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INTRODUCTION

Fetal programming associated with intrauterine growth restriction (IUGR) leads to lifelong deficits in growth and metabolic function (Hales and Barker, 2013). IUGR arises when fetuses respond to poor in utero conditions by developing adaptations that repartition nutrients to critical tissues and away from skeletal muscle (Yates et al., 2012, 2018). This fetal programming is beneficial in utero but leads to persistent reductions in muscle mass and glucose homeostasis in offspring (DeFronzo et al., 1981). Recent studies by our laboratory in sheep and rats demonstrate that maternal inflammation during gestation induces fetal inflammatory adaptations that impair growth and disrupt muscle glucose metabolism (Cadaret et al., 2017, 2018). IUGR fetal skeletal muscle exhibits indicators of enhanced inflammatory sensitivity, which could disrupt glucose uptake and oxidation (Yates et al., 2016; Cadaret et al., 2018). Enhanced inflammatory responsiveness would help explain growth and metabolic deficits observed in IUGR offspring. We hypothesize that fetal programming induced by maternal inflammation persists in offspring and contributes to impaired growth and glucose metabolism at 30 d. Therefore, the objective of this study was to determine whether sustained maternal inflammation induced by bacterial endotoxin at 0.7 gestation leads to fetal programming that contributes to deficits in growth and glucose metabolism in offspring.

MATERIALS AND METHODS

Animals and Experimental Design

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Nebraska–Lincoln, which is an AAALAC International accredited institution. Timed-mated Polypay ewes were treated (iv) with saline (control, $n = 8$) or 0.1 µg/kg bodyweight (BW) of bacterial lipopolysaccharide ($n = 6$; *Escherichia coli* O55:B5; MilliporeSigma) every third day from d 100 to 115 of gestation to produce maternal inflammation-induced IUGR (MI-IUGR) lambs. Lambs were weaned at birth, fed colostrum, and transitioned to a commercial milk replacer (ad libitum). BW and growth metrics were measured at birth and 30 d. At 25 ± 2, indwelling catheters and a perivascular flow probe were surgically placed in hindlimb femoral arteries and veins as previously described (Limesand et al., 2007; Camacho et al., 2017). Glucose-stimulated insulin secretion (GSIS) and hyperinsulinemic-euglycemic clamp (HEC) studies were performed at 28 ± 2 and 29 ± 2 d, respectively. Lambs were

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killed at 30 ± 2 d. Brains were weighed, and primary hindlimb skeletal muscle was isolated for ex vivo metabolic studies.

Glucose-Stimulated Insulin Secretion

A square-wave hyperglycemic clamp was performed as previously described (Yates et al., 2012; Camacho et al., 2017). Three baseline samples were collected at 5-min intervals prior to an intravenous glucose bolus (150 mg/kg BW) and continuous variable-rate glucose infusion to produce steady-state hyperglycemia ~2-fold greater than baseline. Thirty minutes after clamping hyperglycemia, three additional blood samples were collected at 5-min intervals. Blood was centrifuged (14,000 × *g*, 2 min, 4 °C) and plasma insulin concentrations were determined via ELISA (Ovine Insulin; Alpco). Intra- and inter-assay coefficient of variation were less than 10%. Whole blood was analyzed for glucose concentration using an ABL90 FLEX (Radiometer).

Hindlimb Glucose Metabolism

Hindlimb glucose utilization and oxidation rates at baseline and during HEC were quantified as previously described (Cadaret et al., 2018). Lambs were infused with U-[^{14}C]-glucose tracer (18.75 µCi/ml; PerkinElmer) at 1 ml/h for 40 min. Then, arterial and venous blood samples were collected simultaneously at 5-min intervals (four total). Lambs were then infused with 33% dextrose at a variable rate and insulin (Humulin-R; Eli Lilly) at 4 mU/kg/min to produce steady-state hyperinsulinemia and euglycemia. After HEC was achieved for 2 h, simultaneous venous and arterial samples were again taken at 5-min intervals as described earlier. To measure glucose oxidation, blood was added to microcentrifuge tubes containing 2 M HCl to release CO_2. These were suspended inside sealed 20-ml scintillation vials containing 1 M NaOH to capture CO_2. After 24 h at 32 °C, the microcentrifuge tube was removed and Ultima Gold scintillation fluid (PerkinElmer) was added to each vial. Concentrations of $^{14}CO_2$ from each blood sample were quantified via liquid scintillation using a Beckman Coulter 1900. Hindlimb glucose utilization was calculated as the difference between arterial and venous glucose concentrations normalized to femoral blood flow rate and hindlimb weight at necropsy. Hindlimb glucose oxidation rates were quantified in triplicate from the difference between venous and arterial ^{14}C-specific activities normalized to femoral blood flow rate and hindlimb weight at necropsy. The amount of glucose oxidized was calculated in nanomol using the specific activity of the infused radiolabeled glucose.

