CORTICOSTERONE ALTERS MATERNO-FETAL GLUCOSE PARTITIONING AND INSULIN SIGNALLING IN PREGNANT MICE

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KEY POINTS SUMMARY

- Glucocorticoids regulate fetal and adult glucose metabolism, in part by influencing the actions of insulin. However, their effects on materno-fetal glucose partitioning remain largely unknown.
- In this study, when pregnant mice were given the natural glucocorticoid, corticosterone, plasma insulin concentrations and liver insulin-signalling increased but blood glucose concentration was normal.
- However, in the placenta, glucose transport was reduced in association with lower activity of some insulin signalling proteins, depending on the day of pregnancy and maternal food intake.
- In both liver and placenta, there was increased expression of the *Redd1* (*Ddit4*) gene when plasma corticosterone was raised.
- The results show that maternal glucocorticoids interact with signalling pathways in the placenta to limit materno-fetal glucose partitioning.

ABSTRACT

Glucocorticoids affect glucose metabolism in adults and fetuses, but their effects on materno-fetal glucose partitioning remain unknown. This study measured maternal hepatic glucose handling and placental glucose transport together with insulin signalling in these tissues in mice drinking corticosterone either from day (D) 11 to D16 or D14 to D19 of pregnancy (term=D21). On the final day of administration, corticosterone-treated mice were hyperinsulinaemic ($P<0.05$) but normoglycaemic compared to untreated controls. In maternal liver, there was no change in glycogen content or glucose-6-phosphatase activity but increased *Slc2a2* glucose transporter expression in corticosterone-treated mice, on D16 only ($P<0.05$). On D19, but not D16, transplacental ^{3}H-methyl-D-glucose clearance was reduced by 33% in corticosterone-treated dams ($P<0.05$). However, when corticosterone-treated animals were pair-fed to control intake, to prevent the corticosterone-induced increase in food consumption, ^{3}H-methyl-D-glucose clearance was similar to the controls. Depending upon gestational age, corticosterone treatment increased phosphorylation of the insulin-signalling proteins Akt and glycogen synthase-kinase 3β in maternal liver ($P<0.05$) but not placenta ($P>0.05$). Insulin receptor and insulin-like growth factor type I receptor abundance did not differ with treatment in either tissue. Corticosterone upregulated the stress-inducible mechanistic target of rapamycin (mTOR) suppressor, *Redd1* in liver (D16 and D19) and placenta (D19), in *ad libitum* fed animals ($P<0.05$). Concomitantly, hepatic protein content and placental weight were reduced on D19 ($P<0.05$), in association with altered abundance and/or phosphorylation of signalling proteins downstream of mTOR. Taken together, the data indicate that maternal glucocorticoid excess reduces fetal growth partially by altering placental glucose transport and mTOR signalling.

ABBREVIATIONS

4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; D, day of pregnancy; GLUT, glucose transporter; GSK-3β, glycogen synthase kinase 3β; IGF1R, insulin-like growth factor type I receptor; IR, insulin receptor; mTOR, mechanistic target of rapamycin; p70 S6 kinase, p70 ribosomal protein S6 kinase; PF, pair-fed

INTRODUCTION

Glucocorticoids have an important role in regulating metabolism in adult animals (McMahon *et al.*, 1988). Glucocorticoids in the fetal circulation also affect fetal glucose metabolism, particularly glycogen deposition and mobilization in fetal tissues during late gestation (Fowden & Forhead, 2011). However, whether maternal glucocorticoid concentrations influence the materno-fetal supply of glucose remains unknown. Glucose is supplied to the fetus by facilitated diffusion across the placenta, down the materno-fetal glucose concentration gradient (Battaglia & Meschia, 1988). As the glucose requirement of the growing fetus increases through pregnancy, alterations in the gradient and placental transport capacity ensure that the glucose supply meets this demand (Molina *et al.*, 1991; Sakata *et al.*, 1995; Yamaguchi *et al.*, 1996; Ehrhardt & Bell, 1997). Glucocorticoids are well placed to influence materno-fetal glucose supply as their bioavailability changes throughout pregnancy with increasing maternal and fetal adrenal secretion and tissue-specific changes in activity of the 11β hydroxysteroid dehydrogenases, which increase (type I) or decrease (type II) the local availability of active glucocorticoids (Barlow *et al.*, 1974; Condon *et al.*, 1997) Moreover, maternal glucorticoid availability rises in response to environmental stressors that alter growth *in utero*, including excess light and/or heat, physical restraint, infection and dietary restriction of calories and protein (Ward & Weisz, 1984; Montano *et al.*, 1991; Lesage *et al.*, 2001; Asiaei *et al.*, 2011; Belkacemi *et al.*, 2011; Sferruzzi-Perri *et al.*, 2011; Cottrell *et al.*, 2012). However, whether alterations in materno-fetal glucose partitioning underlie the reduction in birth weight when maternal glucocorticoids are elevated during pregnancy (Seckl, 2004) also remains unknown.

