Fetal hyperglycemia acutely induces persistent insulin resistance in skeletal muscle

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This paper is part of a thematic section on 30 Years of the Developmental Origins of Health and Disease. The guest editors for this section were Sean Limesand, Kent Thornburg and Jane Harding.

Abstract

Offspring exposed in utero to maternal diabetes exhibit long-lasting insulin resistance, though the initiating mechanisms have received minimal experimental attention. Herein, we show that rat fetuses develop insulin resistance after only 2-day continuous exposure to isolated hyperglycemia starting on gestational day 18. Hyperglycemia-induced reductions in insulin-induced AKT phosphorylation localized primarily to fetal skeletal muscle. The skeletal muscle of hyperglycemia-exposed fetuses also exhibited impaired in vivo glucose uptake. To address longer term impacts of this short hyperglycemic exposure, neonates were cross-fostered and examined at 21 days postnatal age. Offspring formerly exposed to 2 days late gestation hyperglycemia exhibited mild glucose intolerance with insulin signaling defects localized only to skeletal muscle. Fetal hyperglycemic exposure has downstream consequences which include hyperinsulinemia and relative uteroplacental insufficiency. To determine whether these accounted for induction of insulin resistance, we examined fetuses exposed to late gestational isolated hyperinsulinemia or uterine artery ligation. Importantly, 2 days of fetal hyperinsulinemia did not impair insulin signaling in murine fetal tissues and 21-day-old offspring exposed to fetal hyperinsulinemia had normal glucose tolerance. Similarly, fetal exposure to 2-day uteroplacental insufficiency did not perturb insulin-stimulated AKT phosphorylation in fetal rats. We conclude that fetal exposure to hyperglycemia acutely produces insulin resistance. As hyperinsulinemia and placental insufficiency have no such impact, this occurs likely via direct tissue effects of hyperglycemia. Furthermore, these findings show that skeletal muscle is uniquely susceptible to immediate and persistent insulin resistance induced by hyperglycemia.

Key Words
- skeletal muscle
- pregnancy
- diabetes
- developmental origins of health and disease
- insulin signaling
- glucose metabolism
- ontogeny

Introduction

Offspring born from pregnancy complicated by diabetes have a lifelong increased risk of insulin resistance. In humans, such offspring have a 4- to 8-fold higher risk, beyond genetic risk, to develop type 2 diabetes, metabolic syndrome and/or obesity (Dabelea et al. 2000, 2008, Franks et al. 2006, Clausen et al. 2008, 2009,
Fraser & Lawlor 2014). Thus, the diabetes-altered fetal environment induces a long-lasting change in offspring susceptibility to adverse metabolic outcomes. Likewise in animals, adult offspring that were exposed to maternal diabetes exhibit insulin resistance, as demonstrated in multiple models (Simmons et al. 2001a, Segar et al. 2009, Khalyfa et al. 2013, Kahraman et al. 2014, Latouche et al. 2014, Yokomizo et al. 2014, Blue et al. 2015). However, the underlying mechanisms that produce insulin resistance are not clearly understood. In fact, the immediate fetal molecular perturbations induced by maternal diabetes have received little experimental attention to date.

Diabetes during pregnancy exposes the fetus to multiple perturbations during a critical development period. These perturbations extend beyond hyperglycemia and include maternal ketosis, altered lipids and amino acids, altered placental hemodynamics, maternal inflammation and systemic oxidative stress (Wijendran et al. 1999, Aerts & Van Asche 2001, Cetin et al. 2005, Gin et al. 2006, López-Tinoco et al. 2013, Ryckman et al. 2015, Shang et al. 2015, Zhu et al. 2015, Abdul Aziz et al. 2016, Khosrow beygi et al. 2016, Saad et al. 2016). Although hyperglycemia is the defining aspect of diabetes, the role of maternal hyperglycemia itself in altering fetal metabolic programming has not been well isolated. In fact, some non-hyperglycemic factors independently modulate offspring insulin resistance risk (Mathias et al. 2014, Crume et al. 2015, Shomonov-Wagner et al. 2015, Umekawa et al. 2015). A longstanding obstacle to understanding the role of hyperglycemia has been lack of a technique to isolate glucose exposure from other concurrent systemic maternal perturbations during diabetes. The localized fetomaternal hyperglycemia rat model circumvents these confounders by selectively delivering glucose to fetuses in the left uterine horn, while using right uterine horn fetuses as internal euglycemic controls (Yao et al. 2010, Baack et al. 2014, Gordon et al. 2015). Importantly, this model both isolates the effect of maternal hyperglycemia and allows precise temporal control of hyperglycemia exposure.

