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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

Jessica F Briffa[1#], Sogand S Hosseini[1#], Melanie Tran[1], Karen M Moritz[2] James SM Cuffe[2,3*] and Mary E Wlodek[1*]

[1]Department of Physiology, The University of Melbourne, Victoria 3010, Australia

[2]School of Biomedical Sciences, The University of Queensland, St. Lucia, QLD, 4072, Australia

[3]School of Medical Science, Menzies Health Institute Queensland, Griffith University, Southport, QLD, 4222, Australia

[#]Co-first authorship

[*]Co-senior authorship

To whom correspondence should be address: Professor Mary Wlodek, Department of Physiology, The University of Melbourne, Parkville, VIC 3010, Australia. Phone: 61 3 9035 9923 Fax: 61 3 8344 5818 email: m.wlodek@unimelb.edu.au

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.

Keywords – growth restriction, stress, placenta, glucose transporter, fetal programming

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 (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 hr 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 programmed 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.

Methods

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 hrs (E19). Unstressed counterparts were not exposed to physiological measurements and were unhandled apart from routine animal husbandry purposes. On E20, dams were anesthetized (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].

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.

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].

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].

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 Leondards, NSW, Australia). All data are presented as mean ± SEM and a $P < 0.05$ was assessed as being statistically significant.

Results

Maternal characteristics

We have previously demonstrated that uteroplacental insufficiency surgery reduced F1 litter size and induced growth restriction [12, 13]. F1 Restricted offspring remained smaller throughout lactation (PN35), but caught up to Controls by mating [12, 13]. F1 Control and Restricted females were of a similar body weight during pregnancy on E20, and maternal weight was not affected by Stress [12, 13].

Effect of sex on F2 parameters.

Initial analysis of F2 fetal and placental weights, and gene expression demonstrated minimal sex effects across the groups. Specifically, fetal and placental weights were reduced in females compared to males, whereas relative kidney weight was increased in females compared to males regardless of maternal birth weight or stress (data not shown). Irrespective of maternal birth weight and stress, females had marginally lower gene expression of *Hsd11b2*, *Igf2* and *Slc2a1* (Control only) compared to males (data not shown). However, given the small magnitude of change this is unlikely to be of biological significance.

F2 body and organ weights

F2 fetal weights and crown-rump lengths were reduced in fetuses of F1 Restricted mothers, irrespective of Stress (Table 2). Maternal stress increased F2 fetal head width,

irrespective of maternal birth weight (Table 2). F2 litter size was unaffected by maternal birth weight and stress (Table 2). Placental weight of F2 fetuses was reduced if their mother was Stressed, which resulted in an increased fetal-to-placental weight ratio (Table 2). Relative F2 heart and liver weights were reduced in fetuses whose mother was Stressed, with no changes in relative lung, kidney and brain weights (Table 2).

Maternal and fetal metabolic parameters

Stressed mothers tended to have reduced plasma insulin (Figure 1A; $P = 0.057$) but had increased non-fasted plasma glucose concentrations (Figure 1B). Similarly, F2 fetuses from Stressed mothers had reduced plasma insulin and increased plasma glucose concentrations (Figures 1C and 1D). F2 fetuses from Restricted mothers had reduced proportions of β-cells and islets per pancreas (Figures 1E and 1F). Regardless of maternal birth weight, maternal stress increased the proportion of β-cells per pancreas compared to Unstressed counterparts (Figure 1E).

Placental stress genes

F2 litters from Restricted mothers had reduced placental *Nr3c1* and *Hsd11b1* expression (Figures 2A and 2C). Additionally, *Nr3c1* was reduced in placentae from Stressed mothers (Figure 2A). Independent of maternal birth weight, stress reduced placental *Nr3c2*, *Hsd11b2*, Corticotropin releasing hormone receptor 1 (*Crhr1*) and O-linked N-acetylglucosamine (GlcNAc) transferase (*Ogt*) expression (Figures 2B and 2D-F).

