Gender differences in post-natal growth and renal development in offspring of rabbit mothers with chronic secondary hypertension.

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Running Title: Impact of maternal hypertension on offspring

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Abstract

Previously we demonstrated that adult blood pressure was increased in offspring of rabbit mothers with chronic secondary renal hypertension. Our study identified sex-specific differences in the programming of hypertension, with female not male offspring having increased blood pressure at 30 weeks of age. The aim of this study was to characterise the maternal hypertension during pregnancy to determine potential programming stimuli. Further, we examined the impact of chronic maternal hypertension on offspring birth weight, nephron number and renal noradrenaline content (as an index of renal innervation density). Three groups of mothers and their offspring were studied; two-kidney, one-wrap (2K-1W, n = 9 mothers) hypertensive, two-kidney, two-wrap (2K-2W, n = 8) hypertensive, and a sham–operated group (n = 9). Mean arterial blood pressure was increased by ~20 mmHg throughout pregnancy in both hypertensive groups as compared to sham mothers (P<0.001). Plasma renin activity (PRA; P<0.05) and aldosterone (P<0.05) levels were increased during gestation in the 2K-1W, but not the 2K-2W mothers. Birth weight was increased by ~20% in offspring of both groups of hypertensive mothers (P<0.001), though this was associated with a reduction in litter size. Renal noradrenaline content was increased (~40%, P<0.05) at 5 weeks of age in female 2K-1W offspring as compared to sham offspring. Glomerular number was not reduced in offspring of either group of hypertensive mothers, however glomerular tuft volume was reduced in female 2K-2W offspring (P<0.05), indicative of a reduction in glomerular filtration surface area. In conclusion, the two models of renal hypertension produced differential effects on the offspring. The impact of a stimulated maternal renin-angiotensin system in the 2K-1W model of hypertension may influence development of the renal sympathetic nerves and contribute to programming of adult hypertension.
Introduction

Chronic hypertension during pregnancy is an increasing problem as women are tending to have children later in life when the incidence of obesity, diabetes and renal disease associated hypertension is increased. The babies of women with chronic hypertension not only have acute problems perinatally, but more disturbingly, may have an increased long-term burden in the form of future cardiovascular risk (22, 53). There is now compelling evidence, both epidemiological and experimental, to support the hypothesis that events occurring in fetal life can have life-long consequences for the health of the adult (3, 24). The importance of this issue has been recently highlighted in a NHLBI working group report recommending increased focus on the study of hypertensive disorders in pregnancy and in particular the possibility of future cardiovascular risk to offspring (51).

Previously, we demonstrated that adult blood pressure was increased in offspring of rabbit mothers with pre-existing mild secondary hypertension (2K-1W) (13). Our study identified sex-specific differences in the programming of hypertension, with female not male offspring having increased blood pressure at 30 weeks of age. However, in this study neither maternal blood pressure during pregnancy or birth weight was determined. Therefore in the current study we documented mean arterial pressure (MAP) and plasma renin activity (PRA) in a 2K-1W model of hypertension throughout pregnancy. Furthermore, we incorporated a second model of maternal hypertension in our studies, the 2-kidney, 2-wrap model (2K-2W), with the aim of producing a more severe degree of hypertension in the mothers, as previously reported in males (10, 11). We hypothesised that the increase in MAP in 2K-2W mothers would be greater than the 2K-1W model and that the degree of elevation in blood pressure would be maintained in both hypertensive models during pregnancy.
Low birth weight has been associated with adult hypertension in several models of adverse intrauterine environments, though accumulating evidence suggests that altered fetal development can also occur independently of low birth weight (45). It is well recognised that hypertension in pregnancy is associated with low birth weight in humans (22, 53). Therefore, we also addressed the hypothesis that birth weight would be reduced in the offspring of these hypertensive pregnancies.

Two potential mechanisms that might contribute to the programming of hypertension in adulthood were also examined. A reduced nephron endowment / filtration surface area has been linked to adult hypertension (32). Thus nephron number was determined, stereologically, in kidneys from 5 week old offspring, an age when nephrogenesis is complete (42). We hypothesised that glomerular number would be decreased by maternal hypertension. Strong evidence implicates overactivity of the renal sympathetic nerves in the pathogenesis of hypertension (20, 28). Studies in the spontaneously hypertensive rat (SHR) have shown altered renal nerve development (25). Total noradrenaline levels were increased in SHR when compared to controls, consistent with increased sympathetic innervation density (25). We therefore also aimed to identify whether the renal sympathetic innervation was altered in the offspring of hypertensive mothers; a possible mechanism by which offspring are rendered vulnerable to developing increased blood pressure in later life. It was hypothesised that renal noradrenaline content would be increased (indicative of an increased sympathetic innervation) in the offspring of hypertensive mothers.
Methods

Animals

English crossbred rabbits were used. Experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved in advance by the Monash University Standing Committee on Ethics in Animal Experimentation.

