compensate for blood collection. Three baseline blood samples were collected within the 20 minutes preceding the dextrose bolus at 5–7 minute intervals. The square-wave hyperglycemic clamp was initiated with a dextrose bolus of 1.19 ± 0.04 mmol/kg estimated fetal weight followed by a constant infusion of 33% dextrose to increase and maintain fetal arterial plasma glucose concentration at 2.5 ± 0.1mmol/L, which produces a near-maximal GSIS response (Green *et al.* 2011). During the hyperglycemic clamp, three fetal arterial plasma samples were collected at 45, 50, and 55 min during steady state conditions when concentrations varied less than $\pm 6\%$ of the mean and showed no systematic trend with time. Averages for baseline and hyperglycemia steady state periods were calculated for comparisons. Maximal insulin secretion responsiveness was measured with a follow-on arginine bolus (0.5 mmol/kg estimated fetal weight) as described previously (Gresores *et al.* 1997; Green *et al.* 2012).

Biochemical analysis

Fetal arterial blood samples were collected and prepared for analysis as described previously (Chen *et al.* 2014). Fetal blood samples were collected for blood gasses, pH, and oximetry measurements with an ABL 725, (temperature-corrected to 39.1°C; Radiometer, Copenhagen, Denmark). Plasma glucose and lactate concentrations were measured immediately with a YSI model 2700 SELECT Biochemistry Analyzer (Yellow Springs

Instruments, Yellow Springs, OH). Plasma samples for insulin and NE measurements were stored at −80°C. Insulin concentrations were measured with an ovine insulin ELISA (ALPCO Diagnostics, Windham, NH) that had a 9.1% inter-assay and 8.5% intra-assay coefficients of variation. NE concentrations were measured by Noradrenaline ELISA (Labor Diagnostika Nord GmbH & Co.KG, Nordhorn, Germany) that had a 22.5% inter-assay and 19.6% intra-assay coefficients of variation.

Necropsy and islet isolation

After GSIS-5d, animals were recovered for 3–5 hours before ewes and fetuses were euthanized with an overdose of sodium pentobarbital (86 mg/kg) and phenytoin sodium (11 mg/kg, Euthasol; Virbac Animal Health, Fort Worth, TX). Fetuses and fetal organs were dissected and weighed. The hepatic portion of pancreas was collected for islet isolation (Limesand *et al.* 2006). The splenic portion of pancreas was fixed in 4% paraformaldehyde overnight and prepared for histological analysis as described previously (Cole *et al.* 2009).

Islets were isolated after the fetal pancreas was digested in Krebs-Ringer Buffer (KRB) with Collagenase V (0.425 mg/mL; Sigma) and DNase I (2 μU/mL; Roche Diagnostics, Mannheim, Germany) as described previously (Limesand *et al.* 2006; Chen *et al.* 2014; Rozance *et al.* 2015). Isolated islets were washed in KRB containing 0.5% BSA (KRB-BSA). Islets were cultured overnight in RPMI-1640 medium (Sigma-Aldrich) supplemented with 2% fetal bovine serum, 2.8 mmol/L glucose, and penicillin–streptomycin (50 U and 50 mg; Sigma-Aldrich) at 37°C with 95% O_2-5% CO_2 (Limesand *et al.* 2006; Green *et al.* 2012).

Islet static incubations

Fetal islets from control (n = 6) and NE fetuses (n = 6) were washed twice in KRB-BSA and a third time in KRB-BSA supplemented with 10 μmol/L forskolin (Sigma-Aldrich). All media was equilibrated to 37°C and gassed with 95% O_2-5% CO_2. Ten islets were handpicked (3–4 replicates/condition for each fetus) and incubated at 37°C in 1 mL KRB-BSA-forskolin media with high (11.1 mmol/L) and no (0 mmol/L) glucose, high glucose plus NE (0.1 nmol/L – 10 μmol/L), and 1.1 mmo/L glucose plus 30 mmol/L KCl. After a 1-hour static incubation, islets were pelleted by centrifugation (800 x g) at 4°C for 3 min. The media was removed and insulin was extracted from islets with acid-ethanol (1 M HCl in 70% ethanol) to determine the islet insulin content. The effectiveness of NE in inhibiting insulin secretion was determined by the half-maximal inhibitory concentration (IC_{50}) using the dose response equation (log (inhibitor) vs. normalized response; Prism 6, GraphPad Software, La Jolla, CA).

