Dietary-induced gestational iron deficiency inhibits postnatal tissue iron delivery and postpones the cessation of active nephrogenesis in rats

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Abstract

Gestational iron deficiency (ID) can alter developmental programming through impaired nephron endowment, leading to adult hypertension, but nephrogenesis is unstudied. Iron status and renal development during dietary-induced gestational ID (<6 mg Fe kg⁻¹ diet from Gestational Day 2 to Postnatal Day (PND) 7) were compared with control rats (198 mg Fe kg⁻¹ diet). On PND2–PND10, PND15, PND30 and PND45, blood and tissue iron status were assessed. Nephrogenic zone maturation (PND2–PND10), radial glomerular counts (RGCs), glomerular size density and total planar surface area (PND15 and PND30) were also assessed. Blood pressure (BP) was measured in offspring. ID rats were smaller, exhibiting lower erythrocyte and tissue iron than control rats (PND2–PND10), but these parameters returned to control values by PND30–PND45. Relative kidney iron (μg g⁻¹ wet weight) at PND2–PND10 was directly related to transport iron measures. In ID rats, the maturation of the active nephrogenic zone was later than control. RGCs, glomerular size, glomerular density, and glomerular planar surface area were lower than control at PND15, but returned to control by PND30. After weaning, the kidney weight/rat weight ratio (mg g⁻¹) was heavier in ID than control rats. BP readings at PND45 were lower in ID than control rats. Altered kidney maturation and renal adaptations may contribute to glomerular size, early hyperfiltration and long-term renal function.

Keywords

anemia; development; developmental programming; glomeruli; hypertension; kidneys; nutrition

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Introduction

Half the pregnant women in the US exhibit insufficient iron stores and half these pregnancies progress to anaemia by term because of an eightfold greater iron need in the last trimester (O'Brien *et al.* 2003; Scholl 2005). The fetus is susceptible to nutritional iron depletion during late gestation (Rao and Georgieff 2002; Lozoff *et al.* 2006), coincident with active glomerulogenesis in kidney development. If sufficiently severe, gestational iron deficiency (ID) reduces the oxygen-carrying capacity of the uteroplacental unit and impedes fetal growth rate (i.e. intrauterine growth restriction (IUGR)). Described by the Barker hypothesis, similar to other causes of IUGR, gestational ID may lead to several adult-onset diseases, including chronic hypertension, a major contributor to premature cardiovascular deaths (Godfrey and Barker 2000). The detrimental consequences of gestational ID on the fetal brain (Beard *et al.* 2003; Lozoff *et al.* 2006; Rao *et al.* 2013), liver and placenta (Gambling *et al.* 2009) have been studied and reported; however, precisely how dietary-induced gestational ID affects kidney iron content, renal cellular differentiation, glomerulogenesis and tubular elongation remains unknown. Adult rats born with gestational ID (Lewis *et al.* 2002; Gambling *et al.* 2003) developed hypertension physiologically related to disturbed renal renin–angiotensin system signalling (Lewis *et al.* 2002). However, current knowledge is limited about how disturbances in glomerulogenesis mechanistically affect renal programming of blood pressure (BP).

Once active glomerulogenesis ceases, no new glomeruli are made (Yeung 2006). Glomerulogenesis is incomplete until Postnatal Day (PND) 7 in term rats or 36 weeks gestation in humans (Dunnill and Halley 1973; Haycock 1998; Yeung 2006). Precisely how iron regulates genes in the active nephrogenic zone remains unknown (Gambling *et al.* 2003). In an extreme example, Belgrade rats with a disabled iron transporter, namely divalent metal transporter-1 (DMT-1), exhibited evidence of a nephron deficit at PND9 and ultimately developed very disturbed microscopic morphology (Veuthey *et al.* 2014); however, this model did not report any changes in the timing of the cessation of active nephrogenesis. Dietary-induced gestational ID is more physiologically similar to the human condition of maternal ID and studying term gestational ID rats is a convenient *ex utero* model to study renal development. It is known that IUGR caused by other stressors can lower nephron endowment, which overworks the kidneys, a process known as hyperfiltration, ultimately leading to hypertension (Mackenzie and Brenner 1995). Rat studies show that inadequate tissue iron delivery during fetal (Lisle *et al.* 2003) or early postnatal life (Drake *et al.* 2009) reduces nephron numbers and ultimately causes hypertension (Lewis *et al.* 2002; Gambling *et al.* 2003; McArdle *et al.* 2014). Several studies, including those in extremely ID Belgrade rats, found a nephron deficit (Veuthey *et al.* 2014), but did not present data about the timing of the cessation of the active nephrogenic zone. However, the precise chain of developmental events linking ID to disrupted kidney development, hyperfiltration and hypertension is not known. To our knowledge, no prior study has chronologically examined early postnatal kidney development to measure tissue iron levels and the role of iron in the maturation of the active nephrogenic zone and ultimately nephron allotment. Thus, we sought to examine the critical temporal links between iron, renal development and hypertension in rats by examining blood and tissue

iron status and renal development during the first 6 weeks of life. We tested the hypothesis that dietary-induced gestational ID would deplete circulating and renal tissue iron, impair kidney development and ultimately provide a model to examine developmental programming of BP.

