Antihypertensive treatment in early postnatal life modulates prenatal dietary influences upon blood pressure in the rat

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ABSTRACT

Epidemiological evidence from diverse human populations, supported by experimental evidence from animal models, suggests that maternal nutrition during pregnancy is an important fetal programming influence upon cardiovascular disease. Experiments with a low-protein-diet model of rat pregnancy suggest a role for the renin–angiotensin system in the programming mechanism, since fetal undernutrition permanently elevates pulmonary and plasma angiotensin-converting enzyme activity. Long-term beneficial effects of captopril on blood pressure in this model require further investigation in order to clarify the role of angiotensin II. Pregnant rats were fed a control diet containing 18% (w/w) casein as the protein source or a low-protein diet containing 9% (w/w) casein. Between the ages of 2 and 4 weeks postnatally, mothers and their pups were treated with losartan or nifedipine. All pups in the study had blood pressure determined at 4 and 12 weeks of age using a tail cuff. Animals exposed to the low-protein diet in utero and not subjected to drug treatment had elevated blood pressure relative to control rats (mean increase of 27 mmHg; \( P < 0.001 \)). Treatment of rats exposed to the control diet in utero with either nifedipine or losartan between 2 and 4 weeks of age did not alter their blood pressure. Nifedipine had no effect upon the blood pressure of low-protein-exposed pups, but losartan prevented the blood pressure elevation in these animals. Between 4 and 12 weeks of age, blood pressure increased significantly in all groups (\( P < 0.001 \)). The pattern of blood pressure among the groups was identical to that observed at 4 weeks, suggesting that the observed early effects of losartan would be maintained into adult life. The data are consistent with the hypothesis that angiotensin II plays a major role in the prenatal programming of hypertension. The action of angiotensin II at the AT₁ receptor between 2 and 4 weeks of age may be critically up-regulated by fetal factors, including exposure to glucocorticoids of maternal origin.

INTRODUCTION

A broad range of epidemiological evidence from diverse populations throughout the world indicates associations between impaired fetal growth and non-communicable diseases of adult life [1–4]. Individuals of lower birth weight, or exhibiting evidence of disproportionality (thinness or large head circumference in proportion to body size) at birth are at increased risk of hypertension, diabetes and coronary heart disease. While growth in utero is determined primarily by genetics and may be constrained by maternal size, maternal nutritional status is a major influence upon fetal growth patterns. Nutrition has thus been proposed to be an important programming influence upon cardiovascular disease [5–7]. The findings of epidemiological studies are strongly
supported by studies of animals. In rats and guinea pigs, maternal undernutrition in pregnancy has been demonstrated to modify the blood pressure of the resulting offspring [8]. Studies in our laboratory have particularly focused upon the effects of maternal low-protein diets in rat pregnancy. Fetal exposure to relatively mild maternal protein restriction both retards late-gestation growth, resulting in disproportion at birth, and elevates blood pressure by 15–30 mmHg from the age of weaning and into adulthood [9–13].

Experiments with the low-protein-diet model of rat pregnancy suggest that a number of different mechanisms may play a role, either separately or in concert, in the programming of adult blood pressure. The apparent hypertension appears to be the product of increased peripheral resistance. Pulse rate is generally lower in low-protein-exposed rats and gross heart morphology is normal, suggesting that cardiac output is not elevated [8]. Nutritional effects upon renal morphometry [13] may occur independently, or as a result of increased exposure of the fetal tissues to maternal glucocorticoids [14]. The renin–angiotensin system appears to be central to the programming mechanism. In humans, activities of plasma renin are elevated at birth in growth-retarded infants and are inversely related to birthweight in adult men [15,16]. In rats, fetal undernutrition has little effect upon plasma renin activity or angiotensin II concentrations [12], but permanently elevates pulmonary and plasma angiotensin-converting enzyme (ACE) activity [17].

