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Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: studies with the 11 β-hydroxysteroid dehydrogenase inhibitor carbenoxolone

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Summary Recent human epidemiological studies have linked low birth weight with a substantially increased risk of non-insulin-dependent diabetes mellitus in later life. These data suggest that the intrauterine environment plays a crucial role in determining later glucose homeostasis, but the mechanism is unknown. We have proposed that exposure of the fetus to excess maternal glucocorticoids may underpin the epidemiological findings. Normally placental 11 β-hydroxysteroid dehydrogenase type 2 (11 β-HSD-2) protects the fetus from the normally higher maternal levels of glucocorticoids by inactivating corticosterone and cortisol to inert 11-keto products. Here we show that administration of carbenoxolone, an inhibitor of placental 11 β-HSD 2, to pregnant rats, leads to a significant reduction in average birth weight (20 % fall). At 6 months of age, the male offspring of carbenoxolone-treated pregnancies had similar weights to controls, but showed significantly higher fasting plasma glucose (6.0 ± 0.3 vs 4.8 ± 0.2 mmol/l; $p < 0.01$) and exhibited significantly greater plasma glucose (10 % higher) and insulin (38 % higher) responses to an oral glucose load. These effects of carbenoxolone require intact *maternal* adrenal glands suggesting that inhibition of feto-placental 11 β-HSD 2 is key. These data support the notion that deficiency of placental 11 β-HSD, by exposing the fetus to excess maternal glucocorticoids, reduces growth and predisposes to hyperglycaemia in later life. [Diabetologia (1996) 39: 1299–1305]

Keywords Glucocorticoids, 11 β-hydroxysteroid dehydrogenase, carbenoxolone, diabetes mellitus, birthweight.

Recent epidemiological studies in a range of human populations have shown that low birth weight and other markers of an adverse intrauterine environment (low ponderal index, thinness at birth) are associated with a much higher incidence of cardiovascular disease [1, 2], hypertension [3–6] and non-insulin-dependent diabetes mellitus [5, 7] in subsequent adult life. These relationships are independent of classical adult risk factors (obesity, smoking, excessive alcohol intake, social class) which are indeed additive to the effects of early life [8, 9]. Importantly, this is not merely disease following very low birth weight or prematurity, but reflects a graded relationship across the normal range of term birth weights [9]. The association between low birth weight and later alterations in glucose tolerance has now been shown in several distinct populations [5, 7, 10–14] and these findings have prompted suggestions that specific events in prenatal development may programme later metabolic responses and, in particular, the control of blood pressure and glucose metabolism [5]. The mechanisms of such prenatal programming are undefined, but Hales et al. [5] has proposed that early nutrition is instrumental (the thrifty phenotype hypothesis).

Recently, we proposed an alternative mechanism: that exposure of the fetus to maternal glucocorticoids

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Abbreviations: 11 β-HSD, 11 β-hydroxysteroid dehydrogenase; OGTT, oral glucose tolerance test.

might explain the link between low birth weight and later disease, notably high blood pressure [15]. Glucocorticoids have well-described hypertensive and hyperglycaemic effects in the adult [16] and administration during pregnancy is known to reduce birth weight of animals and humans [17–19]. In addition, glucocorticoids are involved in the development and maturation of various fetal organ systems [20–22] and both glucocorticoids [23] and other steroid hormones [24] exert permanent "programming" effects which have been invoked to explain the links between early life events and later disease. Thus, steroid hormones act during specific periods of prenatal and postnatal development to organise or "imprint" permanent patterns of tissue responses which persist throughout life [23, 24]. Indeed, treatment of pregnant rats with the synthetic glucocorticoid dexamethasone, in a low dose which only modestly reduces birthweight, produces permanent rises in blood pressure in the adult offspring [25].