Whole-islet calcium dynamics

Islets were incubated overnight on glass coverslips pre-coated with human fibronectin (10 mg/L in PBS; BD Biosciences, Bedford, MA, USA; (Green *et al.* 2011)). Islets from control and NE fetuses (n = 3/treatment) were washed at 4°C for 10 min with glucose-free Hank's Balanced Salt Solution (HBSS; 5 mmol/L KCl, 0.3mmol/L KH_2PO_4, 138 mmol/L NaCl, 0.2mmol/L $NaHCO_3$, 0.3mmol/L Na_2HPO_4, 20 mmol/L HEPES, 1.3 mmol/L $CaCl_2$ and 0.4mmol/L $MgSO_4$, pH 7.4) and then loaded with fura-2 acetoxymethylester (2.5 μmol/L;

Molecular Probes) in glucose-free HBSS with 0.0025% pluronic acid for 20 min at 4°C and 20 min at 25°C. The islets were rinsed, incubated in HBSS with 1.1 mmol/L glucose for 10 min at 37°C, and mounted onto the stage of an Olympus IX-70 microscope. Fura-2 was alternately excited at 340 and 380 nm and emitted light (508 nm) captured on a Photometrics CH-250 camera as described previously (Lynch *et al.* 2008). Images were acquired at 0.5–1 minute intervals in the following series of media conditions: low glucose (1.1 mmol/L) for 5 min; high glucose (11.1 mmol/L) for 20 min; 30 mmol/L KCl for 5 min; and 10 µmol/L ionomycin for 4 min. Data were analyzed for 25–67 fields of view obtained from 2–6 islets per fetus (coefficient of variation between islets = 6.1%).

Histology of fetal pancreatic endocrine cells

Pancreatic tissue sections (6 µm) were cut from frozen OCT-embedded control (n = 7) and NE (n = 6) fetuses and prepared for histological evaluation as described previously (Cole *et al.* 2009; Leos *et al.* 2010). Endocrine cells were identified with guinea pig anti-porcine insulin (1:500; Dako, Carpinteria, CA), mouse anti-porcine glucagon (1:500; Sigma-Aldrich), rabbit anti-human somatostatin (1:500; Dako), and rabbit anti-human pancreatic polypeptide (1:500; Dako). Immunocomplexes were detected with affinity-purified secondary antiserum conjugated to Cy2 (rabbit), Texas Red (mouse), or 7-amino-4-methylcoumarin-3-acetic acid (AMCA, guinea pig; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:500 in PBS with 1% BSA. Fluorescent staining was visualized on a Leica DM5500 microscope system and digitally captured with a Spot Pursuit 4 Megapixel CCD camera (Diagnostic Instruments, Sterling Heights, MI). Morphometric analysis was performed with ImagePro 6.0 software (Media Cybernetics, Silver Spring, MD). Positive areas were determined from 25 fields of view (FOV = 0.39 mm^2) on two pancreas sections per animal separated by 120µm intervals (total area = 19.5 mm^2; coefficient of variation between sections = 10.7%).

Statistical analysis

Differences between treatments (control and NE) for animal weights, daily intake, islet IC$_{50}$, and pancreas morphology were analyzed by one-way ANOVA (treatments) using the general linear means procedure (SAS 9.4, SAS Institute Inc., Cary, NC) and differences were determined with Student's t test. Repeated measurement for biochemical, hematological, and hormone from daily samples, GSIS studies, GPAIS studies, and *ex vivo* islet experiments were analyzed by ANOVA using Proc MIXED with fetus as the random effect (SAS 9.4). Main effects within GSIS studies were treatment, period, and their interaction. Main effects for GPAIS within treatment were study, sample time, and their interaction. When the overall ANOVA was significant ($α = 0.05$), individual means were separated with a least significant difference test. Data are presented as the mean ± standard error mean.

Results

Animal weights and daily intake

Maternal body weights were not different between control (56.8 ± 3.9 kg) and NE ewes (52.8 ± 3.3 kg). During the treatment, average daily feed and water intake were similar in control (1.8 ± 0.1 kg and 7.5 ± 0.9 L) and NE ewes (1.9 ± 0.1 kg and 7.2 ± 0.6 L). Fetal age

at necropsy was similar in control (140 ± 1 dGA, 3 male and 4 female) and NE fetuses (141 ± 1 dGA, 7 male and 1 female). Fetal weight was not different between control (3.96 ± 0.23 kg) and NE fetuses (4.02 ± 0.20 kg).

