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Role of glucocorticoids in programming of maternal diet-induced hypertension in the rat

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A rat model of hypertension induced by in utero *exposure to maternal low protein diets has previously been described. Low protein exposed rat pups are of lower weight at birth and have large associated placentas. Such animals are proposed, therefore, to mirror individuals in the human population perceived to be at greater risk of cardiovascular disease in adulthood. Recent work has suggested that maternal glucocorticoids may programme this increased risk of later disease. The role of glucocorticoids in programming the hypertensive state was assessed by administration of the 11β-hydroxylase inhibitor, metyrapone, to pregnant rats consuming 18 (control) or 9% (low protein) casein diets. At day 14 of pregnancy, fetuses and placentas of low protein-fed rats were significantly larger than those of controls. Metyrapone significantly inhibited corticosterone synthesis in both dietary groups, and attenuated the more rapid growth of fetus and placenta in the low protein fed group. Systolic blood pressures of rats exposed to the low protein diet* in utero *were significantly higher (29 mmHg) than those exposed to the control diet. Metyrapone abolished the hypertensive state of low protein exposed rats, but in the control group significantly elevated blood pressure by 15 mm Hg. Maternal and fetal glucocorticoid interactions* in utero *clearly have an important role in determining future regulation of blood pressure. Maternal-diet induced hypertension in the rat would appear to be a glucocorticoid-dependent phenomenon.* (J. Nutr. Biochem. 7:173–178, 1996.)

Keywords: glucocorticoids; hypertension; rat; intrauterine programming; maternal diet

Introduction

Recent epidemiological studies have emphasised the importance of the environment *in utero* and maternal/fetal interactions as determinants of disease in adulthood. Low birthweight and increased placental weight are strong predictors of future risk of hypertension,[1–3] diabetes,[4] chronic lung disease[5] and mortality from ischaemic heart disease.[6–9] These observations have proved robust, in a number of different populations[3,10] despite possible confounding social[11] and genetic factors.[12] Less than optimal maternal nutrition is a known causal factor in human fetal growth retardation[13] and has been proposed to underly the observed association between growth *in utero* and later disease.[14–16]

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The precise molecular mechanisms responsible for the programming of hypertension and other non-communicable diseases of adulthood by maternal dietary factors *in utero*, remain to be determined. A role for glucocorticoids has been advanced, as the treatment of pregnant rats with dexamethasone induces hypertension in the resulting offspring.[17,18] Low placental activity of the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD), which protects the fetus from maternal glucocorticoid influences, is associated with lower fetal weight and increased placental size in both rats[17,18] and humans (Lindsay et al., unpublished observations).

A rat model in which the feeding of low protein diets during pregnancy induces hypertension,[19,20] has recently been described. Though clearly supporting a role for maternal dietary factors in the programming of hypertension, this model also provides evidence of glucocorticoid involvement. Placental 11βHSD activity is lower in rats consuming low protein diets,[21] raising the prospect that over-

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exposure of the fetus to maternal glucocorticoids irreversibly alters homeostatic mechanisms responsible for the normal regulation of blood pressure (Figure 1). Recent evidence suggests long term modulation of the hypothalamic-pituitary-adrenal axis by low protein diets. Adult animals exposed to 9% casein *in utero* display elevated activities of glucocorticoid-inducible enzymes and altered patterns of type II receptor distribution (Langley-Evans et al. unpublished observations).

In the present study we describe the modulatory action of pharmacological blockade of glucocorticoid synthesis, upon the effects of maternal diets of differing protein content on the blood pressures of young adult rats. Metyrapone is used as an inhibitor of the enzyme 11β-hydroxylase in the corticosterone synthetic pathway.[22,23] This agent is not specifically antiglucocorticoid in its action, but it has been demonstrated to attenuate both maternal and fetal corticosteroid production in pregnant rats,[23] lacks the adverse effects on the maternal system associated with cortexolone or RU486 treatment, and avoids the problems associated with surgical adrenalectomy in pregnant animals.

Methods and materials

Chemicals

All chemicals were of analytical grade and were purchased from Sigma (Poole, UK). Dietary materials were obtained from Special Diet Services (SDS) Ltd (Cambridge, UK).

Animals

All the animal experiments were performed in accordance with license number PPL90/00430 granted under the Home Office Animal Act, 1986. A total of 36 adult female Wistar rats were used in the study to generate fetal material and 47 weanling offspring. Adult rats for breeding were housed individually, whereas weanling rats were housed in pairs. Cages were made of wire mesh and kept in a room maintained at 24°C on a 12-h light cycle. All rats had free access to food and water.

