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Effect of maternal iron restriction during pregnancy on renal morphology in the adult rat offspring

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In rats, maternal anaemia during pregnancy causes hypertension in the adult offspring, although the mechanism is unknown. The present study investigated the renal morphology of adult rats born to mothers who were Fe-deficient during pregnancy. Rats were fed either a control (153 mg Fe/kg diet, *n* 7) or low-Fe (3 mg/kg diet, *n* 6) diet from 1 week before mating and throughout gestation. At delivery, the Fe-restricted (IR) mothers were anaemic; the IR pups were also anaemic and growth-retarded at 2 d of age. At 3 and 16 months, systolic blood pressure in the IR offspring (163 (SEM 4) and 151 (SEM 4) mmHg respectively, *n* 13) was greater than in control animals (145 (SEM 3) and 119 (SEM 4) mmHg respectively, *n* 15, $P<0{\cdot}05$). At post mortem at 18 months, there was no difference in kidney weight between treatment groups, although relative kidney weight as a fraction of body weight in the IR offspring was greater than in control animals ($P<0{\cdot}05$). Glomerular number was lower in the IR offspring (11·4 (SEM 1·1) per 4 mm^2, *n* 13) compared with control rats (14·8 (SEM 0·7), *n* 15, $P<0{\cdot}05$). Maternal treatment had no effect on glomerular size, but overall, female rats had smaller and more numerous glomeruli per unit area than male rats. When all animals were considered, inverse relationships were observed between glomerular number and glomerular size ($r-0{\cdot}73$, *n* 28, $P<0{\cdot}05$), and glomerular number and systolic blood pressure at both 3 months ($r-0{\cdot}42$, *n* 28, $P<0{\cdot}05$) and 16 months of age ($r-0{\cdot}64$, *n* 28, $P<0{\cdot}05$). Therefore, in rats, maternal Fe restriction causes hypertension in the adult offspring that may be due, in part, to a deficit in nephron number.

Kidney: Programming: Fetus

A number of epidemiological and experimental studies have shown that maternal nutrition during pregnancy has effects on fetal development that, in turn, have consequences for the health of the offspring in adulthood (Hoet & Hanson, 1999). In rats, maternal undernutrition and diets deficient in protein or Fe cause growth retardation in the pups at birth, and high blood pressure in the offspring in later life (Crowe *et al.* 1995; Lewis *et al.* 2001*b*; Vehaskari *et al.* 2001). The mechanisms responsible for the intra-uterine programming of hypertension are unclear, but may involve changes in renal growth and development.

In man and rodents, nephrogenesis is completed during fetal and early postnatal life respectively, and the total number of nephrons established at this time cannot be increased thereafter (Wintour, 1997). Nephron number is related to size at birth over the normal range of birth weights (Merlet-Benichou *et al.* 1994), and in man and other species, intra-uterine growth retardation is associated with a relatively low number of nephrons (Hinchliffe *et al.* 1992; Merlet-Benichou *et al.* 1994; Bains *et al.* 1996; Bassan *et al.* 2000). Nephron number has also been shown to be an important determinant of blood pressure in adulthood (Brenner *et al.* 1988). Low numbers of nephrons, and hence a reduced filtration surface area, limit the ability of the kidney to excrete Na and maintain normal extracellular fluid volume and blood pressure (Brenner *et al.* 1988). Therefore, the intra-uterine environment may influence blood pressure in adult life through effects on the developing kidney. Indeed, a reduction in nephron number has been observed in the hypertensive offspring of rats fed a low-protein diet throughout pregnancy (Langley-Evans *et al.* 1999). Whether this mechanism is common to other models of intra-uterine programming, such as maternal Fe restriction, is unknown.

The aims of the present study were, therefore, to determine: (1) the effect of maternal Fe restriction during pregnancy on the renal morphology of the adult offspring; (2) whether changes in renal morphology might be responsible for the high blood pressure seen in these animals.

Abbreviation: IR, iron-restricted.

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Methods

Animals

All experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Thirteen female Wistar rats were fed either a control (153 mg/Fe kg diet, *n* 7; K4447.01, Hope Farms, Woerden, Holland) or low-Fe diet (3 mg/kg diet, *n* 6; K4447.00, Hope Farms) from 1 week prior to mating and throughout pregnancy. The compositions of the two diets were identical except for the addition of 150 mg Fe (as iron subcarbonate)/kg to the control diet. The diets contained 175 g crude protein (N × 6·25; as vitamin-free casein)/kg, 52 g crude fat (as soyabean oil)/kg and 662 g sugar and starch/kg with a gross energy content of 16 MJ/kg (Hope Farms).