Mothers

Nulliparous female rabbits (n = 26, 16 ± 1 weeks of age, 2.7 ± 0.1 kg) were housed individually in a room maintained at a temperature of 23-25 °C with a 12h light/dark cycle. Rabbits had free access to water and were fed high fibre, low starch rabbit pellets ad libitum (Glen Forrest Stockfeeders, WA, Australia).

Arterial blood pressure and body weight were measured in rabbits on a control day. Catheters were placed in the central ear arteries under local anesthetic (Xylocaine; Astra Pharmaceuticals, NSW, Australia) and conscious arterial pressure was measured for 30 minutes (13). An arterial blood sample (total volume ~5 ml) was then collected for hematocrit, plasma creatinine, plasma renin activity (PRA) and aldosterone measurement. Surgery was then performed on three groups of rabbits; 2 kidney, 1 wrapped (2K-1W, n = 9) and 2 kidney, 2 wrapped (2K-2W, n = 8) hypertensive and sham-operated (n = 9) normotensive rabbits. In brief, anesthesia was induced with propofol (10 mg/kg IV; Diprivan, Zeneca) and maintained with isoflurane (Forthane, Abbot). In all animals incisions were made on both flanks to expose left and right kidneys. In the sham-operated group the kidneys were untouched. In the 2K-1W model the left kidney was wrapped in cellophane (15 x 12 cm) and in the 2K-2W group both kidneys were wrapped in cellophane
(10, 11, 13). Four weeks following surgery at gestational age 0 (GA0) blood pressure and blood samples were repeated after which the rabbits were mated with a normotensive male. Blood pressure and blood sampling measurements were then repeated at GA18 and GA28 and 5 weeks post-partum.

**Offspring**

Kittens were spontaneously delivered between 31-32 days gestation. Total number of offspring (both live and stillborn) and birth weight was recorded. On the day of birth kidneys were collected from 2 kittens from each litter, which were killed by anesthetic overdose (Pentobarbitone 25 mg/kg i.p.). Kidneys were collected, weighed, snap frozen in liquid nitrogen and stored at –70 °C for later noradrenaline content assay.

The remaining offspring were weighed weekly and at 5 weeks of age arterial blood pressure was measured in the conscious animals via an intra-arterial catheter inserted into the central ear artery. Arterial blood pressure, hematocrit, plasma creatinine and PRA measurements were made as described above in the mothers. The rabbit offspring were than anesthetised with pentobarbitone (60 mg/kg, i.p.) and the kidneys exposed via a midline incision. Ligatures were placed around the left renal vessels, the kidney excised, weighed and snap frozen for later noradrenaline content measurement. A catheter was placed in the lower abdominal aorta below the remaining right kidney in preparation for fixation. The kidney, was cleared by rinsing with phosphate buffer and then perfusion fixed at arterial pressure with 4% paraformaldehyde in 0.1M phosphate buffer for ten minutes as previously described (12).

**Glomerular Stereology**

Right kidneys were cut in half and then into quarters, each quarter was then sliced into 1.5
mm slices. Due to the existing variation in size of slices, they were arranged from smallest to largest and every tenth slice taken for further sampling, with the first slice chosen at random. Tissue blocks were processed and embedded in glycolmethacrylate (Technovit 7100, Haraeus Kulzer Gmbh, Germany). Blocks were exhaustively sectioned at 20 μm with every 10th and 11th section sampled, the first of which was chosen at random from the first 10 sections. Sections were stained with periodic acid-Schiff reagent (PAS).

Total kidney volume was estimated by the Cavalieri principle. In brief, every 10th section was placed under a microfiche with a superimposed grid (3 x 3 mm) and points falling on kidney tissue counted. The following formula was used to calculate kidney volume (V_{kid}):

\[
V_{\text{kid}} = F_1 \times F_2 \times t \times a(p) \times P_s
\]

Where \( F_1 \) is the inverse of the first sampling fraction (\( F_1 = 10; \) i.e. every 10th slice was sampled) and \( F_2 \) is the inverse of the second sampling fraction (\( F_2 = 10, \) i.e. every 10th pair of sections was analysed), \( t \) is the average section thickness, \( a(p) \) is the area associated with each grid point, and \( P_s \) is the total number of points hitting kidney tissue.

Total glomerular number (\( N_{\text{glomer}, \text{kid}} \)) was estimated using physical dissectors as described in detail previously (19). In brief, total glomerular number was estimated using:

\[
N_{\text{glomer}, \text{kid}} = 10 \times 10 \times PS/2PF \times Q
\]

where 10 was the inverse of the first sampling fraction (1/10 of the slices) and 10 was the inverse of the second section sampling fraction (1/10 of the sections), \( PS/2PF \) was the fraction of the section area used for counting glomeruli, and \( Q \) was the actual number of glomeruli counted. Approximately, 100-150 glomeruli were counted for each kidney.