Protocol

Thirty-six virgin, female rats weighing 200 to 250 g were fed diets containing either 18% casein (control diet; $n = 18$ rats) or 9% casein (low protein diet; $n = 18$ rats) for a period of 14 days to habituate them to the feeding regimen. The full composition of each diet is shown in *Table I*. At the end of the 14-day period, the rats were mated and thereafter maintained on the same diets. The detection of a mucous plug on a white paper sheet placed under the wire floor of the cage was taken as an indicator of conception, and the following day noted as day 1 of pregnancy.

Nine rats from each dietary group were injected twice daily with 5 mg/kg body weight metyrapone suspended in arachis oil. A preliminary study using doses of 5, 10, or 20 mg/kg body weight/day metyrapone indicated that the twice daily administration of 5 mg/kg body weight had no adverse effect upon the outcome of pregnancy (i.e., no reduction of litter size or fetal growth). Injections were administered subcutaneously, in a total volume of 0.1 ml, at 0900 and 1600 each day until day 14 of pregnancy. The remaining nine animals in each group were injected with 0.1 ml arachis oil twice daily. All animals were provided with access to 0.9% saline in addition to drinking water to maintain salt balance. On day 14 of pregnancy four rats in each group were sacrificed. The 0900 dose of oil or drug was administered and at 1100 the rats were rapidly killed by cervical dislocation and blood samples obtained for analysis of maternal corticosterone concentrations. Fetal corticosterone concentrations could not be measured as insufficient blood could be collected, even on a pooled litter basis. Fetal and placental material was carefully weighed.

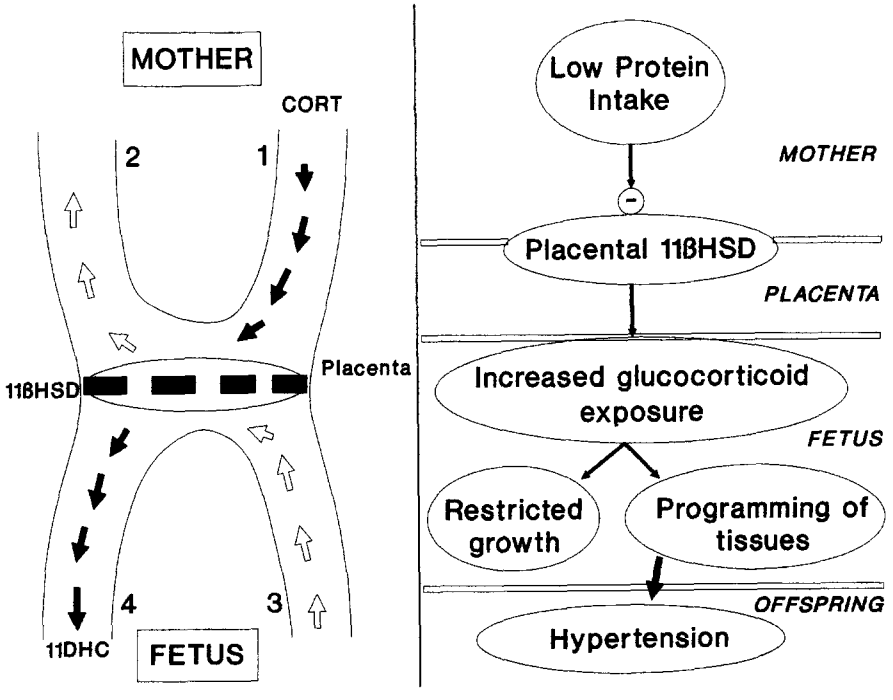

Figure 1 Placental 11βHSD protects the fetus against programming effects of maternal glucocorticoids: modulation by nutritional factors. 11βHSD in the placenta metabolises active glucocorticoids to inactive forms by an NAD-dependent mechanism. Inactivated steroid moves freely across the placenta into the fetal circulation. Maternal undernutrition reduces 11βHSD activity and lowers the protective screen for the fetus. 1–uterine artery 2–uterine vein 3–umbilical artery 4–umbilical vein CORT–corticosterone (rat), cortisol (human) 11DHC–11 dehydrocorticosterone (rat), cortisone (human).

Table 1 Composition of diets

	Dietary Protein	
	18	9
	g/100 g diet	
Casein	18.0	9.0
Cornstarch	42.5	48.5
Fibre	5.0	5.0
Sucrose	21.3	24.3
Choline chloride	0.2	0.2
Methionine	0.5	0.5
AIN-76 Mineral mix	2.0	2.0
AIN-76 Vitamin mix	0.5	0.5
Corn oil	10.0	10.0

Diets were given to the animals as dried balls of 60–100 g weight. AIN-76 mixes were supplied by Special Diet Services (UK).

