Growth restriction before or after birth reduces nephron number and increases blood pressure in male rats

Mary E. Wlodek[1], Kerryn Westcott[1], Andrew L. Siebel[1], Julie A. Owens[2] and Karen M. Moritz[3]

[1]Department of Physiology, University of Melbourne, Parkville, Victoria, Australia; [2]School of Paediatrics and Reproductive Health, Disciplines of Obstetrics and Gynaecology, University of Adelaide, Adelaide, South Australia, Australia and [3]School of Biomedical Sciences, University of Queensland, St Lucia, Queensland, Australia

Impaired growth *in utero* predicts a low nephron number and high blood pressure later in life as does slowed or accelerated growth after a normal birth weight. We measured the effects of early postnatal growth restriction, with or without prenatal growth restriction, on blood pressure and nephron number in male rat offspring. Bilateral uterine artery and vein ligation were performed to induce uteroplacental insufficiency (Restricted) on day 18 of pregnancy. Postnatal growth restriction was induced in a subset of sham operated control animals by reducing the number of pups at birth to that of the Restricted group (Reduced Litter). Compared to Controls, Restricted pups were born smaller while Reduced Litter pups weighed less by postnatal day 3 and both groups remained lighter throughout lactation. By 10 weeks of age all animals were of similar weight but the Reduced Litter rats had elevated blood pressure. At 22 weeks, Restricted but not Reduced Litter offspring were smaller and the blood pressure was increased in both groups. Restricted and Reduced Litter groups had fewer glomeruli and greater left ventricular mass than Controls. These results suggest that restriction of both perinatal and early postnatal growth increase blood pressure in male offspring. This study also demonstrates that the early postnatal period is a critical time for nephron endowment in the rat.

Kidney International (2008) **74,** 187–195; doi:10.1038/ki.2008.153; published online 23 April 2008

KEYWORDS: nephron endowment; hypertension; uteroplacental insufficiency; postnatal growth restriction; perinatal growth restriction

Correspondence: *Mary E. Wlodek, Department of Physiology, University of Melbourne, Parkville, Victoria 3010, Australia. E-mail: m.wlodek@unimelb.edu.au*

Received 12 December 2007; revised 14 January 2008; accepted 6 February 2008; published online 23 April 2008

Poor intrauterine growth results in small for gestational age babies and low birth weight predicts an increased risk of developing many adult onset diseases, including hypertension.[1] It is thought that the fetus, upon exposure to a suboptimal intrauterine environment, makes adaptations, which increase short-term survival. These adaptations impact on normal growth and functional development increasing predisposition to later disease.[2] Growth in the early postnatal period has also been implicated to independently affect later health and result in disease development.[3,4] Accelerated growth in early infancy has been shown to be associated with the later development of coronary artery disease[5] and type II diabetes,[6] although other studies suggest that slowed postnatal growth, especially during the first year, can lead to increased risk of coronary heart disease[7] and insulin resistance.[8] Together, these studies suggest that factors that affect prenatal and/or postnatal growth, including nutrition, are important in determining later cardiovascular and metabolic health.

In animal studies, maternal dietary manipulations (low protein or calorie restriction during pregnancy) restrict fetal growth and produce offspring that have a reduced nephron number[9] and develop hypertension as adults.[10,11] Of particular concern is that a congenital nephron deficit has been implicated as a mechanism through which a prenatal perturbation may result in later hypertension.[12] However, previous studies have not assessed the effects of an impaired postnatal lactational environment, a period when nephrogenesis continues in the rodent. Of interest is that a pregnancy-induced lactation deficit may persist even when the mother is returned to a normal diet at birth. Although it is evident that offspring undergo variable postnatal growth following maternal nutritional modulation, the role of this in the development of later hypertension has not been studied extensively.

In Western society, much of the fetal growth restriction that occurs reflects placental insufficiency and impaired uteroplacental blood flow. Uteroplacental insufficiency and growth restriction, either by bilateral uterine artery and vein ligation[13,14] or aortic clip,[15–18] have been experimentally

induced in several species, including the rat. We have utilized the bilateral uterine vessel ligation model to examine fetal growth and development.[14,18,19] Although this model has previously been thought to affect intrauterine growth only, we have demonstrated that uteroplacental insufficiency impairs mammary development and the lactational environment and thus reduces postnatal nutrition and growth of the offspring.[19] Experimentally induced uteroplacental insufficiency also reduces the number of pups at birth.[14,18] To control for this reduction in litter size on postnatal growth, we have incorporated a second control group in our studies in which the number of pups from a control litter is reduced on day 1 to match the number of offspring in the growth-restricted (Restricted) group. We previously demonstrated that this results in postnatal growth restriction in pups born of normal weight.[19] In some studies, a 60–70% reduction in litter size soon after birth increases milk delivery to individual offspring, resulting in increased early postnatal growth and the development of adult obesity.[20–22] In comparison, we have previously used a more modest reduction in the number of pups (from approximately 10 to 5) to match that resulting from uterine artery ligation and have found that this leads to impaired nutrition postnatally.[20–22] This may be due to the reduced number of pups decreasing the suckling stimulus to the dam, which in turn decreases milk production and pup growth.[19,23] Thus, we have used this litter size reduction to induce postnatal growth restriction alone.