Nearly all animal studies to date examining fetally programmed insulin resistance have focused on the physiologic and molecular perturbations later rather than early in life (Simmons et al. 2001a, Segar et al. 2009, Khalyfa et al. 2013, Latouche et al. 2014, Yokomizo et al. 2014, Blue et al. 2015). Although these later life findings are crucial to understanding the biology underlying offspring complications, they may not identify the primary molecular mechanisms, in that secondary and tertiary pathology from other postnatal factors may be at play. Understanding the earliest molecular perturbations may be essential to understanding the mechanisms that program offspring metabolic risk.

The aim of this study was to better understand the mechanisms by which diabetes exposure induces offspring insulin resistance, in part by characterizing the earliest impacts on insulin action and signaling. To accomplish our aim, we developed in vivo techniques to study rodent fetus glucose disposition, insulin sensitivity and insulin signaling. We examined three distinct aspects of fetal diabetes exposure: fetal hyperglycemic exposure, uteroplacental insufficiency and fetal hyperinsulinemia. Isolated uteroplacental insufficiency was examined because fetal hypoxia results from maternal diabetes, owing to a relative uteroplacental insufficiency via mechanisms that may include structural placental changes and/or increased fetal metabolic demand (Wirde st et al. 1981, Daskalakis et al. 2008, Taricco et al. 2009, Escobar et al. 2013). Isolated fetal hyperinsulinism was studied because hyperglycemic exposure often increases fetal insulin (Gordon et al. 2015). We hypothesized that fetal exposure to late-gestational hyperglycemia would induce immediate tissue-specific insulin resistance, which would persist into later life. We found that fetuses exposed to hyperglycemia rapidly developed skeletal muscle insulin resistance while still in utero. The insulin signaling defects persisted through postnatal life, localized to skeletal muscle. Uteroplacental insufficiency and fetal hyperinsulinism did not have these effects, suggesting that fetal hyperglycemia may directly and persistently impact fetal skeletal muscle.

Research design and methods

Animals and breeding

All procedures were performed within the regulations of Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Iowa Institutional Animal Care and Use Committee. Rodents were housed in a temperature controlled, 12-h light-darkness cycled animal care facility with free access to water and regular chow. Surgery was performed under inhalational isoflurane anesthesia after at least 24 h of acclimation to the facility. Post-operative analgesia included topical bupivacaine applied immediately after wound closure and subcutaneous buprenorphine (0.05 mg/kg) provided twice daily. For experiments conducted to study postnatal
outcomes, 5 mg progesterone (mixed with 1 mL of peanut oil) was administered intraperitoneally 24 h after surgery to prevent preterm labor thus allowing Caesarean delivery at 21-day gestation. All procedures involving fetal manipulation or collection were performed in a fashion alternating between the two uterine horns to account for any time-dependent changes in environment.

Localized feto–maternal hyperglycemia

A vascular catheter draining into the left uterine artery was placed in timed pregnant Hsd:Sprague Dawley SD rats (Envigo, Indianapolis, IN, USA) as described (Yao et al. 2010), including placement of ligatures on the iliac, superior gluteal and hypogastric trunk arteries. Catheters were secured within a Covance Infusion Harness (CIH95, Instech Lab, Plymouth Meeting, PA, USA) and attached to a single channel stainless steel swivel (375/22, Instech Lab). Glucose was infused through the catheter at the rate of 4 mg/min for 48 h during late gestation to expose the left uterine horn fetuses to hyperglycemia. During infusion, the pregnant dams were awake and mobile, with unlimited access to water and food. For studies examining fetal outcomes, infusions were conducted on gestational days (GD) 18–20 in order to allow examination on GD20, prior to the time of parturition on GD21. For studies examining offspring, infusions were conducted on GD 19–21, to allow C-section delivery on GD21. Fetuses delivered by C-section were cross-fostered to healthy mothers.