The remaining five rats in each group were allowed to proceed to full gestation. On giving birth all rats were transferred to a standard laboratory chow diet containing 18.3% protein (CRMX) chow, SDS Ltd). The rats remained on this diet throughout suckling, and the same chow was used to wean the pups at 4 weeks of age. These animals differed, therefore, only in terms of their prenatal dietary experience. At 8 weeks of age a random selection of two to four rats from each litter were removed for determination of blood pressure.

Measurement of blood pressure

Blood pressure was measured using an indirect tail cuff method as previously described.[19] The instrument used was an IITC Model 29 Blood Pressure Monitor with IITC software (Linton Instruments, Diss, UK) to monitor each measurement. This software determined systolic pressure using a preset algorithm, thereby ensuring consistency of measurement. Determinations were performed between 1,000 and 1,100 h in a room maintained at 27°C to which the rats had been previously acclimatized. The same operator performed all measurements and was blinded to the prenatal exposure of the rats. Unlike other indirect measurement systems, with the IITC instrument no further heat shocking is required. Tail cuffs were selected according to the size of each individual rat, and inflated to 300 mm Hg to occlude the tail arterial pulse. Deflation at 3 mm Hg per second allowed determination of systolic pressure as the pulse returned. Consecutive measurements (4–5) were performed on each rat over a period of 5 min. The average systolic pressure was recorded. The coefficient of variation for the measurement is less than 5%.

Determination of plasma corticosterone

Blood samples obtained by heart puncture were centrifuged at 3,000 rpm, 4°C for 15 min. Plasma was removed and stored at −80°C for up to 1 month before analysis. Corticosterone was measured by radioimmunoassay as previously described,[24] using an antibody raised in rabbit (Sigma, UK).

Statistical analysis

All data are presented as mean ± SEM. Where appropriate two-way analysis of variance (ANOVA) was performed, using a Tukey test as a secondary analysis where significant interactions were indicated. A probability of 5% or less was accepted as significant.

Results

Maternal weight gain during the period of feeding and drug treatment was similar in all groups (*Table 2*). At mating all animals were of similar weight (control:arachis oil 269 ± 7 g; control:metyrapone 261 ± 12 g; low protein:arachis oil 267 ± 9 g; low protein:metyrapone 243 ± 7 g). Weight gain over the first 14 days of pregnancy was not significantly impaired by either the low protein diet or drug treatment. Similarly, litter size (*Table 2*) was not compromised by diet or drug treatments. Significant effects of maternal diet and metyrapone were, however, noted with respect to day 14 fetal and placental weights. Animals consuming the low-protein diet had significantly heavier fetuses and placentas than animals consuming the control diet. Metyrapone treatment reduced fetal and placental weights in both dietary groups, but the effect was significantly greater in the low protein fed group ($P < 0.05$), such that after metyrapone treatment fetal and placental weights in the two dietary groups were no longer different.

The efficacy of metyrapone as an inhibitor of maternal corticosterone synthesis was assessed by determining plasma corticosterone concentrations on day 14 of pregnancy. Plasma corticosterone concentrations were similar in arachis oil injected rats consuming 18 and 9% casein diets (18% casein/arachis oil: 22.9 ± 5.2 ng/ml; 9% casein/arachis oil: 27.8 ± 16.6 ng/ml). Metyrapone significantly ($P < 0.05$)

Table 2 Maternal weight gains over 14 days of pregnancy, litter sizes, and day 14 fetal and placental weights

Diet (% casein)	Treatment	Wt gain (g)	Litter size	Fetal wt (mg)	Placental wt (mg)
18	Oil	65 ± 9	13.25 ± 1.0	200 ± 7	220 ± 7
	Metyrapone	63 ± 17	14.00 ± 1.5	140 ± 5*	160 ± 9*
9	Oil	76 ± 8	11.50 ± 2.5	330 ± 29*	280 ± 17*
	Metyrapone	62 ± 5	12.50 ± 1.5	150 ± 3*†	170 ± 8*†

