Maternal inflammation at mid-gestation in pregnant rats impairs fetal muscle growth & development at term.[1]

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ABSTRACT: Intrauterine growth restriction (**IUGR**) is linked to lifelong deficits in muscle mass and metabolic function. Maternal stress increases fetal loss and causes developmental adaptations in fetal muscle, but little is known about the mechanistic role of inflammation in adaptive programming. Therefore, the objective of this study was to determine the effects of sustained maternal inflammation at mid-gestation on fetal mortality, muscle growth, and metabolic parameters at term. Timed-pregnant Sprague-Dawley rats were injected (i.p.) daily with saline (controls) or bacterial endotoxin lipopolysaccharide (**LPS**) on days 9-11 of gestation to induce maternal inflammation and then euthanized just before term (day 20). The average number of fetuses per litter did not differ between treatments, but fetal mass was lower ($P < 0.05$) in LPS rats. Fetal plasma TNFα tended to be greater ($P < 0.10$) and fetal hindlimb muscle TNFR1 and IL6R mRNA tended to be decreased ($P < 0.10$) in LPS rats compared to controls. RNA markers for total macrophages (CD68) and M2 macrophages (CD163) also tended to be decreased ($P < 0.10$) in LPS fetal muscle compared to controls. CD68-positive nuclei were decreased ($P < 0.05$) in LPS fetal muscle but CD168-positive nuclei were not different between treatments. Moreover, myoD-positive nuclei (activated myoblasts) were decreased ($P < 0.05$) and myogenin-positive nuclei (differentiated myoblasts) tended to be increased ($P < 0.10$) in LPS fetal hindlimb muscle compared to controls. Fewer total resident macrophages combined with greater plasma TNFα indicate that M1 fetal macrophages in LPS-treated rats were more productive despite reduced prevalence in skeletal muscle. Moreover, reduced muscle cytokine receptor expression is likely a compensatory response to sustained exposure to high concentrations of circulating cytokines that reduces muscle cytokine sensitivity. Lastly, the reduction in myoD-positive nuclei and increase in myogenin-positive nuclei indicates impaired myoblast function. Because myoblasts facilitate hypertrophic muscle growth, this is likely responsible for the decrease in fetal mass after maternal inflammation. Together, our findings demonstrate that maternal inflammation at mid-gestation causes fetal adaptations in muscle regulation that impair subsequent development and growth.

Key words: fetal hindlimb, inflammatory cytokines

INTRODUCTION

Intrauterine growth restriction (IUGR) is a leading cause of perinatal morbidity and mortality. Low birth weight resulting from preterm birth and/or IUGR is an underlying factor in 60-80% of perinatal death worldwide, and is particularly common in developing countries (UNICEF., 2008). Furthermore, studies have linked IUGR and the associated fetal malnutrition to increased incidence of metabolic syndrome in adult life (Barker et al., 1993; Godfrey and Barker, 2000). The "thrifty phenotype hypothesis" developed by David Barker (Hales et al., 1991) states that IUGR-associated fetal malnutrition forces the fetus to spare nutrients by altering tissue-specific metabolism in order to survive. In utero, adaptive changes disproportionately impact skeletal muscle development, growth, and metabolism (Yates et al., 2016). Skeletal muscle is responsible for the majority of insulin-stimulated glucose utilization, and adaptive restriction in muscle growth capacity helps to spare glucose in the IUGR fetus but result in lifelong deficits in muscle mass and metabolic homeostasis (Brown and Hay, 2016). Skeletal muscle growth requires proliferation, differentiation, and fusion of myoblast into new muscle fibers early in gestation and fusion with existing fibers in the third trimester of pregnancy (Zhu et al., 2004). This process can be impaired by inflammation from resident macrophages within skeletal muscle. Classically-activated M1 macrophages are pro-inflammatory but can polarize to an anti-inflammatory M2 phenotype that inhibits cytokine production and stimulates tissue repair by producing growth factors (Mantovani et al., 2004; Kharraz et al., 2013). The acute effects of inflammatory factors on myoblast function have been investigated in vitro (Frost et al., 1997; Guttridge et al., 2000), and we postulate that inflammatory stress may have similar effects on fetal myoblasts in utero. Impaired myoblast function and the resulting decrease in muscle growth capacity affect long-term metabolic health. Therefore, the objective of this study was to determine the effect of sustained maternal inflammation at mid-gestation on fetal mortality, muscle growth, and metabolic parameters at term.