In an earlier study, treatment of rats exposed to a low-protein diet in utero with the ACE inhibitor captopril between 2 and 4 weeks of age offset the effects of nutritional programming on blood pressure [18]. An interpretation of this finding is that the generation of excess angiotensin II by ACE, or increased sensitivity of the vasculature to angiotensin II in the early postnatal period, is a critical element of the programming mechanism. Captopril is, however, a non-specific agent with effects beyond the renin–angiotensin system. In order to clarify the role of angiotensin II within this rat model of programming, we have studied the effects of a specific angiotensin II receptor antagonist (losartan) and a calcium-channel blocker (nifedipine) upon blood pressure.

**METHODS**

**Animals**

All animal experimentation performed in this study was licensed by the Home Office in accordance with the 1986 United Kingdom Animals Act. A total of 12 virgin female Wistar rats, purchased from Harlan UK, were used to generate the 81 animals used in this experiment. All rats had free access to food and fluids and were housed individually in plastic boxes in a room with a 12 h light/dark cycle and maintained at 22 °C. Six rats were fed a control diet containing 18% (w/w) casein as the protein source, and six rats were fed a low-protein diet containing 9% (w/w) casein. Feeding of both diets began on the day of conception, as indicated by a semen plug on the floor of the mating cage, and ceased at delivery, at which point all dams were fed the same standard laboratory chow diet [CRME, 18.9% (w/w) protein; Special Diet Services, Cambridge, U.K.]. The synthetic diets were isocaloric, and their full composition has been published elsewhere [10].

**Drug treatments**

Between the ages of 2 and 4 weeks postnatally, mothers and their pups were treated with losartan (100 mg/l; kindly donated by Merck, Sharpe and Dohme) or nifedipine (100 mg/l; Sigma), administered through the drinking water. This protocol was used previously with captopril, as reported by Sherman and Langley-Evans [18]. Litters were randomly assigned to the treatment groups, with two litters from each dietary group allocated to each of the control, nifedipine and losartan treatments. A total of 41 pups exposed to 18% casein and 40 pups exposed to 9% casein were thus allocated to the control (16 and eight respectively), nifedipine (11 and 17 respectively) and losartan (14 and 15 respectively) groups.

Losartan is a specific angiotensin II receptor antagonist, which was selected in order to investigate specific programming actions of the renin–angiotensin system on the basis of earlier captopril experiments. Unlike captopril, losartan has no effects on bradykinin and other kinins, and other cardiovascular regulators have not been reported to be influenced by the agent. Nifedipine is a dihydropyridine calcium-channel blocker with negligible long-term effects upon the renin–angiotensin system.

The administration of the drugs began at a time when the pups first began to take small quantities of liquid from feeder bottles. At this point, however, most fluid came from suckling. As the drugs are excreted in milk, some provision of drug to the pups would have occurred through this route [19]. The actual dose administered to the pups is thus difficult to determine and would have increased over the 2 weeks of treatment as independent drinking became established. Doses of both drugs calculated from the total fluid intakes of mothers and all pups in a litter would have been approx. 20 mg·kg⁻¹·day⁻¹ (maximum). These drug doses were consistent with published reports [20,21]. Clinical dosage of nifedipine lies between 20 and 80 mg/day in the treatment of human hypertension.

**Measurement of blood pressure**

Systolic blood pressure was determined using an IITC Model 229 Blood Pressure monitor as reported pre-
viously [18]. This tail-cuff method has been extensively validated and refined to reduce possible stress-related effects and observer subjectivity [18]. All pups in the study had their blood pressure determined at 4 and 12 weeks of age.

**Statistical analysis**

All data are expressed as means ± S.E.M. Gender had no significant effect on the blood pressure of the animals, and as a result data are presented for males and females combined. Two-way analysis of variance (ANOVA) was performed on the data from 4- and 12-week-old animals, considering drug treatment and maternal diet as the independent variables. An independent-samples *t* test was performed as a post hoc test where ANOVA indicated significant (*P* < 0.05) interactions.

**RESULTS**

Early postnatal treatment with losartan or nifedipine had no gross effects upon the animals. As shown in Table 1, rats exposed to a low-protein diet *in utero* were significantly heavier at 4 weeks of age than control rats. Rats exposed to the control maternal diet (18% casein) and treated with nifedipine or losartan had similar body weights at 4 weeks to the untreated control group. Losartan treatment in low-protein-exposed rats resulted in a significantly lower body weight at 4 weeks. A decrease in weight in the nifedipine-treated rats failed to achieve statistical significance. At 12 weeks of age, for both males and females, body weights were mostly similar in all six groups of rats (Table 1). The exception to this was that rats exposed to the 18% casein diet and treated with nifedipine 8–10 weeks previously were significantly heavier than untreated control rats.