Normally, exposure of the fetus to the much higher levels of glucocorticoids in the maternal blood is minimised by a placental enzyme 11 β-hydroxysteroid dehydrogenase type 2 (11 β-HSD-2). This enzyme catalyses the rapid conversion of active, receptor-binding physiological glucocorticoids (cortisol in humans, corticosterone in rats) to inert 11-keto derivatives (cortisone, 11 dehydrocorticosterone) while the type 1 isoform of the enzyme (11 β-HSD-1) is present in the liver but not placenta, and favours the reverse reaction. 11 β-HSD-2 is highly expressed in the placental syncytiotrophoblast [26] and maintains a gradient of cortisol from the maternal to the fetal circulation [27] (Fig. 1). 11 β-HSD-2 activity in the placenta is directly related to birth weight both in rats [25] and humans [28, 29]. Patients bearing mutations of the gene encoding 11 β-HSD-2 have low birth weight [30]. These data are in keeping with the hypothesis that fetal glucocorticoid exposure is of importance in determining birthweight; however, any relationship between placental 11 β-HSD-2 and glucose tolerance in later life is unclear. We have therefore examined the effect of administration to pregnant rats of carbenoxolone, a potent inhibitor of 11 β-HSD-2 [26], on birth weight and later glucose tolerance in the adult offspring.

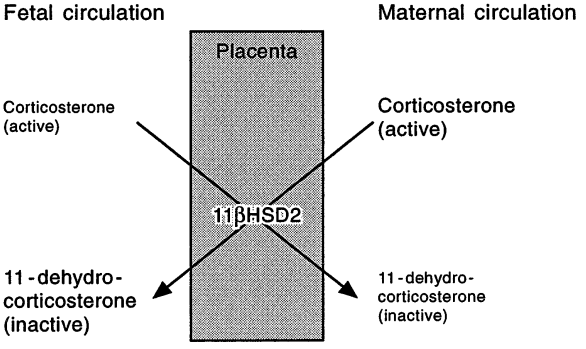

Fig. 1. Corticosterone in the maternal circulation (derived from the maternal adrenal), is converted in the placenta to the inactive form 11-dehydrocorticosterone by action of 11 β HSD 2

Materials and methods

Carbenoxolone treatment in adrenal intact animals. Female Wistar rats (200–250 g, Harlan UK Ltd, Bicester, UK) were maintained under conditions of controlled lighting (lights on 07.00–19.00 hours) and temperature (22 °C) and allowed free access to food (standard rat chow; 56.3 % carbohydrate, 18.3 % protein, NaCl 0.7 %; B. S. & S. Scotland Ltd. Edinburgh, UK) and tap water. In all experiments standards conforming to "The Principles of Animal Care" (NIH publication No. 85-23, revised 1985) were followed. The rats were time-mated and then given either carbenoxolone (12.5 mg/day in 4 % ethanol-saline, 0.1 ml, s. c., Sigma, Poole, Dorset, UK) or vehicle alone (CON) throughout pregnancy. At birth, the offspring were weighed and then no further treatment was given (to mothers or pups).

Carbenoxolone treatment in adrenalectomised animals. Non-pregnant female rats underwent adrenalectomy by the dorsal approach under halothane anaesthesia and thereafter were given saline to drink. Controls were sham-operated (SHAM) and drank water. Blood (for plasma corticosterone estimation) was subsequently taken at 09.00 hours by tail tipping to assess the completeness of adrenalectomy. Adrenalectomised (ADX) animals were time-mated 8–15 days after surgery and treated throughout pregnancy with carbenoxolone (ADX + CBX; 12.5 mg/day, s. c.) or vehicle alone (ADX). Sham-adrenalectomised controls received vehicle.

Measurement of maternal blood pressure, glucose and corticosterone. A separate cohort of adrenal-intact pregnant rats were time-mated and then given either carbenoxolone (CBX; 12.5 mg/day in 4 % ethanol-saline, 0.1 ml, s. c.) or vehicle alone (CON) throughout pregnancy, as above. To assess blood pressure of the dam, a cannula was inserted into the right carotid artery under halothane anaesthesia and the animals allowed to recover for at least 72 h. Blood pressure was measured directly in conscious, unrestrained animals using a pressure transducer (Lectromed Multitrace 2; UK) for 10 min on three separate occasions. Samples for assessment of glucose and corticosterone were obtained from the same animals at 09.00 hours at least 72 h after cannulation. The coefficient of variation for the repeated measures of blood pressure was 6.9 % for mean arterial pressure.