MATERIALS AND METHODS

Animals and experimental design.

Animal use and care was approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln. Animal studies were performed at the University of Nebraska-Lincoln Animal Science Complex, which is accredited by the American Association for

Accreditation of Laboratory Animal Care. From day 9 to 11 of gestation, time-mated Sprague-Dawley rats (Envigo, Indianapolis, IN) received daily i.p. injections of saline (n = 6) or 100μg/kg BW lipopolysaccharide (LPS, n = 7) from *E. coli* 055:B5 (Sigma-Adlrich). Rats were weighed daily, and maternal blood was collected and rectal temperature recorded throughout the treatment period. On day 20 of gestation, dams were euthanized by decapitation under heavy isoflurane anesthesia. Fetal mass and number were recorded and maternal and fetal blood samples were collected. Fetal hindlimbs were collected from three randomly-selected fetuses per litter. For each fetus one hindlimb was fixed in 4% PFA and the other was snap-frozen.

Blood analysis.

Maternal and fetal blood glucose concentrations were determined at necropsy (Bayer Glucose Meter). Plasma was isolated by centrifugation (14,000 x g, 2 min) and TNFα concentrations were determined by Quantikine ELISA kit (R&D Systems) as previously described (hui Seo et al., 2017). Inter-assay CV was less than 10%.

Gene expression.

Droplet digital PCR. RNA was extracted from fetal hindlimb (30 mg) via RNeasy Fibrous Tissue Mini Kit (Qiagen), quantified by spectrophotometry (NanoDrop Technologies), and reverse transcribed via QuantiTect Reverse Transcription Kit (Qiagen). Primers for PCR were designed and droplet digital PCR (ddPCR) was performed with the QX200 ddPCR System (BioRad). Each reaction contained Evagreen Supermix, 10μM of each primer, and 1 μl of cDNA template. Droplets were generated in a QX200 Droplet Generator with Droplet Generator Oil, transferred to a PCR plate, sealed, and placed in a C1000 Touch Thermal Cycler. Samples were activated (95°C for 5 min), denatured for 40 cycles (95°C for 30 s), annealed and extended for 40 cycles (60°C for 1 min), and stabilized (4°C for 5 min and 90°C for 5 min). Finally, droplets were read on the QX200 Droplet Reader and results were analyzed with QuantaSoft Software to obtain copies/μl for genes of interest. Results for CD68, CD163, TNFR1, IL6R, insulin receptor β, and adrenergic receptors β1 and β2 were then normalized to the Ywhaz gene, which was shown to be stable across treatment groups.

Quantitative PCR. Expression of CD68 and CD163 was also measured via qPCR by the UNL Veterinary Diagnostic Center. RNA was extracted and cDNA was generated as described above. Template cDNA was standardized to 100 ng/reaction. Relative mRNA expression was determined using Power SYBR Green PCR Master Mix kits (Applied Biosystems) and ran on the Fast 7500 real-time PCR System. Samples were initially denatured (10 min at 95°C), followed by 40 cycles of 95°C for 15s, and an annealing and extension phase at 60°C for 1 min. mRNA expression was determined in triplicate from cDNA and normalized to the concentration of the housekeeping gene β-actin the using $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry

Fixed fetal hindlimbs were embedded in OCT Compound (Thermo-Fisher) and 8μm cross sections were cut and mounted on glass microscope slides. Slides were dried at 37°C for 30 min and then thrice washed in PBS + 0.5% Triton-X-100. Antigen retrieval was performed by boiling slides in 10 mM citric acid for 20 minutes. Non-specific binding was blocked with 0.5% NEN blocking buffer (Perkin-Elmer) at room temperature for 1 hr. Slides were then incubated overnight at 4°C with primary antibodies diluted in PBS + 1% Bovine Serum Albumin. Negative controls were incubated without primary antibody. Sections were stained with rabbit antibody against myf5 (1:100; Santa Cruz), and mouse antibodies against myoD (1:200, Dako) and myogenin (1:250, Abcam) to identify nuclei expressing these myogenic factors. Macrophage profiles were determined by co-staining for total macrophages (CD68, 1:50; Abcam) and M2 macrophages (CD163, 1:100; Abcam). All nuclei were identified by counterstaining with DAPI (1:2000, Sigma-Aldrich). Immunocomplexes were detected with Alexa Fluor 594 (1:2000; Cell Signaling) or Alexa Fluor 488 (1:1000). Staining was visualized on an Olympus IX73 and digital micrographs were captured with a DP80 microscope camera (Olympus). Images were analyzed with CellsSens Dimension software to determine proportions of positive nuclei within fetal skeletal muscle sections. Animal identifications and treatments were encoded to eliminate bias.

