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

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22 **Running head:** Corticosterone alters materno-fetal glucose partitioning

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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

75 **INTRODUCTION**

76 Glucocorticoids have an important role in regulating metabolism in adult animals (McMahon 77 *et al.*, 1988). Glucocorticoids in the fetal circulation also affect fetal glucose metabolism, 78 particularly glycogen deposition and mobilization in fetal tissues during late gestation 79 (Fowden & Forhead, 2011). However, whether maternal glucocorticoid concentrations 80 influence the materno-fetal supply of glucose remains unknown. Glucose is supplied to the 81 fetus by facilitated diffusion across the placenta, down the materno-fetal glucose 82 concentration gradient (Battaglia & Meschia, 1988). As the glucose requirement of the 83 growing fetus increases through pregnancy, alterations in the gradient and placental transport 84 capacity ensure that the glucose supply meets this demand (Molina *et al.*, 1991; Sakata *et al.*, 85 1995; Yamaguchi *et al.*, 1996; Ehrhardt & Bell, 1997). Glucocorticoids are well placed to 86 influence materno-fetal glucose supply as their bioavailability changes throughout pregnancy 87 with increasing maternal and fetal adrenal secretion and tissue-specific changes in activity of 88 the 11β hydroxysteroid dehydrogenases, which increase (type I) or decrease (type II) the local 89 availability of active glucocorticoids (Barlow *et al.*, 1974; Condon *et al.*, 1997) Moreover, 90 maternal glucorticoid availability rises in response to environmental stressors that alter 91 growth *in utero*, including excess light and/or heat, physical restraint, infection and dietary 92 restriction of calories and protein (Ward & Weisz, 1984; Montano *et al.*, 1991; Lesage *et al.*, 93 2001; Asiaei *et al.*, 2011; Belkacemi *et al.*, 2011; Sferruzzi-Perri *et al.*, 2011; Cottrell *et al.*, 94 2012). However, whether alterations in materno-fetal glucose partitioning underlie the 95 reduction in birth weight when maternal glucocorticoids are elevated during pregnancy 96 (Seckl, 2004) also remains unknown.

97

98 The availability of maternal glucose for transfer to the fetus is maintained, in part, by reduced 99 peripheral glucose uptake by the maternal tissues and an enhanced rate of glucose production 100 by the maternal liver, which increases in size during pregnancy (Mottola & Christopher, 101 1991; Nolan & Proietto, 1996; Saad *et al.*, 1997; Bustamante *et al.*, 2010). In the liver, 102 glycogenolysis and gluconeogenesis produce glucose, which is released into the circulation 103 via the high-capacity glucose transporter GLUT2, encoded by the *Slc2a2* gene. In turn, 104 glucose crosses the placenta by facilitated diffusion, primarily via the transporters GLUT1 105 (*Slc2a1*) and GLUT3 (*Slc2a3*), although as many as five isoforms have been identified in the 106 mammalian placenta (Zhou & Bondy, 1993; Limesand *et al.*, 2004; Jones *et al.*, 2013). 107 GLUT1 is also expressed at low levels in the liver, where it facilitates basal glucose uptake 108 (Olson & Pessin, 1996). Synthetic glucocorticoids, such as dexamethasone, are known to