Blood was taken from the pregnant rats by tail venepuncture on day 21 of gestation (term day 22). On the day of delivery, all rats were transferred onto the control diet. The rat pups remained with their mothers until weaning. On the second day after birth, blood was collected from three pups culled from a subset of the litters (four control and three Fe-restricted (IR) rats). All of the maternal and neonatal blood samples taken were analysed for haematology: the Fe-restricted rats and their pups were anaemic with lower haemoglobin, packed cell volume and erythrocyte counts than the control animals ($P<0{\cdot}005$ in all cases, Table 1). The IR pups were also growth-retarded at 2 d of postnatal age ($P<0{\cdot}005$, Table 1). The numbers of pups per litter that died naturally within 3 d of delivery were similar in the control and IR animals (Table 1). Further culling occurred at 3 d after birth in order to standardise the size of each litter to eight offspring. At 21 d of postnatal age, two male and two female pups from each litter were weaned onto the control diet. In one IR litter, no male pups survived to weaning and four female pups were used in the study instead. A number of rats died of natural causes before post mortem at 18 months of age. Therefore, a total of twenty-eight offspring were used in the following study: fifteen offspring from the control rats (five male, ten female) and thirteen offspring from the IR rats (five male, eight female). All of the rats in the study were housed individually, and food and water were available *ad libitum*.

Physiological measurements

At 3 and 16 months of age, blood pressure was measured in the offspring by the indirect tail-cuff method. The rats were accustomed to the Perspex restraining tubes on three occasions over the previous 2 weeks. Five recordings of systolic blood pressure were made from each rat by the same investigator blind to the treatment groups. The highest and lowest blood pressure measurements were excluded and the three remaining values were averaged.

Tissue collection

At 18 months of age, all of the offspring were killed by CO_2 inhalation and weighed. One kidney from each animal was weighed and fixed in formaldehyde (100 ml/l). The kidneys were embedded in paraffin wax, and 7 μm longitudinal sections were stained with haematoxylin and eosin.

Histological analysis

Using a microscope imaging system, glomerular number and glomerular diameter were determined in five fields of view, each with a unit area of 4 mm^2, from three sections of kidney. Therefore, a total of fifteen fields of view were analysed from each animal. In addition, renal cortical width, expressed as a percentage of the total medullary and cortical width, was measured in three sections of each kidney. Tubulo-interstitial damage was assessed on an arbitrary scale where values of 1, 2 or 3 corresponded to mild, moderate or severe respectively. Tubulo-interstitial damage included dilation and atrophy of the tubular epithelium, interstitial inflammation and fibrosis, and the presence of

Table 1. Neonatal body weight and mortality at 2 d of age, and haematological values in control and iron-restricted (IR) rats, and their offspring, within 2 d of delivery†

(Mean values with their standard errors)

	Control		IR	
	Mean	SEM	Mean	SEM
Maternal blood values at day 21 of gestation‡				
Haemoglobin (g/l)	131	4	100*	3
Packed cell volume (%)	39	1	28*	2
Erythrocyte count (× 10^9/l)	6·59	0·21	5·19*	0·09
Neonatal blood values at 2 d of age§				
Haemoglobin (g/l)	125	3	55*	5
Packed cell volume (%)	37	1	17*	2
Erythrocyte count (× 10^9/l)	2·48	0·11	1·75*	0·08
Neonatal bodyweight (g)	6·2	0·4	4·8*	0·2
Early neonatal loss (pups (*n*) per litter)	2·4	0·9	2·4	1·0

Mean values were significantly different from those of the control animals (unpaired *t* test): *$P<0{\cdot}005$.

† For details of diets and procedures, see p. 34.

‡ Control *n* 7, IR *n* 6.

§ Control *n* 12, IR *n* 9.

eosinophilic luminal casts. All measurements were made by the same investigator, who was blind to the treatment groups.