Ex Vivo Skeletal Muscle Glucose Metabolism

Primary skeletal muscle glucose uptake and oxidation rates were quantified as previously described (Cadaret et al., 2017). The flexor digitorum superficialis muscle of each lamb was dissected longitudinally into ~800 mg strips before incubation in Krebs–Henseleit bicarbonate buffer (KHB) containing (basal), or 5 mU/ml insulin. Glucose uptake was measured in triplicate by incubating muscle in KHB containing 1 mM [^{3}H]2-deoxyglucose and 1 mM [^{14}C]-mannitol for 20 min. Glucose oxidation was measured in triplicate by incubating muscle in KHB containing [^{14}C-U]D-glucose for 2 h, before capturing $^{14}CO_2$ in NaOH for 2 h as previously described (Cadaret et al., 2017).

Statistical Analysis

All data were analyzed using the MIXED procedure in SAS (SAS Institute, Cary, NC) with lamb as the experimental unit. In vivo and ex vivo metabolic data were analyzed for effects due to experimental group, period (or incubation condition), and the interaction. Period or condition was treated as a repeated variable. For in vivo metabolic studies, samples within each period were averaged for each lamb. Similarly, technical replications were averaged for each lamb in ex vivo studies. Morphometric data were analyzed for effects due to treatment. All data are presented as means ± SEM.

RESULTS

Lamb Growth Metrics

At birth, BW tended to be less ($P < 0.1$) and average daily gain (ADG) from birth to d 30 was less ($P < 0.05$) in MI-IUGR lambs than controls (Table 1). At 30 d, BW was ~20% less ($P < 0.05$) in MI-IUGR lambs than controls. Brain weight or BW was ~19% greater ($P < 0.05$) in MI-IUGR lambs compared to controls.

In Vivo Metabolic Studies

Basal and GSIS did not differ between groups (Figure 1). Insulin-stimulated hindlimb glucose rates did not differ between groups (data not shown). However, hindlimb insulin-stimulated glucose oxidation was ~70% less ($P < 0.05$) in MI-IUGR lambs compared to controls (Figure 2).

Ex Vivo Skeletal Muscle Glucose Metabolism

Basal and insulin-stimulated glucose uptake rates in primary skeletal muscle from MI-IUGR lambs was less ($P < 0.05$) than skeletal muscle from controls (Figure 3A). Incubation with insulin increased ($P < 0.05$) glucose uptake in skeletal muscle from all lambs. Glucose oxidation rates were less ($P < 0.05$) in MI-IUGR muscle than controls under basal and insulin-stimulated conditions (Figure 3B). Insulin increased ($P < 0.05$) glucose oxidation in skeletal muscle from all lambs.