Statistical analysis.

All data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute) to determine treatment effects. Dam was the experimental unit. Skeletal muscle mRNA concentrations from ddPCR are expressed as copies per copy of Ywhaz. Macrophage mRNA concentrations analyzed by qPCR were normalized to β-actin and are expressed relative to the controls. All data are expressed as means ± standard error. Proportions of nuclei positive for myogenic factors and macrophage markers were determined from an average of 250 and 850 positive nuclei, respectively, counted across 18 fields of view.

RESULTS

Morphometrics and blood analysis.

The number of fetuses per litter did not differ between control and LPS-treated rats, but fetal mass was reduced ($P < 0.10$) in LPS fetuses (71.4±2.8 and 60.8±2.8, respectively). Maternal blood glucose was reduced ($P < 0.05$) in LPS-treated rats 12 hours after the first daily injection but did not differ from controls otherwise (data not shown). Maternal plasma TNFα was greater ($P < 0.05$) in LPS-treated rats six hours after the first daily injection but did not differ from controls otherwise (data not shown). Fetal blood glucose at necropsy did not differ between treatments (data not shown), but LPS rats tended to have greater ($P < 0.10$) fetal plasma TNFα than controls (0.02±0.26 and 0.83±0.29 pg/ml, respectively).

Skeletal muscle gene expression.

When measured by qPCR, CD68 mRNA expression was decreased ($P < 0.05$) in LPS fetal muscle but CD163 mRNA did not differ between treatments (Figure 1). When measured by ddPCR, CD68 mRNA concentrations tended to be decreased ($P < 0.10$) and CD163 concentrations were decreased ($P < 0.05$) in LPS fetal muscle compared to controls. TNFR1 and IL6R mRNA tended to be reduced ($P < 0.10$) in LPS fetal muscle as well (Figure 2). No differences were observed for insulin receptor or β adrenergic receptor gene expression.

Skeletal muscle immunohistochemistry.

CD68-positive nuclei/μm^2 tended to be decreased ($P < 0.10$) in LPS fetal muscle compared to controls but CD163-positive nuclei/μm^2 were not different (Figure 3). Likewise, the proportion of CD68-positive nuclei–to–CD163-postive nuclei did not differ between treatments (0.94±0.20 vs. 1.24±0.24, respectively). MyoD-positive nuclei/μm^2 were decreased ($P < 0.05$) and myogenin-positive nuclei tended to be greater ($P < 0.10$) in LPS fetal hindlimb muscle (Figure 4), but myf5-postive nuclei/μm^2 did not differ between treatments (128.4±28.5 vs. 92.0±24.1, respectively).

DISCUSSION

In the present study, we show that reduced fetal growth is a consequence of maternal inflammation at mid-gestation. This decrease in fetal mass near term appears to be the result of restricted skeletal muscle growth capacity, as reduced myoD and increased myogenin in hindlimb muscle was indicative of impaired myoblast function. Fetuses from LPS-treated dams had higher concentrations of circulating TNFα near term, which indicates that greater inflammation may be responsible for reductions in myoblast-induced fetal skeletal muscle growth. Additionally, increased circulating cytokines were accompanied by decreases in skeletal muscle TNFR and IL6R mRNA, which together with decreased myoD and increased myogenin indicate a compensatory decrease in cytokine sensitivity due to chronically high circulating inflammatory cytokines. These findings indicate that sustained maternal inflammation at mid-gestation impairs fetal skeletal muscle growth near term due to changes in myoblast responsiveness to critical cytokine regulation.

