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Maternal growth restriction and stress exposure in rats differentially alters expression of components of the placental glucocorticoid barrier and nutrient transporters

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ABSTRACT

The placenta plays a major role in the development of fetal growth restriction, which affects 10% of pregnancies and contributes to chronic adult disease risk. We have reported that female rats born small develop cardiometabolic dysfunction only during pregnancy. The physiological tests performed during pregnancy induced a maternal stress response as indicated by increased maternal corticosterone concentrations. This stress effected placental growth compared to females who were unhandled during pregnancy. Maternal stress and growth restriction independently program F2 offspring metabolic dysfunction. This study investigated the effects of maternal stress and growth restriction on placental and fetal metabolic parameters that may contribute to F2 offspring metabolic disease. Maternal growth restriction reduced F2 fetal weight whilst maternal stress reduced placental weight. Stressed mothers had reduced insulin and increased glucose concentrations, changes that were reflected in the fetus. Fetal β-cell number was reduced by maternal growth restriction, but was increased by stress exposure. Maternal growth restriction reduced placental *Slc2a1, Igf2, Slc38a2* and *Nr3c1* gene expression. Maternal stress decreased the expression of *Slc2a1, Igf2, Slc38a2, Nr3c1, Slc2a3, Slc2a4, Nr3c2, Hsd11b2, Crhr1* and *Ogt*. Maternal birth weight effects on fetal weight were likely due to changes in placental nutrient transporter and *Igf2* expression. On the contrary, maternal stress induced a systemic effect by altering maternal metabolic parameters, placental gene expression and fetal glucose and insulin concentrations. This study highlights the importance of informing pregnant women on effective ways to cope with stress during pregnancy to prevent adverse long-term disease outcomes in their children.

1. Introduction

Fetal growth restriction complicates 10% of pregnancies and is a major risk factor for the transgenerational programming of cardiometabolic disease. Uteroplacental insufficiency is the primary cause of fetal growth restriction in Western society and is often due to insufficient blood flow to the fetal-placental unit or impaired placental function. Key placental functions include regulating nutrient transport and production of growth factors including insulin-like growth factors (IGF1 and 2), which mediates placental and fetal growth. Epidemiological and experimental studies have implicated dysregulation of these systems in mediating fetal growth restriction and impairing fetal organ development, resulting in programmed disease [1–3]. Glucose and amino acids are the primary energy substrates utilized by the developing fetus and are transported across the placenta by glucose (Slc2a1, Slc2a3 and Slc2a4) and amino acid transporters (Slc38a1), which are also modulated by the IGF-system.

Stress is inevitable throughout life, making it common for women to experience stress during pregnancy. Maternal stress dysregulates placental nutrient transport and growth factor production, which can reduce fetal growth [4,5]. Stress elicits these effects through the production of glucocorticoids, such as cortisol

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(humans) and corticosterone (rats). Glucocorticoids bind to the glucocorticoid (Nr3c1) and mineralocorticoid receptors (Nr3c2) to regulate a number of genes including those involved with nutrient transport and cellular growth. Given their vital role in maintaining a healthy pregnancy [6], the placenta metabolizes excess glucocorticoids by the actions of 11 beta-hydroxysteroid dehydrogenase type 2 (Hsd11β2) [7]; although this "glucocorticoid barrier" can be saturated at high glucocorticoid concentrations. Previous studies have demonstrated that growth restricted fetuses have an impaired placental "glucocorticoid barrier" [8], altered IGF concentrations [9] and dysregulated expression of amino acid [10] and glucose transporters [11]. However, no studies to date have investigated if these changes are also apparent in placenta associated with the next generation (F2).

We have previously demonstrated that F1 growth restricted females only develop disease during pregnancy [12]. Specifically, growth restricted females developed glucose intolerance and glomerular hypertrophy during pregnancy and F2 birth weight was reduced [12], suggesting that growth restriction may be passed onto the next generation. However, F2 growth restriction only occurred when F1 dams were exposed to a range of physiological protocols (blood pressure measurements, glucose tolerance testing and 24 h metabolic cage) compared to an unhandled cohort [12,13]. Subsequent analysis of maternal plasma corticosterone concentrations demonstrated that these physiological measurements induced a stress response and this was associated with F2 growth restriction [13]. We have recently demonstrated that maternal growth restriction and maternal stress independently program adult F2 metabolic outcomes [14]. However, no studies to date have investigated the placental mechanisms that may explain these findings. Therefore, the aim of this study was to characterize the effects of maternal growth restriction and stress on the placental nutrient transport system, IGF system, glucocorticoid activity regulators and fetal metabolic parameters that may contribute to the adult F2 metabolic dysfunction.