Measurement of placental 11 β-HSD activity. Placental 11 β-HSD-2 activity was assessed in separate groups of carbenoxolone and control-treated pregnant animals by infusion of [^{3}H] corticosterone to achieve steady state and subsequent extraction of [^{3}H] corticosterone and [^{3}H] 11-dehydrocorticosterone from the blood of the dams, fetal tissues and placenta. On days 17–21 of gestation animals (5 control, 3 treated with carbenoxolone 12.5 mg/day throughout pregnancy) were subjected to halothane anaesthesia and the left carotid and right jugular vein were cannulated. Animals were given a priming dose of [^{3}H] corticosterone (3 μCi in 0.6 ml 0.9 % NaCl; Amersham, Amersham, UK) and followed by a constant infusion of 0.15 $\mu Ci \cdot min^{-1} \cdot 30\ \mu l^{-1} \cdot min^{-1}$ for a total of 80 min via the

jugular catheter. Samples of arterial blood (300 μl with replacement of volume with 0.9% NaCl) were obtained at 20, 40, 60 and 80 min of infusion to ensure steady-state and blood pressure and pulse rate, measured as above (Lectromed Multitrace 2). At 80 min, placental and fetal tissues were removed and frozen in liquid nitrogen. Steroids were extracted with ethyl acetate from three placentae and matched fetuses from each animal and separated by thin layer chromatography [31]. Activity of 11 β-HSD-2 was assessed by the increase in [^{3}H] 11-dehydrocorticosterone from arterial blood to placental tissue.

Oral glucose tolerance test (OGTT). At 6 months of age male offspring underwent an OGTT. Animals were fasted from 16.00 hours the day before and 2 g/kg glucose (as a 0.5 g/ml solution) was given by gavage between 08.00 and 09.00 hours the next morning. Blood was taken by tail tipping at 0, 30, 60, 90 and 120 min, centrifuged immediately and the plasma stored at – 70 °C.

Glucose, insulin and corticosterone assays. Plasma glucose was determined by an enzymatic (glucose oxidase) method using a Beckman Synchron CX3 multichannel analyser (Beckman Instruments Ltd, High Wycombe, UK). The intra-assay and inter-assay coefficients of variation were less than 1% and 2.2%, respectively. Plasma insulin was determined as previously described [32] using rat insulin standards (Novo Nordisk, Copenhagen, Denmark) and iodinated insulin (Lifescreen, Watford, UK). The intra-assay and inter-assay coefficients of variation of this method are less than 10% throughout the range. Corticosterone was estimated using a radioimmunoassay [33]. The intra-assay coefficient of variation was 3.8%.

Statistical analysis

All data are expressed as mean ± SEM. Data were compared using unpaired Student's *t*-tests or one or two-way ANOVA followed by Newman-Keuls post-hoc multiple comparisons test, where appropriate. Values were considered significant when p was less than 0.05.

Results

Effect on birth weight of carbenoxolone in adrenal intact and adrenalectomised rats. Administration of carbenoxolone to adrenally intact pregnant rats led to a 20% reduction in offspring birth weight (CBX 4.54 ± 0.08 g, $n = 35$; CON 5.68 ± 0.07 g, $n = 39$; $p < 0.01$; Fig. 2) but did not affect litter size (CON 9.7 ± 1.1, $n = 4$; CBX 8.7 ± 1.7, $n = 4$) or the length of gestation (CON 22.2 ± 0.2 days; CBX 22 ± 0.1 days). Adrenalectomy reduced plasma corticosterone levels in pregnant rats to less than 60 nmol/l, compared to 898 ± 103 nmol/l in the sham-operated controls. Adrenalectomy itself was associated with a 9% reduction in offspring birth weight, but there was no additional effect of carbenoxolone on birth weight in adrenalectomised animals (Fig. 2). Adrenalectomy, with or without carbenoxolone, did not affect litter size (SHAM 7.2 ± 1.3, $n = 5$; ADX 8 ± 0, $n = 4$; ADX + CBX 7.3 ± 2.7, $n = 3$) or the length of gestation (SHAM 22.2 ± 0.4 days; ADX 22 ± 0 days; ADX + CBX 21.7 ± 0.9 days). Adrenalectomised and sham-operated rats showed similar weight gains through pregnancy (SHAM 84 ± 7 g; ADX 83 ± 15 g; ADX + CBX 66 ± 18 g).