Daily fetal blood parameters

Prior to chronic infusions, fetal plasma glucose, insulin, NE, and blood PaO_2 were not different between experimental groups (Figure 1). During the 7-day infusion, average NE concentrations were higher (9.26 ± 0.87 ng/mL vs. 0.88 ± 0.11 ng/mL, $P < 0.01$), insulin concentrations were lower in NE-fetuses compared to controls (0.12 ± 0.01 µg/L vs. 0.43 ± 0.02 µg/L, $P < 0.01$), and glucose concentrations were not different. NE fetuses also had higher blood PaO_2 than controls (27.3 ± 0.5 mmHg vs. 20.8 ± 0.3 mmHg, $P < 0.01$). There was no treatment by day interaction for NE, glucose, insulin, or PaO_2 during this period.

During the 5 days post-infusion, average plasma NE concentrations (2.46 ± 0.41 ng/mL vs. 0.61 ± 0.07 ng/mL; $P < 0.01$) and blood PaO_2 (23.0 ± 0.4 mmHg vs. 20.1 ± 0.4 mmHg, $P < 0.01$) remained elevated in NE fetuses compared to controls, respectively, but both measurements were lower ($P < 0.05$) after infusion than during infusion (Figure 1). Glucose and insulin concentrations were not different between treatments after the chronic infusion ended. There was no treatment by day interaction for any of these variables.

Fetal glucose-stimulated insulin secretion

By design, glucose concentrations were increased to 2.5 ± 0.1 mmol/L ($P < 0.01$) from baseline during the hyperglycemic period of all three GSIS studies (Figure 2). Baseline and hyperglycemic glucose concentrations were not different between treatments in any GSIS studies. The glucose infusion rate to maintain the hyperglycemic clamp also was not different between treatment groups (Table 1).

In the GSIS-pre study, baseline insulin concentrations at either period were not different between treatments and increased ($P < 0.001$) with hyperglycemia similarly in both treatment groups (Figure 2). Baseline insulin concentrations also were not different between NE and control fetuses in GSIS-1d or GSIS-5d studies, but insulin concentrations during the hyperglycemic periods were greater ($P < 0.05$) in NE fetuses than controls in both studies. Insulin secretion responsiveness (difference between baseline and hyperglycemic insulin concentrations) was not different among three GSIS studies in control fetuses. In NE fetuses, however, insulin secretion responsiveness was greater ($P < 0.01$) in GSIS-1d (1.46 ± 0.36 µg/L) and GSIS-5d (1.77 ± 0.44 µg/L) compared to GSIS-pre (0.66 ± 0.12 µg/L).

Blood gas, pH, hematocrit, plasma lactate, and plasma NE were not different between treatments in GSIS-pre, although differences in period means were found; there was no treatment by period interaction (Table 1). In GSIS-1d, NE fetuses had greater PaO_2 (25.4 ± 0.5 mmHg vs. 20.0 ± 0.3 mmHg, $P < 0.01$), blood oxygen content (4.0 ± 0.1 mmol/L vs. 3.1 ± 0.1 mmol/L, $P < 0.05$) and NE concentrations (3.33 ± 0.42 ng/mL vs. 0.61 ± 0.05 ng/mL, $P < 0.01$) than controls, respectively. The blood $PaCO_2$ values were lower in NE fetuses than controls (46.7 ± 0.5 mmHg vs. 51.0 ± 0.3 mmHg, $P < 0.01$), while pH, hematocrit, and lactate were not different. In GSIS-5d NE remained greater than controls (1.54 ± 0.14 ng/mL vs. 0.62 ± 0.07 ng/mL, $P < 0.05$; Table 1).

Fetal glucose-potentiated arginine-induced insulin secretion

GPAIS was enhanced in NE fetuses following infusion but controls were unaffected (Figure 3). Average GPAIS insulin concentrations in NE fetuses were greater ($P < 0.01$) in GPAIS-1d (4.02 ± 0.39 μg/L) and GPAIS-5d (3.64 ± 0.35 μg/L) compared to GPIAS-pre (2.66 ± 0.19 μg/L), which was similar to all three control GPAIS studies.

Islet responsiveness

Insulin secretion rates from isolated fetal sheep islets were measured in the presence of 11.1 mmol/L glucose and various NE concentrations (Figure 4A). Maximum glucose-stimulated insulin secretion was greater in NE islets than control islets (2.20 ± 0.27 ng/islet/h vs. 1.38 ± 0.24 ng/islet/h, $P < 0.05$). Minimum insulin secretion rates with NE inhibition were not different between NE (0.74 ± 0.18 ng/islet/h) and control islets (0.68 ± 0.15 ng/islet/h), and these rates were also not different from incubations with no glucose. In islet incubations with 30 mmol/L KCl, insulin secretion rates were greater ($P < 0.05$) than other conditions but not different between treatments (NE, 8.84 ± 1.47 ng/islet/h vs. control, 5.66 ± 1.91 ng/islet/h). The IC_{50} concentration was greater ($P < 0.05$) for NE than control islets (Figure 4B). Insulin contents of isolated islets were not different between NE and control fetuses (Figure 4C).