Maternal data are shown as means ± SEM for $n = 10$ observations. Two-way ANOVA indicated no significant effect of metyrapone treatment or maternal diet upon maternal weight gain or litter size. Fetal and placental data are shown as mean ± SEM for 34 to 52 observations. Two way analysis of variance indicated significant effects of diet upon fetal weight ($F = 17.8$, $P < 0.001$) and placental weight ($F = 14.2$, $P < 0.0001$). Metyrapone had significant effects upon fetal weight ($F = 22.11$, $P < 0.0001$) and placental weight ($F = 62.06$, $P < 0.0001$). Maternal diet and metyrapone interacted to influence both fetal weight ($F = 4.24$, $P < 0.05$) and placental weight ($F = 7.17$, $P < 0.01$). *indicates significantly different to 18% casein exposed oil treated group ($P < 0.05$). †indicates significantly different to 9% casein exposed oil treated group ($P < 0.05$).

lowered plasma corticosterone concentrations by approximately 90% in both groups of animals (18% casein/metyrapone: 3.2 ± 0.7 ng/ml; 9% casein/metyrapone: 2.5 ± 1.0 ng/ml).

The effects of maternal dietary protein restriction and metyrapone treatment upon the systolic blood pressure of the offspring was assessed when the pups were 8 weeks old. *Table 3* shows the combined body weights and systolic blood pressures of male and female offspring. Although males were significantly heavier than females (males 163 ± 5 g; females 133 ± 3 g, $P < 0.001$), no significant sex differences in blood pressure were observed (males 150 ± 5 mm Hg; females 141 ± 6 mm Hg) and so only the combined data is shown. No relationship was noted between body weight and blood pressure (Spearmans coefficient R = 0.035, Pearsons coefficient $r = 0.043$), indicating that appropriate cuff sizes were selected to match each animals size. At 8 weeks of age both male and female rats exposed to 18% casein diet *in utero* were heavier than rats exposed to 9% casein. Metyrapone had no significant effect on body weight at 8 weeks (*Table 3*). Systolic blood pressures of low protein exposed rats were significantly elevated (29 mmHg) relative to pups exposed to the control diet. Metyrapone treatment had opposing effects on rats exposed to the different maternal diets. In rats exposed to 18% casein *in utero* metyrapone treatment led to an elevated (15 mmHg) blood pressure than in vehicle treated controls. Contrastingly, in the low protein exposed group metyrapone alleviated the maternal diet-induced hypertension. Blood pressure in these animals remained 10 mmHg above the pressures observed in vehicle treated 18% casein-exposed rats, but the difference was no longer statistically significant.

Discussion

The findings of Barker et al. in recent years have indicated a role for maternal nutrition in pregnancy in determining a wide range of degenerative diseases of adulthood, in the offspring.[1,4–9] These observations have profound implications for public health, and it is now necessary to determine the precise mechanisms of maternal dietary programming, the nature of the nutrients involved, and the critical periods in pregnancy associated with programming of specific pathologies. For this purpose a rat model has been developed, with a primary focus on hypertension.[19] In this model the feeding of low protein diets during pregnancy induces hypertension in the resulting offspring. The hypertensive state is of early onset, is apparently lifelong and is associated with lower birthweight and increased placental size.[19,20,25] These observations strongly mirror the findings of human epidemiological studies.

Unlike other strategies used for retarding growth in animals, for example surgical ligation of the uterine artery,[26] which also lead to higher blood pressure in the offspring, the dietary manipulation used in the present study was relatively mild. The precise nature of the dietary restriction and the response of the dams to the low-protein diet is extensively discussed elsewhere.[19,20,25] The typical weight gain of low-protein fed rats in the present study, and the normal litter sizes observed, highlight the low severity of the protocol. The magnitude of the blood pressure difference observed between 18 and 9% casein exposed arachis oil treated pups (29 mm Hg) was consistent with our previous studies.[19,20,25] The purpose of the present study was to examine the proposed link between maternal diet and glucocorticoid action in programming the observed hypertension *in utero*.[21]

The paradigm of pharmacological adrenalectomy using metyrapone was originally proposed and validated in non-pregnant rats by Plotsky and Sawchenko.[22] In the current study the modification of the method for use in pregnant rats[23] was further adapted. A dose of 10 mg/kg body weight/day was shown in preliminary trials to be optimal for survival of the fetuses in utero. Metyrapone inhibits 11β-hydroxylase in both maternal and fetal adrenals[23] and thereby suppresses corticosterone production on both sides of the placenta. In the present study maternal plasma corticosterone concentrations in metyrapone treated animals were 90% lower than in controls, consistent with other studies.[23] Fetal corticosterone was not determined as the animals were too small to obtain samples. It is assumed, however, that metyrapone-exposed fetuses were subject to very low or entirely absent corticosteroid levels, as at day 14 the fetal adrenal is non-functional and metyrapone crosses the placenta.[23]