Methods

Animal methods

The University of Wisconsin–Madison and University of Minnesota Animal Care and Use Committees approved this protocol. The dietary model used in the present study, adopted from our collaborators, intended to simulate ID in pregnancy due to low dietary intake. Female timed-pregnant bred in-house Sprague-Dawley dams (Charles River Laboratories, Wilmington, MA, USA) were fed either an iron-sufficient (198 mg kg⁻¹ iron) control rodent diet or an ID (<6 mg kg⁻¹ iron) rodent diet (Harland Teklad, Madison, WI, USA) from Gestational Day 2 to PND7, the approximate equivalent to term human birth in respect to kidney development. At PND7, the ID lactating dams were switched to the iron-sufficient diet, as reported previously (Siddappa *et al.* 2003; Rao *et al.* 2007; Tran *et al.* 2012). On PND1, litters were culled to 10 pups. Placentas collected during delivery were frozen immediately in liquid nitrogen and stored at –80°C for later western blot analysis (Sun *et al.* 2013). Dams and pups were not disturbed until PND2, but were then weighed daily. Starting at PND2, several pups were randomly selected for daily blood and tissue harvests, maintaining litter size at 10. Litter size was maintained at 10 pups throughout lactation by cross-fostering and was consistent between groups and litters. No deaths occurred during the study apart from culling. On PND2–PND10, PND15, PND30 and PND45, pups were killed using lethal isoflurane anaesthesia, followed immediately by cardiac puncture for blood and then by midline dissection for tissue harvest.

Blood analyses

Blood was collected in EDTA and no additive microtainer tubes (BD, Franklin Lakes, NJ, USA) and analysed for complete blood counts using a Poch100i analyser (Sysmex, Mundelein, IL, USA). The zinc protoporphyrin to heme ratio (ZnPP/H) was measured using a 206D haematofluorometer (Aviv Biomedical, Lakewood, NJ, USA) after washing with phosphate buffered saline to remove interfering pigments (Blohowiak *et al.* 2008). The ZnPP/H ratio was used to measure incomplete iron incorporation into haemoglobin because, under iron-limited conditions, zinc substitutes for iron in the protoporphyrin ring. Whole blood was centrifuged at 3000g for 4 min at 48°C to obtain plasma and serum, which was frozen at –80°C for subsequent analysis of plasma C-reactive protein (CRP; Rat CRP ELISA; Immunology Consultants, Newberg, OR, USA) and plasma ferritin (Rat Ferritin ELISA; Immunology Consultants). Serum total iron binding capacity (TIBC) and transferrin (Tf) saturation (Iron/UIBC; Pointe Scientific, Canton, MI, USA) were determined. Plasma creatinine was analysed using the Quantichrom creatinine assay kit (BioAssay Systems, Hayward, CA, USA).

Placental western blot analyses

Western blot analysis was performed on frozen placentas. Placental tissue was homogenised in lysis buffer (4 mM $\text{NaN}_2\text{O}_7 \cdot \text{H}_2\text{O}$, 50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, 5 $\mu\text{g mL}^{-1}$ leupeptin, 5 $\mu\text{g mL}^{-1}$ aprotinin and 0.04% microcystin) and centrifuged at 16 000g for 15 min at 48°C. After protein quantification, samples underwent electrophoresis on an 18-well 4%–20% gradient Tris-HCl sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (BioRad, Hercules, CA, USA) at 150 V for 90 min, and were then electrically transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Monoclonal antibodies against transferrin receptor 1 (TfR1) CD71 (1: 200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β -actin antibody (1: 3000; Cell Signaling, Danvers, MA, USA) were used. Membranes were visualised with an enhanced chemiluminescence detection kit (Thermo Scientific, Waltham, WA, USA). Films were developed and quantified using densitometry.

Tissue non-heme iron analyses

Kidney and liver weights were obtained when rats were killed and tissues were frozen and then stored at -80°C for non-heme iron analysis. After homogenisation in iron-free water (v/v) with an iron-free pestle homogenizer, non-heme iron concentration ($\mu\text{g g}^{-1}$ wet weight) and tissue total non-heme iron allotment (μg) were quantified as described previously (Rebouche *et al.* 2004; Sun *et al.* 2013).