Cytosolic calcium concentrations at 1.1 mmol/L glucose were not different between NE and control islets (340/380 ratio of 0.66 ± 0.01 in NE and 0.67 ± 0.06 in controls). High glucose concentrations and 30 mmol/L KCl increased intracellular calcium, but means were not different between treatments (0.71 ± 0.01 in NE vs. 0.73 ± 0.07 in control for high glucose, and 1.24 ± 0.08 in NE vs. 1.46 ± 0.20 in control for KCl).

Pancreatic endocrine cell area

There were no differences in the total area staining positive for insulin (β-cells), glucagon (α-cells), or the combination of somatostatin (δ-cells) and pancreatic polypeptide cells (F-cells) between treatments (Table 2).

Discussion

Fetal stress associated with placental dysfunction chronically elevates catecholamines (Okamura *et al.* 1990; Greenough *et al.* 1990). In this study, we demonstrate that sustained exposure to elevated NE directly produces lasting adaptations in fetal β-cells. The compensatory increase in insulin secretion responsiveness to glucose and arginine was still observed five days after cessation of the NE infusion. Furthermore, we confirmed enhanced insulin secretion to glucose was intrinsic to the islets following isolation. Although the increased β-cell responsiveness to glucose and arginine was not explained by differences in β-cell mass, insulin content, or intracellular calcium signaling, the β-cells remained desensitized to adrenergic stimulation, which supports previous findings that show lower expression of adrenergic receptors and signaling molecules (Chen *et al.* 2014). Previously, IUGR fetuses with elevated catecholamines exhibited improved β-cell responsiveness during adrenergic blockade, indicating adaptive programming effects from chronic adrenergic stimulation are present (Leos *et al.* 2010; Macko *et al.* 2013). In these previous studies, we

found the compensatory increase in insulin secretion responsiveness immediately after reducing the adrenergic stimulation, whereas here we show the enhancement was maintained for five days following removal of elevated adrenergic stimulation indicating that the effects were programmed.

Enhanced insulin secretion responsiveness to secretagogues and adrenergic desensitization represent two functional adaptations in the β-cell that persisted well beyond the end of NE infusion. These findings expand our previous work where we observed a compensatory increase in insulin secretion three hours after terminating the NE infusion and also showed lower expression of α_2-ARs and $G_{\alpha i-2}$ protein in fetal sheep islets. It is likely that these two effects are interrelated because recent genetic studies reveal that overexpression of α_{2A}-AR causes impaired docking and exocytosis of insulin granules (Rosengren *et al.* 2010; Rosengren *et al.* 2012; Straub & Sharp 2012). However, this tonic inhibition of insulin release was alleviated with α_2-AR antagonists (Rosengren *et al.* 2010; Yang *et al.* 2012). Conversely, α_{2A}-AR deficient mice not only lack inhibitory actions in pancreatic β-cell but also are hyperinsulinemic and hypoglycemic (Fagerholm *et al.* 2004; Savontaus *et al.* 2008). The α_2-ARs desensitization in pancreatic β-cells found in the present study may, in part, explain the compensatory increase in β-cell responsiveness following cessation of hypercatecholaminemia because of the loss of tonic inhibition by adrenergic receptors.

Alternative mechanisms for enhanced GSIS and GPAIS responsiveness in the NE fetus may include increased metabolic coupling to oxidative phosphorylation or regulation of secretory granule exocytosis. We have previously shown that uncoupling protein 2 (UCP2) mRNA expression is reduced in NE islets following acute recovery overnight culture *in vivo* (Chen *et al.* 2014). In β-cells, UCP2 is a negative regulator of insulin secretion because it decreases the proton-motive force in the inner mitochondria membrane to circumvent ATP synthesis (Zhang *et al.* 2001; Affourtit & Brand 2006; Affourtit & Brand 2008). Conversely, a reduction of UCP2 as identified in islets of NE fetuses enhances insulin secretion (Zhang *et al.* 2006; Robson-Doucette *et al.* 2011). The reduction in UCP2 expression in NE islets may be a direct effect of adrenergic stimulation or an indirect effect because adrenergic stimulation decrease rates of glucose metabolism (Laychock & Bilgin 1987; Laychock 1989; Dalgaard 2012). Transcriptome expression analysis by high-throughput RNA sequencing of NE islets revealed significant increases in chloride channel, calcium activated, family member 1 (CLCA1) and FXYD domain containing ion transport regulator 2 and significant decreases in solute carrier family 9 member A2 (Na^+/H^+ Exchanger 2) and inositol 1,4,5-trisphosphate receptor type 2 in NE islets compared to controls after the acute recovery (unpublished data). Although additional work is necessary to determine the role of these ion channels in the NE islets, there is evidence that chloride flux across the β-cell membrane can modulate exocytotic capacity and alter depolarization kinetics (Barg *et al.* 1999; Barg *et al.* 2001; Thevenod 2002).