The availability of maternal glucose for transfer to the fetus is maintained, in part, by reduced peripheral glucose uptake by the maternal tissues and an enhanced rate of glucose production by the maternal liver, which increases in size during pregnancy (Mottola & Christopher, 1991; Nolan & Proietto, 1996; Saad *et al.*, 1997; Bustamante *et al.*, 2010). In the liver, glycogenolysis and gluconeogenesis produce glucose, which is released into the circulation via the high-capacity glucose transporter GLUT2, encoded by the *Slc2a2* gene. In turn, glucose crosses the placenta by facilitated diffusion, primarily via the transporters GLUT1 (*Slc2a1*) and GLUT3 (*Slc2a3*), although as many as five isoforms have been identified in the mammalian placenta (Zhou & Bondy, 1993; Limesand *et al.*, 2004; Jones *et al.*, 2013). GLUT1 is also expressed at low levels in the liver, where it facilitates basal glucose uptake (Olson & Pessin, 1996). Synthetic glucocorticoids, such as dexamethasone, are known to

increase hepatic glycogen content and activity of the final, rate-limiting glucogenic enzyme, glucose-6-phosphatase in pregnant rats and sheep (Klepac, 1985; Franko *et al.*, 2007). Moreover, placental GLUT expression is altered by maternal treatment with synthetic glucocorticoids in pregnant rats (Hahn *et al.*, 1999; Langdown & Sugden, 2001). Thus, when glucocorticoid concentrations are raised inappropriately by stress or exogenous administration, alterations in hepatic release and placental transport of glucose may alter the fetal supply and thus contribute to fetal growth restriction induced by these hormones.

Glucocorticoids may affect both the maternal liver and placenta by interacting with the insulin-signalling pathway. Insulin can act via either the insulin receptor (IR) or the related insulin-like growth factor type I receptor (IGF1R). The metabolic effects of activation of either receptor are mediated by phosphorylation of the serine/threonine kinase Akt (Taniguchi *et al.*, 2006). In the liver, phosphorylated Akt stimulates glycogen synthesis through glycogen synthase-kinase 3β (GSK-3β) and inhibits gluconeogenic enzyme expression. Akt also activates the mechanistic target of rapamycin (mTOR) pathway, which promotes protein synthesis via phosphorylation of the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and p70 ribosomal protein S6 kinase (p70 S6 kinase). Dexamethasone enhances the suppressive effect of insulin on hepatic glucose production but induces peripheral insulin resistance in pregnant rats (Holness & Sugden, 2001). In non pregnant rats, dexamethasone also impairs insulin receptor signalling in the liver (Saad *et al.*, 1993) and attenuates insulin stimulated phosphorylation of Akt and GSK-3β in skeletal muscle (Ruzzin *et al.*, 2005). Moreover, dexamethasone inhibits protein synthesis in muscle, in part by upregulating the mTOR supressor, regulated in development and DNA-damage responses 1 (*Redd1*, also known as *Ddit4*) (Wang *et al.*, 2006; McGhee *et al.*, 2009; Britto *et al.*, 2014).

Whilst both IR and IGF1R are present in the placenta in rodents and other species (Posner, 1974), transplacental glucose transport is not directly insulin-sensitive, as GLUT4 is not expressed in the trophoblast (Hay, 2006). However, the mitogenic, anti-apoptotic and anabolic actions of the insulin signalling cascade may indirectly influence the fetal nutrient supply by influencing cellular proliferation or transporter protein abundance in the placenta (Mandl *et al.*, 2002; Jansson *et al.*, 2003). Certainly, the expression and phosphorylation level of insulin-receptor-Akt signalling proteins are reduced in the small placentae of human infants with intrauterine growth restriction (Laviola *et al.*, 2005; Yung *et al.*, 2008).

Moreover, when placental growth restriction is induced by dexamethasone administration in rats or dietary manipulations in larger animals, phosphorylation of Akt and of proteins of the mTOR pathway is decreased, in association with a reduction in glucose transporter protein on the trophoblast membrane (Ain *et al.*, 2005; Rosario *et al.*, 2011; Kavitha *et al.*, 2014).

The aim of this study was to determine whether elevated maternal concentrations of the natural glucocorticoid, corticosterone, influence hepatic glucose handling and the materno-fetal glucose supply in the pregnant mouse. Mice were given exogenous corticosterone at a dose designed to produce plasma corticosterone concentrations similar to those reported previously in undernourished or light/heat stressed dams (Montano *et al.*, 1991; Sferruzzi-Perri *et al.*, 2011) . One group of animals was given corticosterone from day (D)11, when the definitive placenta is established, to D16, when it attains its maximum weight (term is D21) (Coan *et al.*, 2004). A second group was given corticosterone between day D14 and D19 of pregnancy, the period characterised by most rapid absolute growth of the fetus. We specifically hypothesized that corticosterone would alter the capacity of the liver to release glucose and the capacity of the placenta to transport it to the fetus. Moreover, we determined whether these changes were associated with alterations in insulin-signalling within the tissues.

MATERIALS AND METHODS

Animals

All procedures were conducted under the Animals (Scientific Procedures) Act 1986. C57BL6/J mice were group housed, in 12 hr:12 hr light:dark conditions with *ad libitum* access to water and standard chow (Rat and Mouse Number 3 Breeding, Special Diet Services). Female mice (6-8 weeks old) were mated overnight with stud males and the day of copulatory plug detection designated day (D) 1 of pregnancy. As described in detail previously (Vaughan *et al.*, 2012), pregnant females were randomly allocated to receive corticosterone treatment (corticosterone 21-hemisuccinate, Q1562-000, Steraloids Inc, Newport, RI, USA; 200 μg ml^{-1} in drinking water) *either* from D11 to D16 (n=20) *or* from D14 to D19 (n=31) (Fig. 1). Mean corticosterone dose was 83 ± 9 μg g^{-1} day^{-1} in dams treated from D11 to D16 and 81 ± 6 μg g^{-1} day^{-1} in those treated later in pregnancy. Controls (n=74) drank tap water throughout. Daily *ad libitum* food intake per mouse was significantly greater than control values during corticosterone treatment from D14 to D19 (5.2 ± 0.1 g day^{-1}, n=4 cages; controls 4.3 ± 0.1 g day^{-1}, n=8 cages, P<0.05), but not D11 to D16 (4.3 ± 0.1 g day^{-1}, n=6 cages; controls 4.1 ± 0.2 g day^{-1}, n=15 cages) (Vaughan *et al.*, 2012). Consequently, a subset of animals (n=14) were corticosterone treated and limited to the average food intake of control mice between D14 and D19. Mice were studied on D16 or D19, according to the methods detailed below.