Kidney microscopic morphometric analyses

Right kidneys were fixed in 10% formalin and embedded in paraffin blocks. Mid-sagittal sections were cut to 10 μm widths and subsequently stained with haematoxylin and eosin for histology. Photomicrographs were captured digitally using a SPOT Insight Camera (Diagnostic Instruments, Sterling Heights, MI, USA) and a Nikon Eclipse 50i/55i (Melville, NY, USA). Morphometric parameters were examined in a blinded manner and determined using SPOT Advanced software (Diagnostic Instruments). The presence of an active nephrogenic zone was examined by the identifying presence or absence of immature C- and S-shaped bodies near the capsule, as described previously (Sun *et al.* 2013). Mean radial glomerular counts (RGCs) were determined from eight different $\times 10$ cortical photomicrographs (Rodriguez *et al.* 2004, 2005; Drake *et al.* 2009). Generations were identified by parallel lines drawn perpendicular to a mid-kidney medullary ray starting at the corticomedullary junction, proceeding out to the capsule, with every glomerulus traversing a line (Drake *et al.* 2009). Glomerular surface area and glomerular density (mean number of glomeruli per mm^2 of cortex; Drake *et al.* 2009; Sun *et al.* 2013) measurements were determined in $\times 10$ fields merged to reconstitute the whole cortex in Adobe Photoshop CS3 software (Adobe Systems, San Jose, CA, USA) and a 45° angle or 'pie' slice drawn from the geometric centre of the kidney.

BP analyses

After weaning and 1 week before readings were recorded, conscious rats were acclimated daily to the tail-cuff CODA Non-Invasive Blood Pressure System (Kent Scientific,

Torrington, CT, USA; Feng and DiPetrillo 2009). Rats were placed in an appropriate-sized animal nose cone holder and acclimated on a heating pad and room temperature, both at 26°C, for 5 min for tail vasodilation. Cross-sectional and serial readings were taken in accordance with manufacturer recommendations, set to produce 10 consecutive readings and analysed if greater than five systolic BP readings were physiologically probable. The lowest and highest readings were then excluded, with the mean of the remaining values recorded.

Statistical analyses

Statistical analyses comparing differences between the control and ID group were performed using one-way analysis of variance (ANOVA) followed by a post hoc Student–Newman–Keuls' test. Analysis of covariance (ANCOVA) was used to examine interactions of slopes between the control and ID groups. Western blot optical density (OD) was analysed as fold of control and further normalised against the OD of β -actin and expressed as fold of control. The presence of a nephrogenic zone was analysed by Fisher's exact test. Regressions were performed to test normality, and linear regression analysis was used to examine relationships between parameters. Data are presented as the mean $\pm$ s.e.m. The level of significance was established *a priori* at $P < 0.05$ (two-tailed).

Results

Rats of both sexes were studied. At each postnatal age from PND2 to PND10, 8–30 control rats (11 separate litters) and 6–15 ID rats (11 separate litters) were studied. In addition, 23 control (five litters) and 21 ID rats (four litters) were studied on PND15, 19 control (four litters) and 13 ID rats (five litters) were studied on PND30 and 11 control (three litters) and 10 ID rats (four litters) were studied on PND45. Bodyweights at PND2 were not significantly different ($P = 0.2$); however, body-weights from PND3 to PND10 in the gestational ID group were lighter than in the control group ($P < 0.05$; Fig. 1a). Both control and ID rats doubled their PND2 weights by PND7. At PND15 and PND30, ID rats remained growth restricted compared with age-matched controls ($P < 0.05$). Catch-up growth was attained by PND45. Mean kidney weight trended lower in ID rats throughout the first 10 days, but was lighter in the ID than control group by PND10 ($P < 0.05$; Fig. 1b). At PND15 and PND30, ID kidney weights were lighter than the control group ($P < 0.05$) but became heavier ($P < 0.05$) at PND45 (Fig. 1b). When expressed as kidney weight relative to rat weight (mg g^{-1}), the groups did not differ until PND7, with the kidney weight: bodyweight ratio higher in the ID than control group on PND7–9 ($P < 0.05$), comparable to that in the control group on PND10, PND15 and PND30 and higher again in the ID than control group on PND45 ($P < 0.04$). The relationship between relative wet kidney weight plotted against rat weight was not constant, but was positive in both groups, exhibiting a steeper slope over PND2–PND10 in the ID group ($r^2 = 0.338$, $P < 0.0001$) than in the control group ($r^2 = 0.164$, $P < 0.0007$; Fig. 1c).