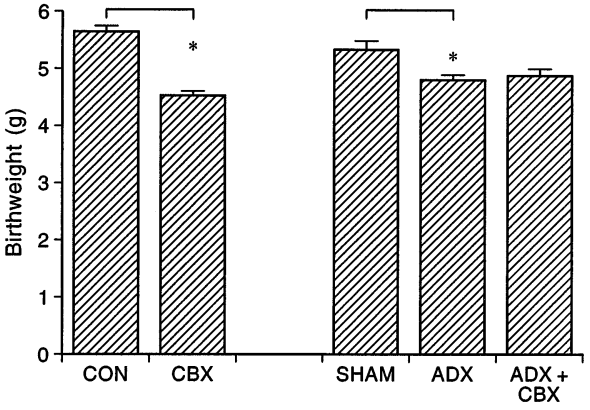

Fig. 2. Effect of carbenoxolone on birthweight. Birth weight (g) of the offspring of pregnant rats treated with vehicle control (CON $n = 39$), carbenoxolone (CBX $n = 35$), sham adrenalectomy (SHAM $n = 36$), adrenalectomy (ADX $n = 24$) and adrenalectomy and carbenoxolone (ADX + CBX $n = 22$) throughout pregnancy. * $p < 0.05$ compared with control

Effect of carbenoxolone on maternal blood pressure, glucose and corticosterone. Treatment with carbenoxolone had no significant effect on *maternal* blood pressure (measured directly in unrestrained rats) on days 18–20 of pregnancy (CON, systolic 117 ± 2 mm Hg and diastolic 86 ± 5 mm Hg, $n = 4$; CBX, systolic 119 ± 3 mm Hg and diastolic 77 ± 4 mm Hg, $n = 3$). Similarly, neither maternal plasma glucose (CON, 5.3 ± 0.3 mmol/l, $n = 4$; CBX, 6.4 ± 0.3 mmol/l, $n = 4$) nor maternal plasma corticosterone at 09.00 hours (CON 692 ± 172 nmol/l; CBX 647 ± 101 nmol/l) were significantly altered by carbenoxolone.

Effect of carbenoxolone on placental 11 β-HSD in vivo. Maternal blood concentrations of [^{3}H] corticosterone and [^{3}H] 11-dehydrocorticosterone were similar in control and carbenoxolone groups at 20, 40, 60 and 80 min (two-way ANOVA), indicative of isotopic steady-state. Carbenoxolone did not alter the metabolic clearance rate for corticosterone (CON 11.8 ± 0.9 $n = 5$; CBX 11.0 ± 1.3 ml/min, $n = 3$) and infused pregnant animals had similar blood pressures (mean arterial pressure CON 77.2 ± 4.5; CBX 82 ± 5.3 mm Hg). There was no difference with treatment in the percentage of total [^{3}H] steroids as corticosterone in maternal blood at steady-state (CON 90.8 ± 2.1; CBX 89.5 ± 3.4%). Carbenoxolone treatment reduced placental 11 β-HSD activity (activity as percentage of matched control: CON 100 ± 9.4; CBX 63.5 ± 8.6%; $p < 0.05$) and led to an increase in

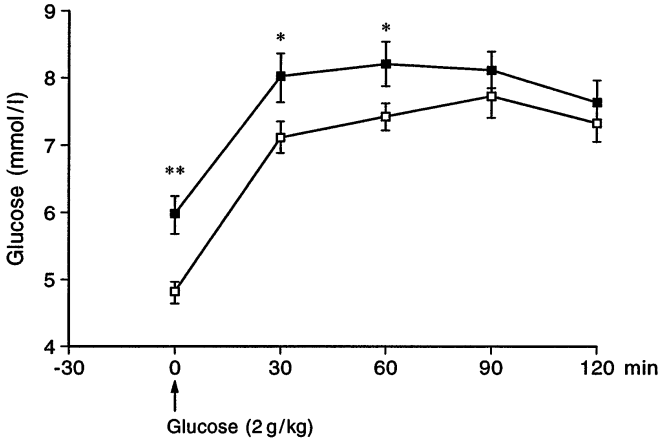

Fig. 3. Plasma glucose response to an oral glucose load in offspring at 6 months. Pregnant rats received vehicle (—□— CON $n = 18$) or carbenoxolone (- - -■- - - CBX $n = 14$). Treatment was only during pregnancy. Repeated measures ANOVA, $F = 5.93$, $p < 0.05$. ** $p < 0.01$; * $p < 0.05$, unpaired t-test vs control for individual timepoints