Materno-fetal ^{3}H-methyl-D-glucose clearance and tissue collection

On the study day, all mice were anaesthetized with an intraperitoneal injection of fentanyl-fluanisone and midazolam in water (1:1:2, 10 μg ml-1, Janssen Animal Health, High Wycombe, UK). In a subset of animals (n=41), unidirectional materno-fetal clearance of the non-metabolisable analogue, ^{3}H-methyl-D-glucose (MeG, NET379001MC, specific activity 2.22 TBq $mmol^{-1}$) was measured at both ages using previously described techniques (Sibley *et al.*, 2004). In all mice, a blood sample was taken from the heart into a chilled, EDTA-coated tube. The mouse was then killed by cervical dislocation and weighed before and after removal of the uterus. The maternal liver and individual placentae were dissected out, weighed and flash frozen in liquid nitrogen. Blood samples were centrifuged and the separated plasma stored at -20°C. In animals given MeG, the time (<4 min) from injection of the tracer to collection of the blood sample was recorded and MeG content was measured in maternal plasma and in whole fetuses digested in Biosol (National Diagnostics, Hull, UK)

using liquid scintillation counting (Optiphase Hisafe and LKB Wallac 1216 Rackbeta, both Perkin Elmer, Cambridge, UK).

Blood glucose and plasma hormone measurements

Glucose concentration was measured in whole blood at the time of collection using a hand-held glucometer (One-Touch, Orthoclinical Diagnostics, High Wycombe, UK). Corticosterone, insulin, and insulin-like growth factor I (IGF-I) concentrations were measured in EDTA-plasma.

Corticosterone was measured using a commercially available radioimmunoassay (#07-120102, MP Biomedicals, Orangeburg, NY, USA) as described previously (Vaughan *et al.*, 2012). Intra-assay and interassay coefficients of variation for three pools of quality controls were 7.3% (mean 193.9 ng ml^{-1}) and 6.9% (mean 248.7 ng nl^{-1}), respectively, and the lower detection limit of the assay was 7.7 ng ml^{-1}. Rodent-specific enzyme-linked immunosorbent assays (ELISA) were used to measure insulin (Crystal Chem Inc, Downers Grove, IL, USA) and IGF-I (R&D Systems Europe Ltd, Abingdon, UK). In all cases, intra-assay coefficient of variation was less than 7%. The limits of detection of the assays were 0.1 ng ml^{-1} for insulin and 3.5 pg ml^{-1} for IGF-I.

Glycogen and protein content, and glucose-6-phosphatase activity

Glycogen content and glucose-6-phosphatase activity were determined per gram of maternal liver (n=5-8 per treatment group), as described previously (Franko *et al.*, 2007). Briefly, for the determination of glycogen, liver homogenates in ice-cold water (0.1ml of 100 mg/ml homogenate, in duplicate) were incubated with amyloglucosidase (70U) for 10 minutes. Subsequently, the reaction mixture was deproteinised (zinc sulphate and barium hydroxide) and centrifuged before glucose concentration was measured in the supernatant using a Yellow Springs glucose analyser (2300 Statplus) (Franko *et al.*, 2007). For the determination of glucose-6-phosphatase activity, homogenates made in 250 µM sucrose solution (0.1ml of 10 mg/ml homogenate) were incubated with glucose-6-phosphate (25 µM) for 10 minutes. The reaction was stopped by the addition of trichloracetic acid-ascorbic acid solution and then centrifuged. Inorganic phosphate was determined colorimetrically in the supernatant through the addition of ammonium molybdate, in the presence of arsenite-citrate reagent (Fowden *et al.*, 1993). For both assays, non-enzymatic production of glucose or phosphate was

determined for each homogenate and subtracted from the enzymatic production. Liver and placenta protein content was determined using the Lowry assay (Forhead *et al.*, 2003).

Signalling protein abundance and phosphorylation

Western blotting was used to determine the total abundance and phosphorylation level of signalling proteins both in maternal liver and placentae (n=5-9 per group, one placenta per litter). Protein extracts were resolved by electrophoresis and transferred to a nitrocellulose membrane for interrogation. Equal protein loading was verified by Ponceau staining (Romero-Calvo *et al.*, 2010). Antibodies to the following targets were used: insulin receptor β, IGF-I receptor β (both 1:400 dilution, from Santa Cruz Biotechnology, Inc. Dallas, TX, USA), Akt total and phosphorylated (Ser473), glycogen synthase kinase 3β total and phosphorylated (Ser21/9), 4E-binding protein total and phosphorylated (Thr37/46), and p70 S6 kinase total and phosphorylated (Thr389) (all 1:1000 dilution, from Cell Signalling Technology, Danvers, MA, USA). The intensity of antibody binding was visualised using the enhanced chemiluminescence reaction (Amersham) and quantified with Image J.

Gene expression

Quantitative real time PCR was used to measure gene expression in maternal liver and in the placenta second closest in weight to the mean of each litter (n=6 per treatment, per age). Expression of *Slc2a1* (Taqman gene expression assay Mm00441473_m1). *Slc2a2* (Mm00446229_m1), *Slc2a3* (Mm00441483_m1), *Nr3c1* (Mm00433832_m1), *Hsd1* (Mm00476182_m1), *Hsd2* (Mm01251104_m1), *Redd1* (Mm00512504_g1), *Vegf* (Mm01281449_m1), *Igf2* (Mm00439564_m1) and the placental specific *Igf2P0* transcript (primer and probe sequences in (Coan *et al.*, 2010)) were normalised to the geometric mean of *Hprt1* (Mm00446968_m1) and *Sdha* (Mm01352366_m1) using the ΔΔCt method (all Taqman assays from Applied Biosystems, Warrington, UK using the 7500 Fast System).