109 increase hepatic glycogen content and activity of the final, rate-limiting glucogenic enzyme,
110 glucose-6-phosphatase in pregnant rats and sheep (Klepac, 1985; Franko *et al.*, 2007).
111 Moreover, placental GLUT expression is altered by maternal treatment with synthetic
112 glucocorticoids in pregnant rats (Hahn *et al.*, 1999; Langdown & Sugden, 2001). Thus, when
113 glucocorticoid concentrations are raised inappropriately by stress or exogenous
114 administration, alterations in hepatic release and placental transport of glucose may alter the
115 fetal supply and thus contribute to fetal growth restriction induced by these hormones.
116
117 Glucocorticoids may affect both the maternal liver and placenta by interacting with the
118 insulin-signalling pathway. Insulin can act via either the insulin receptor (IR) or the related
119 insulin-like growth factor type I receptor (IGF1R). The metabolic effects of activation of
120 either receptor are mediated by phosphorylation of the serine/threonine kinase Akt
121 (Taniguchi *et al.*, 2006). In the liver, phosphorylated Akt stimulates glycogen synthesis
122 through glycogen synthase-kinase 3β (GSK-3β) and inhibits gluconeogenic enzyme
123 expression. Akt also activates the mechanistic target of rapamycin (mTOR) pathway, which
124 promotes protein synthesis via phosphorylation of the eukaryotic translation initiation factor
125 4E binding protein 1 (4E-BP1) and p70 ribosomal protein S6 kinase (p70 S6 kinase).
126 Dexamethasone enhances the suppressive effect of insulin on hepatic glucose production but
127 induces peripheral insulin resistance in pregnant rats (Holness & Sugden, 2001). In non
128 pregnant rats, dexamethasone also impairs insulin receptor signalling in the liver (Saad *et al.*,
129 1993) and attenuates insulin stimulated phosphorylation of Akt and GSK-3β in skeletal
130 muscle (Ruzzin *et al.*, 2005). Moreover, dexamethasone inhibits protein synthesis in muscle,
131 in part by upregulating the mTOR supressor, regulated in development and DNA-damage
132 responses 1 (*Redd1*, also known as *Ddit4*) (Wang *et al.*, 2006; McGhee *et al.*, 2009; Britto *et*
133 *al.*, 2014).
134
135 Whilst both IR and IGF1R are present in the placenta in rodents and other species (Posner,
136 1974), transplacental glucose transport is not directly insulin-sensitive, as GLUT4 is not
137 expressed in the trophoblast (Hay, 2006). However, the mitogenic, anti-apoptotic and
138 anabolic actions of the insulin signalling cascade may indirectly influence the fetal nutrient
139 supply by influencing cellular proliferation or transporter protein abundance in the placenta
140 (Mandl *et al.*, 2002; Jansson *et al.*, 2003). Certainly, the expression and phosphorylation level
141 of insulin-receptor-Akt signalling proteins are reduced in the small placentae of human
142 infants with intrauterine growth restriction (Laviola *et al.*, 2005; Yung *et al.*, 2008).

143 Moreover, when placental growth restriction is induced by dexamethasone administration in
144 rats or dietary manipulations in larger animals, phosphorylation of Akt and of proteins of the
145 mTOR pathway is decreased, in association with a reduction in glucose transporter protein on
146 the trophoblast membrane (Ain *et al.*, 2005; Rosario *et al.*, 2011; Kavitha *et al.*, 2014).
147
148 The aim of this study was to determine whether elevated maternal concentrations of the
149 natural glucocorticoid, corticosterone, influence hepatic glucose handling and the materno-
150 fetal glucose supply in the pregnant mouse. Mice were given exogenous corticosterone at a
151 dose designed to produce plasma corticosterone concentrations similar to those reported
152 previously in undernourished or light/heat stressed dams (Montano *et al.*, 1991; Sferruzzi-
153 Perri *et al.*, 2011) . One group of animals was given corticosterone from day (D)11, when the
154 definitive placenta is established, to D16, when it attains its maximum weight (term is D21)
155 (Coan *et al.*, 2004). A second group was given corticosterone between day D14 and D19 of
156 pregnancy, the period characterised by most rapid absolute growth of the fetus. We
157 specifically hypothesized that corticosterone would alter the capacity of the liver to release
158 glucose and the capacity of the placenta to transport it to the fetus. Moreover, we determined
159 whether these changes were associated with alterations in insulin-signalling within the
160 tissues.

161 **MATERIALS AND METHODS**

162 **Animals**

163 All procedures were conducted under the Animals (Scientific Procedures) Act 1986.
164 C57BL6/J mice were group housed, in 12 hr:12 hr light:dark conditions with *ad libitum*
165 access to water and standard chow (Rat and Mouse Number 3 Breeding, Special Diet
166 Services). Female mice (6-8 weeks old) were mated overnight with stud males and the day of
167 copulatory plug detection designated day (D) 1 of pregnancy. As described in detail
168 previously (Vaughan *et al.*, 2012), pregnant females were randomly allocated to receive
169 corticosterone treatment (corticosterone 21-hemisuccinate, Q1562-000, Steraloids Inc,
170 Newport, RI, USA; 200 μg ml^{-1} in drinking water) *either* from D11 to D16 (n=20) *or* from
171 D14 to D19 (n=31) (Fig. 1). Mean corticosterone dose was 83 ± 9 μg g^{-1} day^{-1} in dams treated
172 from D11 to D16 and 81 ± 6 μg g^{-1} day^{-1} in those treated later in pregnancy. Controls (n=74)
173 drank tap water throughout. Daily *ad libitum* food intake per mouse was significantly greater
174 than control values during corticosterone treatment from D14 to D19 (5.2 ± 0.1 g day^{-1}, *n*=4
175 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},
176 *n*=6 cages; controls 4.1 ± 0.2 g day^{-1}, *n*=15 cages) (Vaughan *et al.*, 2012). Consequently, a
177 subset of animals (n=14) were corticosterone treated and limited to the average food intake of
178 control mice between D14 and D19. Mice were studied on D16 or D19, according to the
179 methods detailed below.
180