Fetal mass was decreased late in gestation following sustained maternal inflammation, likely due to decreases in skeletal muscle mass. We attribute decreased skeletal muscle growth to impaired myoblast function, as myoD was decreased and myogenin was increased in fetuses from LPS-treated dams. The absence of myoD results in impaired myoblast function, as myoblasts will continue to proliferate rather than exiting the cell cycle and fusing with fibers (Rudnicki et al., 1993). The combination of decreased myoD and increased myogenin indicates a greater percentage of differentiated myoblasts but a reduced percentage of active proliferating myoblast. This imbalance in MRF expression represents an imbalance in the myogenic cell population and, ultimately, a deficient muscle growth capacity. Additionally, maternal inflammation resulted in higher concentrations of TNFα in fetal plasma, well after treatment ended. TNFα has been shown to impede differentiation and fusion of myoblasts at high concentrations (Miller et al., 1988). High fetal inflammatory cytokines reduced TNFR and IL6R in skeletal muscle, indicating a reduced sensitivity to these important regulators of skeletal muscle. Reduced sensitivity impedes the ability of cytokines to elicit a regulatory effect on myoblasts and causes an imbalance between proliferation and differentiation by allowing precocious differentiation.

Interestingly, increased circulating TNFα concentrations and decreased skeletal muscle cytokine sensitivity was not the result of greater numbers of resident macrophages, as mRNA and protein markers showed decreased total macrophage populations, but no difference in M2 macrophage population in fetal hindlimb after maternal inflammation. However, these fetal macrophages are still producing greater amounts of inflammatory cytokines 10 days after maternal inflammation has subsided. Although it is not certain whether these inflammatory cytokines are from placental or fetal origin, the temporal spacing between induction of maternal inflammation and fetal plasma TNFα response suggest they are not of maternal origin. Moreover, since fetal TNFα is greater despite decreased macrophage number, it appears that fetal M1 macrophages have increased activity. As a compensatory action for chronic exposure to inflammatory cytokines in LPS fetuses, the major cytokine receptors were decreased in skeletal muscle. Inflammatory cytokines bind to these receptors and activate the NF-κB signaling pathway to upregulate gene expression of additional inflammatory cytokines, chemokines, and other inflammatory factors (Pahl, 1999). Activation of this pathway is responsible for the inhibitory effect of cytokines on myoblast differentiation and thus reduced sensitivity at the level of the receptor may explain the increase in myoblast differentiation and concurrent reduction in proliferating myoblast.

Fetal inflammation may be a direct response to maternal inflammation or may be indirectly caused by placental insufficiency. Maternal TNFα was acutely elevated in response to LPS treatment but did not differ from controls at day 20 of gestation when fetuses were collected, and in fact was not different on the second and third days of LPS administration. Fetal circulating TNFα was increased at term, suggesting fetal inflammation is most likely due to placental insufficiency. Additionally, previous studies found that inflammatory cytokines do not typically pass the placental barrier (Aaltonen et al., 2005), indicating that all cytokines within the fetus and amniotic fluid are of conceptus origin.

In conclusion, maternal inflammation at mid-gestation results in decreased fetal mass due to impaired myogenesis that is still apparent at term. We demonstrate that impaired myogenesis is due to myoblast dysfunction as evident by decreased myoD and increased myogenin. Moreover, myoblast dysfunction is the likely result of increased inflammatory cytokines and the resultant decreased sensitivity to these cytokines. The inflammatory response may be due to amplified activity of M1 macrophages, as

macrophage number was actually decreased in fetal skeletal muscle after maternal inflammation. Together, our findings show that maternal inflammation induces fetal adaptive responses that interrupt myoblast regulation, causing myoblast dysfunction and impaired skeletal muscle development and growth.