The mean systolic blood pressure values of the 4-week-old rats are shown in Figure 1. Animals exposed to the low-protein diet and not subjected to drug treatment had blood pressures that were significantly elevated above those of control rats (maternal 18% casein diet) (increase of 27 mmHg; *P* < 0.001). Treatment of rats exposed to the control diet *in utero* with either nifedipine or losartan between 2 and 4 weeks of age did not alter their blood pressures. Similarly, nifedipine had no effect upon the blood pressures of low-protein-exposed pups, which remained 28 mmHg above control pressures. Losartan, however, appeared to prevent the blood pressure elevation in low-protein-exposed rats, such that the 9% casein/losartan group had similar blood pressures to those of the controls exposed to the 18% casein diet *in utero*.

Between 4 and 12 weeks of age, blood pressures increased significantly in all groups of rats (*P* < 0.001). As shown in Figure 2, the pattern of blood pressures observed at 4 weeks of age was preserved 8 weeks later, when all animals were fully mature. All rats exposed to the 18% casein maternal diet *in utero*, regardless of drug treatment, had similar systolic pressures. Low-protein-exposed rats that were either untreated or treated with nifedipine had blood pressures that were elevated relative to those of the 18% casein controls (increase of 32–35 mmHg; *P* < 0.001). However, the blood pressures of low-protein-exposed rats that had been treated with losartan between 2 and 4 weeks of age were comparable with those of control rats exposed to the 18% casein maternal diet. No significant relationships were observed

**Table 1** Body weights of rats at 4 and 12 weeks of age

Data are mean body weights ± S.E.M. for (*n*) observations per group. Data at 4 weeks of age show male and female rats combined, as ANOVA indicated no effect of sex on body weight at this time. At 12 weeks male and female weights differed significantly. ANOVA indicated that, at 4 weeks, weight was influenced by diet (*P* < 0.01) and an interaction of diet and drug treatment (*P* < 0.05). At 12 weeks, weight was influenced by an interaction of diet and drug treatment (*P* < 0.05). * Indicates significantly different from rats exposed to an 18% casein diet *in utero* with no drug treatment (*P* < 0.05); † indicates significantly different from rats exposed to a 9% casein diet *in utero* with no drug treatment (*P* < 0.05); ‡ indicates significantly different from rats of the alternative dietary exposure on same drug treatment (*P* < 0.05).

<table>
<thead>
<tr>
<th>Maternal diet (% casein, w/w)</th>
<th>Drug treatment</th>
<th>4 weeks</th>
<th>12 weeks</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>18</td>
<td>None</td>
<td>300 ± 8 (5)</td>
<td>184 ± 2 (11)</td>
</tr>
<tr>
<td>18</td>
<td>Nifedipine</td>
<td>330 ± 15 (4)‡</td>
<td>197 ± 3 (7)‡</td>
</tr>
<tr>
<td>18</td>
<td>Losartan</td>
<td>300 ± 7 (9)</td>
<td>177 ± 6 (5)</td>
</tr>
<tr>
<td>9</td>
<td>None</td>
<td>320 ± 292 (2)</td>
<td>184 ± 4 (6)</td>
</tr>
<tr>
<td>9</td>
<td>Nifedipine</td>
<td>300 ± 5 (9)</td>
<td>182 ± 9 (8)</td>
</tr>
<tr>
<td>9</td>
<td>Losartan</td>
<td>291 ± 4 (8)</td>
<td>181 ± 3 (7)</td>
</tr>
</tbody>
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Systolic blood pressure (SBP) was determined in 4-week-old rats that had been exposed to a control (18% casein) or low-protein (9% casein) diet in utero and then treated or not with losartan (LOS) or nifedipine (NIF) between 2 and 4 weeks of postnatal age. All data are shown as means ± S.E.M. The numbers of rats studied were: 18% casein diet: control, n = 16; LOS, n = 14; NIF, n = 11; 9% casein diet: control, n = 8; LOS, n = 15; NIF, n = 17. *Indicates significantly different from all 18% casein groups (P < 0.01).