In our previous study using chronic 7-day NE infusion, we observed hyperglycemia and increased arterial blood oxygen tension in NE fetuses during the treatment. In the present study, we maintained fetal glucose concentrations at normal values to eliminate the confounding influence of chronic hyperglycemia, which was previously demonstrated to negatively influence insulin secretion (Carver *et al.* 1995; Green *et al.* 2012). Here, we

confirm that the NE-induced compensation in fetal sheep islets is independent of the fetal glucose concentrations. Moreover, when we compare the NE fetal cohorts and the PI-IUGR fetal sheep cohort that is hypoglycemic (Leos *et al.* 2010; Macko *et al.* 2013), we can exclude an influence of fetal glucose concentrations for the compensatory enhancement in β-cell function following chronic NE exposure because it is present in fetuses with hypo-, hyper-, and euglycemic conditions. Similarly, oxygen tension is not expected to affect these results because they persist in models with different fetal oxygenation. Acutely, in normal sheep fetuses, higher blood PaO_2 did not augment GSIS (Jackson *et al.* 2000), and when placental insufficiency-induced IUGR fetuses were oxygenated to normal levels from hypoxemia, insulin secretion was enhanced independently of norepinephrine concentrations (Macko *et al.* 2016).

Plasma NE concentrations of NE fetuses remained 5.3-fold and 2.5-fold higher than controls at one and five days after terminating NE infusion, respectively. Chronic NE exposure or physiological hypoxic challenges in fetal sheep and lambs inhibit catecholamine clearance rates in the placenta and lung, which partially explain the prolonged elevation (Chappell *et al.* 1991; Bzoskie *et al.* 1995; Bzoskie *et al.* 1997). Also, if α_2-ARs on the adrenal chromaffin cells were desensitized increased rates of NE synthesis would be expected, because the adrenal glands possess short feedback inhibition similar to sympathetic neurons (Brede *et al.* 2003; Moura *et al.* 2006). Despite the elevation of NE, NE fetuses still had significantly higher β-cell responsiveness, indicating that the new levels of catecholamines are below effective concentrations to inhibit insulin secretion.

Persistence in enhanced insulin secretion responsiveness resulting from chronically elevated NE *in utero* begins to explain the common occurrence for transient hyperinsulinemic hypoglycemia seen in infants born small for gestational age or with perinatal stress (Collins *et al.* 1990; Duvanel *et al.* 1999; Stanley *et al.* 2015). The baseline insulin and glucose concentrations in NE fetuses are equivalent to controls, but glucose concentrations will increase after delivery. If the increased β-cell sensitivity continues postnatally, it is anticipated that hyperinsulinemia will pursue and lower neonatal glucose concentrations. Hyperinsulinemia and hypoglycemia will be exacerbated by adrenergic receptor desensitization because counter regulatory mechanisms for glucose homeostasis will be reduced (Hawdon *et al.* 1993; Rozance & Hay, Jr. 2010; Chen *et al.* 2010). Although management of asymptomatic hypoglycemia is controversial, reoccurring episodes are associated with poor neurodevelopment and growth deficits (Duvanel *et al.* 1999; Rozance & Hay, Jr. 2016). Therefore, while the symptoms may resolve, there are potential long term consequences to transient hyperinsulinemic hypoglycemia in neonate that may result from chronically elevated NE.

In conclusion, chronic elevation of NE concentration, not hyperglycemia, produces a compensatory enhancement in β-cell responsiveness to insulin secretagogues after terminating the NE infusion in fetal sheep. Improved insulin secretion was intrinsic to pancreatic islets, which maintained α_2-AR desensitization six days after termination of NE infusion, which may contribute to the manifestation of persistent adaptation in β-cells. Although previous studies in PI-IUGR fetuses and lambs support peripheral adrenergic receptor desensitization that impairs the ability of catecholamines to mobilize free fatty

acids (Chen *et al.* 2010; Yates *et al.* 2012b; Yates *et al.* 2016), this is the first study to describe programming effects from NE in β-cells. Importantly, the β-cell dysfunction created by NE-infusion begins to explain the potential variability in small-for-gestational age infants because sustained exposure to high catecholamine levels is found in IUGR infants. The enhanced β-cell responsiveness induced by chronic high catecholamine concentrations in the fetus will create an imbalance in the insulin disposition index that may impair the maturation of glucose homeostasis.