Mean glomerular tuft (\( V_{\text{glomer}} \)) and renal corpuscle (\( V_{\text{corp}} \)) volumes were estimated as:
\[ V_{\text{glom}} = \frac{V_{(\text{glom,kid})}}{N_{V_{(\text{glom,kid})}}}, \]

and

\[ V_{\text{corp}} = \frac{V_{(\text{corp,kid})}}{N_{V_{(\text{glom,kid})}}}, \]

Where \( V_{(\text{glom,kid})} \) and \( N_{V_{(\text{glom,kid})}} \) are volume density and numerical density, respectively, of glomeruli in kidney and \( V_{(\text{corp,kid})} \) is the volume density of renal corpuscles in kidney (31).

Total glomerular tuft (\( V_{\text{glom(tot)}} \)) and renal corpuscle (\( V_{\text{corp(tot)}} \)) volumes were also estimated as:

\[ V_{\text{glom(tot)}} = V_{\text{glom}} \times N_{\text{glom,kid}} \]

and

\[ V_{\text{corp(tot)}} = V_{\text{corp}} \times N_{\text{glom,kid}} \]

**Noradrenaline Assay**

In offspring at birth and 5 weeks of age noradrenaline content was determined in the left kidney by HPLC, as previously described (43).

**Statistical Analysis**

One-way ANOVA with tukey post-hoc comparisons were performed to test for differences between the groups on the control day in the mothers (see Table 1) and to compare differences in glomerular number and noradrenaline content between the groups of offspring. One-way repeated ANOVA was used to test for statistical differences between the maternal groups during gestation, with factors time (\( P_T \); GA0, GA18, GA28 and 5 weeks post-partum) and group (\( P_G \); sham vs. 2K-1W; sham vs. 2K-2W; or 2K-1W vs. 2K-2W) and their interaction (\( P_{GT} \)). P values were conservatively adjusted using the
Greenhouse-Geisser correction (36). $P < 0.05$ was considered to be statistically significant.

**Results**

**Mothers**

There were no significant differences in any variable measured between the groups of female rabbits (prospective mothers) at entry to the study (Table 1).

Mean arterial pressure (MAP) was significantly greater in both the 2K-1W ($P_G < 0.001$) and 2K-2W ($P_G < 0.01$) groups as compared to the sham-operated group throughout gestation and the post-partum period (fig 1). Surprisingly, in the two models of renal hypertension the increase in blood pressure was not different. MAP rose by $21 \pm 5$ mmHg ($P < 0.001$) and $24 \pm 3$ mmHg ($P < 0.001$) in both the 2K-1W and 2K-2W groups 4 weeks post-surgery, respectively. Comparison between the 2K-1W and 2K-2W groups revealed no statistical difference in MAP between the 2 models of hypertension at any time. There was a significant dip in MAP throughout gestation in all groups reaching a nadir at GA28 (fig 1). The decrease in MAP from GA0 to GA28 was of a similar degree being $18 \pm 6$ mmHg, $15 \pm 5$ mmHg and $15 \pm 2$ mmHg in sham, 2K-1W and 2K-2W groups, respectively. MAP rebounded to pre-pregnancy levels at 5 weeks post-partum in all groups following birth (fig 1). Heart rate was not significantly different between the groups throughout the study. Hematocrit was not significantly different between the groups though a significant fall in hematocrit ($\sim 4\%$) was observed during gestation in all groups.

Plasma renin activity was significantly greater in the 2K-1W hypertensive mothers throughout gestation and the post-partum period as compared to the sham-operated group (fig 1, $P_G = 0.03$). There was no difference in the PRA levels between the 2K-2W and
sham groups (fig 1). PRA was also significantly greater in the 2K-1W group as compared to the 2K-2W group throughout gestation and the post-partum period ($P_G = 0.03$). Plasma aldosterone rose significantly during gestation in the sham-operated group increasing by $250 \pm 110\%$ ($P < 0.05$) and $110 \pm 87\%$ ($P < 0.05$) at GA18 and GA28 as compared to GA0, respectively. The plasma aldosterone level was significantly increased at GA0 in the 2K-1W group ($P_G = 0.03$) as compared to the sham group during pregnancy, but not in the 2K-2W mothers (fig 1).

Plasma creatinine levels were significantly elevated by $\sim 15\%$ in both models of hypertension following surgery and throughout gestation, though the degree of increase was not different between the 2 models as compared to the sham group (fig 1). The degree of increase in plasma creatinine in the 2K-1W and 2K-2W groups was not further altered during pregnancy (fig 1).