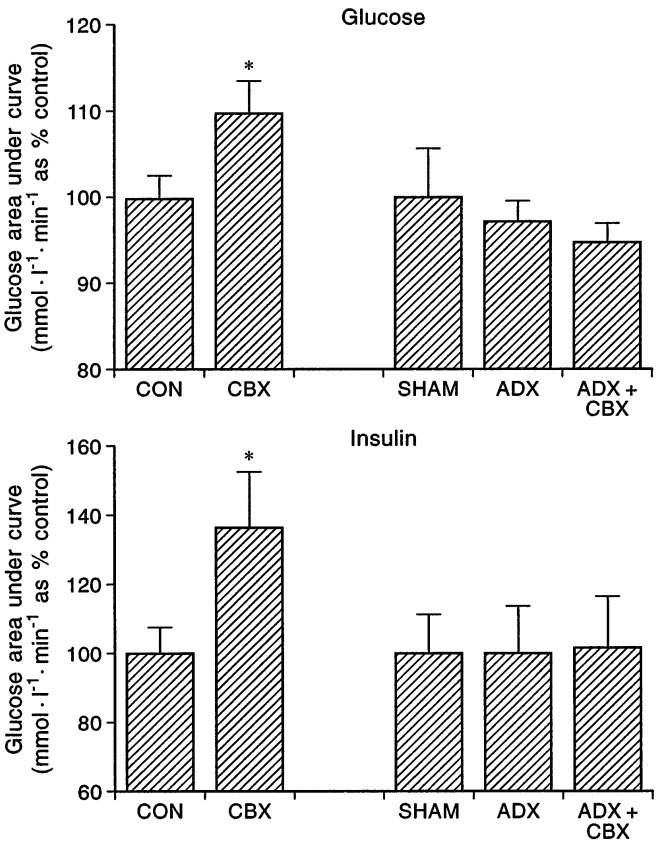

Fig. 4. Glucose and insulin responses to an oral glucose load in offspring of carbenoxolone treated rats. Area under curve for glucose and insulin in 6-month-old offspring of rats treated in pregnancy with vehicle control (CON $n = 18$), carbenoxolone (CBX $n = 14$), sham adrenalectomy and vehicle (SHAM $n = 8$), adrenalectomy and vehicle (ADX $n = 8$) and adrenalectomy with carbenoxolone (ADX + CBX $n = 8$). * $p < 0.05$ compared with control

[^{3}H] corticosterone as a percentage of total [^{3}H] -steroids in fetal tissues (CON 65.6 ± 1.6; CBX $74.2 \pm 2.0\%$; $p < 0.05$).

Adult offspring response to an oral glucose load. At 6 months of age, the male offspring of adrenal intact rats treated with carbenoxolone during pregnancy displayed higher fasting plasma glucose levels (CON 4.8 ± 0.2; CBX 6.0 ± 0.3 mmol/l; $p < 0.01$). The plasma glucose response to an oral glucose load was also higher in the offspring of carbenoxolone-treated pregnancies (CON vs CBX repeated measures ANOVA, $f = 5.93$, $p = 0.02$, Fig. 3). The area under the glucose curve across the OGTT was significantly (10%) higher for the offspring of carbenoxolone-treated pregnancies and the response of insulin in this group was also significantly (38%) greater (Fig. 4). Body weights were similar at 6 months in all groups.

Maternal adrenalectomy prevented the effect of carbenoxolone on offspring glucose tolerance. There was no difference in glucose tolerance in the offspring of the adrenalectomised pregnant females with or without carbenoxolone in terms of either basal glucose or the response of plasma glucose or insulin (Fig. 4).

Discussion

Carbenoxolone is known to act *in vitro* as an inhibitor of placental 11 β-HSD-2 [26]. Here we demonstrate that administration of carbenoxolone inhibits placental 11 β-HSD-2 activity *in vivo* and allows increased passage of maternal corticosterone to the fetus, at least within the last week of pregnancy. Further, carbenoxolone treatment reduces birthweight, an effect similar to that observed with dexamethasone [25], a synthetic glucocorticoid which is a poor substrate for 11 β-HSD-2 [26, 34]. Finally, the adult offspring of carbenoxolone-treated dams display altered glucose tolerance, with both higher fasting glucose and increased glucose and insulin responses to an oral glucose load in adulthood. The importance of 11 β-HSD-2 in limiting fetal exposure to maternal glucocorticoid [27, 35] is supported both by the effect of carbenoxolone to increase [^{3}H] corticosterone access to the fetal circulation and the observation that the effects on both birthweight and offspring glucose tolerance are dependent on the presence of intact maternal adrenal glands. This suggests that carbenoxolone does not have direct effects on the dam, but acts via increased exposure of the feto-placental unit to maternal glucocorticoids.