DISCUSSION

The results of this study demonstrate that sustained maternal inflammation at 0.7 gestation yields IUGR and growth deficits that persist after birth. Furthermore, fetal programming responses to maternal inflammation impaired skeletal muscle glucose metabolism, which also persist after birth. In addition to exhibiting asymmetrical growth restriction at birth, MI-IUGR lambs grew ~22% slower, making them ~20% smaller at 30 d. Greater brain or BW in MI-IUGR lambs was consistent with asymmetrical growth and brain-sparing, which is a hallmark characteristic of IUGR (Limesand et al., 2007). Maternal inflammation yielded less severe IUGR with a less pronounced effect on BW at birth than the maternal hyperthermic model (Yates et al., 2018). However, our findings indicate that MI-IUGR growth deficits persist in offspring. It is worth noting that MI-IUGR lambs did not exhibit postnatal catch up growth over the first 30 d after birth in this study. Nevertheless, our MI-IUGR lambs exhibited substantially less glucose oxidation within hindlimb tissues and in isolated hindlimb skeletal muscle. Although hindlimb tissues in MI-IUGR lambs used normal amounts of glucose, ex vivo studies indicated that skeletal muscle-specific glucose uptake was impaired. We did not observe evidence of β-cell dysfunction in this study, which contrasted with the results of our fetal studies in this model of IUGR (Cadaret et al., 2018). These findings indicate that sustained maternal inflammation at 0.7 gestation has a substantial negative effect on fetal programming of glucose metabolism that mirrored other IUGR models (Limesand et al., 2007; Brown et al., 2015). They further show that adaptive fetal programming responses to maternal inflammation persist in offspring at 30 d and affect muscle tissue responsiveness to insulin. Reductions in muscle glucose oxidation could be due to the developmental shift toward anaerobic glycolysis (Brown et al., 2015; Yates et al., 2018), which produces lactate for nutrient repartitioning via the Cori cycle. We hypothesized in previous studies that this shift results from programming of skeletal muscle inflammatory signaling that occurs as a result of maternal inflammation (Cadaret et al., 2018; Posont et al., 2018). Such alterations appear to enhance skeletal muscle sensitivity to inflammatory cytokines, which would help to explain in part the growth and metabolic pathologies we observed in this study (Posont et al., 2018; Yates et al., 2018).

Table 1. Growth measurements for MI-IUGR lambs

Variable	Control	MI-IUGR	*P* value
Birthweight, kg	4.42 ± 0.16	3.61 ± 0.43	0.09
30 d BW, kg	13.88 ± 0.37	11.06 ± 0.92	0.03
ADG, kg/day	0.32 ± 0.01	0.25 ± 0.02	0.03
30 d Brain/BW	5.91 ± 0.15	7.23 ± 0.34	0.02

Values are expressed as means ± SE. ADG = average daily gain.

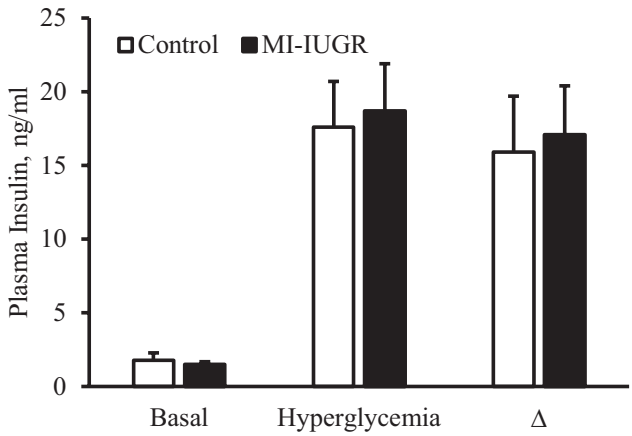

Figure 1. Plasma insulin concentrations in MI-IUGR lambs at 30 d of age under basal and steady-state hyperglycemic conditions as well as the difference between periods. Square-wave hyperglycemic clamps were performed in controls ($n = 8$) and MI-IUGR ($n = 6$) lambs. *Means differ ($P < 0.05$) from controls within the period.

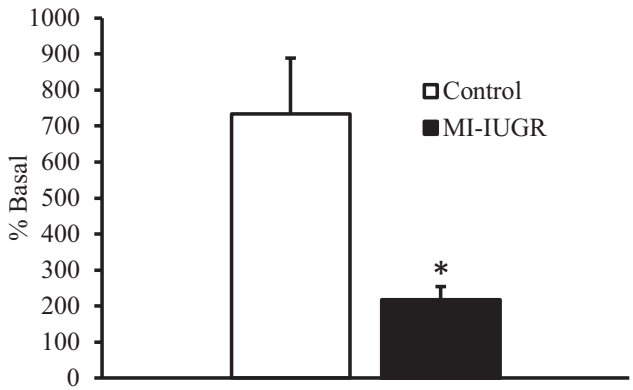

Figure 2. Hindlimb-specific insulin-stimulated glucose oxidation rates in MI-IUGR lambs at 30 d of age. Square-wave hyperinsulinemic-euglycemic clamps were performed in controls ($n = 8$) and MI-IUGR ($n = 6$) lambs. *Means differ ($P < 0.05$) from controls.