Unilateral uterine artery ligation

The left or right lower abdominal quadrant was incised in GD18 rats to gain access to the left or right uterine artery respectively. The ipsilateral artery was ligated with 3-0 silk suture and the abdominal wall closed. The contralateral artery was not manipulated and the contralateral horn was injected with regular insulin (Novolin R, Novo Nordisk Inc) or saline (negative control). Fetal tissues were collected 20 min thereafter and snap frozen using a Beckman LS6000 Scintillation Counter (GMI, Ramsey, MN, USA). Tissue sample absorbance was also examined in supernatants after precipitation with 10% of ATS-3 General Rodent Tattoo System Pigment (AIMS Inc, Hornell, NY, USA) or an equivalent amount of diluent and pigment, using an insulin syringe with a 31G × 5/16” needle (Easy Touch, Houston, TX, USA). Over 1–2 days of action, the detemir dose of 0.1 units amounts to an average insulin dose of 50–100 mU/kg/min in a typical 0.7 gram fetus. This insulin exposure is hyperinsulinemic, less than a magnitude higher than what is used for high-dose hyperinsulinemic clamps in adult rodents. Note that because the fetus is constantly supplied with glucose by the placenta, there is no concern for severe sustained hypoglycemia. In fact, much higher doses of insulin have been administered to rodent fetuses: for example, injection of five units insulin into gestational day 17 fetuses (Ogata et al. 1988) amounting to ~900 mU/kg/min. The experimental treatment administered (detemir or diluent) was encoded by the laterality of the flank tattoo. Both uterine horns were placed back in abdominal cavity and the abdominal wall closed. Fetuses were euthanized 48 h after injection or allowed to be delivered spontaneously and cared for by the same mother.

Biochemical analyses

Fetal blood was collected via decapitation before placenta detachment. Tail blood was collected from 21-day-old pups at 0, 15, 30, 60 and 120 min during glucose tolerance testing. Fetal and 21-day-old pups blood glucose levels were measured using One Touch Ultra meter (LifeScan Inc., Milpitas, CA, USA). Serum insulin concentrations were measured using Ultrastensitive Rat Insulin ELISA kit (#90060, Crystal Chem, Downers Grove, IL, USA).

Radiolabeled glucose uptake

Two hours after completion of glucose infusion, pregnant mothers were given 350 microcuries of 2-[1,2-3H(N)]-deoxy-d-glucose (3H-2DG) over 30 s via inferior vena cava injection while alternate fetuses in both uterine horns were injected with regular insulin (Novolin R, Novo Nordisk Inc) or saline (negative control). Fetal tissues were collected 20 min thereafter and snap frozen immediately. Tissue samples were dissolved in 4N KOH overnight and the supernatants were neutralized. The 3H content was measured by liquid scintillation counting using a Beckman LS6000 Scintillation Counter (GMI, Ramsey, MN, USA).
Ba(OH)$_2$ plus ZnSO$_4$ (Halseth et al. 2000) to remove 2-deoxyglucose-6-phosphate. Tissue 2DG-6-P content was calculated by determining the difference of radioactivity with and without Ba(OH)$_2$: ZnSO$_4$ precipitation, and then normalized to tissue weight and reported as that relative to normalized 2DG-6-P content in brain (Sawatzke et al. 2015).

**In vivo insulin signaling**

Insulin administered at maximal stimulating doses, roughly 200 units/kg (Li et al. 2015), was used to acutely activate insulin signaling. To study tissue insulin-stimulated responses, fetuses received 0.5 unit regular insulin (Novolin R, Novo Nordisk Inc) or saline intraperitoneally while remaining in utero. For postnatal studies, 21-day-old pups were given five units regular insulin or saline via inferior vena cava injection. Skeletal muscle (muscle from whole leg for fetuses, soleus for 21-day-old pups), liver and brown fat tissues were collected and snap frozen 15 min after injection.

**Immunodetection**

Tissues were homogenized on ice and centrifuged at 10,000 g at 4°C for 4 min. Supernatant content of AKT/phospho-AKT, PDK1/phosphor-PDK1, MTOR/phospho-MTOR and PTEN/phospho-PTEN was measured using western blotting as previously reported (Baack et al. 2014, Gordon et al. 2015). Polyclonal antibodies recognizing AKT (9272, 1:500), phosphorylated AKT Ser 473 (9271, 1:500), PDK1 (3062, 1:1000), phosphorylated PDK1 Ser241 (3438, 1:1000), PTEN (9188, 1:1000), phosphorylated PTEN Ser380/Thr382/383 (9549, 1:1000), MTOR (2983, 1:1000) and phosphorylated MTOR Ser2448 (5536, 1:1000) were purchased from Cell Signaling Technology, Inc. Anti-β-ACTIN antibody was purchased from Sigma-Aldrich (A5441). Immunodetected protein quantity was determined by measuring pixel intensity of immunoblot bands using ImageJ software (NIH). *In vivo* insulin-stimulated AKT phosphorylation was calculated by dividing phosphorylated AKT over total AKT. Samples from the same mother were run and analyzed on the same blot so as to preserve the natural within-mother pairing of samples. Total IRS-1 and tyrosine phosphorylated IRS-1 protein content was quantified using MSD Insulin Signaling Panel Kit (total protein – K15152C, phospho-protein panel: K15151C, Mesoscale Discovery, Rockville, MD, USA). Phospho-AKT (Ser 473), GSK3-β (Ser 9) and p70s6 kinase (Thr421/Ser424) were measured using MSD AKT Signaling Whole Cell Lysate Kit (K15115D-1). Fetal tissue lysates were prepared following manufacturer’s instruction and loaded to 96-well plates at a protein concentration of 20µg/well. The 96-well plates were read with Meso QuickPlex SQ 120 (Mesoscale Discovery).