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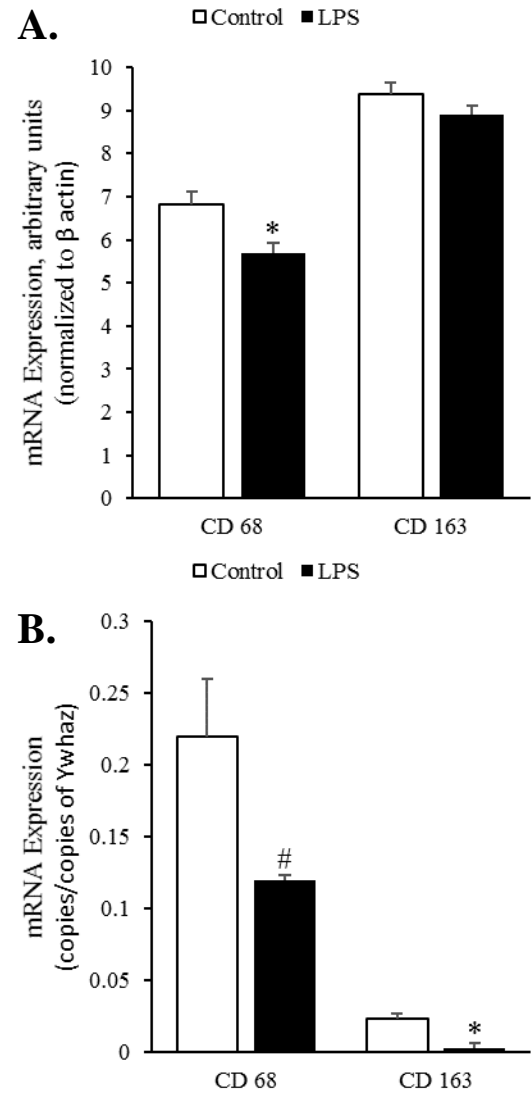
Figure 1. Gene expression analysis for total and M2 macrophage markers in fetal hindlimb as measured by qPCR (A) and ddPCR (B) * means differed ($P < 0.05$) between control and LPS fetuses. # means tended to differ ($P < 0.10$) between control and LPS fetuses.
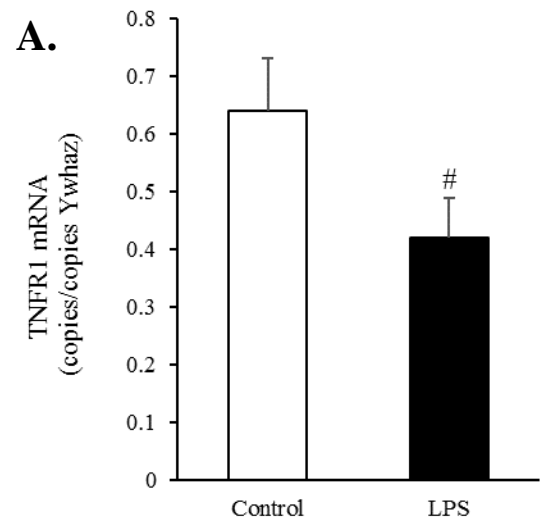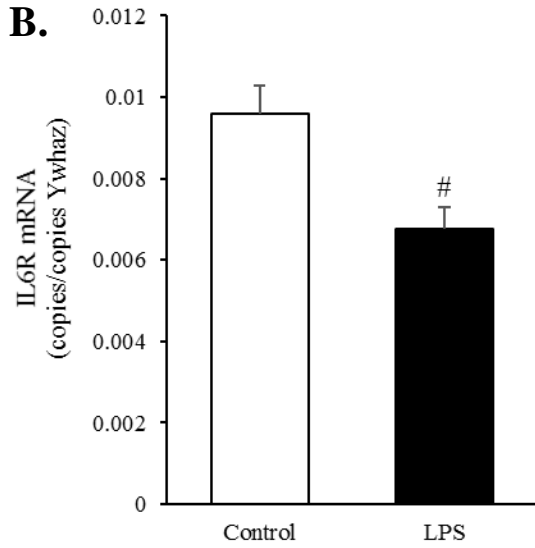
Figure 2. Gene expression analysis (ddPCR) of TNFR1 (A) and IL6R (B) in fetal hindlimb after maternal inflammation. # means tended to differ ($P < 0.10$) between control and LPS fetuses.
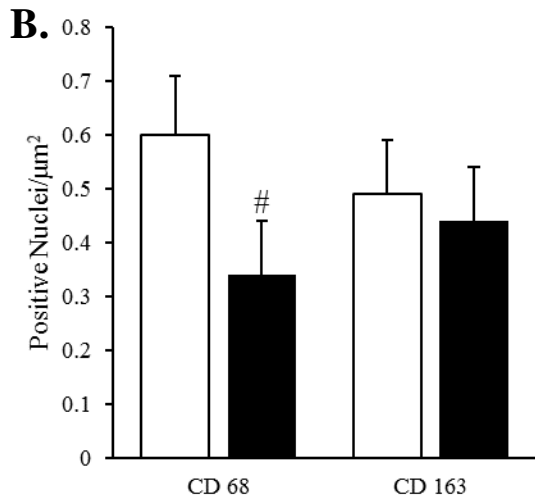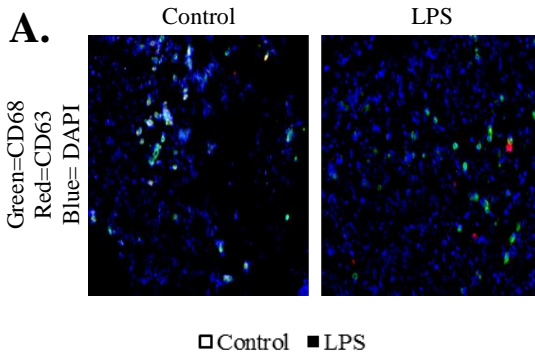
Figure 3. A. Immunostaining of markers for total (CD68) and M2 (CD163) macrophages in cross-sections of fetal hindlimb muscle after maternal inflammation. A. Representative