Statistics

All data are presented as mean ± SEM. Separate statistical comparisons were made at D16 and D19. Results were considered significant when $P<0.05$. At D16, the control group and corticosterone treated group were compared by unpaired Student's t-test. At D19, the three groups (control, D11-14 corticosterone, D14-19 corticosterone *ad lib* and D14-19

corticosterone pair-fed) were compared by one-way ANOVA; when there was an overall effect of treatment, individual groups were compared pairwise with a Bonferroni *post hoc* test. Where measurements were made for individual fetuses within a litter (fetal and placental weight, MeG clearance), each measurement was considered a replicate so litter means were calculated such that the number of subjects was the number of litters.

RESULTS

Hormone levels and biometry

Exogenous corticosterone treatment raised plasma corticosterone concentrations, as reported previously (Table 1, Vaughan et al, 2012). However, when corticosterone treated dams were pair-fed to control food intake, the rise in plasma corticosterone was smaller than in *ad libitum* fed dams and did not differ significantly from control values. Plasma insulin was also elevated by corticosterone treatment over either age range, irrespective of food intake, but blood glucose concentration did not differ between treatment groups (Table 1). Maternal plasma IGF-I concentration was unaffected by corticosterone treatment at D16 and D19 (Table 1).

Corticosterone treatment from D11 to D16 reduced the weight of the pregnant mouse on D16 (Table 2). Specifically, the gravid uterus was lighter in corticosterone-treated animals whereas maternal carcass weight did not differ significantly, compared to controls (Table 2). When corticosterone was given from D14 to D19, there was no effect of treatment on either total body weight, uterus weight or carcass weight on D19, even when corticosterone treated mice were pair-fed to controls (Table 2). However, when weights were expressed as a percentage of whole body weight, there was an overall increase in the percentage weight of maternal carcass and decrease in percentage weight of the uterus following corticosterone administration at both D16 and D19 (Table 2).

Corticosterone reduced fetal weight by 8% and 19% at D16 and D19, respectively (Table 2). Placental weight was also reduced by corticosterone treatment at both ages, regardless of maternal food intake (Table 2). The number of viable fetuses per litter was unaffected by maternal treatment (Table 2).

Maternal liver

Glucogenic capacity

Following corticosterone administration from D11 to D16, the maternal liver was lighter as a percentage of carcass weight, although not as an absolute value compared to controls (Table 3). At D19, neither absolute nor percentage liver weight was affected by corticosterone treatment. Neither hepatic glycogen content nor glucose-6-phosphatase activity were affected by treatment at either age. However, there was a significant decrease in hepatic protein

content in D14-19 corticosterone-treated, pair fed animals, relative to those receiving corticosterone and eating *ad libitum* (Table 3).

Gene expression

Hepatic *Slc2a1* glucose transporter expression was significantly lower than controls in animals given corticosterone from D14 to D19 but not from D11 to D16 (Fig 2). Conversely, *Slc2a2* expression was significantly elevated in animals given corticosterone from D11 to D16 but not from D14 to D19 (Fig. 2). Abundance of the *Nr3c1* glucocorticoid receptor in the liver of the pregnant mouse was reduced by glucocorticoid treatment at both gestational ages (Fig. 2). Furthermore, corticosterone-treated animals had a significantly lower hepatic expression of the glucocorticoid-activating *Hsd1* gene, compared to controls, at D16 but not at D19. *Hsd2* abundance was too low to quantify accurately in liver tissue. Hepatic expression of the *Redd1* gene was increased ~4-fold in *ad libitum* fed, corticosterone-treated dams at both D16 and D19 but was not different from controls in corticosterone treated, pair-fed animals at D19 (Fig. 2).

Signalling protein abundance and phosphorylation

Corticosterone treatment did not significantly alter hepatic expression of the insulin receptor β or the insulin-like growth factor type I receptor β, compared to controls at either age (Fig. 3). Downstream of the receptors, total Akt but not serine-473 phosphorylated Akt was reduced by corticosterone treatment at D16 (Fig. 3). In contrast, phosphorylated but not total Akt was increased in the corticosterone-treated, pair-fed group. The ratio of phospho to total Akt was increased in all corticosterone treated groups, irrespective of age or food intake during treatment. Serine-9/21 phosphorylated GSK-3β was also increased during corticosterone treatment at both ages, regardless of maternal food intake. Total GSK-3β abundance was affected by treatment on D19 only, when it was higher in the corticosterone-treated, *ad libitum* fed group than in either the corticosterone-treated, pair-fed animals or the untreated controls. Although neither total nor phosphorylated 4E-BP1 were affected by corticosterone, there was a significant effect of treatment on the phosphorylation of p70 S6 kinase at threonine-389 on D19, which was again highest in the D14 to D19 treated, *ad libitum* fed group.

Placenta

Materno-fetal MeG clearance

There was an overall effect of treatment on transplacental methyl-D-glucose clearance at D19 (P=0.04), but not D16 (P>0.05, Fig. 4). Compared to D19 controls, both materno-fetal clearance and fetal accumulation of the tracer was lower in those mice given corticosterone from D14 to D19 (Fig. 4). However, limiting food intake to control levels in corticosterone-treated dams abolished the effect on placental glucose transport at D19.