Statistical analysis

All results are presented as mean values with their standard errors. Statistical differences between the measurements were determined by maternal treatment and sex of the offspring using two-way ANOVA followed by the Tukey test. Relationships between the variables were assessed by linear regression. Differences where $P<0{\cdot}05$ were regarded as significant.

Results

Systolic blood pressure

At both 3 and 16 months of age, systolic blood pressure in the IR offspring was significantly greater than that measured in the control rats ($P<0{\cdot}05$ in both cases, Fig. 1). There were no differences in systolic blood pressure between the male and female animals of each treatment group at either age (Fig. 1).

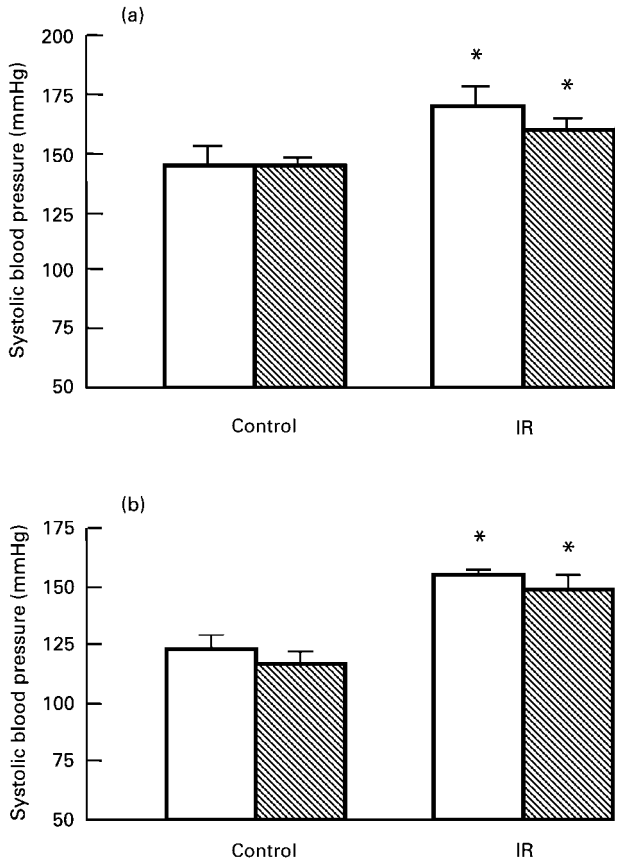

Fig. 1. Systolic blood pressure in control (male *n* 5, female *n* 10) and iron-restricted (IR; male *n* 5, female *n* 8) offspring at (a) 3 months and (b) 16 months of age. □, Male; ▨, female. For details of diets and procedures, see p. 34. Values are means with their standard errors shown by vertical bars. There was a significant effect of maternal treatment (two-way ANOVA): $P<0{\cdot}001$. Mean values were significantly different from those of the control offspring of the same sex (Tukey test): $^*P<0{\cdot}05$.

Body and kidney weights

At 18 months of age, the body weight of the rats was significantly affected by both maternal treatment and the sex of the offspring ($P<0{\cdot}05$ in both cases, Table 2). The IR offspring remained significantly lighter than the control offspring, and the male rats were significantly heavier than the female rats in both treatment groups (Table 2).

The kidney weights of the rats did not differ with maternal treatment, although the kidneys from the male animals were significantly heavier than those from the female animals ($P<0{\cdot}001$, Table 2). When expressed as % body weight, the relative kidney weight was significantly greater in the IR rats than in the control rats ($P<0{\cdot}05$, Table 2), but was not influenced by the sex of the offspring.

Renal morphology

Glomerular number, measured per unit area, in the IR offspring was significantly lower than that in the control animals ($P<0{\cdot}005$, two-way ANOVA, Fig. 2(a)). The difference in glomerular number between the IR and control offspring was significant for the female rats ($P<0{\cdot}05$, Tukey test, Fig. 2(a)) and just outside statistical significance for the male rats ($P=0{\cdot}06$, Tukey test, Fig. 2(a)). In addition, significantly more glomeruli were observed in the female rats than in the male rats, regardless of maternal treatment ($P<0{\cdot}001$, Fig. 2(a)). Maternal treatment had no effect on glomerular size, but overall, female rats had significantly smaller glomeruli than the male offspring ($P<0{\cdot}005$, Fig. 2(b)). There were no effects of maternal treatment or the sex of the offspring on the relative size of the renal cortex (Table 2). A greater degree of tubulo-interstitial damage was observed in the male rats compared with the female rats of the study ($P<0{\cdot}01$, Table 2), but this was not influenced by maternal treatment (Table 2).