Acknowledgments

Abbreviations

AR	adrenergic receptor
dGA	days of gestational age
$\mathbf{[Ca^{2+}]_i}$	intracellular calcium concentration
GPAIS	glucose-potentiated arginine-induced insulin secretion
GSIS	glucose stimulated insulin secretion
$\mathbf{IC_{50}}$	half-maximal inhibitory concentration
KRB	Krebs-Ringer buffer
NE	norepinephrine
PI-IUGR	placental insufficiency-induced intrauterine growth restriction

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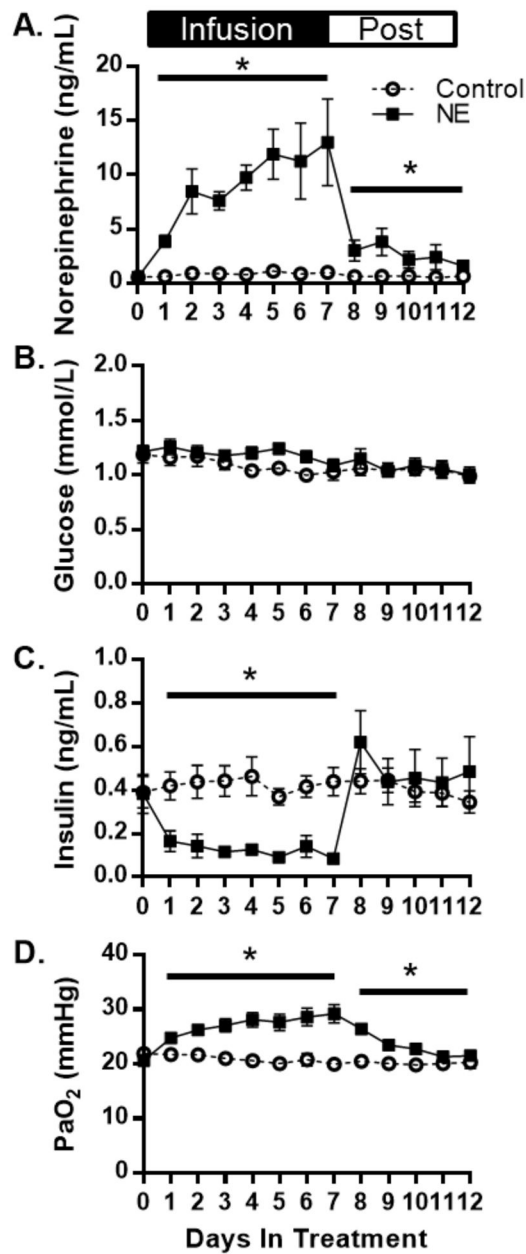
Figure 1. NE Infusion Inhibits Insulin Concentrations
Daily arterial means are presented for control (circles, n = 7) and NE fetuses (squares, n = 8). Values for plasma norepinephrine (A), plasma glucose (B), plasma insulin (C) and partial pressure of blood oxygen (PaO$_2$, D) were determined for each day of treatment (x-axis). Difference ($P < 0.05$) during the infusion and post-infusion periods are identified with an asterisk.
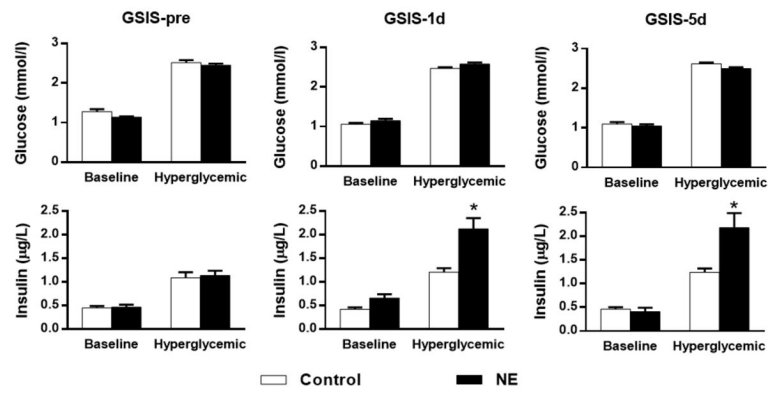
Figure 2. Persistent Enhancement of GSIS in NE fetuses
Plasma glucose and insulin concentrations are presented for the three GSIS studies, which are labeled above the graphs: before (GSIS-pre), 1 day after (GSIS-1d) and 5 days after (GSIS-5d) the chronic infusion. Baseline and hyperglycemic steady state period means for control (n = 6–7) and NE fetuses (n = 8) were analyzed within each GSIS study. Differences ($P < 0.05$) between control and NE fetuses within the period are identified (*).
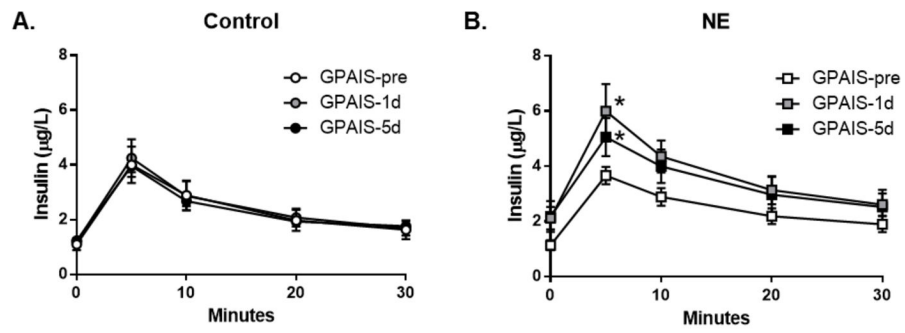
Figure 3. Persistent Enhancement of GPAIS in NE Fetuses