Gene expression

Placental expression of both glucose transporter genes, *Slc2a1* and *Slc2a3* was increased in corticosterone treated mice on D16 (Fig. 5). However, on D19 *Slc2a1* and *Slc2a3* expression was similar in all groups. Conversely, placental *Redd1* expression was increased in *ad libitum* fed, corticosterone treated dams on D19 but not D16. Pair-feeding corticosterone-treated dams between D14 and D19 limited the increase in *Redd1* expression in the placenta such that there was no significant difference from control values (Fig. 5). The growth factor *Vegf* was more highly expressed in the placenta after corticosterone treatment only on D19. There was no change in *Igf2* or *Igf2P0* expression with corticosterone treatment at either age (Fig. 5). Moreover, there was no significant difference in placental expression of *Nr3c1*, *Hsd1* and *Hsd2* between control and corticosterone-treated animals, as reported previously (Vaughan *et al.*, 2012).

Signalling protein abundance and phosphorylation

Placental insulin receptor and IGF-I receptor expression levels did not differ with maternal treatment at either D16 or D19 (Fig. 6). On D16 corticosterone significantly reduced phosphorylation of Akt at the serine-473 residue, although total protein abundance was similar to controls. Total Akt abundance was lower in corticosterone-treated, pair-fed animals in particular compared to controls at D19, but the phospho form was not affected by treatment. Neither total expression nor phosphorylation of GSK-3β in the placenta was altered by corticosterone at either age (Fig. 6). There was no change in abundance or phosphorylation of 4E-BP1 or p70 S6 kinase at D16. However, on D19 there was a tendency for corticosterone treatment to increase total 4E-BP1 abundance, irrespective of food intake, without affecting abundance of the threonine-37/46 phosphorylated form. Consequently, there was a significant decrease in the phospho-4E-BP1:total 4E-BP1 ratio on D19 (P=0.048, Fig. 6C). At this age, corticosterone also caused a decrease in threonine-389 phosphorylation of p70 S6 kinase relative to controls, when expressed as a ratio to the total abundance of the protein. Total protein content per g placenta did not differ significantly between treatment

groups at D16 (control 70 ± 4 mg/g, corticosterone 57 ± 5 mg/g, P=0.085) or D19 (control 67 ± 12 mg/g, corticosterone, *ad libitum fed* 64 ± 5 mg/g, corticosterone, pair-fed, 63 ± 7 mg/g, P>0.1).

DISCUSSION

This study is the first to demonstrate that *in vivo* placental glucose transport is reduced in mice given exogenous corticosterone in late pregnancy. Plasma insulin concentration and hepatic insulin signalling were elevated in corticosterone treated dams but there was no effect on maternal hepatic glucogenic capacity or blood glucose concentration. Contrastingly, depending upon the timing of corticosterone administration, there were alterations in placental Akt-mTOR signalling consistent with reduced protein synthesis and the observed placental growth restriction. Although the precise physiological changes in the mother and placenta depended upon maternal food intake, ultimately maternal glucocorticoid excess led to a reduction in feto-placental growth.

When pregnant mice were given exogenous corticosterone in their drinking water, plasma corticosterone concentrations were significantly higher in dams fed *ad libitum* compared to those restricted to control food intake, even though the total amount of corticosterone drunk was the same (Vaughan *et al.*, 2012). With reduced food availability, the noctural period of food and water intake may have finished earlier in the night in pair-fed dams, allowing more time for exogenous corticosterone to be cleared from the plasma before measurements were made at a fixed time the following morning. Certainly, previous studies in rats have shown that limiting food availability alters the normal circadian rhythm of endogenous corticosterone secretion (Gallo & Weinberg, 1981). In contrast, maternal plasma insulin concentration was increased independently of the increase in food intake. This indicates that oral corticosterone administration had a physiological effect in corticosterone-treated, pair-fed dams even though their plasma corticosterone concentrations were similar to controls on the study day. Plasma insulin levels are also raised when the synthetic glucocorticoid, dexamethasone, is given to pregnant rats (Holness & Sugden, 2001) or pregnant women (Ahmad *et al.*, 2006). Furthermore, dexamethasone raises plasma insulin concentration when given to non-pregnant rats by intravenous infusion (Saad *et al.*, 1993) but causes hyperphagia only when infused centrally (Zakrzewska *et al.*, 1999). This suggests that mice treated with corticosterone in the present study were hyperinsulinaemic as a consequence of peripheral insulin resistance rather than due to greater nutrient intake. Although maternal corticosterone did not affect blood glucose concentration in the present study, measurements here were made in the fed state; thus absorption of glucose from the gut may have obscured changes in endogenous production or clearance. Indeed, previous studies have shown hyperglycaemia in fasted (Holness & Sugden, 2001) but not fed (Franko *et al.*, 2010), dexamethasone-treated

pregnant rats. Whilst the natural state of insulin resistance in late pregnancy favours distribution of resources to the gravid uterus, corticosterone-treated dams exhibited a preferential decrease in weight of the uterus and contents, rather than of maternal tissues. In part, this may be attributable to direct inhibition of fetal growth by corticosterone crossing the placenta (Fowden *et al.*, 1996) but may also be due to the decreased placental transport of glucose, seen in this study, and of amino acids, observed previously (Vaughan *et al.*, 2012).