DISCUSSION

The concept that fetal exposure to undernutrition is able to determine a lifelong increase in risk of hypertension and coronary heart disease is strongly supported by both epidemiological data and animal experiments. The model used in the present study has yielded highly consistent effects of a maternal low-protein diet in rat pregnancy upon the blood pressure of the resulting offspring [8–14,17,18,26,27]. In keeping with the finding that individuals in the human population who are of low birthweight have increased blood pressure, rats exposed to low-protein diets in utero are of low to normal weight at birth and have blood pressures significantly above those of control animals.

Earlier work with the model has sought to determine the possible contribution of the renin–angiotensin system to the elevated blood pressures of low-protein-exposed rats. Such animals have normal plasma renin activities and low to normal angiotensin II concentrations [12]. ACE activity is elevated in lungs and plasma, and the raised blood pressures of adult animals are reversibly treatable using captopril [17]. Interestingly, we have found that treatment of low-protein-exposed rats with ACE inhibitors in early postnatal life lowers their blood pressure in the long term [18].

The effects of captopril in previous studies [17,18] were suggestive of an important role for angiotensin II in the prenatal programming of hypertension. However, those findings may have been confounded by virtue of the non-specificity of captopril to the renin–angiotensin system and by the fact that, although untested, antihypertensive drugs not directed at this system may be equally effective. In the present work we have addressed these points by administering either a specific AT1-receptor antagonist or a second antihypertensive drug which exerts limited effects on the renin–angiotensin system. The results obtained suggest that the conclusions drawn from experiments with captopril are valid, and that angiotensin II does indeed play a key role in the prenatal programming of hypertension.

The nature of the homoeostatic mechanisms that regulate blood pressure determines that virtually all antihypertensive treatments exert some effects upon the renin–angiotensin system. Nifedipine was selected for use in the present study because its interactions with the renin–angiotensin axis are limited, and in some studies totally absent. Kusaka et al. [22] reported that treatment of hypertensive subjects with nifedipine had no effect upon humoral markers of renin–angiotensin activity, although clearly this overlooks possible effects at the
level of specific tissues. Conversely, nifedipine has been reported to exert an acute stimulatory effect upon renin activity [23]. If such an effect were to operate in the present experiment, the nifedipine data would remain an interesting comparison with the effects of losartan, in terms of renin–angiotensin stimulation relative to antagonism of the angiotensin axis.

Some effects of the drug protocols on body weight were observed. As has been observed previously with this rat model [12], low-protein exposure produced rats that were larger than control animals at 4 weeks of age. Losartan treatment significantly reduced body weight in low-protein-exposed rats, but not in animals exposed to a control maternal diet (18% casein). Although body weight and blood pressure are frequently related in rats, the weight-lowering effect of losartan does not appear to explain the diet-specific antihypertensive effect, as no significant correlation was observed between body weight and systolic blood pressure. Indeed, at 12 weeks of age the $r^2$ analysis suggested that body weight variation explained only 1% of the variation in blood pressure. In further support of this argument, it should be noted that, at 12 weeks of age, low-protein-exposed rats that had been treated with losartan in early life remained normotensive and had body weights similar to those of untreated rats exposed to 9% casein diets.

The findings of the present study add further weight to the observation that the low-protein-diet model of blood pressure programming in the rat bears striking similarities to the spontaneously hypertensive rat (SHR) strain. In SHRs, as with the present model, early treatment with ACE inhibitors such as captopril, or with losartan, can set the blood pressure of the animals at a lower level than would be normal for the strain [20,24–26]. Other antihypertensive drugs, as with nifedipine in the present study, do not exert the same long-term effect on blood pressure in SHRs as those targeted at the renin–angiotensin system.