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

182 On the study day, all mice were anaesthetized with an intraperitoneal injection of fentanyl-
183 fluanisone and midazolam in water (1:1:2, 10 μg ml-1, Janssen Animal Health, High
184 Wycombe, UK). In a subset of animals (n=41), unidirectional materno-fetal clearance of the
185 non-metabolisable analogue, ^{3}H-methyl-D-glucose (MeG, NET379001MC, specific activity
186 2.22 TBq $mmol^{-1}$) was measured at both ages using previously described techniques (Sibley
187 *et al.*, 2004). In all mice, a blood sample was taken from the heart into a chilled, EDTA-
188 coated tube. The mouse was then killed by cervical dislocation and weighed before and after
189 removal of the uterus. The maternal liver and individual placentae were dissected out,
190 weighed and flash frozen in liquid nitrogen. Blood samples were centrifuged and the
191 separated plasma stored at -20°C. In animals given MeG, the time (<4 min) from injection of
192 the tracer to collection of the blood sample was recorded and MeG content was measured in
193 maternal plasma and in whole fetuses digested in Biosol (National Diagnostics, Hull, UK)

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

196 **Blood glucose and plasma hormone measurements**

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

201 Corticosterone was measured using a commercially available radioimmunoassay (#07-
202 120102, MP Biomedicals, Orangeburg, NY, USA) as described previously (Vaughan *et al.*,
203 2012). Intra-assay and interassay coefficients of variation for three pools of quality controls
204 were 7.3% (mean 193.9 ng ml^{-1}) and 6.9% (mean 248.7 ng nl^{-1}), respectively, and the lower
205 detection limit of the assay was 7.7 ng ml^{-1}. Rodent-specific enzyme-linked immunosorbent
206 assays (ELISA) were used to measure insulin (Crystal Chem Inc, Downers Grove, IL, USA)
207 and IGF-I (R&D Systems Europe Ltd, Abingdon, UK). In all cases, intra-assay coefficient of
208 variation was less than 7%. The limits of detection of the assays were 0.1 ng ml^{-1} for insulin
209 and 3.5 pg ml^{-1} for IGF-I.

210

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

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

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

227

228 **Signalling protein abundance and phosphorylation**

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

240 **Gene expression**

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

250

251 **Statistics**

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

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

261 **RESULTS**

262 **Hormone levels and biometry**

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

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

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

284 **Maternal liver**

285 *Glucogenic capacity*

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

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

293 *Gene expression*

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

305 *Signalling protein abundance and phosphorylation*

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

320 **Placenta**

321 *Materno-fetal MeG clearance*

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

327 *Gene expression*

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

339 *Signalling protein abundance and phosphorylation*

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

354 groups at D16 (control 70 ± 4 mg/g, corticosterone 57 ± 5 mg/g, $P=0.085$) or D19 (control 67
355 ± 12 mg/g, corticosterone, *ad libitum fed* 64 ± 5 mg/g, corticosterone, pair-fed, 63 ± 7 mg/g,
356 $P>0.1$).

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

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

396 Oral corticosterone treatment did not alter liver glycogen content or G6pase activity, in
397 contrast to previous studies in pregnant animals using the synthetic glucocorticoid,
398 dexamethasone (Klepac, 1985; Franko *et al.*, 2007). Downregulation of both the
399 glucocorticoid receptor (*Nr3c1*) and the glucocorticoid-activating isoform of 11β-
400 hydroxysteroid dehydrogenase (*Hsd1*) may mean that local corticosterone bioactivity was not
401 elevated sufficiently to alter liver enzyme expression, in the present study. Alternatively, the
402 net effect on hepatic carbohydrate metabolism may have been due to the balance of elevated
403 plasma corticosterone and increased insulin signalling. In contrast to the effect of
404 glucocorticoid treatment in the non-pregnant rat (Saad *et al.*, 1993), there was no
405 downregulation of hepatic IR or IGF1R expression here. Indeed, the phosphorylation state of
406 signalling proteins downstream of the insulin receptor broadly reflected a normal response to
407 elevated insulin. Both Akt and GSK-3β were hyperphosphorylated during corticosterone
408 treatment, which would be expected to suppress gluconeogenic enzyme expression.
409 Nonetheless, corticosterone treatment did increase hepatic *Slc2a2* glucose transporter gene
410 expression on D16. *Slc2a2* gene expression is linked to glucose availability in cultured
411 hepatocytes (Rencurel *et al.*, 1996) and known to be transcriptionally regulated by other
412 hormones *in vivo* (Weinstein *et al.*, 1994). Whilst corticosterone treatment also tended to
413 decrease hepatic *Slc2a1* expression, the transport capacity and absolute abundance of GLUT1
414 is low in the liver (Olson & Pessin, 1996), meaning it is unlikely to influence glucose release.
415 Although insulin signalling was not measured in other maternal tissues in the current study,
416 previous investigations have consistently shown that glucocorticoid treatment impairs
417 skeletal muscle insulin sensitivity and peripheral glucose uptake in pregnant and non-
418 pregnant rodents, independently of glucose transporter abundance (Haber & Weinstein, 1992;
419 Holness & Sugden, 2001). Thus, adaptations in both the liver and extra-hepatic tissues tend to
420 increase glucose availability for transfer to the fetus and are unlikely to contribute to reduced
421 fetal growth when maternal glucocorticoids are raised.
422