Oral corticosterone treatment did not alter liver glycogen content or G6pase activity, in contrast to previous studies in pregnant animals using the synthetic glucocorticoid, dexamethasone (Klepac, 1985; Franko *et al.*, 2007). Downregulation of both the glucocorticoid receptor (*Nr3c1*) and the glucocorticoid-activating isoform of 11β-hydroxysteroid dehydrogenase (*Hsd1*) may mean that local corticosterone bioactivity was not elevated sufficiently to alter liver enzyme expression, in the present study. Alternatively, the net effect on hepatic carbohydrate metabolism may have been due to the balance of elevated plasma corticosterone and increased insulin signalling. In contrast to the effect of glucocorticoid treatment in the non-pregnant rat (Saad *et al.*, 1993), there was no downregulation of hepatic IR or IGF1R expression here. Indeed, the phosphorylation state of signalling proteins downstream of the insulin receptor broadly reflected a normal response to elevated insulin. Both Akt and GSK-3β were hyperphosphorylated during corticosterone treatment, which would be expected to suppress gluconeogenic enzyme expression. Nonetheless, corticosterone treatment did increase hepatic *Slc2a2* glucose transporter gene expression on D16. *Slc2a2* gene expression is linked to glucose availability in cultured hepatocytes (Rencurel *et al.*, 1996) and known to be transcriptionally regulated by other hormones *in vivo* (Weinstein *et al.*, 1994). Whilst corticosterone treatment also tended to decrease hepatic *Slc2a1* expression, the transport capacity and absolute abundance of GLUT1 is low in the liver (Olson & Pessin, 1996), meaning it is unlikely to influence glucose release. Although insulin signalling was not measured in other maternal tissues in the current study, previous investigations have consistently shown that glucocorticoid treatment impairs skeletal muscle insulin sensitivity and peripheral glucose uptake in pregnant and non-pregnant rodents, independently of glucose transporter abundance (Haber & Weinstein, 1992; Holness & Sugden, 2001). Thus, adaptations in both the liver and extra-hepatic tissues tend to increase glucose availability for transfer to the fetus and are unlikely to contribute to reduced fetal growth when maternal glucocorticoids are raised.

Certainly, corticosterone had an effect on hepatic protein content, which was specifically reduced in the livers of corticosterone-treated, pair fed animals. Although this may be a direct catabolic effect of corticosterone, reduced phosphorylation of p70 S6 kinase suggests that there may also be a reduction in mTOR-stimulated protein translation in the livers of these mice. Paradoxically, expression of the mTOR suppressor *Redd1* was increased only in the livers of *ad libitum* fed, not pair fed, corticosterone-treated mice, which did not show any evidence of reduced protein content. The mechanism of altered hepatic protein content may therefore be more complex in pair-fed animals, when food intake is not increased to balance the catabolic effect of the glucocorticoid.

The reduction in materno-fetal glucose clearance in D19 pregnant mice given corticosterone is in agreement with previous *in vivo* studies in sheep showing that steady state fetal glucose uptake is reduced when fetal cortisol concentration is increased (Ward *et al.*, 2004). In contrast, when pregnant rats are given dexamethasone for two days near term, there is no change in uterine uptake of a non-metabolisable glucose tracer (Norris *et al.*, 2011), perhaps reflecting the differing effects of the natural and synthetic glucocorticoids, or the more prolonged period of glucocorticoid administration in the present study. Mouse dams given corticosterone and pair-fed to control food intake did not exhibit a reduction in placental glucose transport, even though fetal weight was reduced to the same extent as in *ad libitum* feeding animals. In part, the apparent interaction between corticosterone and dietary restriction in influencing materno-fetal glucose clearance in pair-fed dams may relate to their lower prevailing plasma corticosterone but may also reflect an independent influence of nutrition on placental glucose transporter abundance, as changes in placental capacity for glucose transport have been demonstrated previously in pregnant mice and rats fed less than the normal caloric intake (Lesage *et al.*, 2002; Coan *et al.*, 2010). Fetal growth restriction in the corticosterone-treated, pair fed animals may have been due to reduced amino acid supply, as placental methylaminoisobutyric acid clearance is known to be decreased in the corticosterone-treated mice, irrespective of food intake (Vaughan *et al.*, 2012). Indeed, previous observations show that natural variations in fetal weight are more closely related to placental transport of amino acids than glucose at D19 (Coan *et al.*, 2008). Moreover, although glucose transport capacity per gram of placenta was not affected in the pair-fed animals, reduced absolute placental weight and vascularity are likely to have impaired net fetal glucose uptake. The reduced size of the corticosterone-treated placenta may be explained, in part, by lower abundance or activity of the Akt protein at both D16 and D19.

Akt expression is also reduced in rat placentae growth restricted by maternal dexamethasone administration (Ain, 2005), although this was accompanied by reduced *Igf2* expression, unlike the present study. Unlike previous studies in dexamethasone-treated rats rats (Hewitt *et al.*, 2006), placental *Vegf* expression was increased by corticosterone, suggesting that there may be an attempt to compensate for the reduction in blood vessel density reported previously in these mice (Vaughan *et al.*, 2012). The more marked direct effect of corticosterone on D19 than D16 placentae may reflect the lower placental expression of the glucorticoid metabolising isoform of 11β-hydroxysteroid dehydrogenase (*Hsd2*) in later pregnancy (Condon *et al.*, 1997).

The observed changes in materno-fetal MeG clearance in corticosterone-treated dams at D19 were not related to placental expression of the glucose transporter genes, *Slc2a1* and *Slc2a3*, which did not differ with treatment at this age. On the other hand, *Slc2a1* and *Slc2a3* were upregulated by corticosterone treatment on D16, without a change in MeG clearance. These adaptations in placental glucose transport may also relate to changes in expression of glucose transporter genes *Slc2a8* and *Slc2a9*, which have been shown recently to be regulated by endocrine signals in the mouse placenta (Jones *et al.*, 2013). However, post-transcriptional mechanisms may also have a role in regulating placental nutrient transport when glucocorticoids are raised. Certainly, adaptations in placental System A amino acid transport capacity have previously been shown to be temporally dissociated from *Slc38a* amino acid transporter expression in corticosterone treated mice (Vaughan *et al.*, 2012). In the present study, placental glucose transport capacity was specifically reduced when maternal plasma corticosterone concentration and placental *Redd1* expression were raised in the *ad libitum* fed, corticosterone treated mice. In pair-fed, corticosterone treated dams, when plasma corticosterone and placental *Redd1* expression were similar to controls, MeG clearance was not decreased even though the alterations in maternal insulin signalling and feto-placental growth suggested that these dams had received a physiologically effective dose of corticosterone. Thus, placental *Redd1* expression was closely related to both the prevailing maternal corticosterone concentration and glucose transport across the placenta.