Insulin concentrations are presented for the GPAIS studies in control (A, n = 6–7) and NE fetuses (B, n = 8). The arginine bolus is administered at time 0. GPAIS studies were conducted before (GPAIS-pre), 1 day after (GPAIS-1d) and 5 days after (GPAIS-5d) the chronic treatment. There was no study by time interaction. Study differences (*; $P < 0.05$) for the average insulin concentration were observed in the NE fetuses but not controls.
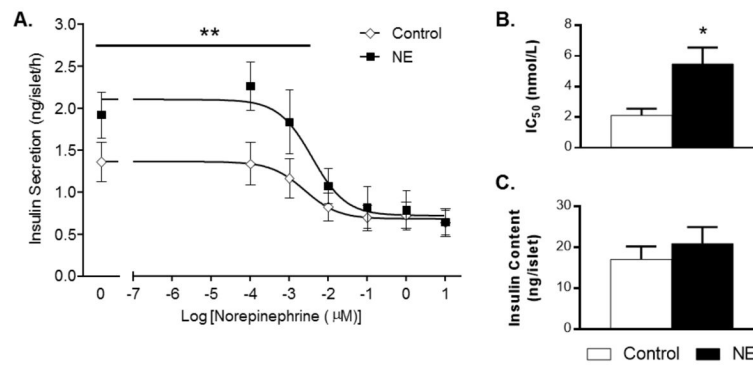
Figure 4. Enhanced Glucose Stimulated Insulin Secretion in NE Islets
Pancreatic islets were isolated from control and NE fetuses (n = 6 animals/treatment). A) Insulin secretion rates were measured in static incubations that contain 11.1 mmol/L glucose and various NE concentrations that ranged from 0–10 μmol/L (y-axis). The bar with asterisks indicate that the maximum insulin secretion rate was significantly different ($P < 0.01$) between control and NE islets. B) The half maximal inhibitory concentration (IC_{50}) was calculated for each treatment. Values represent means ± SEM, and the asterisks indicates a difference ($P < 0.05$) between treatment groups. C) Islet insulin contents were not different between treatments.

Table 1

Glucose infusion, blood gas, lactate, and norepinephrine concentrations during GSIS studies.