Erythrocyte iron was assessed by haematocrit (Hct) and ZnPP/H ratios. In ID rats, Hct percentages were 26%–34% lower than in age-matched control rats from PND2 to PND10 ($P < 0.05$), as well as 27% lower ($P < 0.05$) at PND15, before reaching control levels at PND30 (Table 1). The nadir in Hct differed, occurring at PND3 in the control group and on

PND8 in the ID group. In addition, ZnPP/H ratios were higher in ID than control rats at PND2 and PND10 ($P < 0.05$). At PND15, the ZnPP/H ratio was higher in the ID group, but overcorrected at PND30 and PND45, becoming lower ($P < 0.05$) compared with age-matched controls (Table 1). The liver, an erythropoietic organ in suckling rats, exhibited fewer erythropoietic pockets at PND9 in ID compared with control rats (Fig. 2), despite a marked rise in plasma iron delivery in the ID group at PND9–PND10. TIBC levels were higher in ID than control rats at PND9–PND10 ($P < 0.05$) after return of maternal dietary iron, although initially did not differ between groups. In addition, TIBC levels remained higher at PND15 ($P < 0.05$) in the ID group, becoming comparable to levels in the control group by PND30 and PND45 (Table 1). In ID rats, Tf saturation was lower than in control rats from PND2 to PND10 and on PND15 ($P < 0.05$); however, Tf saturation was comparable to control levels by PND30 and PND45 (Table 1). Confirming that the model caused maternal adaptation to improve fetal iron delivery, placental TfR protein expression was higher in gestational ID ($P < 0.001$) compared with control (Fig. 3).

In addition to erythrocyte and transport iron, storage iron was assessed. In both groups, mean liver non-heme iron concentrations fell from PND2 to PND9, with levels lower in ID than control rats ($P < 0.05$) until levels in the ID group reach control levels on PND10. Although similar at PND15 and PND30, ID liver iron concentrations at PND45 were lower in the ID than control group ($P < 0.05$; Fig. 4a). Similarly, ferritin, the plasma index of storage iron, was much lower in ID pups than in control pups, until overcorrecting and increasing at PND30 ($P < 0.05$) compared with control before equalising at PND45 (Table 1). Because ID causes placental and fetal inflammatory responses, levels of the liver acute phase protein CRP were examined. CRP levels were variable in both iron groups, increased after weaning and differed only at PND30 when CRP was higher in the ID than control group ($P < 0.05$; Table 1). Mean kidney non-heme iron concentration was between 45% (PND2) and 60% (PND10) lower in the ID compared with control group ($P < 0.05$), with a marked fall at PND3 in both groups. At PND15, PND30 and PND45, kidney iron concentration in ID rats did not differ from that in the control group (Fig. 4b). Combining PND2–PND10 data from both groups, plasma ferritin levels were directly related to liver iron concentrations ($r^2 = 0.57$, $P < 0.0001$; Fig. 4c). Although PND2–PND10 liver iron was also directly related to Tf saturation ($r^2 = 0.42$, $P < 0.0001$), stepwise regression found only the relationship between liver iron plasma ferritin remained significant ($r^2 = 0.65$, $P < 0.0001$). Combining both groups from PND2 to PND10 (Fig. 4c), kidney iron concentration was directly related to Tf saturation ($r^2 = 0.45$, $P < 0.0001$). Although PND2–PND10, kidney iron was also related to plasma ferritin ($r^2 = 0.34$, $P < 0.0001$), stepwise regression found only the relationship between Tf saturation and kidney iron concentration ($r^2 = 0.08$, $P < 0.03$) remained significant.

Nephrogenesis and the timing of the disappearance of the nephrogenic zone were studied. Distorted and immature C- and S-shaped glomerular bodies were found in the active nephrogenic zone in 91% of the ID group at PND10, whereas all nephrogenic zones in control group were mature by PND7 ($P < 0.05$; Fig. 5). At PND15, the ID group exhibited immature and more closely spaced glomeruli with fewer mean RGCs than control. However, at PND30, RGCs appeared more evenly dispersed and were morphologically similar to those in the control group (Fig. 6a). Although mean glomerular size was smaller in the ID group at

PND15 ($P < 0.05$), it did not differ from that in the control group at PND30 (Fig. 6b). Glomerular planar surface area as a ratio of cortical area was smaller in the ID group at PND15 ($P < 0.05$), but did not differ from the control group at PND30 (Fig. 6c). At PND15, cortical depths were smaller in the ID group ($P < 0.05$), but did not differ from control at PND30 (Fig. 6c).

As a functional marker of developmental programming, mean plasma creatinine levels tended to be higher in ID compared with control rats at PND45 (1.24 ± 0.06 vs 1.15 ± 0.09 mg dL⁻¹; $P = 0.16$). In addition, tail-cuff systolic BP was assessed in rats at PND39–PND45, with ID rats exhibiting a lower BP than control rats ($P < 0.05$; Fig. 7a). As anticipated, males at this postnatal age tended to have higher BP than females in both groups (Fig. 7b).

Discussion

Herein, we comprehensively describe circulating and tissue iron status during a critical phase of renal development in an established model of dietary-induced gestational and early postnatal ID. This work is the first to define the effect of iron on perinatal renal development. It was found that dietary-induced gestational ID produced growth-restricted, iron-depleted and anaemic offspring that, after repletion, experienced an overcorrection of both erythrocyte iron and plasma ferritin levels. Liver and kidney iron metabolism underwent differential regulation, with the nadir of kidney iron in ID offspring temporally coinciding with the point when glomerulogenesis normally ends and prolonging the active nephrogenic zone. In addition, ID rats in young adulthood exhibited a lower, not higher, BP.