There are many potential mechanisms whereby carbenoxolone treatment might act to produce glucose intolerance in later life. Maternal factors clearly contribute to the fetal environment and potentially

to later disease. In particular, the supply of metabolic fuels from mother to fetus has been proposed to have long-term effects on offspring metabolism [36] and this hypothesis has found support in the adverse effects of maternal hyperglycaemia on offspring glucose handling in human populations [37–39] and animals models [40–43]. In our model, however, there was no increase in *maternal* blood glucose with carbenoxolone, rendering this potential mechanism unlikely. Similarly, other indirect effects mediated via maternal sodium retention and hypertension appear not to be involved in the action of carbenoxolone in this study. It seems more probable that carbenoxolone acts directly on the placenta or fetus to exert its actions. The lack of effect of carbenoxolone on birth weight or offspring glucose control in adrenalectomised rats, strongly points to a mechanism requiring maternal glucocorticoids. This is likely to be inhibition of placental and/or fetal tissue 11 β-HSD, which would increase fetal exposure to (maternal) corticosterone. Other enzymes are also affected by carbenoxolone in vitro [44, 45], although the concentrations required are much higher than the nanomolar K_i for 11 β-HSD [26] and are unlikely to be achieved in vivo [46]. Moreover, it is more difficult to conceive how effects on other enzymes might be dependent upon maternal adrenal products.

Glucocorticoids might act in a number of ways in the developing animal to provoke later glucose intolerance. Glucocorticoids inhibit insulin release [47] and islet beta-cell replication in vitro [48, 49]. Thus, increased exposure to maternal glucocorticoids may permanently reduce beta-cell mass, later expressed as impaired glucose tolerance. Equally, glucocorticoids may act to programme hormonal responses or metabolic pathways. Glucocorticoids exert important maturational effects on adrenergic receptor systems [50, 51] and a variety of key metabolic enzymes [22, 52]. These include phosphoenolpyruvate carboxykinase, the rate-limiting enzyme in gluconeogenesis, which is directly and potently regulated by glucocorticoids at the level of transcription [53]. Early exposure to glucocorticoids might programme these systems to alter permanently carbohydrate metabolism. Alternatively, prenatal and immediate postnatal stress (and glucocorticoids) are well-documented to programme increased hypothalamic-pituitary-adrenal axis activity producing glucocorticoid hypersecretion throughout life [54, 55]. Such an effect is indeed observed after prenatal dexamethasone exposure [56] and might of course contribute to hyperglycaemia. Finally, glucocorticoids may act indirectly by influencing fetal or placental expression of key growth factors. In this regard glucocorticoids regulate the synthesis of insulin-like growth factors 1 and 2, many of their binding proteins and both receptor subtypes in the fetus and placenta [57, 58].

Clearly excess glucocorticoid exposure is not the only determinant of birth weight. Maternal adrenalectomy also reduces birth weight, but importantly the offspring of adrenalectomised pregnancies, although smaller, do not display alterations in glucose handling. Obviously not all manipulations that attenuate birth weight programme hyperglycaemia, a notion supported by the human epidemiological data which emphasise the importance of thinness (low ponderal index) rather than merely birth weight *per se* [1, 7, 59].

Undernutrition in early life has also been suggested to be of critical importance in determining fetal growth and later disease [8]. This is supported by the demonstration of impaired endocrine pancreatic function following protein malnutrition during prenatal [60] or early postnatal life [61] in animal models. It is therefore intriguing to note that maternal protein restriction during rat pregnancy also attenuates placental 11 β-HSD activity [62]. Thus, although many of the molecular details require elucidation, these data support the hypothesis that placental 11 β-HSD-2 deficiency, by allowing increased fetal exposure to maternal glucocorticoids, plays a key role in mediating the effects of deleterious genetic [30] and maternal environmental factors upon feto-placental growth and the programming hyperglycaemia and diabetes in adult life.

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