Placental nutrient transporter genes

Igf1 expression was not altered by maternal birth weight or stress (Figure 3A). F2 litters from Restricted mothers had reduced placental *Igf2* and *Slc38a2* expression, which were further reduced if their mother was Stressed (Figures 3B and 3F). Placental *Slc2a1* gene expression was reduced in F2 litters whose mothers were Restricted and Unstressed. Maternal stress additionally reduced *Slc2a1* (Figure 3C), *Slc2a3* and *Slc2a4* expression (Figures 3D and 3E).

Discussion

We have previously demonstrated that maternal growth restriction and stress independently program age-specific F2 metabolic disease [14]. This study has, for the first time, investigated the placental adaptations that may regulate these programming outcomes.

We demonstrated that F2 fetuses from F1 growth restricted mothers were smaller at E20, which was not exacerbated by maternal stress. In contrast, maternal stress reduced placental weight and increased the fetal-to-placental weight ratio. The reduction in F2 fetal weight following maternal growth restriction and the reduced placental weight associated with maternal stress were likely mediated by different pathways. Specifically, the maternal birth weight effects were likely due to changes in placental nutrient transporters and *Igf2* expression. Whereas, maternal stress induced a systemic effect by altering maternal corticosterone [11], glucose and insulin concentrations, placental gene expression and fetal glucose and insulin concentrations.

Metabolic health

The maternal stress induced increase in maternal corticosterone [13] likely contributed to the increased maternal plasma glucose and reduced insulin concentrations currently reported. Chronic stress reduces glucose stimulated insulin secretion [20], which is mediated by corticosterone [21]. In the current model, the stress induced changes in corticosterone, glucose and insulin all likely contribute to the placental adaptations observed. Glucose can regulate transcription [22] of glucose transporters [23] and IGF-1 [24] and affect post translational protein modifications through O-GlcNAcylation [25]. Insulin may similarly regulate placental adaptations and interact with glucocorticoids or oxygen to alter placental growth [26]. Interestingly, in the current study, fetal glucose and insulin concentrations reflected maternal concentrations, suggesting that the placental adaptations had little impact on transplacental glucose transport. Insulin is largely unable to pass through the placenta [27], as such it is likely that adaptations in fetal insulin concentrations and β-cell number are mediated by maternal glucocorticoids [20]. These stress induced changes in fetal metabolic health likely contributed to the adult disease phenotypes previously reported [13]. Studies have shown that pancreatic islets from IUGR fetuses have impaired glucose-induced regulation of insulin biosynthesis [28]. We now demonstrate that β-cell number and islets per pancreas are reduced in growth restricted fetuses from mothers born small. The mechanisms that contribute to this outcome are unclear, but may be a consequence of impaired growth over the entirety of pregnancy as opposed to an event that occurs towards term.

Placental stress system

Maternal stress and being born small independently effected genes involved in placental stress-responsiveness and glucocorticoid metabolism. A reduction in *Nr3c1* and *Hsd11b1* in

response to maternal growth restriction is likely a beneficial adaptation to minimize the impact of stress on placental function. Unsurprisingly, stress altered the expression of 5 of the 6 stress-responsive genes, including *Nr3c1* and *Nr3c2*. The stress induced changes reported are similar to our model of maternal hypoxia in mice (E14.5-E18.5) where corticosterone concentrations were increased at E18.5 and placental *Nr3c1* and *Nr3c2* were reduced [5]. Interestingly, these results are in contrast to our model of corticosterone administration to pregnant mice (E12.5-E15; term = 19 days), which upregulates *Nr3c1* without affecting *Nr3c2* [4]. These differences are likely due to intermittently increased corticosterone concentrations following maternal stress compared to daily administration of corticosterone [4]. Interestingly, *Hsd11b1* was unaffected by stress while *Hsd11b2* was reduced, a finding again similar to our hypoxia model [5]. If this reduction in *Hsd11b2 mRNA* translates to a functional change in enzyme activity, glucocorticoids may readily pass to the fetus impairing fetal growth and development. Ogt is a marker of cellular stress [29] with the direction of change dependent on the type of stress experienced. In mice, corticosterone administration increases OGT [30], whereas chronic variable stress in mice reduces it [29]. It is unsurprising that *Ogt* is reduced in our model, given the reduction in many other components of the stress pathway. Many stress-related genes regulate nutrient transport and cellular uptake in trophoblast cells [31], which can affect placental and/or fetal growth. Specifically, CRHR1 has been shown to regulate placental glucose transporters [32]. Therefore, the reduction in *Crhr1* by maternal stress may contribute to the reduced placental growth by altering glucose uptake. These changes in placental gene expression may implicate stress-responsive pathways in offspring disease, particularly the programmed adrenal adaptations previously reported [14].