Body weights were not different between the three groups at the commencement of the study (Table 1). All three groups of rabbits gained weight following surgery. Normotensive sham rabbits gained significantly more weight ($+610 \pm 128$ g) than the 2K-1W ($+278 \pm 41$ g, $P < 0.05$) and 2K-2W ($+320 \pm 64$ g, $P < 0.05$) hypertensive rabbits by GA0 following surgery. Body weight remained significantly lower in the 2K-1W hypertensive mothers than the sham-operated mothers throughout pregnancy, though the rate of increase was not different; $0.52 \pm 0.05$ kg vs. $0.59 \pm 0.04$ kg, respectively from GA0 to PP (fig 1; $P_{GT} = 0.9$). In the 2K-2W group, body weight was also significantly less ($P_g = 0.03$), than the sham-operated group throughout pregnancy. However, the rate of increase during pregnancy was also significantly less in the 2K-2W ($0.34 \pm 0.05$ kg) as compared to the sham group, from GA0 to PP (fig 1; $P_{GT} = 0.007$).
**Offspring**

*At birth and 5 weeks of age*

A total of 149 kittens were born to 26 dams (Table 2). Birth weight was significantly greater (~25%) in male and female offspring in both models of hypertensive mothers as compared to the offspring of normotensive sham-operated mothers (fig 2). By 5 weeks of age differences in body weight were no longer apparent in all offspring except the female offspring of 2K-2W mothers, which were significantly smaller than the age matched offspring of sham mothers (fig 2). Litter size and sex ratio for each group are given in Table 2. Litter size was significantly reduced in the 2K-1W (P < 0.05), as compared to the sham group offspring. Litter size was also smaller in the 2K-2W group offspring but did not reach statistical significance (Table 2). When litter size was included as a covariate in the analysis of birth weight, a significant effect of litter size was observed but birth weight was no longer different between the groups (fig 3). Organ to body weight ratios were not significantly different at birth in offspring of both models of hypertension as compared to the offspring of sham-operated mothers (data not shown). There was no difference in the ratio of male to female offspring between the groups (Table 2).

Conscious intra-arterial blood pressure was not significantly different between the sham, 2K-1W and 2K-2W offspring at 5 weeks of age (fig 4). PRA was significantly reduced in both the 2K-1W (~28%; P < 0.05) and 2K-2W (~27%; P < 0.05) offspring at 5 weeks of age as compared to the offspring of sham-operated mothers (fig 4).

**Glomerular number**

Glomerular number was determined in right kidneys from female offspring at 5 weeks of age; an age when nephrogenesis is complete in the rabbit. Estimated nephron number
averaged 160,803 ± 11,838, 172,470 ± 13,746 and 172,198 ± 19,529 in the sham, 2K-1W and 2K-2W female offspring, not significantly different. Glomerular tuft and corpuscle volumes were also not significantly different between the 2K-1W and sham groups (Table 3). However, mean glomerular tuft volume (P = 0.04), total glomerular tuft volume (P = 0.04) and total corpuscle volume (P = 0.04) were significantly smaller in the 2K-2W as compared to the sham offspring (Table 3). Mean corpuscle volume was smaller but this did not reach statistical significance (P = 0.1).

Renal noradrenaline content

Renal noradrenaline content was determined as an index of renal sympathetic innervation density. At birth total noradrenaline content was ~20% lower in female (P = 0.02) and ~15% lower in male (not significant; P = 0.2) 2K-1W offspring as compared to sham offspring. When corrected for kidney weight the decrease in noradrenaline was ~30% (per g KW) in the female (P = 0.01) and male (P = 0.04) 2K-1W offspring compared to sham offspring (fig 5). At 5 weeks of age both total noradrenaline and noradrenaline per gram kidney weight was ~40% higher (P = 0.05) in female but not male 2K-1W offspring compared to sham offspring. (fig 5). No significant differences in noradrenaline content were seen between the 2K-2W and sham offspring (fig 5). At birth renal noradrenaline content was greater in female than male offspring of sham-operated mothers (fig 5). By 5 weeks of age noradrenaline content was on longer significantly different between the sexes in the sham offspring (fig 5).

Discussion

MAP was elevated throughout pregnancy in both 2K-1W and 2K-2W models of hypertension. Activation of the renin-angiotensin-system (RAS) was also evident, with
PRA and plasma aldosterone significantly increased in the 2K-1W, but not the 2K-2W mothers. These two models of chronic maternal hypertension differentially affected the offspring. Birth weight was not reduced in offspring from either model of maternal hypertension, but renal noradrenaline content (an index of renal innervation) was increased in the 5 week old female 2K-1W offspring, while a reduction in glomerular volume (indicative of filtration surface area) was indicated in the 5 week old female 2K-2W offspring. Potentially, both these alterations in offspring development may increase the risk of hypertension in later life.