It can be argued that the lack of any effect of nifedipine weakens the assertion that the effect of losartan indicates a specific role for angiotensin II in the early-life programming of hypertension. One of the aims of the study was to establish whether a decrease in blood pressure caused by any means in low-protein-exposed rats could have a long-term effect. Calcium antagonists were selected for this purpose, as there are few drugs in other classes that have as limited an interaction with the renin–angiotensin system. Nifedipine was the drug of choice as it is excreted in milk [19] and could thus be administered to the 2-week-old rat pups. The lack of an effect is unlikely to be a product of timing or route of administration. Studies in humans suggest that nifedipine-treated women produce milk containing the drug at similar concentrations to those observed in plasma. The dosage of nifedipine in the present study was equivalent to that in other rat studies [21] and could be considered to be high, on a per kg body weight basis, relative to clinical doses. The lack of an acute effect of nifedipine on blood pressure at 4 weeks is largely inexplicable, although it is possible, if angiotensin II plays a key role in the early setting of blood pressure, that the proposed stimulatory effect on renin activity [23] offset the antihypertensive effects of calcium antagonism. To address the issue of non-specific effects of blood pressure reduction in the long term, it may be necessary to consider a larger battery of antihypertensive agents, across a broad spectrum of drug classes, for example $\beta$-blockers, calcium antagonists and centrally acting agents.

The data are strongly supportive of a specific role for angiotensin II in determining the higher blood pressure of rats exposed to maternal undernutrition in fetal life. Previous studies have failed to demonstrate any clear elevation of angiotensin II concentrations in either 4-week-old or mature animals [12,17]. ACE activity is, however, elevated in the lungs and plasma of low-protein-exposed rats. The long-term effects of losartan appear to suggest that the action of angiotensin II at the AT$_1$ receptor is critical between 2 and 4 weeks of age. As earlier work has suggested low to normal angiotensin II concentrations in low-protein-exposed rats at 4 weeks of age, we infer that increased action at the receptor is a product of either higher-affinity AT$_1$ sites or an increase in receptor number in the vasculature and other tissues. Local renin–angiotensin systems in brain, heart and kidney may have a critical role in determining blood pressure. Preliminary data from cannulated, anaesthetized rats suggests that animals exposed to low-protein diets in utero are more sensitive to lower concentrations of infused angiotensin II [27], although it is unclear which target tissues mediate angiotensin-induced increases in resistance.

There is a considerable body of evidence implicating glucocorticoids in the programming of blood pressure by the maternal diet [14]. The elevated blood pressures of low-protein-exposed rats depend not only on an intact adrenal gland in postnatal life [28], but also on maternal glucocorticoid synthesis during pregnancy [29]. Overexposure of fetal tissues to maternal glucocorticoids is an essential step in the programming mechanism. Low-protein-exposed rats remain hypersensitive to glucocorticoids into adult life, and have increased glucocorticoid receptor numbers at several sites, including the vasculature [11]. Glucocorticoids may up-regulate the actions of the renin–angiotensin system at the level of angiotensinogen synthesis, ACE and, importantly, the AT$_1$ receptor [14,30]. Intrauterine sterile exposure may thus establish an increased sensitivity to angiotensin II in early postnatal life, which in turn establishes lifelong raised blood pressure. Although not analysed in the present study, the expression of AT$_1$ receptors in the kidneys and other tissues is currently under consideration in control and low-protein-exposed rats. Preliminary
data suggest that renal expression is up-regulated in the hypertensive low-protein group (A. Trowern and C. B. Whorwood, unpublished work). Further work will establish the effects of early losartan treatment on receptor expression.

Harrap [31] has argued that data of this nature obtained with the SHR strain and in our previous work using captopril [18] may be suggestive of a critical window in the early postnatal period during which the adult blood pressure might be modified. Certainly, the present data support the assertion that intrauterine influences upon blood pressure, determined by maternal lifestyle and nutritional status, could be reversed by appropriate and carefully targeted antihypertensive treatment. Recent findings by Holemans et al. [32] similarly suggest that fetal influences per se may not alter adult blood pressure, but instead alter postnatal vascular responsiveness and hence determine interactions with the environment encountered in adult life. Further investigation of such interactions between the intrauterine environment and postnatal factors, throughout the lifespan, must become a major public health priority for the future.

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