**Statistical analysis**

The statistical significance of differences between means of maternal measures was assessed by Student’s *t*-test. The impact of fetal interventions on fetal and offspring outcomes was assessed by paired *t*-test weighted by the total number of fetuses/offspring examined per mother, as implemented in the weights package in R (version 3.3.3, The R Foundation for Statistical Computing). This approach accounted for the natural pairing by mother and the inherently unbalanced number of replicates per mother per treatment since the number of fetuses per mother and per uterine horn varies naturally. The weighted paired *t*-test approach was used only for single interventions (i.e. simple comparison of two groups). For 2 × 2 experimental designs assessing fetal or offspring outcomes, linear mixed-effects modeling was used treating the two interventions as mixed effects with potential interaction and treating mothers as a random effect thus effectively accounting for individual fetuses/offspring as within-mothers repeated measures. This was accomplished using the R package inlme4, with *P* values calculated using type-III ANOVA tables as implemented in the car R package. Results of glucose tolerance tests were assessed by calculating the area under the curve (AUC) using the trapezoidal rule, followed by weighted, paired *t*-test comparing the two groups. Two-sided *P* values were computed and significance was defined as *P* < 0.05. 95% Confidence intervals (CI95) were calculated from the standard errors estimated by the weighted paired *t*-test for single intervention experiment and from the inlme4 package for 2 × 2 experiments. To help visualize within-mother pairing in figure panels which compare fetal treatments, data which originate from the same mother have the same color and are connected by a line. Thus, within-mother pairings are encoded by color and lines connecting the mean observed values. Note that colors are not conserved between figure panels and panels often represent different maternal cohorts. All data include results from both genders. Bars represent means ± standard errors of measurement (s.e.m.), calculated per mother for maternal outcomes and per fetus for fetal outcomes.
Results

Hyperglycemia rapidly induces insulin resistance in fetal skeletal muscle

Rat fetuses were exposed to 48 h of hyperglycemia via infusion of glucose into the left uterine artery on gestational days 18–20 (Fig. 1A). Maternal serum glucose was unchanged during infusion (Fig. 1B). By contrast, during infusion, glucose-exposed fetuses experienced moderate hyperglycemia, whereas control fetuses remained euglycemic (Fig. 1C, CI95 = +21 to 147 mg/dL).

Note that the glucoses in Fig. 1B and C should not be directly compared because they were obtained under differing conditions: 1B while the mothers were awake and ambulatory, whereas 1C samples were collected during maternal general anesthesia and laparotomy, which caused maternal glucose concentrations to rise. Previous work showed that fetal insulin is increased upon 24 h hyperglycemic exposure using this model (Gordon et al. 2015). However, by 48 h of hyperglycemic infusion fetal insulin levels were not significantly different than in euglycemic fetuses (Fig. 1D, CI95 = −1.2 to +2.1 ng/mL) despite fetal hyperglycemia.

To assess fetal insulin sensitivity, the uptake of maternally delivered tritiated 2-deoxyglucose ($^{3}$H-2DG) into fetal tissues was measured with and without acute fetal insulin treatment (Fig. 2A). Levels of $^{3}$H-2DG in maternal serum underwent the expected rapid peak after infusion and subsequent decay (Fig. 2B). Fetal tissue content of tritiated 2-deoxyglucose-phosphate ($^{3}$H-2DG-P) was measured 20 min after maternal $^{3}$H-2DG infusion. When considered across both hyperglycemia- and euglycemia-exposed fetuses, insulin had no statistically significant impact on tissue $^{3}$H-2DG-P content. However, there was a significant reduction in $^{3}$H-2DG-P content in hyperglycemia- compared to euglycemia-exposed fetuses when considered across both saline and insulin treatment groups (Fig. 2C, CI95 = −0.49 to −0.01). The lower uptake of $^{3}$H-2DG-P into hyperglycemia-exposed fetal muscle suggested the possibility of impaired insulin sensitivity in this tissue.