**Mothers**

Several maternal factors may have influenced fetal and post-natal development, some of which were differentially effected in the models of renal hypertension utilized in the current study. The similar degree of hypertension (~20 mmHg) in the two models was unexpected, as was the indication that renal function was reduced to a similar extent (~20% increase in plasma creatinine levels) since we have previously reported in males an increased severity of hypertension and reduction in renal function in the 2K-2W model as compared to the 2K-1W model (10, 11, 15). The reasons for the similar increase in blood pressure in these models in the females are unknown, though it is well recognised that hypertension is attenuated in females as compared to males in many experimental models of hypertension (49). This increase in blood pressure may influence fetal development and programme adult blood pressure. However, against this argument a previous study in which blood pressure was increased by central infusion of aldosterone during pregnancy, did not increase blood pressure in adult offspring (27). Compromised renal function, with altered extracellular fluid homeostasis in maternal plasma may in turn influence fetal development (21). In many cases, human hypertension is accompanied by renal insufficiency, which
may contribute to disturbances in extracellular fluid homeostasis and an altered hormonal milieu, as observed in our model.

Differential changes in the renin-angiotensin-aldosterone system were observed in the 2 models of hypertension. The 2K-1W mothers had elevated PRA and plasma aldosterone levels during gestation while the 2K-2W mothers did not. Neither renin or angiotensin II cross the placenta (5). However, the uteroplacental circulation has a local RAS that plays important roles in placental angiogenesis and in modulating placental production of cytokines, growth factors and vasoactive substances, which also influence fetal development (47). It is our premise that due to the elevated maternal RAS activity, the placental RAS may be down regulated, thereby altering production of placental factors important for both placental and fetal development. It is also possible that elevated Angiotensin II (Ang II) may result in reduced uterine blood flow. Previous studies where chronic Ang II was infused into pregnant rabbits (4) and ewes (41) have confirmed this. Evidence also suggests that uteroplacental perfusion is reduced in humans (53) and animal models (30, 39) with chronic hypertension. Normally, the uterine artery is particularly insensitive to Ang II due to the predominance of angiotensin type 2 receptors (AT₂R) during pregnancy (52); however following chronic Ang II infusion uterine artery AT₂R density decreases which may reduce uterine blood flow (6). Thus placental nutrient transfer may be affected due to reduced uterine blood flow via this mechanism during pregnancy when maternal Ang II levels are increased.

Ang II has also been shown to decrease 11beta-hydroxysteroid dehydrogenase (11βHSD₂) in cultured human placental cells acting via AT₂ receptors (34). If 11βHSD₂ is decreased in our model this would potentially increase fetal exposure to maternal glucocorticoids, which have been shown to influence the growth and development of the
fetus (18). Aldosterone can cross the placenta and may therefore influence fetal
development directly. Minuth and colleagues (44) have investigated differentiation of the
beta-type intercalated cells in the collecting ducts of newborn rabbit kidney by labeling the
cultured epithelia with peanut agglutinin (PNA). They demonstrated that incubation of new
born kidney collecting duct with aldosterone increased the number of PNA labelled cells to
72% as compared to 8% in the control. Although the concentration of aldosterone used in
the culture was much higher than normal physiological levels, one can speculate that long
term exposure to elevated aldosterone (as seen in our 2K-1W model) may result in altered
renal development.

Another potential mechanism by which fetal development may have been altered is
through malnutrition. The hypertensive mothers were relatively undernourished compared
to the normotensive sham mothers as less weight was gained following surgery to induce
hypertension. This was aggravated in the 2K-2W mothers, and the rate of increase in
body weight during pregnancy was less in the 2K-2W mothers and this was associated
with lower post-natal growth rates in the offspring of these pregnancies. Nutrient restriction
is the most commonly used model of an adverse intrauterine environment and has been
shown to programme adult hypertension in many studies (33) and may contribute to
programming of offspring in our studies, particularly in the 2K-2W model.

Thus chronic renal hypertension is a complex model in which many adverse stimuli may
potentially have an adverse impact on fetal development with long-term consequences for
the adult. The rabbit as a model in these studies has many advantages. We have shown
that the hemodynamic changes that occur in a rabbit during pregnancy very closely
resemble those that occur in human pregnancy. The rabbit is also comparable to the
human in that its placentation is similar. The rabbit, like humans has a discoid
hemochorial placenta, the primary difference between the two being that the exchange surface is of a villous nature in the human placenta, but is labyrinthine in the rabbit placenta (9, 23). Each fetus has its own individual placenta with a single trophoblast layer separating maternal and fetal blood in the placental labyrinth (9). The visceral yolk sac (a pre-placental organ) and the extraembryonic membranes more closely resemble those in the human than do those of rats and mice (23). Also, the sequence of organ development (Carnegie stages) is similar in the rabbit and human (23). PRA in the sham operated normotensive rabbits increased towards late gestation (P < 0.08). Again this is similar to the situation during normal pregnancy in humans, sheep and rats, when PRA rises progressively, reaching a peak at birth and decreasing thereafter (54). Aldosterone levels, in line with the elevated PRA also increased towards late gestation in the normotensive rabbits, in agreement with studies in humans (55).