Iron is vital for neonatal growth because half the iron needed for infant growth and development is normally acquired before birth (Lozoff *et al.* 2006). The ID rats in the present study were growth restricted and exhibited poor iron status, similar to humans born after severe gestational ID (Gambling *et al.* 2003, 2009; Lisle *et al.* 2003). With dam milk providing the sole source of iron to pups until weaning, ID rat size did not equal that of control pups for at least 1 month after return of maternal dietary iron and at least 15 days after anaemia corrected. However, in the first 10 days, the relationship between rat weight and relative kidney weight in ID rats was steeper than control (i.e. progressively higher relative kidney weights as the ID rats aged). It is unclear whether this increased relative kidney weight is due to oedema or fluid retention or a greater growth rate.

This work is novel because it studied erythropoietic parameters during recovery from dietary-induced gestational ID. Suckling rats born to ID mothers exhibited poorer erythrocyte iron incorporation, circulating iron and iron stores that slowly recovered after returning maternal dietary iron. This compares with only one previous study that created ID during weaning and followed erythropoiesis during the recovery, which interestingly also found an overcorrection (i.e. higher Hct in young adulthood; Hegde *et al.* 2011). After maternal iron repletion, the circulating iron (TIBC) in ID suckling pups rose to control levels, but Hct, erythrocyte iron incorporation, ferritin levels and kidney iron levels did not equalise until after weaning. When there is limited iron availability, iron is prioritised for erythropoiesis at the expense of developing tissues, such as the kidneys (Georgieff *et al.* 1995).

This poor recovery rate may be from the lack of contribution of the liver, an organ that is normally erythropoietic in neonatal rats. Even with lower demands accompanying fetal growth restriction and slower postnatal growth rate, lower liver iron concentrations in gestational ID pups were consistent with findings in human placental insufficiency-induced IUGR (Georgieff *et al.* 1995). The present study observed an increase in both liver iron and ferritin immediately after birth in controls, likely from iron derived from erythrocyte breakdown (Rao and Georgieff 2007) or potentially from rat colostrum, reflecting the liver's role as a storage depot for future growth. At PND45, liver iron levels in ID rats dropped below control levels, a key finding representing either the altered programming of liver stores or potentially an enhanced delivery to erythrocytes, as evidenced by a lower ZnPP/H ratio. The present study found improved erythrocyte iron delivery and a tendency for a higher Hct at PND45, consistent with research finding that ID created in older weanling rats also programmed higher Hct at the expense of poorer liver iron status upon recovery from ID (Hegde *et al.* 2011).

Placental adaptation also occurred in gestational ID with a 1.5-fold higher placental TfR expression, accompanying the markedly lower neonatal liver iron. This finding was consistent with previous work in rats describing that when liver iron concentrations fall by 50%, placental TfR is upregulated from falling liver hepcidin (Gambling *et al.* 2009). As expected, liver iron concentration was more highly correlated with plasma ferritin than with Tf saturation, supporting the use of ferritin as a surrogate index of storage iron. Although neonatal hepcidin levels were not measured, a logical assumption is that hepcidin, the master iron regulator, controls neonatal liver iron, as well as plasma ferritin levels.

The present study was the first to observe that dietary iron affects neonatal renal development in rats. Renal iron quickly dropped after birth, but was relatively protected at 50% of control levels, compared with liver iron at less than 25% of controls. Tissue iron content in the kidney was more strongly related to the circulating iron index, Tf saturation, than plasma ferritin, and renal iron levels recovered more quickly than hepatic iron. Liver iron was strongly related to plasma ferritin and regulated to prevent iron overload through hepcidin, known to regulate liver iron uptake and release (Park *et al.* 2001; Pietrangelo 2007). However, our data support that hepcidin may be of relatively minor importance in the neonatal kidney (Smith and Thevenod 2009), with Tf-bound iron playing a major role through its presence in the glomerular filtrate and binding to TfR on the distal tubular epithelia (Smith and Thevenod 2009). Differences in renal versus liver iron processing are consistent with previous findings in fetal sheep (Sun *et al.* 2013) and in DMT-1-mutated iron-deficient Belgrade rats (Veuthey *et al.* 2014), but contrast with descriptions of hepcidin regulating renal iron metabolism in adult mice (Moulouel *et al.* 2013). Finding that fetal and neonatal kidneys exhibit major developmental differences in iron regulation compared with adults is highly novel, hypothesis generating and would be important to explore in order to understand the susceptibility of immature kidneys to developmental defects.