To better assess insulin actions in this tissue, the impact of hyperglycemic exposure on insulin-stimulated AKT phosphorylation was assessed; Figure 3A shows relevant experimental design. There was a significant impairment in insulin-stimulated AKT phosphorylation in hyperglycemia-infused fetal skeletal muscle (Fig. 3B, CI95 = −0.55 to −0.007). Tyrosine phosphorylation of IRS-1 in skeletal muscle exhibited trends toward an increase in insulin-stimulated vs saline-treated fetuses across both the hyperglycemia- and euglycemia-exposed groups.
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Figure 2
Effects of transient hyperglycemia on fetal skeletal muscle insulin sensitivity. (A) Experimental timeline. Glucose was infused into the left uterine artery for 48 h during late gestation. The hyperglycemic infusion was discontinued. Two hours later tritiated 2-deoxyglucose (2DG) was administered maternally and fetuses were administered insulin or saline. Fetal tissues were collected 20 min thereafter. (B) Maternal serum scintillation count after 2DG injection (n = four mothers), error bars represent per mother s.e.m. (C) Fetal skeletal muscle insulin-stimulated tritiated 2-deoxyglucose uptake, relative to that in fetal brain, after 48 h exposure to euglycemia or hyperglycemia. (n = 5–9 fetuses/group from three mothers). *P < 0.05 for overall difference between euglycemia and hyperglycemia groups. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0455.

Figure 3
Effects of transient hyperglycemia on fetal skeletal muscle insulin signaling. (A) Experimental timeline. Glucose was infused into the left uterine artery for 48 h during late gestation. While the infusion was continued, regular insulin or saline was administered to fetuses and fetal tissues were collected 15 min thereafter. (B) Fetal skeletal muscle in vivo insulin (INS) stimulated AKT phosphorylation in fetuses exposed to euglycemia (EG) or hyperglycemia (HG) (n = 27 fetuses/group from 11 mothers). *P < 0.05 for difference between the two groups. Representative western blots shown on right of panel. (C) Tyrosine phosphorylated IRS-1 expression measured using MSD Multiplex Assay (n = 10–14 fetuses/group from six mothers) in saline and insulin-stimulated fetuses. (D) Relative protein expression of signaling molecules proximal to AKT measured using MSD Multiplex Assay. (Total PDK, phospho PDK, total MTOR, phospho-MTOR and total PTEN n = 6–9 fetuses/groups from three mothers, and phospho-PTEN n = 4–6 fetuses/group from two mothers). A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0455.

(Fig. 3C, CI95 =−0.002 to +0.03), though this increase did not reach statistical significance (P = 0.096). However, the response to insulin did not differ between the euglycemia and hyperglycemia-exposed groups (Fig. 3C, CI95 =−0.01 to +0.02). Furthermore, there were no changes among several proteins that influence upstream aspects of insulin signaling. Namely, there were no changes in skeletal muscle PTEN, PDK-1, mTOR or their phosphorylation states induced by fetal hyperglycemic exposure (Fig. 3D).

Hyperglycemia rapidly alters insulin signaling in fetal brown fat and liver

The impacts of fetal hyperglycemic exposure on insulin action in brown adipose tissue and liver were also examined. In fetal brown adipose tissue, hyperglycemic exposure produced similar trends in glucose uptake (Fig. 4A) as observed in skeletal muscle, though there were no statistically significant differences between the groups.
GSK phosphorylation levels were reduced in hyperglycemia compared to euglycemia-exposed fetuses across conditions (Fig. 5B, CI95 = −0.65 to +0.03). Likewise, phosphorylation of AKT in liver showed similar reductions (Fig. 5A, CI95 = −0.67 to +0.06), though this did not reach statistical significance (P=0.10).
Fetal hyperinsulinemia has no acute impact on insulin signaling

As previously reported, hyperglycemia-exposed fetuses develop hyperinsulinemia after 24h of infusion (Gordon et al. 2015) though this is not sustained at 48h (Fig. 1D). Maternal diabetes and hyperglycemia can also induce fetal hyperinsulinemia in humans and other animal models (de Ménibus & Mallet 1980, White et al. 2015), though the impact of isolated fetal hyperinsulinemia on development of insulin resistance is unknown. It is possible that transient hyperinsulinemia could modulate fetal tissue insulin sensitivity. For example, hyperinsulinemia induces insulin resistance in a variety of cell culture models (Andreozzi et al. 2011). To test this directly in vivo during fetal maturation, mouse fetuses were administered, in utero, detemir, a long-acting neutral-pH insulin or diluent as control (Fig. 6A). Insulin sensitivity was then assessed 48h after insulin detemir administration, by measuring the acute response to regular insulin fetal injection. In both detemir and diluent administered fetuses, regular insulin produced a rapid and significant reduction in fetal blood glucose (Fig. 6B), indicative of intact insulin action. There were no statistically significant differences in glucoses between detemir- and diluent-treated fetuses, nor was there a statistical difference in their responses to acute insulin (Fig. 6B). Consistent with this, insulin-stimulated AKT phosphorylation was not statistically