Placental nutrient transport

A number of nutrient transporters and growth factors were reduced in placentae of fetuses from mothers born small and in dams exposed to stress. While reduced placental *Igf2* in growth restricted dams may contribute to the reduced fetal weight, the reduction in *Igf2* following maternal stress may contribute to the reduced placental weight. Placental IGF2 is secreted into the fetal circulation where it can contribute to β-cell development and function [33]. Indeed, research has demonstrated that fetal hyperglycemia can impair IGF signaling in pancreatic islets, leading to transgenerational glucose intolerance [33]. Placental IGF2 production can be regulated by insulin and glucocorticoids, and a reduction in placentally-derived IGF2 reduces passive nutrient transport and dysregulates amino acid and glucose

transporters [34]. *Slc2a1* was reduced by both maternal growth restriction and stress, a finding similarly observed in chronic stress [35] and maternal hypoxia [5], suggesting impaired placental glucose transport. In the current study, neither *Slc2a3* nor *Slc2a4* were effected by maternal growth restriction, but both were reduced by maternal stress. This stress-induced decrease in glucose transporters are likely a placental adaptation to the increased maternal and fetal glucose concentrations in attempt to prevent the glucose-induced alterations in β-cell number and fetal insulin concentrations.

Sex-specific differences

Fetal sex had only minimal effects on placental gene expression, with no effects on other parameters measured, which contrasts with other studies in similar models [5, 36, 37]. Furthermore, it was expected that sex-specific placental adaptations may contribute to the sexually dimorphic disease outcomes in F2 offspring [14]. We suggest that the relatively large effects induced by maternal growth restriction and stress may outweigh any subtle impact of fetal sex on placental outcomes.

Conclusion

This study demonstrates the placental adaptations in response to maternal growth restriction and stress, which likely precede the programmed F2 metabolic deficits. Mothers born small have fetuses with reduced placental *Igf2*, *Slc2a1* and *Slc38a2* expression that may contribute to reduced fetal size. Maternal stress did not exacerbate these effects, but independently altered maternal and fetal metabolic function, decreased placental size and altered the expression of nutrient transport, fetal growth and stress genes. It is likely that the stress-induced changes to glucose and insulin concentrations contributed to the placental and fetal outcomes, highlighting the multifaceted impact of maternal stress. Therefore, providing women with better ways to cope with stress during pregnancy is imperative to prevent the long-term disease risk for both the mother and child.

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Figure Legends

Figure 1. The effect fetal growth restriction and maternal stress during pregnancy has on maternal and second generation (F2) metabolic function on embryonic day 20 (n = 6-9 per group, with n = 1 representing 1 litter). Maternal plasma insulin (A) and glucose (B), fetal plasma insulin (C) and glucose (D). Proportions of β-cells (E) and islets (F) in the fetal pancreas in Unstressed (left pane) and Stressed (right pane) litters. Data were analyzed with a two-way ANOVA to determine differences between Treatment and Stress, and are expressed as mean ± SEM, where ns is not significant. Control litters denoted by white open bars; Restricted litters denoted by black closed bars.

Figure 2. The effect fetal growth restriction and maternal stress during pregnancy has on second generation (F2) placental labyrinth stress genes on embryonic day 20 (n = 5-7 per group, with n = 1 representing 1 litter). Placental labyrinth gene expression of *Nr3c1* (A), *Nr3c2* (B), *Hsd11b1* (C), *Hsd11b2* (D), *Crhr1* (E) and *Ogt* (F) in Unstressed (left pane) and Stressed (right pane) litters. Data were analyzed with a two-way ANOVA to determine differences between Treatment and Stress, and are expressed as mean ± SEM, where ns is not significant. Control litters denoted by white open bars; Restricted litters denoted by black closed bars.