Overall, when values from all animals were combined, irrespective of maternal treatment or the sex of the offspring, significant inverse relationships were observed between glomerular number at 18 months of age and systolic blood pressure at both 3 months ($r-0{\cdot}42$, n 28, $P<0{\cdot}05$) and 16 months of age ($r-0{\cdot}64$, n 28, $P<0{\cdot}001$, Fig. 3). Significant relationships between glomerular number and systolic blood pressure at 16 months of age were evident in both groups of male ($r-0{\cdot}81$, n 10, $P<0{\cdot}005$) and female ($r-0{\cdot}62$, n 18, $P<0{\cdot}01$) offspring. The degree of tubulo-interstitial damage was also positively correlated with systolic blood pressure at 16 months of age ($r+0{\cdot}47$, n 28, $P<0{\cdot}05$). However, multiple linear regression showed that glomerular number, rather than renal damage, was the best predictor of systolic blood pressure in these animals. In addition, in all animals, glomerular number was significantly and inversely related to glomerular size ($r-0{\cdot}73$, n 28, $P<0{\cdot}001$, Fig. 4); when analysed by sex of the offspring, this relationship

Table 2. Body and kidney weights, and relative kidney weight, renal cortical width and degree of tubulo-interstitial damage in the control and iron-restricted (IR) offspring at 18 months of age‡

(Mean values with their standard errors)

	Control				IR				Statistical significance of effect (two-way ANOVA)		
	Male (*n* 5)		Female (*n* 10)		Male (*n* 5)		Female (*n* 8)				
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Treatment	Sex	Interaction
Body weight (g)	821·0	56·9	523·7*	36·3	698·3	46·4	395·5*†	29·5	$P<0·05$	$P<0·001$	NS
Kidney weight (g)	3·10	0·22	1·90*	0·09	3·42	0·34	1·87*	0·22	NS	$P<0·001$	NS
Relative kidney weight (%)	0·38	0·03	0·37	0·02	0·50	0·06	0·48†	0·05	$P<0·05$	NS	NS
Relative renal cortical width (%)	37·6	3·7	34·9	1·1	36·2	1·1	39·0	1·1	NS	NS	NS
Tubulo-interstitial damage (arbitrary values)	2·10	0·21	1·32*	0·10	2·47	0·33	1·85	0·29	NS	$P<0·01$	NS

Mean values were significantly different from those of the male offspring of the same maternal treatment (Tukey test): *$P<0·05$.
Mean values were significantly different from those of the control offspring of the same sex (Tukey test): †$P<0·05$.
‡ For details of diets and procedures, see p. 34.
NS, not significant.