In contrast to the current findings, corticosterone reduces placental amino acid transport in both *ad libitum* and pair fed dams (Vaughan *et al.*, 2012). Corticosterone, therefore, appears to affect placental transport of glucose and amino acids by different mechanisms. Since the ratio of phosphorylated to total 4E-BP1 and p70 S6 kinase abundance was reduced in

corticosterone-treated placentae irrespective of food intake in the current study, placental amino acid transport appears to be more directly related to inhibition of mTOR than placental glucose transport. Certainly, 4E-BP1 and p70 S6 kinase hypophosphorylation is associated with reduced amino acid transporter protein expression in the placentae of rat dams deprived of protein during pregnancy (Rosario *et al.*, 2011). Collectively, these observations suggest that corticosterone reduces placental glucose transport via *Redd1* independently of mTOR while it acts to lower placental amino acid transport by inhibition of mTOR dependent translation or translocation of transporters to the cell membrane. Indeed, the placental adaptation in signalling downstream of mTOR induced by corticosterone overexposure may be indirect and mediated by the maternal hyperinsulinaemia observed in both the *ad libitum* fed and pair-fed groups of dams. The precise effects of corticosterone on placental nutrient transport, therefore, depend on both its actual circulating concentration and its longer term actions on maternal metabolism and other metabolic hormone concentrations. Fetal sex may also be an important factor in determining the effect of maternal corticosterone on materno-fetal nutrient transport, as the normal permeability of the placenta to natural glucocorticoids is known to differ between male and female conceptuses (Montano *et al.*, 1993).

Overall, the data show that increased concentrations of natural glucocorticoids in the pregnant mother may limit materno-fetal glucose partitioning and thus fetal growth by reducing placental glucose transport capacity. Moreover, whilst natural glucocorticoids increase insulin signalling and glucose transporter expression in the maternal liver, they impair insulin signalling in the placenta with possible consequences for the activity of other nutrient transport systems. As glucose is the major substrate of fetal growth and oxidative metabolism, these effects are likely to impact upon the size and metabolic phenotype of the infant at birth. However, the interaction of glucocorticoids with other nutrient sensing pathways in the placenta may represent a target for interventions to rescue fetal growth when maternal glucocorticoids are raised during pregnancy.

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Competing interests

The authors have no competing interests

Author Contributions

ORV and ALF conceived of and designed the experiments. All authors collected analysed and interpreted the data. ORV, ANS-P and ALF drafted the article and revised it critically for important intellectual content. All authors approved the final version of the manuscript.

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TABLES

Table 1 Mean ± SEM plasma hormone and blood glucose concentrations in control and corticosterone-treated pregnant mice

	D16		D19		
	Con	Cort D11-16	Con	Cort D14-19	Cort D14-19 +PF
Corticosterone	n=23	n=12	n=24	n=10	n=9
ng ml^{-1}	**632 ± 60**	**1142 ± 134***	**714 ± 41^{a}**	**1143 ± 176^{b}**	**810 ± 74^{ab}**
Insulin	n=7	n=7	n=12	n=7	n=5
ng ml^{-1}	**1.1 ± 0.3**	**40.3 ± 5.8***	**1.1 ± 0.3^{a}**	**20.6 ± 4.5^{b}**	**16.5 ± 8.3^{b}**
IGF-I	n=6	n=6	n=8	n=5	n=4
ng ml^{-1}	208 ± 21	193 ± 12	219 ± 25	155 ± 13	216 ± 10
Glucose	n=32	n=18	n=35	n=16	n=14
mM	9.7 ± 0.4	10.5 ± 0.7	9.2 ± 0.3	9.5 ± 0.7	10.2 ± 0.6

Groups were compared by Student's t-test at D16, * denotes $P<0.05$ vs Con. Groups were compared by one-way ANOVA at D19, $^{a\ b}$ denote significantly different values by Bonferroni *post hoc* comparisons ($P<0.05$). Values in bold indicate significant overall effect of treatment. *n* values are given in table. PF indicates corticosterone-treated animals pair-fed to control food intake.

Table 2 Mean ± SEM maternal, fetal and placental biometry in control and corticosterone-treated pregnant mice

	D16		D19		
	Con	Cort D11-16	Con	Cort D14-19	Cort D14-19 +PF
	n=32	n=20	n=42	n=17	n=14
Body weight g	**29.6 ± 0.5**	**27.8 ± 0.4***	34.6 ± 0.6	34.3 ± 1.0	32.2 ± 0.9
Hysterectomised g	23.2 ± 0.4	22.8 ± 0.4	23.2 ± 0.3	24.7 ± 0.7	23.1 ± 0.6
% total body weight	**78.6 ± 0.6**	**80.8 ± 0.5***	**67.5 ± 0.7[a]**	**72.1 ± 1.1[b]**	**71.9 ± 1.1[b]**
Uterus g	**6.4 ± 0.2**	**5.3 ± 0.1***	12.2 ± 1.0	9.6 ± 0.5	9.1 ± 0.5
% total body weight	**21.4 ± 0.6**	**19.2 ± 0.5***	**32.5 ± 0.7[a]**	**27.9 ± 1.1[b]**	**28.1 ± 1.1[b]**
Fetus mg	**390 ± 4**	**361 ± 5***	**1178 ± 11[a]**	**959 ± 15[b]**	**937 ± 29[b]**
Placenta mg	**101 ± 1**	**95 ± 1***	**89 ± 1[a]**	**78 ± 1[b]**	**78 ± 1[b]**
Number of fetuses	7.0 ± 0.4	6.4 ± 0.2	6.7 ± 0.2	6.9 ± 0.3	6.4 ± 0.4

Groups were compared by Student's t-test at D16, * denotes $P<0.05$ vs Con. Groups were compared by one-way ANOVA at D19, [a b] denote significantly different values by Bonferroni *post hoc* comparisons ($P<0.05$). Values in bold indicate significant overall effect of treatment. *n* values are given in table. PF indicates corticosterone-treated animals pair-fed to control food intake.