Figure 3. The effect fetal growth restriction and maternal stress during pregnancy has on second generation (F2) placental labyrinth nutrient transporter genes on embryonic day 20 (n = 5-7 per group, with n = 1 representing 1 litter). Placental labyrinth gene expression of *Igf1* (A), *Igf2* (B), *Slc2a1* (Glut1; C), *Slc2a3* (Glut3; D), *Slc2a4* (Glut4; E) and *Slc38a2* (F) in Unstressed (left pane) and Stressed (right pane) litters. Data were analyzed with a two-way ANOVA to determine differences between Treatment and Stress, and are expressed as mean ± SEM, where ns is not significant. Significant differences between Treatment groups are indicated by an asterisk (*$P < 0.05$) and differences between maternal stresses are indicated by a hashtag (#$P < 0.05$). Control litters denoted by white open bars; Restricted litters denoted by black closed bar.

Table 1. 'Real-time' PCR gene targets, accession numbers and primer sequences (custom made primers). Succinate dehydrogenase complex, subunit A (*SDHA*), TATA box binding protein (*TBP*) and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

Gene	Accession Number	Forward Primer	Reverse Primer
Nr3c1	NM_012576		
Nr3c2	NM_013131		
Hsd11b1	NM_017080		
Hsd11b2	NM_017081		
Ogt	NM_107107		
Crhr1	NM_030999		
Igf1	NM_178866		
Igf2	NN_031511		
Slc38a2	NM_181090		
Slc2a1	NM_138827		
Slc2a3	NM_017102		
Slc2a4	NM_012751.1	AGTTGGAAAGAGAGCGTCCACTGT	GCTGCAGCACCACTGCAATAATCA
Sdha	NM_130428		
Tbp	NM_001004198		
Actb	NM_031144.3	TCATGAAGTGTGACGTTGACATCCGTAAAG	CCTAGAAGCATTTGCGGTGCACGATGGAGG
Gapdh	NM_017008.3	AGTTCAACGGCACAGTCAAG	GTGGTGAAGACGCCAGTAGA

Table 2. Second generation (F2) litter averages for fetal dimensions, placental weight and relative organ weights on embryonic day (E) 20. Data were analyzed with a two-way ANOVA to determine differences between Treatment and Stress, and are expressed as mean ± SEM, where ns is not significant with n = 9 - 11 litters/group.

	Unstressed		Stressed		Two-way ANOVA		
	Control	**Restricted**	**Control**	**Restricted**	**Treatment**	**Stress**	**Interaction**
F2 Litter Size	11.1±0.6	9.1±0.8	10.2±0.5	10.6±0.8	ns	ns	ns
Fetal and Placental Dimensions							
Fetal weight (g)	1.864±0.025	1.848±0.022	1.889±0.036	1.792±0.022	*P* = 0.050	ns	ns
Crown-rump length (mm)	27.70±0.29	27.18±0.28	27.50±0.22	26.64±0.16	*P* = 0.008	ns	ns
Head length (mm)	9.90±0.08	9.72±0.09	10.16±0.13	9.94±0.11	ns	*P* = 0.034	ns
Head width (mm)	6.72±0.05	6.65±0.06	6.65±0.05	6.59±0.05	ns	ns	ns
Placental weight (g)	0.315±0.004	0.323±0.004	0.282±0.006	0.271±0.007	ns	*P* = 0.0001	ns
Fetal-to-placental weight (ratio)	6.036±0.171	5.761±0.077	6.769±0.142	6.712±0.189	ns	*P* = 0.0001	ns
Relative Organ Weights (%)							
Heart	0.523±0.013	0.523±0.010	0.489±0.023	0.488±0.018	ns	*P* = 0.045	ns
Lung	2.629±0.048	2.485±0.069	2.490±0.044	2.527±0.063	ns	ns	ns
Kidney	0.764±0.016	0.830±0.013	0.762±0.026	0.748±0.039	ns	ns	ns
Liver	7.545±0.099	7.708±0.071	6.959±0.118	6.868±0.069	ns	*P* = 0.0001	ns
Brain	5.804±0.097	5.730±0.080	5.676±0.099	5.547±0.121	ns	ns	ns