**Offspring**

Hypertensive pregnancies in humans, even mild hypertension, are generally associated with reduced birth weight (22, 53), though there are reports of increased birth weight (59-62). It is arguable in this study as to whether the offspring from both models of maternal hypertension had an increased birth weight or the hypertensive mothers had smaller sized litters and therefore relatively larger offspring. Taking litter size into account in the statistical analysis as a covariate removed the difference in body weight between the groups. This supports the conclusion that smaller litter size was responsible for the relative increase in birth weight.

In our original study we demonstrated that at 10 weeks of age there was no difference in MAP (in both sexes) and suppression of PRA in offspring of 2K-1W mothers (13). We now report a similar situation at 5 weeks of age, with no difference in blood pressure and
reduced PRA in the offspring of 2K-1W mothers. In addition we demonstrated similar findings in the offspring of 2K-2W mothers. Whether the reduced PRA reflects altered development and/or function of the renin-angiotensin system or a compensatory response counteracting a rise in blood pressure in the offspring is unknown.

In the female offspring of 2K-1W mothers, renal noradrenaline content was reduced by ~20% at birth but was increased by ~40% at 5 weeks of age, providing evidence of altered renal sympathetic nerve growth and development. These alterations in noradrenaline content occurred predominantly in female offspring, which corresponds with our earlier demonstration that adult blood pressure was increased in female not male 2K-1W offspring at 30 weeks of age (13). No differences in renal noradrenaline content were observed in the offspring of the 2K-2W mothers at birth or 5 weeks of age. This suggests that activation of the renin-angiotensin-aldosterone system in the 2K-1W model of hypertension, not the increase in maternal arterial pressure, may have impinged upon the development of the renal sympathetic nerves. In the male offspring similar trends were seen in noradrenaline content at birth, but were no longer apparent by 5 weeks of age. A possible explanation for this sexually-dimorphic effect may be that the ontogeny of renal sympathetic innervation is different in males and females as suggested by the higher renal noradrenaline content at birth in females as compared to males from normotensive mothers.

Given the compelling evidence implicating the renal sympathetic nerves in the development of essential hypertension (20, 28, 46, 50), altered growth of the renal nerves may underlie the programming of high blood pressure in adult offspring in the 2K-1W model of maternal hypertension. In further support of this hypothesis, altered renal nerve development; with increased noradrenaline content being observed from as early as 1
week of age, has been demonstrated in spontaneously hypertensive rats (25). Also, renal
denervation abolished hypertension in low-birth weight offspring from pregnant rats with
reduced uterine perfusion demonstrating a causative role for the renal nerves in the
development of programmed hypertension (1). However, another study in offspring from
pregnancies with reduced uterine perfusion demonstrated no effect on adult blood
pressure or activity of the sympathetic nervous system (29).

If the kidney’s of female offspring of 2K-1W mothers are hyper-innervated one might
expect that renin release be enhanced, however this was not the case PRA was reduced
in these offspring. We offer two possible explanations for this finding. Firstly, neural
stimulation of PRA may be increased but other mechanisms may be counter-regulating
this effect (ie tubulo-glomerular feedback) in the pre-hypertensive phase in this model.
Secondly, evidence suggests that at least 2 populations of functionally distinct nerves
innervate the kidney (Type I and II) (14, 16, 17, 37, 38). We speculate that if it is Type I
nerves that are increased in the female offspring (predominantly innervating the smooth
muscle of the afferent arteriole) this might also explain why PRA is not increased.
However, there is some evidence to suggest that the renin-angiotensin system is playing a
role in the development of the hypertension at least in the maintenance phase since at 30
weeks of age PRA is normal when you might expect PRA to be suppressed due to the
increase in arterial pressure (13).

How the renin-dependent 2K-1W hypertensive maternal environment might influence renal
nerve growth is not known as few studies have examined the development of the renal
sympathetic nerves in disease models or for that matter under normal conditions (2, 48).
However, much work has focused on the growth and survival of postganglionic
sympathetic neurons in the broader setting of the periphery (7, 26, 35). Briefly, neuronal
network development is guided by a finely orchestrated sequence of attractive and repulsive molecular cues that determine axon extension, neuron survival or removal and generation of dendrites, branches (arborisation) or synapses. These molecular cues, (e.g. vascular endothelial growth factor, nerve growth factor, bone morphogenic proteins, Wnts, Ephrins and Slits to name a few (7, 26, 35) maybe up or down regulated or miss-timed causing an alteration in final development of the sympathetic nervous system.