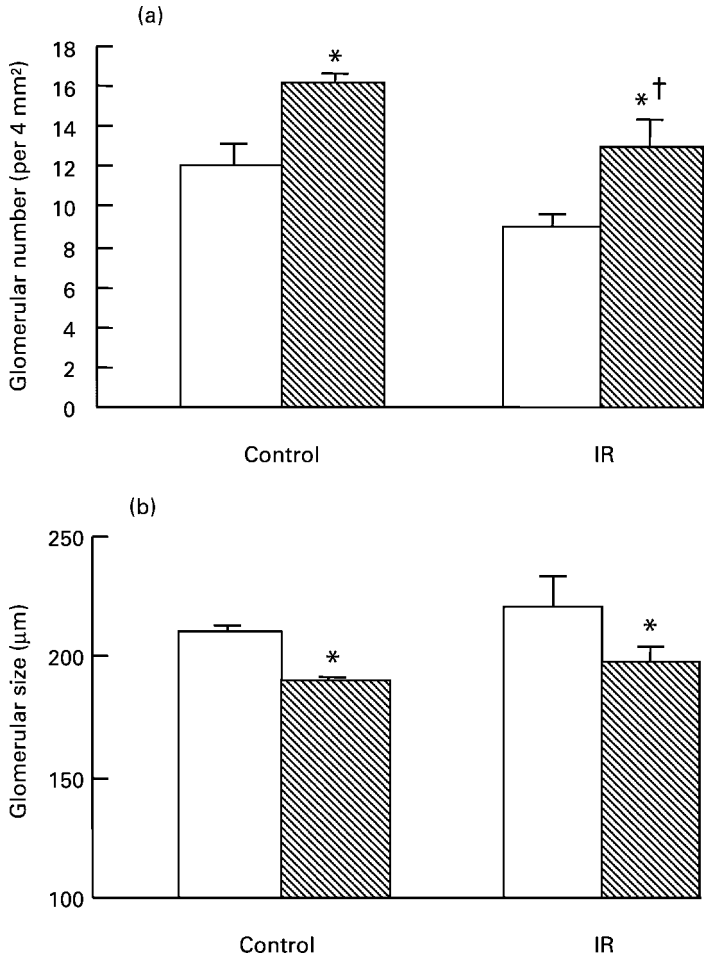

Fig. 2. Glomerular (a) number and (b) size in control (male *n* 5, female *n* 10) and iron-restricted (IR; male *n* 5, female *n* 8) offspring at 18 months of age. □, Male; ▨, female. For details of diets and procedures, see p. 34. Values are means with their standard errors shown by vertical bars. There was a significant effect of maternal treatment and sex of offspring for glomerular number (two-way ANOVA): $P<0·005$. There was a significant effect of sex of offspring for glomerular size (two-way ANOVA): $P<0·005$. Mean values were significantly different from those of male offspring of the same maternal treatment (Tukey test): *$P<0·05$. Mean value was significantly different from that of control offspring of the same sex (Tukey test): †$P<0·05$.

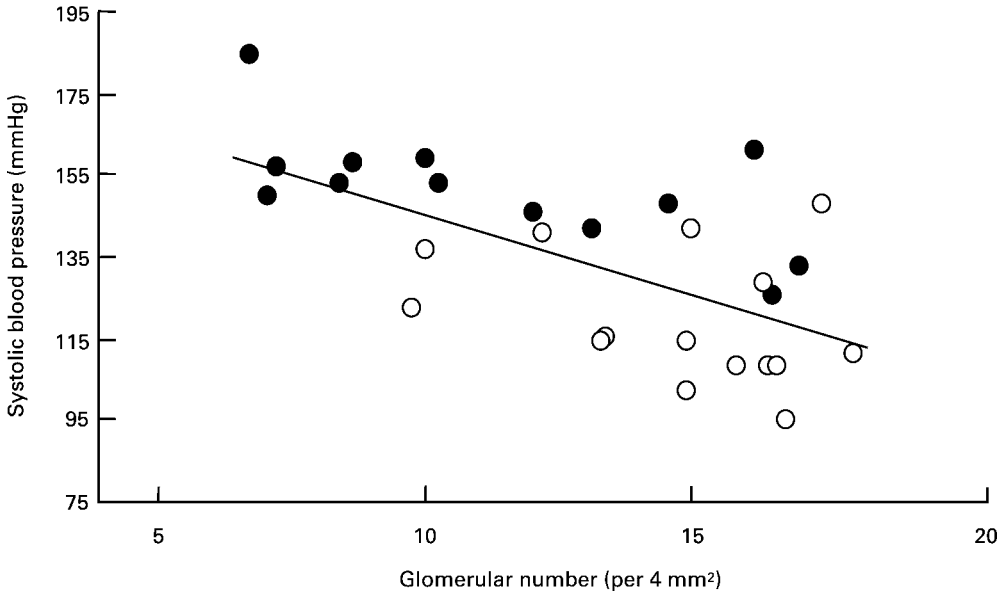

Fig. 3. Relationship between glomerular number at 18 months of age and systolic blood pressure at 16 months of age in control (○) and iron-restricted (●) offspring: $r-0{\cdot}64$, n 28, $P<0{\cdot}001$. For details of diets and procedures, see p. 34.