Figure 6
Impact of fetal hyperinsulinemia on acute insulin signaling. (A) Experimental timeline. Detemir, a long-acting insulin, or control diluent, was administered to fetuses during late gestation to induce chronic hyperinsulinemia (HI). Forty-eight hours later, regular insulin (short acting) or saline was administered to the fetuses, and fetal blood/tissues collected 15 min thereafter to assess insulin signaling. (B) Fetal blood glucose in response to exogenous insulin stimulation (n = 9–15 fetuses/group, eight mothers) after 48h exposure to long-acting insulin (detemir) or control (diluent). *P < 0.05 for decrease in glucose induced by insulin across groups. (C) In vivo insulin-stimulated AKT phosphorylation in fetal skeletal muscle (n = 15–18 fetuses/group from six mothers), (D) fetal brown adipose tissue (n = 14 fetuses/group, seven mothers), and (E) fetal liver (n = 11–14 fetuses/group, five mothers). Representative western blots shown adjacent to panels. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0455.
altered in detemir- vs diluent-injected fetuses when examined in fetal skeletal muscle (Fig. 6C), brown adipose tissue (Fig. 6D) or liver (Fig. 6E).

**Fetal uteroplacental insufficiency does not acutely impair fetal insulin signaling**

Fetoplacental exposure to maternal diabetes and hyperglycemia results in fetal hypoxia, owing to a relative uteroplacental insufficiency via mechanisms that may include structural placental changes and/or increased fetal metabolic demand (Widness et al. 1981, Daskalakis et al. 2008, Taricco et al. 2009, Escobar et al. 2013). Thus, relative uteroplacental insufficiency and fetal hypoxia may have been induced in our rat model of hyperglycemia exposure and thus could have been a mechanism of fetal perturbation. Relevantly, uteroplacental insufficiency induces subsequent offspring insulin resistance, which is thought to involve skeletal muscle (Simmons et al. 2001b, Selak et al. 2003, Gatford et al. 2010). It was thus of interest to determine the impact of uteroplacental insufficiency on fetal skeletal muscle insulin sensitivity. Uteroplacental insufficiency was induced in rats by unilateral uterine artery ligation and fetuses examined 48 h thereafter (Fig. 7A). As expected, fetal rats exposed to uterine artery ligation were significantly smaller (Fig. 7B). However, unlike hyperglycemia-exposed fetuses, uterine artery ligation-exposed fetuses had no impairment in insulin-stimulated AKT phosphorylation (Fig. 7C), and instead exhibited the opposite trend toward enhanced AKT phosphorylation (CI95 = −0.15 to +0.54).

**Impaired skeletal muscle insulin signaling and glucose intolerance in offspring exposed to fetal hyperglycemia but not fetal hyperinsulinism**

Given the rapid induction of fetal insulin resistance during hyperglycemic exposure, it was thus of considerable interest as to whether insulin sensitivity normalized after birth and cessation of the hyperglycemic exposure. To test this, rat pups were birthed by C-section, reared by foster mothers, and their insulin responsiveness or glucose tolerance tested at 21 days of age (Fig. 8A). Interestingly, skeletal muscle insulin sensitivity was impaired at 21 days of age in offspring formerly exposed to hyperglycemia as assessed by insulin-stimulated AKT phosphorylation (Fig. 8B, CI95 = −0.38 to −0.02). By contrast, there was no impairment in insulin-stimulated AKT phosphorylation at 21 days of age in hyperglycemic exposed offspring brown adipose tissue (Fig. 8C) or liver (Fig. 8D). Importantly, offspring exposed to fetal hyperglycemia exhibited a modest but statistically robust degree of glucose intolerance, as among all six paired groups the glucose AUC during glucose tolerance testing was higher in the hyperglycemia-exposed offspring (Fig. 8F, P = 0.03).

Mice offspring exposed to fetal hyperinsulinism were also studied at 21 days of age (Fig. 9A). Interestingly, there was a significant impairment in insulin-stimulated AKT phosphorylation in the liver (Fig. 9C, CI95 = −0.66 to −0.07) but not skeletal muscle (Fig. 9B) of fetally detemir- vs diluent-treated offspring when examined at 21 days of age. These offspring exhibited normal glucose tolerance as indicated by no overall change in AUC (Fig. 9D and E).