The rabbit, like the rat and mouse is altricial (development continues during the postnatal period), thus completion of nephrogenesis occurs around 2-3 weeks after birth (42). A reduction in nephron number has consistently been associated with the programming of adult hypertension in other models (8, 33, 56-58). Glomerular number was estimated, using unbiased stereology, only in female rabbits in this study, as we previously found that only female rabbits had a significant increase in arterial blood pressure by 30 weeks of age (13). We demonstrated that total glomerular number was not reduced in female offspring of either model of chronic hypertension indicating that a reduced nephron endowment is not involved in the programming of hypertension in offspring of hypertensive mothers. However, Bowman’s capsule and glomerular capillary tuft volumes were decreased in female offspring of 2K-2W mothers. Therefore female offspring of 2K-2W mothers had the same number of glomeruli but these glomeruli were on average smaller in size, indicative of a reduction in filtration surface area. A reduction in filtration surface area at birth has been postulated to predispose an individual to hypertension in later life (32, 40). Thus there is evidence of altered renal development in the female offspring of 2K-2W mothers, however considering these animals also demonstrated a reduced bodyweight at 5 weeks of age, the significance of the reduced filtration surface area for adult cardiovascular disease remains to be seen.
In conclusion, birth weights of offspring from two models of hypertension were not reduced. The fact that maternal blood pressure was increased by a similar level in these models but that PRA was only elevated in the 2K-1W mothers may contribute to the differential effects on offspring development observed. We can only speculate whether it was the hypertension, activation of the maternal renin-angiotensin system or their combination that drives programming of high blood pressure in the offspring of 2K-1W mothers. We suggest that permanent structural and/or functional changes in the renal sympathetic control of kidney function in offspring of 2K-1W hypertensive mothers may contribute to the programming of hypertension in adulthood. The reduction in postnatal growth and glomerular volume (indicative of a reduced filtration surface area) in the 2K-2W model of chronic maternal hypertension, warrants future investigation, as it has not been determined if hypertension is programmed in these offspring as adults. Hypertension during pregnancy affects 1 in 10 women with the cause in the majority of cases unknown (51). Our studies show that individuals may be differentially affected during fetal development depending on the aetiology of the maternal hypertension.
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Table and Figure Legends

**Table 1**: Baseline variables (mean ± SEM) measured in nulliparous female rabbits prior to surgery for the induction of hypertension at entry to the study. MAP, mean arterial pressure; HR, heart rate; PRA, plasma renin activity. No statistical differences were observed between the groups (one-way ANOVA with Tukey post-hoc comparisons).

**Table 2**: Total number of births and stillbirths, average litter size, range of litter size and sex ratio of litters from sham-operated normotensive, 2K-1W and 2K-2W hypertensive mothers. Values are mean ± SEM. * P < 0.05 as compared to sham operated normotensive mothers.

**Table 3**: Body weight and variables measured in the right kidney of 5 week old female offspring of sham, 2K-1W and 2K-2W hypertensive mothers, 2K-1W and 2K-2W hypertensive mothers. * P < 0.05 as compared to sham group.

**Figure 1**: Mean arterial pressure, plasma renin activity, plasma aldosterone, plasma creatinine concentration and body weight (mean ± SEM) in sham (solid circles, n = 9), 2 kidney - 1 wrap (2K-1W, open triangles, n = 9) and 2 kidney - 2 Wrap (2K-2W, open squares, n = 8) operated rabbits immediately prior to mating (4 weeks post-surgery) on gestational day 0 (GA0) and on days 18 (GA18) and 28 (GA28) of gestation and at 5 weeks post-partum (PP). Rabbits have a 32 day gestation and offspring are weaned at 5 weeks postpartum. One-way repeated measures ANOVA; factors group (G), time (T) and their interaction (GT). Aldosterone was not measured at PP.

**Figure 2**: Body weight (mean ± SEM) at birth and 5 weeks of age in male and female offspring from sham (black bars, n = 9 litters), 2K-1W (open bars, n = 9 litters) and 2K-2W (grey bars, n = 8 litters) mothers. * P < 0.05 as compared to sham offspring of the same
sex and age.

Figure 3: Average litter birth weight plotted against litter size for offspring of sham-operated (n = 9, black circles), 2K-1W (n = 9, open circles) and 2K-2W (n = 8, grey circles) mothers. ANOVA (group; G) with litter size (LS) as a covariate.

Figure 4: Conscious mean arterial pressure and plasma renin activity (mean ± SEM) at 5 weeks of age in male (n = 6) and female (n = 6) offspring from sham (black bars), 2K-1W (open bars) and 2K-2W (grey bars) mothers. * P < 0.05 as compared to sham offspring of the same sex.