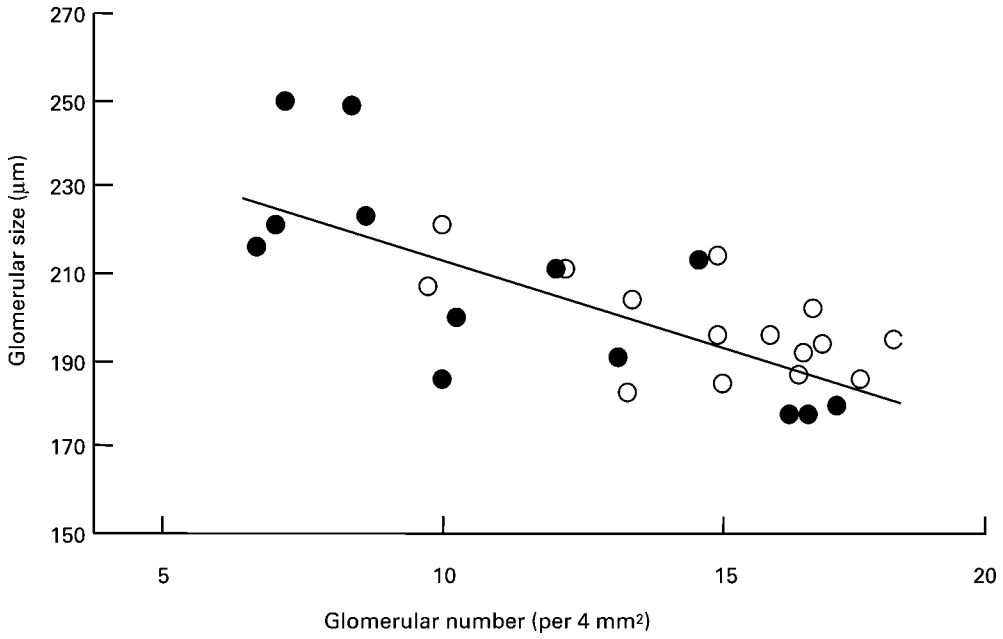

Fig. 4. Relationship between glomerular number and glomerular size in control (○) and iron-restricted (●) offspring at 18 months of age: $r-0{\cdot}73$, n 28, $P<0{\cdot}001$. For details of diets and procedures, see p. 34.

was significant for female ($r-0{\cdot}71$, n 18, $P<0{\cdot}001$) but not male rats ($r-0{\cdot}50$, n 10, $P=0{\cdot}14$).

Discussion

In the present study, maternal Fe restriction during pregnancy caused a decrease in nephron number in the adult rat offspring. In addition, when values from all animals were considered, significant inverse relationships were observed between glomerular number and systolic blood pressure at both 3 and 16 months of age. Therefore, the reduction in nephron number induced by maternal Fe restriction may be responsible, at least in part, for the hypertension seen in these animals.

The nephron deficit observed in the IR offspring is likely to result in a decrease in filtration surface area, as maternal treatment had no effect on glomerular size. However, as a percentage of body weight, the kidneys of the IR offspring were larger than those of the control animals, which indicates that there may have been compensatory renal growth as a consequence of maternal treatment. Furthermore, the significant relationship between glomerular

number and glomerular size observed in all rats suggests that there may be a degree of compensatory glomerular hypertrophy in the animals with a relatively low nephron complement. A positive relationship between glomerular number and glomerular volume has been observed previously in human neonates (Manalich *et al.* 2000). In the present study, the male rats had fewer but larger glomeruli, per unit area, than female animals, regardless of maternal treatment. A lower density of glomeruli in the renal cortex has also been described in male mice (Messow *et al.* 1980). Furthermore, in the present study, a greater degree of tubulo-interstitial damage was observed in the male rats compared with the female animals. This suggests that the male animals may be more susceptible to the development of hypertension, although there were no differences in systolic blood pressure between the sexes in each treatment group at 16 months of age.

There are two principal ways in which maternal Fe restriction can reduce nephron number in the adult offspring. First, it may accelerate the rate of loss of nephrons seen with old age. Overall, the glomerular number measured at 18 months of age may be lower than that first established in early life, and may not be equivalent to the nephron complement in these animals at the time of the blood pressure recordings. However, the value is likely to be representative for the treatment group. The offspring of rats either undernourished or fed a low-protein diet throughout pregnancy show a greater amount of glomerular and tubulo-interstitial damage compared with control animals (Lucas *et al.* 2001; Vehaskari *et al.* 2001). However, in the present study, there was no effect of maternal Fe restriction on the degree of renal damage due to ageing in the offspring at 18 months of age. Second, maternal Fe restriction may influence nephrogenesis in the offspring during both fetal and early postnatal life. In the present study, the mothers were likely to continue to be anaemic during early lactation, and, indeed, the neonates were both growth-retarded and anaemic at 3 d of age. However, maternal Fe restriction has been shown to cause hypertension in adult rat offspring that were cross-fostered to control mothers at birth (Gambling *et al.* 2001*a*). Previous studies have also shown that, despite an increase in the placental transfer of Fe to the fetus, this level of dietary Fe deficiency causes anaemia in the fetuses close to term (Finch *et al.* 1983; Gambling *et al.* 2001*b*). Furthermore, positive relationships between maternal and fetal circulating concentrations of both haemoglobin and ferritin are seen in anaemic women at delivery (Singla *et al.* 1996). Therefore, maternal Fe restriction appears to have an impact upon both fetal and neonatal development.