423 Certainly, corticosterone had an effect on hepatic protein content, which was specifically
424 reduced in the livers of corticosterone-treated, pair fed animals. Although this may be a direct
425 catabolic effect of corticosterone, reduced phosphorylation of p70 S6 kinase suggests that
426 there may also be a reduction in mTOR-stimulated protein translation in the livers of these
427 mice. Paradoxically, expression of the mTOR suppressor *Redd1* was increased only in the
428 livers of *ad libitum* fed, not pair fed, corticosterone-treated mice, which did not show any
429 evidence of reduced protein content. The mechanism of altered hepatic protein content may
430 therefore be more complex in pair-fed animals, when food intake is not increased to balance
431 the catabolic effect of the glucocorticoid.
432
433 The reduction in materno-fetal glucose clearance in D19 pregnant mice given corticosterone
434 is in agreement with previous *in vivo* studies in sheep showing that steady state fetal glucose
435 uptake is reduced when fetal cortisol concentration is increased (Ward *et al.*, 2004). In
436 contrast, when pregnant rats are given dexamethasone for two days near term, there is no
437 change in uterine uptake of a non-metabolisable glucose tracer (Norris *et al.*, 2011), perhaps
438 reflecting the differing effects of the natural and synthetic glucocorticoids, or the more
439 prolonged period of glucocorticoid administration in the present study. Mouse dams given
440 corticosterone and pair-fed to control food intake did not exhibit a reduction in placental
441 glucose transport, even though fetal weight was reduced to the same extent as in *ad libitum*
442 feeding animals. In part, the apparent interaction between corticosterone and dietary
443 restriction in influencing materno-fetal glucose clearance in pair-fed dams may relate to their
444 lower prevailing plasma corticosterone but may also reflect an independent influence of
445 nutrition on placental glucose transporter abundance, as changes in placental capacity for
446 glucose transport have been demonstrated previously in pregnant mice and rats fed less than
447 the normal caloric intake (Lesage *et al.*, 2002; Coan *et al.*, 2010). Fetal growth restriction in
448 the corticosterone-treated, pair fed animals may have been due to reduced amino acid supply,
449 as placental methylaminoisobutyric acid clearance is known to be decreased in the
450 corticosterone-treated mice, irrespective of food intake (Vaughan *et al.*, 2012). Indeed,
451 previous observations show that natural variations in fetal weight are more closely related to
452 placental transport of amino acids than glucose at D19 (Coan *et al.*, 2008). Moreover,
453 although glucose transport capacity per gram of placenta was not affected in the pair-fed
454 animals, reduced absolute placental weight and vascularity are likely to have impaired net
455 fetal glucose uptake. The reduced size of the corticosterone-treated placenta may be
456 explained, in part, by lower abundance or activity of the Akt protein at both D16 and D19.