Glomerulogenesis is complete at 36 weeks gestation in humans and at PND7 in rats (Lisle *et al.* 2003; Gambling *et al.* 2009), after which no new glomeruli are normally formed (Yeung 2006). However, the active nephrogenic zone in ID rats, exposed to the nadir in tissue iron, remained active past PND7, supporting iron's role in kidney development. This contrasts

from our previous work that found delayed cessation of active nephrogenesis in IUGR fetal sheep with higher, not lower, renal iron concentrations (Sun *et al.* 2013). It is also possible that the PND8–10 kidney responded to the rapid rise in iron delivery via higher TIBC in a similar fashion to the fetal sheep (Sun *et al.* 2013). Massive cell division during prolonged glomerulogenesis (Shah *et al.* 2004) may explain, in part, the apparent normalisation of glomerular generation, total glomerular planar surface area and cortical depth between PND15 and PND30 in ID rats. Microscopic morphometry studies were limited by the inability to perform kidney microscopic morphometry by the more sensitive dissector methods, but differences were clearly appreciated. A disproportionately rapid rise in glomerular size in ID rats at PND45 may be maladaptive and caused by early hyperfiltration, as evidenced by a relatively greater kidney weight over time (Zohdi *et al.* 2007), and worsening renal function (i.e. plasma creatinine) at PND45 in ID rats (Miura *et al.* 1994; Schillaci *et al.* 2001).

Hypertension was previously reported in offspring of gestational ID rats (Gambling *et al.* 2003; Lisle *et al.* 2003). In contrast with our hypothesis, BP was lower in ID rats, which can be due to a smaller somatic size but equal weight at PND45. This finding is consistent with a human study describing lower BP in 7-year-old children whose mothers had anaemia and likely better plasma expansion during pregnancy (Brion *et al.* 2008). It is not clear whether this finding will persist into adulthood or whether male–female differences are possible. Timing could explain this finding because our data are consistent with lower BP in 6-week-old female offspring of diet-induced gestational ID rats, whereas BP was higher in comparably-aged male offspring (Gambling *et al.* 2003), despite much work describing the development of hypertension in older rats of both sexes (Lisle *et al.* 2003). Part of the effect could be that young, healthy kidney donors exhibited lower BP after kidney donation during the ensuing hyperfiltration phase, something not observed in older kidney donors (Saxena *et al.* 2006). Greater vasodilation in youth may lower BP readings and is consistent with greater renal nitric oxide levels observed in 6-week-old rats in another IUGR model (Stewart *et al.* 2005). The present study is the first to describe a lower BP in male and female offspring of gestational ID rats, which may be a long-term adaptive mechanism for gestational ID or other IUGR. It may also be the first stage of altered BP control that precedes the development of hypertension.

However, if BP readings are lower, this novel finding is scientifically important and future studies are needed to explore the regulation of BP. Our findings relating to BP could have differed from those reported by Gambling *et al.* (2003) because of their use of a different rat strain, the Rowett Lister Hooded strain, more prolonged use of an ID diet before mating and a diet with 15% of the iron of iron-sufficient (IS) (7.5 vs 50 mg kg⁻¹) compared with Sprague-Dawley rats being fed a shorter-term diet with a greater difference between ID and IS (<6 vs 198 mg kg⁻¹). The present study could have been improved by the use of more precise telemetry methods. The study of timing and degree of maternal iron depletion on kidney development and BP would be most interesting.

Impaired growth due to poor suckling may contribute to renal pathological findings. The present study is limited by a lack of pair feeding, which limits the ability to dissect the specificity of findings to iron nutrition rather than the end result of the growth restriction.

Future studies would benefit with the addition of pair-fed controls and closer assessment of food intake by the dams. However, we found that gastrostomy feeding of an ID artificial formula starting at PND4 sustained normal somatic and organ growth, but resulted in relatively more abnormal histological renal development (Drake *et al.* 2009). In unpublished data (P. J. Kling) using the artificial feeding gastrostomy model, we have found a prolonged, abnormal active nephrogenic zone at PND8–9 in ID pups, supporting that prolonged active nephrogenesis was specific to iron deficit.

In conclusion, offspring of dietary-induced gestational ID with early postnatal ID developed several functional adaptations, including postnatal growth restriction, anaemia and depleted tissue iron. After iron repletion and recovery, we observed an overcompensated delivery of iron for erythropoiesis. Renal iron metabolism differed from liver iron metabolism, with the nadir in renal iron content coinciding with normal timing of nephrogenic zone maturation. This may have altered developmental programming, stimulating a prolongation of active nephrogenesis. Functional implications were found, including larger kidneys (relative to rat weight), a tendency for higher plasma creatinine and programming of lower BP readings in young adult rats of both sexes. It is unknown whether this adaptive programming will lead to hypertension later in life, but several pieces of evidence point towards early hyperfiltration. In addition to maternal IDA, many fetal conditions, including placental insufficiency-induced IUGR and diabetes, deplete fetal tissue iron content (Chockalingam *et al.* 1987), a common condition in premature infants born weeks or months before active nephrogenesis is complete. The novel clinical knowledge gained from this model will shed light on kidney development in at-risk term infants and even more so in premature infants who are at greater risk of impaired kidney development. This novel model shows that iron trafficking in the kidney differs from the liver and this may affect strategies to better protect renal development in premature humans, especially as it relates to elucidation of the hyperfiltration hypothesis set forth by Brenner *et al.* (1988) in term and premature infants. Thus, it can be a valuable tool to help model human disease and further investigate the iron-mediated molecular and pathophysiological processes regulating renal development and BP control.