There are a number of possible mechanisms by which maternal Fe restriction may impair nephrogenesis and lead to a nephron deficit in later life. First, the anaemia seen in the fetuses and neonates in the present study and in other studies may reduce renal oxygenation. Although there is no evidence for fetal hypoxia in this model of maternal Fe restriction, in terms of the tissue expression of genes such as hypoxia-inducible factor and vascular endothelial growth factor (Lewis *et al.* 2001*a*), there may be changes in renal O_2 delivery. Changes in oxygenation are known to influence the extent and timing of nephrogenesis in the developing rat kidney *in vitro* (Tufro-McReddie *et al.* 1997). Second, maternal Fe restriction may influence renal development via changes in circulating glucocorticoid concentration. In the non-pregnant rat, plasma cortisol increases within 10 d of dietary Fe deficiency and is maintained above values in control animals for at least 40 d (Campos *et al.* 1998). Even without a change in the circulating fetal glucocorticoid concentration, the developing kidney may become more sensitive to glucocorticoids with maternal Fe restriction. In rats, a decrease in renal 11β-hydroxysteroid dehydrogenase mRNA (the enzyme that metabolises cortisol and corticosterone) and an increase in glucocorticoid receptor expression have been observed in the fetal and neonatal offspring of animals fed a low-protein diet during pregnancy (Bertram *et al.* 2001). Increased exposure to glucocorticoids *in utero* has previously been shown to impair nephrogenesis. Dexamethasone administration to pregnant rats causes growth retardation and a 60 % reduction in nephron number in the pups at birth, and hypertension in later life (Celsi *et al.* 1998). Changes in the hormonal environment and renal O_2 delivery *in utero* may, in turn, affect the availability of specific growth factors known to be important for normal nephrogenesis (Horster *et al.* 1999). For example, insulin-like growth factor-I and -II have a major role in nephrogenesis, and their bioavailability has been shown to be influenced by glucocorticoids and oxygenation *in utero* (Mosier *et al.* 1987; Rogers *et al.* 1991; Tapanainen *et al.* 1994; Doublier *et al.* 2001). Last, maternal Fe restriction may influence renal development via associated changes in other micronutrients such as vitamin A. In weanling rats, circulating vitamin A levels and retinol utilisation are suppressed by dietary Fe deficiency (Jang *et al.* 2000). Vitamin A deficiency *in utero* is associated with intra-uterine growth retardation and is known to impair nephrogenesis (Rondo *et al.* 1995; Lelievre-Pegorier *et al.* 1998*a*). A positive relationship between plasma vitamin A concentration and number of nephrons has been demonstrated in rat fetuses at day 21 of gestation (Lelievre-Pegorier *et al.* 1998*a*). Indeed, vitamin A treatment can correct the nephron deficit seen in rats born to mothers fed a low-protein diet throughout pregnancy (Lelievre-Pegorier *et al.* 1998*b*). Therefore, the present study shows that maternal Fe deficiency during pregnancy has consequences for the offspring, not only during fetal and neonatal development, but also in adult life. Like other models of intra-uterine programming by maternal nutrition, Fe restriction causes a deficit in nephron number, which may lead to hypertension in the adult offspring. Indeed, the effects of anaemia on the fetal and neonatal kidney may be a mechanism common to a number of experimental models. In rats undernourished or fed a low-protein diet during pregnancy, reductions in packed cell volume and total body Fe content are observed in the fetuses at day 21 of gestation (Vaquero & Navarro, 1996; Barone *et al.* 1998). The findings of the present study highlight the importance of sufficient Fe levels, not only for normal renal development *in utero*, but also for the prevention of programming of hypertensive disease in later life.

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