Figure 5: Renal noradrenaline content expressed per total kidney and corrected per gram kidney wet weight (mean ± SEM) measured at birth and 5 weeks of age in male and female offspring of sham-operated (sham, black bars, n = 9 litters), 2K-1W (open bars, n = 9 litters) and 2K-2W (grey bars, n = 8 litters) mothers. * P < 0.05 as compared to the sham group of the same sex. # P < 0.05 comparing male versus female sham groups. (For each bar; n = 1 male and 1 female randomly selected from each litter).
Table 1: Baseline variable (mean ± SEM) measured in nulliparous female rabbits prior to surgery for the induction of hypertension at entry to the study.

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 9)</th>
<th>2K-1W (n = 9)</th>
<th>2K-2W (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight kg</td>
<td>2.8 ± 0.2</td>
<td>2.7 ± 0.1</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>Age days</td>
<td>108 ± 3</td>
<td>112 ± 4</td>
<td>112 ± 2</td>
</tr>
<tr>
<td>MAP mmHg</td>
<td>77 ± 4</td>
<td>78 ± 5</td>
<td>75 ± 3</td>
</tr>
<tr>
<td>HR beats/min</td>
<td>220 ± 3</td>
<td>218 ± 7</td>
<td>215 ±10</td>
</tr>
<tr>
<td>Hematocrit %</td>
<td>35 ± 2</td>
<td>37 ± 2</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>PRA ng Angl/ml plasma/h</td>
<td>3.0 ± 0.4</td>
<td>2.8 ± 0.3</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>Plasma Aldosterone pg/ml</td>
<td>169 ± 30</td>
<td>196 ± 20</td>
<td>205 ± 34</td>
</tr>
<tr>
<td>Plasma creatinine µmol/l</td>
<td>71 ± 7</td>
<td>77 ± 3</td>
<td>74 ± 6</td>
</tr>
</tbody>
</table>

MAP, mean arterial pressure; HR, heart rate; PRA, plasma renin activity. No statistical differences were observed between the groups (one-way ANOVA with Tukey post-hoc comparisons).
**Table 2:** Total number of births and stillbirths, average litter size, range of litter size and sex ratio of litters from sham-operated normotensive, 2K-1W and 2K-2W hypertensive mothers. Values are mean ± SEM. * P < 0.05 as compared to sham operated normotensive mothers.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>2K-1W</th>
<th>2K-2W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mothers</td>
<td>9</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Total offspring</td>
<td>61</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>Total still births</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Average litter size</td>
<td>6.8 ± 0.7</td>
<td>4.8 ± 0.6 *</td>
<td>5.5 ± 1.9</td>
</tr>
<tr>
<td>Litter size range</td>
<td>3 - 11</td>
<td>1 - 8</td>
<td>4 - 9</td>
</tr>
<tr>
<td>Sex ratio (male : female)</td>
<td>4 ± 1 : 3 ± 1</td>
<td>3 ± 1 : 3 ± 1</td>
<td>3 ± 1 : 3 ± 1</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>2K-1W</th>
<th>2K-2W</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 6</td>
<td>n = 8</td>
<td>n = 7</td>
<td></td>
</tr>
<tr>
<td><strong>Body weight g</strong></td>
<td>920± 60</td>
<td>890 ± 40</td>
<td>760 ± 40 *</td>
</tr>
<tr>
<td><strong>Kidney weight g</strong></td>
<td>5.4 ± 0.8</td>
<td>5.0 ± 0.2</td>
<td>4.5 ± 0.4 *</td>
</tr>
<tr>
<td><strong>Kidney/body weight ratio x 10^{-3}</strong></td>
<td>7.5 ± 1.0</td>
<td>5.6 ± 0.3</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td><strong>Estimated glomerular number</strong></td>
<td>160803±11838</td>
<td>172470±13746</td>
<td>172198±19529</td>
</tr>
<tr>
<td><strong>Mean tuft volume mm^3 x 10^{-4}</strong></td>
<td>4.7± 0.6</td>
<td>3.8 ± 0.4</td>
<td>3.2 ± 0.3 *</td>
</tr>
<tr>
<td><strong>Total tuft volume mm^3</strong></td>
<td>67 ± 10</td>
<td>65 ±7</td>
<td>54 ± 5 *</td>
</tr>
<tr>
<td><strong>Mean corpuscle volume mm^3 x 10^{-4}</strong></td>
<td>5.8 ± 0.9</td>
<td>4.8 ± 0.4</td>
<td>4.3 ± 0.4</td>
</tr>
<tr>
<td><strong>Total corpuscle volume mm^3</strong></td>
<td>91 ± 10</td>
<td>83 ± 9</td>
<td>69 ± 0.4 *</td>
</tr>
</tbody>
</table>
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