The administration of metyrapone was found to significantly attenuate growth of fetus and placenta in both maternal dietary groups. In untreated low protein-fed rats the weights of fetuses and placentas were significantly greater at day 14, than in controls. It would appear therefore, that the growth of low-protein exposed fetuses in early development is accelerated and that low protein feeding elicits early placental hypertrophy. This is in accordance with the suggestion that less than optimal nutrition leads to a rapid trajectory of growth in the fetus from conception.[27] These rapidly growing fetuses are perceived to be more vulnerable to maternal programming effects,[27] presumably as the capacity to supply the high demands of the fetus in critical periods of organogenesis is absent. The attenuation of growth by metyrapone may suggest a role for glucocorticoids in determining fetal and placental growth rates. Metyrapone is, however, not a specific antiglucocorticoid agent

Table 3 Body weights and systolic blood pressures of offspring at 7 weeks of age

Maternal diet (% casein)	Treatment	Body weight (g)	Systolic blood pressure (mm Hg)
18	Oil	156 ± 6	129 ± 5
	Metyrapone	169 ± 18	144 ± 5*
9	Oil	141 ± 4*	158 ± 6*
	Metyrapone	133 ± 5*	139 ± 3†

Data are shown as means ± SEM for n = 8–19 observations. Two-way ANOVA indicated a significant effect of maternal diet on body weight ($F = 33.5$, $P < 0.001$) and blood pressure ($F = 3.95$, $P < 0.05$) Metyrapone treatment interacted with maternal diet to influence systolic blood pressure ($F = 5.22$, $P < 0.05$). *indicates significantly different to 18% casein exposed oil treated group ($P < 0.05$). †indicates significantly different to 9% casein exposed oil treated group ($P < 0.05$).

and a more cautious interpretation is required. In addition to lowering corticosterone levels, the drug treatment would be associated with large rises in circulating adrenocorticotrophin and corticotrophin releasing factor,[22,23] and flux of steroid precursors into androgen, estrogen, and mineralocorticoid pathways, would cause a broad disturbance of the endocrine system. Despite the problems associated with this agent the alternatives are equally complex. Where surgical adrenalectomy is performed the corticosteroid production of the fetal adrenal is sufficient to maintain maternal glucocorticoid concentrations and indeed the fetal adrenal may hypertrophy, leading to increased exposure to steroid in mid-late gestation.[28] With this approach, low protein diet-induced hypertension is not ablated (Langley-Evans, unpublished observation). The antiglucocorticoid RU486 has potent antiprogestin activity and would result in termination of pregnancy[29] and therefore can not be used.

The striking finding of the present study was that blockade of corticosterone synthesis during the first 2 weeks of fetal development, had long-term effects on the blood pressure of the offspring. This observation supports the findings of Benediktsson et al.,[17,18] who demonstrated that dexamethasone treatment of the pregnant rat induces hypertension in the offspring. Clearly, in the low-protein diet exposed rat fetus maternal corticosterone synthesis is a prerequisite for the initiation of the hypertensive state. This would suggest that corticosterone acts at critical periods of development, to irreversibly program homeostatic mechanisms involved in regulation of blood pressure. The critical phase of development during which glucocorticoid blockade appears to alter long-term regulation of blood pressure, appears to lie within the first 2 weeks of pregnancy. In the control dietary group, drug treatment led to an increased blood pressure in later life. This implies that *in utero* corticosteroids may normally provide tonic stimulation of homeostatic mechanisms, thereby ensuring maintenance of normal blood pressure.

Although the present work illustrates a measure of dependency of maternal-diet-induced hypertension on maternal corticosterone production, the study is limited in scope. Further work will be directed toward two particular concerns. Firstly, metyrapone-treated animals will be administered corticosterone to establish whether the effect of the drug is indeed mediated through its inhibition of 11β-hydroxylase. Secondly, the issue of the opposing effects of the drug in the different dietary groups requires resolution. This dietary effect does not reflect maternal corticosterone concentrations, which were lowered to similar levels in both groups. The role of 11βHSD activity in placenta may be of importance. Placental 11βHSD activity in control animals may eliminate the tonic stimulation of the development of homeostatic control systems when metyrapone lowers hormone concentrations to less than 10% of normal. In low-protein fed dams, however, which have lower placental 11βHSD activity,[21] sufficient corticosteroid may reach the fetus to allow normal development of central and renovascular control systems to proceed.

In conclusion we have demonstrated that metyrapone treatment of pregnant rats consuming 18 or 9% casein diets has differential effects on the later blood pressure of the resulting offspring. The data are consistent with the hypothesis that dietary modulation of fetal corticosteroid exposure has a role in the programming of hypertension *in utero*.

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