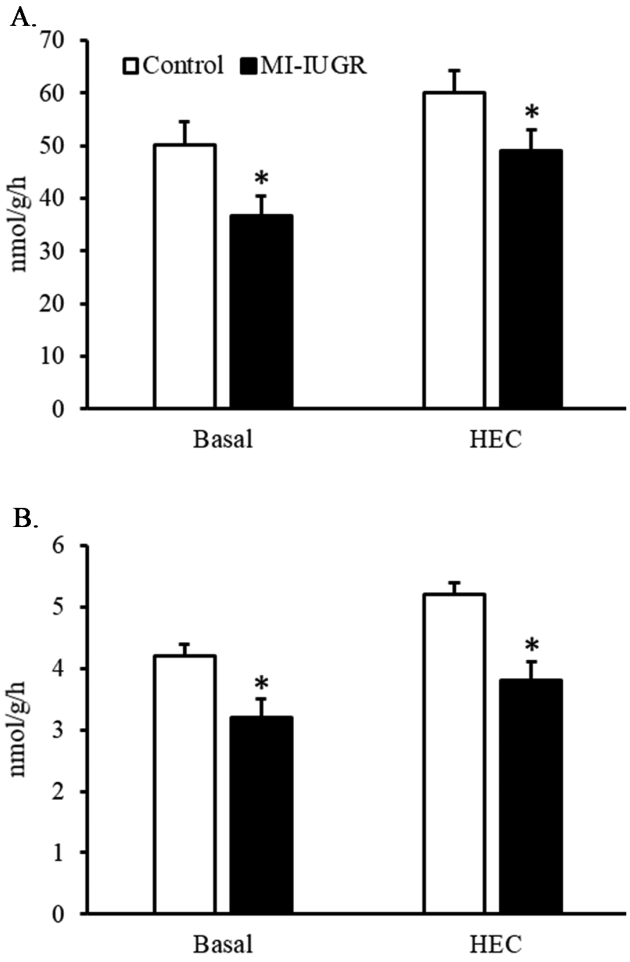

Figure 3. Ex vivo primary skeletal muscle glucose uptake (A.) and oxidation (B.) rates for MI-IUGR lambs at 30 d of age. Primary flexor digitorum superficialis muscle strips were incubated in basal and insulin-spiked (HEC) media. Incubations were performed for controls (n = 8) and MI-IUGR (n = 6) lambs. *Means differ (P < 0.05) from controls within the period.

Inflammatory responsiveness was not measured in this study, but our previous studies found that skeletal muscle from MI-IUGR rats and sheep respond differently than control muscle to the inflammatory cytokine tumor necrosis factor α (Cadaret et al., 2017, 2018). Furthermore, we previously found differences in inflammatory signaling pathways in skeletal muscle and myoblasts of IUGR fetal sheep that coincided with altered proliferation and differentiation rates of these cells (Posont et al., 2018). Further research will determine how the apparent enhancement of these inflammatory pathways might contribute to the persistent MI-IUGR metabolic phenotype observed in this study.

In conclusion, sustained maternal inflammation at 0.7 gestation resulted in IUGR, with moderate effects on postnatal growth but substantial effects on skeletal muscle glucose metabolism in offspring. Poor skeletal muscle metabolic capacity and asymmetrical growth restriction are common characteristics of IUGR offspring in humans and livestock. The results of this study indicated that fetal programming responses to maternal inflammation persist after birth and contribute to deficits in growth and glucose metabolism via unknown mechanisms. These programmed responses may be due to previously observed alterations in inflammatory signaling pathways of skeletal muscle, which could represent intervention targets to improve outcomes in IUGR offspring.

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Conflict of interest statement. The authors have no conflict of interest to declare.

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