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**Figure 7**

Acute fetal effects of 48 h placental insufficiency. (A) Experimental timeline. One uterine artery was ligated during late gestation while the contralateral artery was left intact (control). Forty-eight hours later, insulin or saline was administered to fetuses, and fetal tissues harvested 15 min thereafter. (B) Fetal weight 48 h after induction of placental insufficiency by uterine artery ligation (‘ligate’ group) (n = 43–44 fetuses/group from seven mothers). *P < 0.05 for difference between groups. (C) Fetal skeletal muscle insulin-stimulated AKT phosphorylation 48 h after ligation (n = 12 fetuses/group from six mothers). Representative western blot shown below panel. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0455.
Discussion

Human offspring exposed to maternal diabetes during fetal life have increased risk of insulin resistance in adulthood, beyond genetic risks (Dabelea et al. 2000, 2008, Clausen et al. 2008, 2009). Likewise, animal models of diabetes during pregnancy produce offspring that exhibit insulin resistance (Segar et al. 2009, Blue et al. 2015). Due to the complex pathology induced by diabetes, the role of hyperglycemia alone in this process has been difficult to define. In fact, the direct role of hyperglycemia in producing offspring diabetes risk has been recently questioned (Donovan & Cundy 2015). To our best knowledge, this is the first experimental design examining offspring insulin sensitivity that isolates hyperglycemia’s effects alone in absence of other systemic maternal alterations that otherwise accompany diabetes in pregnancy (Baack et al. 2014). We found most strikingly that fetal exposure to hyperglycemia during late gestation induces immediate and lasting insulin resistance in skeletal muscle, whereas isolated fetal hyperinsulinism or uteroplacental insufficiency did not exert this effect.

The detrimental impact of fetal hyperglycemic exposure on skeletal muscle insulin sensitivity was evident even before birth, at the conclusion of 48h of hyperglycemic exposure. Similarly, human fetuses exposed to maternal glucose intolerance have diminished insulin sensitivity as calculated from umbilical cord blood insulin and glucose (Luo et al. 2010). The induction of skeletal muscle insulin resistance by hyperglycemia is not entirely surprising, as in vivo (Oku et al. 2001, Haber et al. 2003) and in vitro (Kurowski et al. 1999) experiments demonstrate that hyperglycemia acutely induces insulin resistance in mature skeletal muscle. However, fetal insulin signaling in skeletal muscle has received little experimental attention (Anand et al. 2002, Dunlop et al. 2015) such that even the molecular ontogeny of fetal insulin signaling is not well characterized. Fetal skeletal muscle may be at special
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risk for development of permanent insulin resistance. Importantly, the majority of lifelong muscle cells are formed during fetal myogenesis (Du et al. 2010). By contrast, brown adipose tissue (Rehnmark & Nedergaard 1989) and liver (Moreno-Carranza et al. 2018) experience ongoing cellular formation after birth. Thus, it has been hypothesized that adverse exposure during fetal development could exert long-lasting effects on adult muscle insulin sensitivity (Du et al. 2010). Our findings are consistent with this hypothesis, in that impaired insulin signaling persisted in skeletal muscle for at least several weeks after fetal hyperglycemic exposure, whereas insulin signaling remained normal in liver and brown adipose tissue over the same time frame. Our studies herein only address insulin sensitivity but do not address its impact on the observed mild defect in glucose tolerance. It is possible that beta cell defects might contribute to the impaired glucose tolerance we observed in hyperglycemia-exposed offspring at 21 days of age. In fact, exposure to maternal diabetes has also been shown to induce long-lasting beta cell dysfunction and impaired insulin secretion (Gauguiere et al. 1991, Frost et al. 2012).

Fetal exposure to hyperglycemia alone has also been achieved in ovine pregnancy models, which have studied its impact on leucine oxidation, insulin secretion and glucose transporter expression (Liechty et al. 1993, Anderson et al. 2001, Frost et al. 2012). However, we are unaware of studies examining the impact of hyperglycemia alone on insulin signaling in these models. In our model, 48h of hyperglycemic exposure reduced glucose uptake into skeletal muscle and diminished insulin-stimulated AKT phosphorylation (Figures 2 and 3). However, insulin-stimulated IRS1 tyrosine phosphorylation was not affected, indicating that the hyperglycemia-induced defect in the insulin signaling pathway lies between IRS and AKT. This is consistent with findings in mature rat muscle acutely exposed ex vivo to hyperglycemia in which there were impairments in insulin-stimulated AKT phosphorylation but not insulin-stimulated phosphatidylinositol 3-kinase activity (Kurowski et al. 1999). We found no changes in the expression of PDK, PTEN and mTOR, which are proteins that mediate aspects of insulin signaling upstream of AKT. These findings suggest the presence of other molecular mechanisms that mediate the negative impact of hyperglycemia on fetal skeletal muscle insulin signaling. The magnitude of the defect in skeletal muscle insulin signaling at 21 days of age was mild (~25% reduction), though this is similar to other rodent studies of offspring exposed to systemic maternal diabetes/hyperglycemia (Holemans et al. 1997,
Nasu-Kawaharada et al. 2013). Likewise, the impairment in glucose tolerance was mild, which is likewise similar to prior studies in rodent offspring from diabetic pregnancy (Gauguier et al. 1990). We chose 48 h of hyperglycemic exposure in part due to practical considerations related to a non-trivial day-to-day failure rate of these infusions but also because even brief periods of maternal system hyperglycemia induce metabolic effects, which persist at least through the neonatal period (Ktorza et al. 1981). However, it is possible that stronger lasting effects may have been observed after a longer infusion. Furthermore, it is uncertain how these defects would have evolved over time had the offspring been allowed to further mature.