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Fig. 1. Morphometric parameters of control and iron-deficient (ID) rats from Postnatal Day (PND) 2 to PND10 and on PND15, PND30 and PND45. Note, the scale on y-axis for PND15, PND30 and PND45 differs from that for earlier time points. (a) Serial rat weights differed between control and ID groups, except on PND2, PND5 and PND45. (b) Kidney weights differed between control and ID groups on PND4, PND6, PND10, PND15 and PND30. (c) Wet kidney weights relative to rat weight differed between control and ID groups on PND7–9 and PND45. Data are the mean $\pm$ s.e.m. * $P < 0.05$ compared with control. (d) wet kidney

weight as a proportion of rat weight during the first 10 postnatal days was plotted against rat weight and analysed using linear regression. The solid black line represent the regression line for the control group ($r^2 = 0.164$, $P < 0.001$), whereas the dashed line represents the regression line for the ID group ($r^2 = 0.338$, $P < 0.0001$). The slopes differed significantly ($P < 0.01$).

Fig. 2. Erythropoietic pockets located in haematoxylin and eosin-stained (a) control and (b) iron-deficient (ID) liver on Postnatal Day (PND) 9, after return of iron to maternal diet. Scale bar = 100 μ m. Circles indicate erythropoietic pockets. CV, central vein.

Fig. 3. Placental transferrin receptor 1 (TfR1) in control and iron-deficient (ID) dams. (a) Placental samples taken at term analysed for TfR by western blot analysis with β -actin as a loading control. (b) Mean ($\pm$ s.e.m.) optical density (fold of control) of placental TfR expression in control and ID groups. * $P < 0.05$ compared with control.

Fig. 4.

Tissue and storage iron in control and iron-deficient (ID) rats from Postnatal Day (PND) 2 to PND10 and on PND15, PND30 and PND45. Note, the scale on y-axis for PND15, PND30 and PND45 differs from that for earlier time points. (a) Liver non-heme iron concentrations differed between ID and control rats on PND2–PND9 and PND45. (b) Kidney non-heme iron concentrations differed between ID and control rats on PND2–PND10. Data are the mean $\pm$ s.e.m. * $P < 0.05$ compared with control. (c) Combining all PND2–PND10 values, a direct relationship was observed between plasma ferritin and liver iron concentration (logarithmic regression; $r^2 = 0.57$, $P < 0.0001$) and between plasma transferrin (Tf) saturation and kidney iron concentration (linear regression; $r^2 = 0.45$, $P < 0.0001$).

Fig. 5. Maturation of the nephrogenic zone of control and iron-deficient (ID) kidneys, showing haematoxylin and eosin kidney sections from Postnatal Day (PND) 6 to PND8. Scale bar = 50 µm. Mature round glomeruli are found at PND7 in control rats. Active nephrogenic zones, including dense groups of immature glomerular cells with C- and S-shaped bodies are seen only in PND6 kidneys in control rats, but are present in PND6–PND8 kidneys in ID rats.

Fig. 6.

Microscopic morphometric measurements in control and iron-deficient (ID) kidneys at Postnatal Day (PND) 15 and PND30. (a) Radial glomerular generations were lower in ID than control rats on PND15 but not PND30. (b) Mean glomerular size was lower in ID than control rats on PND15. (c) Glomerular planar surface area ratio was lower in ID than controls on PND15 but not PND30. (d) Cortical depth was lower in ID than control rats on PND15 than PND30. Data are the mean $\pm$ s.e.m. * $P < 0.05$ compared with control.

Fig. 7. Resting systolic blood pressure (SBP) in control and iron-deficient (ID) groups from Postnatal Day (PND) 39 to PND45. (a) SBP was lower in ID than control rats. (b) Sex-specific SBP was lower in ID than control for both males and females, although SBP in control females did not differ from that in ID males. Data are the mean $\pm$ s.e.m. * $P < 0.05$ compared with control.