2. Methods

2.1. Animal procedures

All experiments were approved by The University of Melbourne's animal experimentation ethics sub-committee (AEC: 0911289) following the code for the care and use of animals for scientific purposes from the National Health and Medical Research Council (NHMRC) of Australia. Female Wistar Kyoto rats (9–13 weeks) were obtained from the Animal Resource Centre (Canning Vale, WA, Australia) and were maintained under a 12-hr light/dark cycle at constant temperature (19–22 °C) with *ad libitum* access to food and water. Rats were mated and surgery was performed on day 18 of gestation (E18; term = 22 days) [15]. Briefly, F0 pregnant rats were anaesthetized and uteroplacental insufficiency was induced by bilateral uterine vessel (artery and vein) ligation (offspring termed Restricted). Sham surgery was performed with uterine vessels not ligated (offspring termed Control). Dams delivered first generation (F1) offspring naturally at term.

At 18 weeks, F1 Control and Restricted females were mated with a normal male and allocated to undergo a Stressed or Unstressed pregnancy as described previously [12,13]. Briefly, Stressed dams were exposed to physiological stressors including tail-cuff blood pressure, a non-fasted intraperitoneal glucose tolerance test (E18) and placed in a metabolic cage for 24 h (E19). Unstressed counterparts were not exposed to physiological measurements and were unhandled apart from routine animal husbandry purposes. On E20, dams were anaesthetized (Ketamine-50 mg/kg; Ilium Xylazil-10 mg/kg), F2 fetuses removed and dams euthanized and blood collected. Fetuses were weighed (high precision laboratory scale to 1 mg accuracy) and had dimensions measured (horizontal position with calipers to 0.01 mm accuracy) before being decapitated for blood collection (pooled in litters). Placentae (separated into labyrinth and junctional zones), fetal hearts, lungs, kidneys, livers and brains were weighed, snap frozen and stored at −80 °C. Fetal pancreata were rapidly excised and immediately fixed in 10% NBF. For tissue analysis, one male and one female sample was used from each litter, with each sample representing a single animal (i.e. n = 1). To ensure accurate sex was recorded, DNA was extracted from the placental labyrinth and qPCR used to measure the presence/absence of the sex-determining region Y (SRY) gene using a commercially available TaqMan probe (Rn04224592_u1; NM_012772.1) (Life Technologies), as described previously [4].

2.2. Quantitative PCR

RNA was extracted from placental labyrinth tissue using the miRNeasy mini kit (QIAGEN) and treated with DNase (QIAGEN). cDNA was generated from 1 μg of RNA using the RT^2 HT First Strand Kit (QIAGEN) and qPCR was conducted using SYBR green, as described previously [13]. Custom RT^2 Profiler PCR Arrays (QIAGEN) were used to analyze the expression of genes involved in glucocorticoid/stress signaling, nutrient transport and placental growth (Table 1). Additional primers were custom designed (*Slc2a4*, *Actb* and *Gapdh*) (Table 1). All data were normalized to the geometric mean of housekeeping genes (*Actb, Gapdh, Sdha* and *Tbp*). Relative changes in mRNA abundance were quantified using the $2^{-\Delta\Delta CT}$ method and reported in arbitrary units normalized to the Unstressed Control group. Statistical analysis determined that the housekeepers were not affected by Treatment or Stress.

2.3. Fetal pancreatic immunohistochemistry

Fixed fetal pancreata were processed into paraffin blocks and sectioned at 5 μm (n = 8–9/group). Five sections of equal distance apart were immunostained for insulin and random systematic point counting was used to determine the proportion of β-cells and islets per pancreas. As fetal pancreata are too small to accurately weigh, β-cell and islet masses were not calculated [16,17].

2.4. Plasma analysis

Non-fasted maternal and fetal plasma at *post mortem* were analyzed for glucose using enzymatic fluorometric analysis and insulin using a rat insulin radioimmunoassay kit (Merck Millipore; Bayswater, VIC, Australia) as previously described [18,19].

2.5. Statistical analysis

Maternal body weights and glucose and insulin concentrations at *post mortem* were analyzed using a two-way ANOVA with Treatment and Stress as factors. Fetal and placental weights and dimensions, glucose and insulin concentrations, placental gene expression and fetal β-cell/islet number were initially analyzed using a three-way ANOVA with Treatment, Sex and Stress as factors. As Sex had minimal effects on any outcomes in this model, data was pooled and reanalyzed with a two-way ANOVA with Treatment and Stress as factors. If an interaction was present, a Student's t-test was used to determine where the significance lies. If no interaction was present, but there was a main effect for either Treatment or Stress, this was used to identify the main differences in each parameter. ANOVA and t-tests were performed using SPSS Statistics 22 (IBM; St Leonards, NSW, Australia). All data are presented as mean ± SEM and a $P < 0.05$ was assessed as being statistically significant.