micrographs are depicted for control and LPS fetal hindlimb cross sections (8μm). Sections were co-stained for total (green) and M2 (red) macrophages and counterstained with DAPI (blue). B. Analysis of positive total and M2 macrophages. # means differed ($P < 0.10$) between control and LPS fetuses.
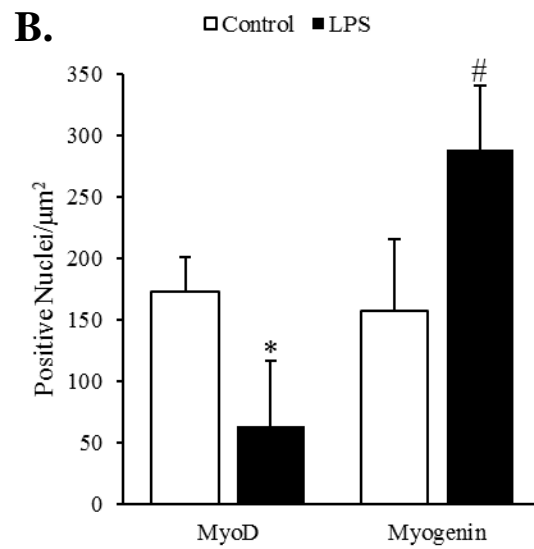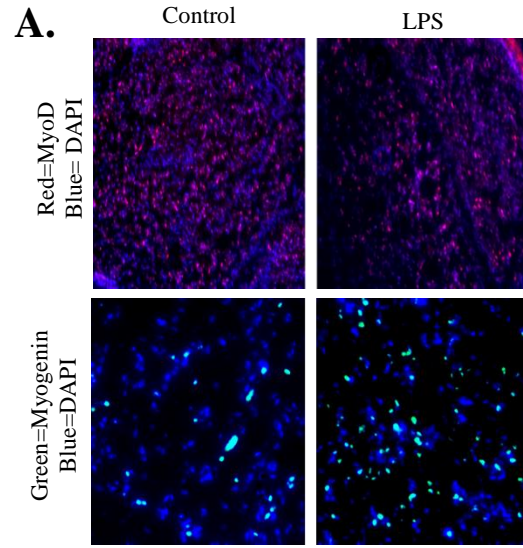
Figure 4. A. Immunostaining of myoD and myogenin in fetal hindlimb muscles after maternal inflammation. Representative micrographs are depicted for control and LPS fetal hindlimb cross sections (8 μm). Sections were stained for myoD (red) or myogenin (green) and counterstained with DAPI (blue). B. Analysis of myoD and myogenin positive nuclei. * means differed ($P < 0.05$) between control and LPS fetuses. # means differed ($P < 0.10$) between control and LPS fetuses.