Table 1

Blood haematopoietic and iron indices in development

Data are the mean ± s.e.m. ZnPP/H, zinc protoporphyrin/heme; TIBC, total iron binding capacity; CRP, C-reactive protein; PND, postnatal day; ID, iron deficiency

	PND2	PND3	PND4	PND5	PND6	PND7	PND8	PND9	PND10	PND15	PND30	PND45
Haematocrit (%)												
Control	30.2 ± 1.2	25.8 ± 0.7	28.7 ± 0.8	30.3 ± 1.6	31.4 ± 0.8	30.4 ± 1.0	29.8 ± 0.5	34.7 ± 1.0	31.1 ± 0.6	30.8 ± 0.5	38.8 ± 0.7	43.4 ± 0.5
Gestational ID	22.3 ± 1.3	20.9 ± 1.4	23.4 ± 1.4	24.5 ± 1.6	22.0 ± 0.9	20.8 ± 2.1	18.4 ± 0.8	22.0 ± 0.9	21.5 ± 1.6	22.5 ± 0.7	38.7 ± 1.6	44.6 ± 0.7
P-value	<0.0001	<0.004	<0.016	<0.023	<0.0001	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	0.9	0.25
ZnPP/H ratio (µmol/mol)												
Control	121.8 ± 6.6	93.4 ± 7.1	111.2 ± 4.9	101.1 ± 4.6	100.7 ± 8.3	94.5 ± 7.7	101.9 ± 8.5	73.7 ± 5.0	77.0 ± 4.4	73.9 ± 4.7	75.8 ± 4.7	71.0 ± 1.9
Gestational ID	386.5 ± 69.2	368.9 ± 60.1	337.3 ± 54.1	245.9 ± 22.8	275.2 ± 13.4	255.0 ± 38.8	212.1 ± 17.6	268.7 ± 33.5	183.8 ± 14.0	156.6 ± 7.6	56.0 ± 2.7	62.3 ± 2.5
P-value	<0.0001	<0.0001	<0.0001	<0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.021	<0.021
TIBC (µmol L ⁻¹)												
Control	60.2 ± 4.1	63.9 ± 4.9	74.6 ± 7.0	84.9 ± 9.9	87.7 ± 5.6	60.8 ± 11.4	79.5 ± 8.5	74.0 ± 4.0	74.1 ± 6.0	76.0 ± 3.1	113.9 ± 3.8	107.6 ± 5.3
Gestational ID	64.1 ± 4.6	60.2 ± 2.7	77.3 ± 1.8	88.3 ± 5.1	92.5 ± 9.0	94.7 ± 6.8	138.5 ± 12.6	111.5 ± 18.0	117.6 ± 16.8	91.4 ± 3.2	113.9 ± 5.2	90.6 ± 9.8
P-value	0.55	0.5	0.75	0.8	0.65	<0.040	<0.003	0.07	<0.035	<0.007	0.99	0.2
Transferrin saturation (%)												
Control	57.1 ± 7.1	34.8 ± 2.9	50.7 ± 4.7	45.9 ± 9.5	47.4 ± 5.3	60.6 ± 7.1	36.5 ± 9.7	49.2 ± 2.1	36.5 ± 6.2	22.1 ± 1.8	72.6 ± 4.0	66.1 ± 4.9
Gestational ID	23.1 ± 2.1	14.3 ± 2.4	9.9 ± 2.7	6.2 ± 2.2	9.6 ± 3.1	15.4 ± 6.3	31.9 ± 6.3	9.8 ± 3.2	14.3 ± 3.1	9.1 ± 1.8	70.7 ± 4.0	53.8 ± 11.1
P-value	<0.0015	<0.0002	<0.0001	<0.005	<0.0003	<0.0015	0.7	<0.0001	<0.01	<0.0004	0.75	0.35
Ferritin (µg mL ⁻¹)												
Control	4129 ± 760	10 715 ± 2449	7638 ± 1326	4391 ± 559	3258 ± 494	3624 ± 690	2684 ± 515	1823 ± 217	1324 ± 198	1102 ± 201	1674 ± 234	1266 ± 224
Gestational ID	2030 ± 306	2885 ± 900	545 ± 74	373 ± 82	1252 ± 302	274 ± 78	246 ± 37	275 ± 67	153 ± 19	333 ± 65	4916 ± 2136	1178 ± 248
P-value	<0.0075	<0.004	<0.0003	<0.0001	<0.0015	<0.0025	<0.0005	<0.0001	<0.0006	<0.0001	<0.025	0.8
CRP (mg 100 mL ⁻¹)												
Control	1.02 ± 0.12	0.68 ± 0.06	1.1 ± 0.20	1.06 ± 0.13	1.11 ± 0.13	1.47 ± 0.11	0.74 ± 0.11	0.87 ± 0.09	0.86 ± 0.09	1.68 ± 0.27	26.9 ± 2.7	35.7 ± 2.7
Gestational ID	0.65 ± 0.07	0.74 ± 0.12	0.86 ± 0.17	0.62 ± 0.14	1.26 ± 0.13	1.44 ± 0.17	0.88 ± 0.09	1.07 ± 0.16	0.71 ± 0.17	0.92 ± 0.08	46.12 ± 3.94	34.68 ± 2.85
P-value	<0.01	0.6	0.45	<0.035	0.4	0.9	0.351	0.3	0.45	<0.003	<0.0008	0.8