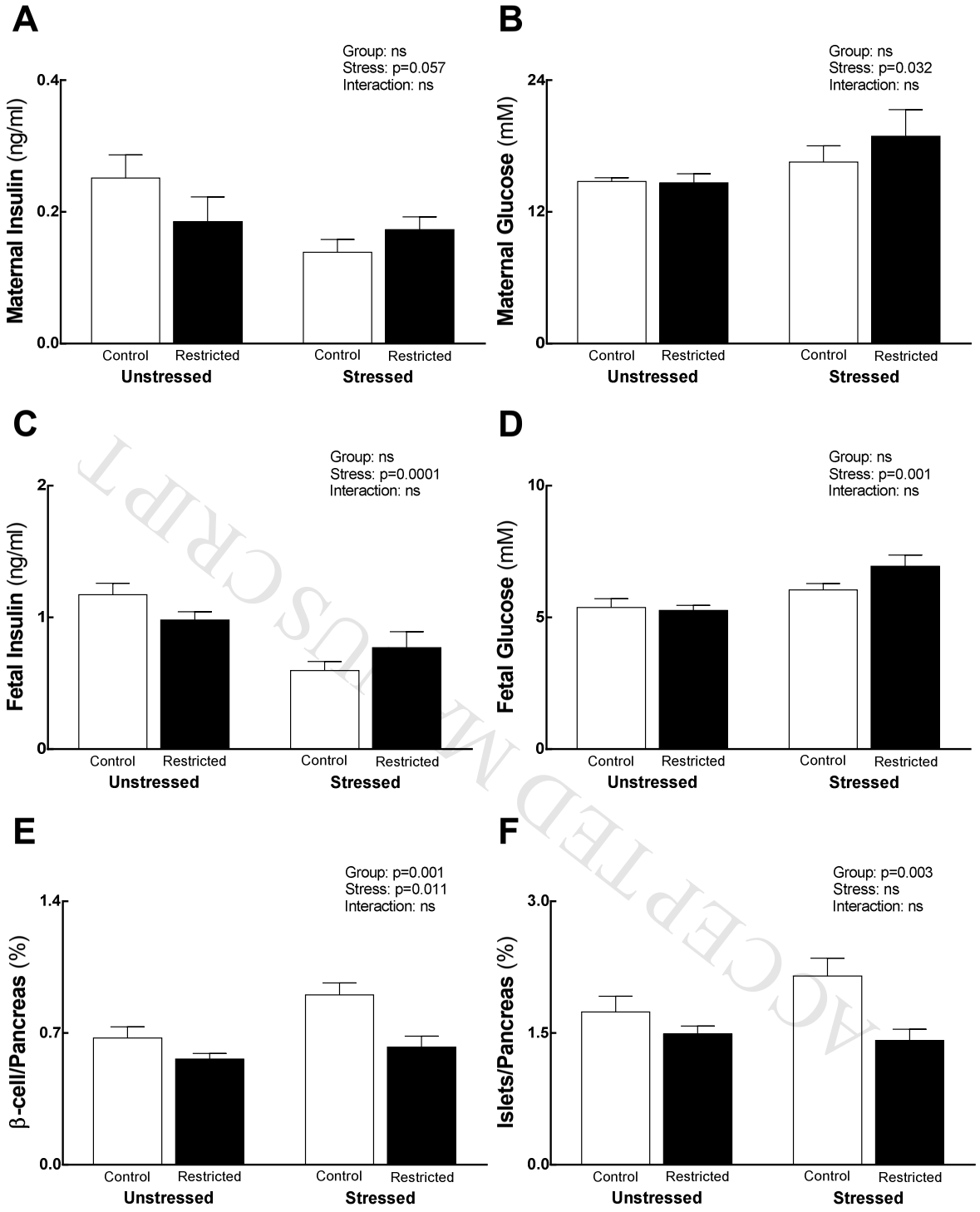

Figure 1

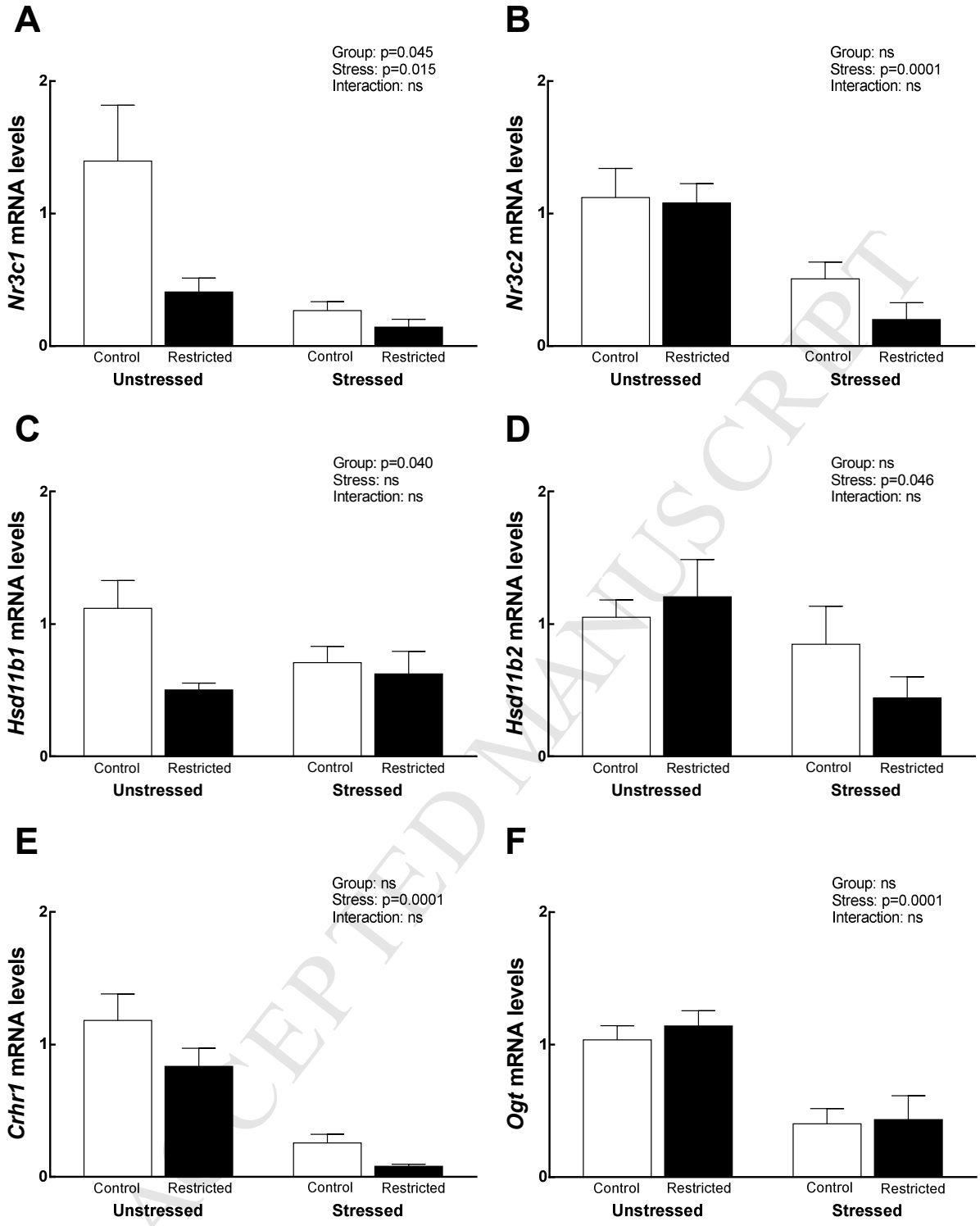

Figure 2

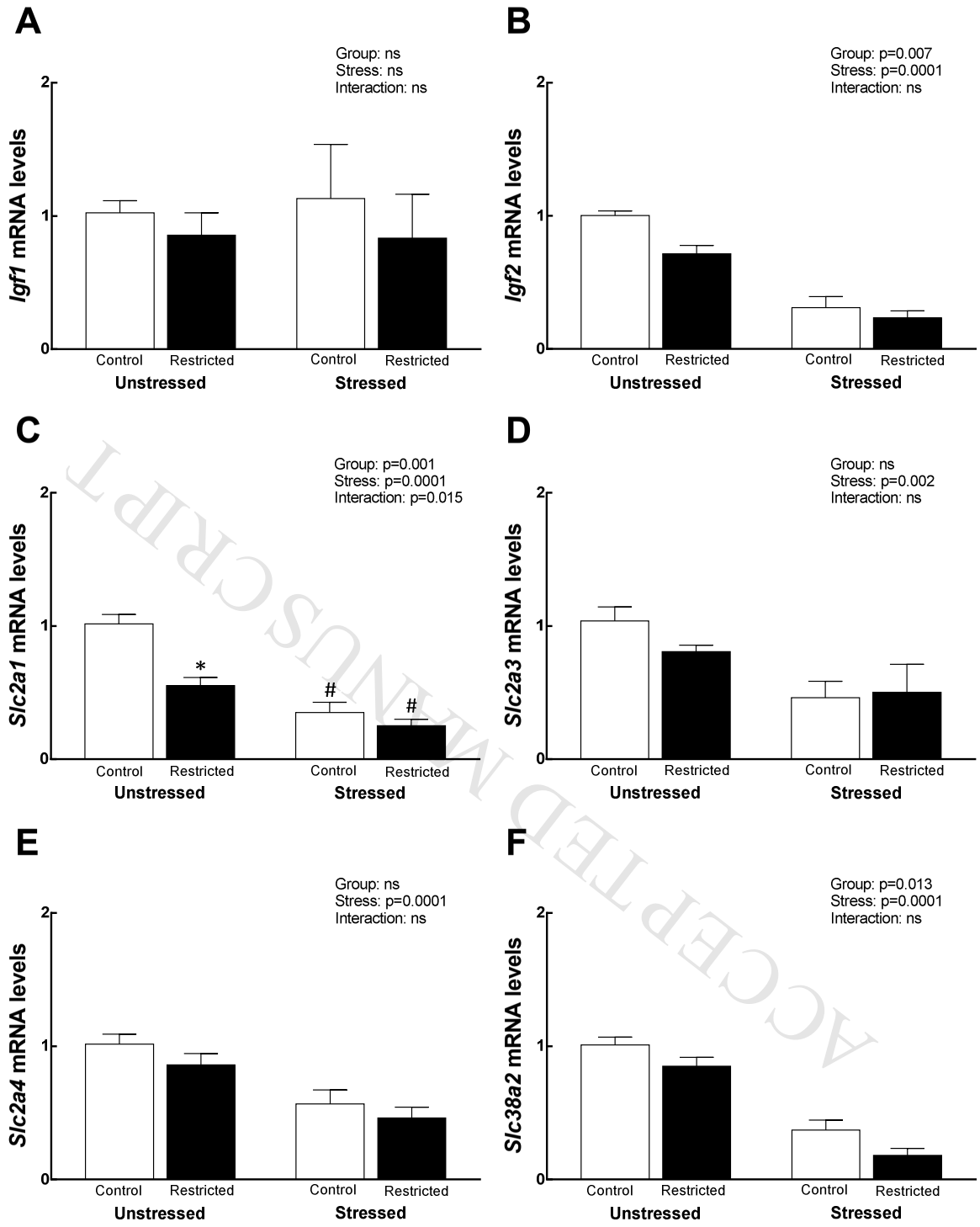

Figure 3

Highlights

- Uteroplacental insufficiency causes fetal growth restriction in the next generation.
- Stress alters maternal and fetal metabolic status and reduces placental growth.
- Fetal and placental outcomes are not exacerbated in stressed growth restricted dams.
- Growth restriction and stress independently alter placental nutrient transporter genes.

Competing Interests

The authors declare no conflicts of interest.