457 Akt expression is also reduced in rat placentae growth restricted by maternal dexamethasone
458 administration (Ain, 2005), although this was accompanied by reduced *Igf2* expression,
459 unlike the present study. Unlike previous studies in dexamethasone-treated rats rats (Hewitt
460 *et al.*, 2006), placental *Vegf* expression was increased by corticosterone, suggesting that there
461 may be an attempt to compensate for the reduction in blood vessel density reported
462 previously in these mice (Vaughan *et al.*, 2012). The more marked direct effect of
463 corticosterone on D19 than D16 placentae may reflect the lower placental expression of the
464 glucorticoid metabolising isoform of 11β-hydroxysteroid dehydrogenase (*Hsd2*) in later
465 pregnancy (Condon *et al.*, 1997).
466
467 The observed changes in materno-fetal MeG clearance in corticosterone-treated dams at D19
468 were not related to placental expression of the glucose transporter genes, *Slc2a1* and *Slc2a3*,
469 which did not differ with treatment at this age. On the other hand, *Slc2a1* and *Slc2a3* were
470 upregulated by corticosterone treatment on D16, without a change in MeG clearance. These
471 adaptations in placental glucose transport may also relate to changes in expression of glucose
472 transporter genes *Slc2a8* and *Slc2a9*, which have been shown recently to be regulated by
473 endocrine signals in the mouse placenta (Jones *et al.*, 2013). However, post-transcriptional
474 mechanisms may also have a role in regulating placental nutrient transport when
475 glucocorticoids are raised. Certainly, adaptations in placental System A amino acid transport
476 capacity have previously been shown to be temporally dissociated from *Slc38a* amino acid
477 transporter expression in corticosterone treated mice (Vaughan *et al.*, 2012). In the present
478 study, placental glucose transport capacity was specifically reduced when maternal plasma
479 corticosterone concentration and placental *Redd1* expression were raised in the *ad libitum*
480 fed, corticosterone treated mice. In pair-fed, corticosterone treated dams, when plasma
481 corticosterone and placental *Redd1* expression were similar to controls, MeG clearance was
482 not decreased even though the alterations in maternal insulin signalling and feto-placental
483 growth suggested that these dams had received a physiologically effective dose of
484 corticosterone. Thus, placental *Redd1* expression was closely related to both the prevailing
485 maternal corticosterone concentration and glucose transport across the placenta.
486
487 In contrast to the current findings, corticosterone reduces placental amino acid transport in
488 both *ad libitum* and pair fed dams (Vaughan *et al.*, 2012). Corticosterone, therefore, appears
489 to affect placental transport of glucose and amino acids by different mechanisms. Since the
490 ratio of phosphorylated to total 4E-BP1 and p70 S6 kinase abundance was reduced in

491 corticosterone-treated placentae irrespective of food intake in the current study, placental
492 amino acid transport appears to be more directly related to inhibition of mTOR than placental
493 glucose transport. Certainly, 4E-BP1 and p70 S6 kinase hypophosphorylation is associated
494 with reduced amino acid transporter protein expression in the placentae of rat dams deprived
495 of protein during pregnancy (Rosario *et al.*, 2011). Collectively, these observations suggest
496 that corticosterone reduces placental glucose transport via *Redd1* independently of mTOR
497 while it acts to lower placental amino acid transport by inhibition of mTOR dependent
498 translation or translocation of transporters to the cell membrane. Indeed, the placental
499 adaptation in signalling downstream of mTOR induced by corticosterone overexposure may
500 be indirect and mediated by the maternal hyperinsulinaemia observed in both the *ad libitum*
501 fed and pair-fed groups of dams. The precise effects of corticosterone on placental nutrient
502 transport, therefore, depend on both its actual circulating concentration and its longer term
503 actions on maternal metabolism and other metabolic hormone concentrations. Fetal sex may
504 also be an important factor in determining the effect of maternal corticosterone on materno-
505 fetal nutrient transport, as the normal permeability of the placenta to natural glucocorticoids
506 is known to differ between male and female conceptuses (Montano *et al.*, 1993).
507
508 Overall, the data show that increased concentrations of natural glucocorticoids in the
509 pregnant mother may limit materno-fetal glucose partitioning and thus fetal growth by
510 reducing placental glucose transport capacity. Moreover, whilst natural glucocorticoids
511 increase insulin signalling and glucose transporter expression in the maternal liver, they
512 impair insulin signalling in the placenta with possible consequences for the activity of other
513 nutrient transport systems. As glucose is the major substrate of fetal growth and oxidative
514 metabolism, these effects are likely to impact upon the size and metabolic phenotype of the
515 infant at birth. However, the interaction of glucocorticoids with other nutrient sensing
516 pathways in the placenta may represent a target for interventions to rescue fetal growth when
517 maternal glucocorticoids are raised during pregnancy.
518

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776 **Competing interests**

777 The authors have no competing interests

778

779 **Author Contributions**

780 ORV and ALF conceived of and designed the experiments. All authors collected analysed
781 and interpreted the data. ORV, ANS-P and ALF drafted the article and revised it critically for
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791

792

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.