Study	Parameter	Period	Control (n = 7)	NE (n = 8)	P-value Treatment	P-value Period
GSIS-pre	Glucose Infusion (mmol/hr)	Hyperglycemia	11.0 ± 1.2	12.5 ± 0.7	NS	
	pH	Baseline	7.33 ± 0.01	7.34 ± 0.01	NS	
		Hyperglycemia	7.31 ± 0.01	7.32 ± 0.01		< 0.01
	PaO$_2$ (mmHg)	Baseline	21.3 ± 0.3	20.9 ± 0.6	NS	
		Hyperglycemia	20.5 ± 0.3	20.9 ± 0.5		< 0.05
	PaCO$_2$ (mmHg)	Baseline	48.8 ± 0.5	49.4 ± 0.4	NS	
		Hyperglycemia	50.3 ± 0.6	50.7 ± 0.5		< 0.01
	Oxygen Content (mmol/L)	Baseline	3.7 ± 0.1	3.7 ± 0.1	NS	
		Hyperglycemia	3.3 ± 0.1	3.5 ± 0.1		< 0.05
	Hematocrit (%)	Baseline	33.3 ± 0.6	34.6 ± 0.4	NS	
		Hyperglycemia	32.9 ± 0.6	34.3 ± 0.3		< 0.01
	Lactate (mmol/L)	Baseline	2.03 ± 0.09	1.94 ± 0.08	NS	
		Hyperglycemia	2.50 ± 0.09	2.33 ± 0.08		< 0.01
	NE (ng/mL)	Baseline	0.50 ± 0.08	0.53 ± 0.08	NS	
		Hyperglycemia	0.61 ± 0.10	0.72 ± 0.13		< 0.05
GSIS-1d	Glucose Infusion (mmol/hr)	Hyperglycemia	14.2 ± 0.9	11.8 ± 1.1	NS	
	pH	Baseline	7.32 ± 0.01	7.32 ± 0.01	NS	
		Hyperglycemia	7.30 ± 0.01	7.30 ± 0.01		< 0.01
	PaO$_2$ (mmHg)	Baseline	20.1 ± 0.5	26.0 ± 0.6	< 0.01	
		Hyperglycemia	19.9 ± 0.5	24.7 ± 0.8		< 0.05
	PaCO$_2$ (mmHg)	Baseline	50.5 ± 0.4	46.1 ± 0.6	< 0.01	
		Hyperglycemia	51.4 ± 0.5	47.2 ± 0.7		< 0.01
	Oxygen Content (mmol/L)	Baseline	3.2 ± 0.2	4.2 ± 0.1	< 0.05	
		Hyperglycemia	3.0 ± 0.1	3.8 ± 0.1		< 0.01
	Hematocrit (%)	Baseline	34.0 ± 1.0	32.3 ± 0.4	NS	
		Hyperglycemia	33.5 ± 0.8	31.8 ± 0.5		< 0.01
	Lactate (mmol/L)	Baseline	2.12 ± 0.13	1.33 ± 0.07	< 0.01	
		Hyperglycemia	2.66 ± 0.13	1.78 ± 0.09		< 0.01

Study	Parameter	Period	Control (n = 7)	NE (n = 8)	P-value Treatment	P-value Period
	NE (ng/mL)	Baseline	0.59 ± 0.06	3.11 ± 0.56	< 0.01	NS
		Hyperglycemia	0.62 ± 0.07	3.55 ± 0.65		
GSIS-5d	Glucose Infusion (mmol/hr)	Hyperglycemia	14.3 ± 1.1	13.7 ± 0.8	NS	
	pH	Baseline	7.32 ± 0.01	7.34 ± 0.01	NS	< 0.01
		Hyperglycemia	7.30 ± 0.01	7.31 ± 0.01		
	PaO$_2$ (mmHg)	Baseline	20.3 ± 0.6	20.7 ± 0.4	NS	< 0.05
		Hyperglycemia	19.3 ± 0.5	20.3 ± 0.5		
	PaCO$_2$ (mmHg)	Baseline	50.7 ± 0.5	51.4 ± 0.3	NS	< 0.01
		Hyperglycemia	51.6 ± 0.6	52.7 ± 0.4		
	Oxygen Content (mmol/L)	Baseline	2.8 ± 0.1	3.1 ± 0.1	NS	< 0.01
		Hyperglycemia	2.5 ± 0.1	2.9 ± 0.1		
	Hematocrit (%)	Baseline	31.3 ± 1.0	30.9 ± 0.6	NS	< 0.01
		Hyperglycemia	30.9 ± 0.9	30.5 ± 0.6		
	Lactate (mmol/L)	Baseline	2.37 ± 0.15	2.04 ± 0.13	NS	< 0.01
		Hyperglycemia	3.11 ± 0.20	2.66 ± 0.10		
	NE (pg/mL)	Baseline	0.56 ± 0.11	1.60 ± 0.17	< 0.05	NS
		Hyperglycemia	0.67 ± 0.09	1.50 ± 0.23		

No significant interactions between treatment and period were identified.

Values are means ± SEM.

NS, not significant.

Table 2

Endocrine pancreas morphometry.

	Control (n = 7)	NE (n = 6)
β-cell area, %	2.22±0.43	2.04±0.26
α-cell area, %	1.48±0.19	1.67±0.28
δ- and PP-cell area, %	0.97±0.10	0.98±0.17
Total Endocrine Area, %	4.66 ± 0.67	4.69 ± 0.61

PP, pancreatic polypeptide.

Values are means ± SEM.