Table 3 Mean ± SEM liver weight and glucogenic capacity in control and corticosterone-treated pregnant mice

	D16		D19		
	Con	Cort D11-16	Con	Cort D14-19	Cort D14-19 +PF
Liver weight	n=32	n=20	n=37	n=16	n=14
mg	1766 ± 42	1641 ± 42	1709 ± 33	1788 ± 78	1781 ± 53
% of carcass	**7.6 ± 0.1**	**7.1 ± 0.1***	7.4 ± 0.1	7.2 ± 0.2	7.7 ± 0.3
Hepatic glucogenic capacity	n=6	n=6	n=8	n=7	n=5
Glycogen mg/g	45.3 ± 2.8	39.3 ± 4.3	48.6 ± 2.7	43.8 ± 4.1	46.2 ±2.8
Glycogen total mg	76.0 ± 6.6	62.4 ± 8.1	80.8 ± 5.9	79.7 ± 8.4	81.3 ± 5.9
G-6-Pase U/g	20.9 ± 2.3	26.6 ± 1.0	26.4 ± 0.6	25.3 ± 1.3	22.8 ± 2.0
G-6-Pase U/mg protein	378 ± 36	406 ± 24	374 ± 27	315 ± 14	377 ± 46
Protein mg/g	56 ± 5	66 ± 2	**73 ± 5^{ab}**	**80 ± 3^{a}**	**62 ± 3^{b}**

Groups were compared by Student's t-test at D16, * denotes $P<0.05$ vs Con. Groups were compared by one-way ANOVA at D19, $^{a\ b}$ denote significantly different values by Bonferroni *post hoc* comparisons ($P<0.05$). Values in bold indicate significant overall effect of treatment. *n* values are given in table. PF indicates corticosterone-treated animals pair-fed to control food intake.

FIGURE LEGENDS

Figure 1

Flow chart illustrating allocation of mice to different experimental treatments, feeding regimens and study days. Grey shading indicates animals given exogenous corticosterone (200 μg ml^{-1} in drinking water). D, day of pregnancy.

Figure 2

Mean ± SEM maternal liver expression of genes involved in glucose transport and glucocorticoid action at (A) day 16 and (B) day 19 of pregnancy. Expression determined by the ΔΔCt method relative to Groups were compared by Student's t-test at day 16, * denotes $P<0.05$ vs Con. Groups were compared by one-way ANOVA at day 19, [a] [b] denote significantly different values by Bonferroni *post hoc* comparisons ($P<0.05$). n=6 per treatment group at each age

Figure 3

Mean ± SEM maternal liver abundance of total and phosphorylated forms of insulin-signalling proteins. (A) Representative blots from each age and treatment group, approximate molecular weights of the protein bands are given to the right of the blot, (B) abundances at day 16, (C) abundances at day 19 of pregnancy. Groups were compared by Student's t-test at day 16, * denotes $P<0.05$ vs Con. Groups were compared by one-way ANOVA at day 19, [a] [b] denote significantly different values by Bonferroni *post hoc* comparisons ($P<0.05$). D16 control n=6, corticosterone-treated n=7; D19 control n=9, corticosterone-treated *ad libitum* n=8, corticosterone-treated pair-fed n=5.

Figure 4

Mean ± SEM placental transport of non-metabolisable ^{3}H-methyl-D-glucose at day 16 and day 19 of pregnancy. (A) Unidirectional materno-fetal clearance per gram of placenta, (B) accumulation per gram of fetus. Groups were significantly different by Student's t-test at day 16. Groups were compared by one-way ANOVA at day 19, † indicates overall $P<0.05$; [a] [b] denote significantly different values by Bonferroni *post hoc* comparisons ($P<0.05$). D16 control n=7, corticosterone-treated n=5; D19 control n=17, corticosterone-treated *ad libitum* n=7, corticosterone-treated pair-fed n=5.

Figure 5

Mean ± SEM placental expression of genes involved in glucose transport and glucocorticoid action, and growth factors at (A) day 16 and (B) day 19 of pregnancy. Expression determined by the ΔΔCt method relative to Groups were compared by Student's t-test at day 16, * denotes $P<0.05$ vs Con. Groups were compared by one-way ANOVA at day 19, [a b] denote significantly different values by Bonferroni *post hoc* comparisons ($P<0.05$). *Nr3c1*, *Hsd1* and *Hsd2* expression data have been reported in a previous publication. n=6 per treatment group at each age.

Figure 6

Mean ± SEM placental abundance of total and phosphorylated forms of insulin-signalling proteins. (A) Representative blots from each age and treatment group, approximate molecular weights of the protein bands are given to the right of the blot, (B) abundances at day 16, (C) abundances at day 19 of pregnancy. Groups were compared by Student's t-test at day 16, * denotes $P<0.05$ vs Con. Groups were compared by one-way ANOVA at day 19, † indicates overall $P<0.05$; [a b] denote significantly different values by Bonferroni *post hoc* comparisons ($P<0.05$). D16 control n=7, corticosterone-treated n=7; D19 control n=6, corticosterone-treated *ad libitum* n=6, corticosterone-treated pair-fed n=4.