In our model, hyperglycemia induces transient fetal hyperinsulinemia at 24 h (Gordon et al. 2015). This fetal hyperinsulinemia is not sustained by 48 h (Fig. 1D) of infusion, consistent with prior observation that maternal diabetes exposure can produce glucotoxicity and degranulation of fetal islets (Aerts & van Assche 1977). Since hyperinsulinemia induces insulin resistance in a variety of cell culture models (Andreozzi et al. 2011), this raised the possibility that hyperinsulinism may have contributed to fetal skeletal muscle insulin resistance. To address this directly, we used a model of chronic hyperinsulinism in the fetal rat through use of a long-acting insulin analog. Interestingly, no acute impact on fetal insulin signaling was observed, whereas postnatal hepatic insulin signaling was impacted at 3 weeks of age. Glucose tolerance AUC was normal. Importantly for the present focus, these results suggest that transient induction of fetal hyperinsulinism is not the mechanism by which hyperglycemia induces acute or chronic skeletal muscle insulin resistance.

Fetuses developing in the context of diabetic pregnancy often exhibit hypoxia and markers of chronic hypoxia (Widness et al. 1981, Daskalakis et al. 2008, Taricco et al. 2009, Escobar et al. 2013) even when fetal weight is increased (Taricco et al. 2009). The origins of the fetal hypoxia are not perfectly understood but are thought to reflect a degree of relative placental insufficiency, related to structural placental abnormalities (Daskalakis et al. 2008, Taricco et al. 2009), increased fetal oxidative metabolism and oxygen consumption induced by hyperglycemia (Philipps et al. 1984, Crandell et al. 1985), fetal overgrowth relative to the placenta (Surányi et al. 2016, Gloria-Bottini et al. 2016) and/or diminished uteroplacental blood flow (Reed et al. 2018). We did not examine the impact of uteroplacental insufficiency or hyperglycemia on placental glucose transport to the fetus. Nonetheless, we thus tested whether uteroplacental insufficiency incurred by uterine artery ligation induces the same acute defects in skeletal muscle insulin signaling as caused by hyperglycemia. We found that uteroplacental insufficiency induced by unilateral uterine artery ligation did not induce insulin resistance, but instead induced a trend toward increased insulin sensitivity consistent with findings in related models from large (Limesand et al. 2007) animals. This is interesting because uterine artery ligation is well described to eventually induce offspring skeletal muscle insulin resistance in the long run (Simmons et al. 2001b, Selak et al. 2003, Gatford et al. 2010) and suggests that the proximal mechanisms by which hyperglycemia vs uteroplacental insufficiency induce offspring skeletal muscle insulin resistance differ.

In conclusion, we find that fetal exposure to isolated maternal hyperglycemia is sufficient to induce insulin resistance, especially in skeletal muscle where insulin signaling was disrupted for at least several weeks after birth when the hyperglycemic exposure ceased. Our results imply that the mechanism by which uteroplacental insufficiency programs later dysmetabolism and diabetes risk differs from the mechanisms induced by hyperglycemia. Finally, our results raise the possibility that fetal skeletal muscle is uniquely at risk for programming of long-lasting insulin resistance. 

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.

Funding
This project was funded by the American Academy of Pediatrics (Marshall Klaus Award to K L K), the University of Iowa Children's Miracle Network (research grant to K L K), the Fraternal Order of Eagle Diabetes Research Center (scholar award to A W N) and the National Institutes of Health through R01-DK115791 (to A W N), R01-DK081548 (to A W N) and R24-DK096518 (to A W N).

Author contribution statement

Acknowledgements
The authors thank Philip Gruppuso, MD, Brown University, for helpful discussion.
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Received in final form 4 November 2018
Accepted 14 November 2018
Accepted Preprint published online 14 November 2018