To date, there has been little investigation into the effect of early postnatal growth restriction as an independent factor contributing to adult disease development, including hypertension. One study in which additional pups were cross-fostered onto a dam at birth to increase litter size and thereby to decrease nutritional supply received by individual pups showed that offspring slow their growth after birth. Only males were examined in that study, and it was found that offspring had a reduced number of enlarged glomeruli in their kidneys.[24] However, the limitation of that study was that blood pressure was not measured. Therefore, the first aim of this study was to compare the effects of perinatal growth restriction (where offspring are exposed to restraint before and after birth) with postnatal growth restriction (due to reduced litter size) alone on blood pressure in male offspring. Furthermore, as nephrogenesis continues after birth in the rat and thus may be influenced by postnatal nutrition and growth, we also aimed to determine the effects of perinatal and postnatal growth on nephron endowment in offspring. Similar to other studies,[14,25] we chose to study male offspring to address these issues, as it is reported that males tend to be more susceptible to the development of hypertension[25] and that the mechanisms controlling blood pressure may be sex specific.[26]

To further elucidate the mechanisms behind the effect of growth restriction on blood pressure, we examined components of the renin–angiotensin system, which play a critical role in renal development and the maintenance of blood pressure and fluid homeostasis. We and others have shown that alterations in the renal and cardiac renin–angiotensin system occur following growth restriction and that these changes correlate with increased blood pressure and altered renal development in offspring.[14,27–29] Specifically, the expression of the angiotensin II type 1 (AT_1) and 2 (AT_2) receptors was examined in renal and cardiac tissue at 6 months of age. Gene expression studies were also performed in the adult to examine markers of tissue remodeling (including collagen, metalloproteinases (MMPs), and the tissue inhibitors of metalloproteinases (TIMPs)), as we expected fibrosis-related disease to be developing in these offspring. We hypothesized that offspring from both restricted and reduced litter groups would have a nephron deficit with altered renal and cardiac gene expression associated with increased blood pressure.

RESULTS

Body weights and growth profiles

Litter size on day 1 along with the body weights of male offspring at days 3, 6, and 35, at week 10, and at postmortem (6 months) are shown in Table 1. On days 3 and 6 after birth, reduced litter male pups weighed less than controls, whereas restricted pups were lighter than both control and reduced litter pups ($P<0.05$). Restricted male rats underwent a degree of accelerated growth between days 6 and 35, reaching weights comparable to those of the reduced litter group at day 35. However, both reduced litter and restricted groups were still lighter than control pups ($P<0.05$). The reduced litter male offspring underwent a period of accelerated growth after weaning so that by 10 weeks of age, their weights were comparable to control animals. Reduced litter offspring were of similar weight to controls at 6 months, whereas restricted offspring remained lighter ($P<0.05$).

Blood pressure

Perinatal or postnatal growth restriction did not alter systolic blood pressure at 5 weeks of age, although animals in the reduced litter group tended to have higher blood pressure (by ~5–8 mm Hg) compared to the control or restricted group (Figure 1a). At 9 weeks of age, the reduced litter offspring had increased blood pressure (by 14 mm Hg) compared to both control and restricted male offspring ($P<0.05$; Figure 1b). At 22 weeks, the restricted and reduced litter groups both had increased blood pressure (by 9 and 7 mm Hg, respectively) compared to controls ($P<0.05$; Figure 1c).

Kidney parameters

Absolute kidney weight in male offspring of the restricted and reduced litter groups was reduced ($P<0.05$; Figure 2a), but not when corrected for body weight (Figure 2b). Kidney volume (measured in five animals per group) was not significantly different between the groups but tended to be lower in the restricted and reduced litter animals (control, $0.88 \pm 0.09\ cm^3$; restricted, $0.74 \pm 0.07\ cm^3$; and reduced litter, $0.79 \pm 0.07\ cm^3$). A reduced glomerular number (by 36

Table 1 | Litter size on day 1 and body weight at days 3, 6, and 35, at 10 weeks, and at 6 months

		Body weight (g)				
	Litter size	Day 3	Day 6	Day 35	Week 10	6 months
Control	10.3 ± 1.3^{b}	6.01 ± 0.2^{c}	8.99 ± 0.2^{c}	97.0 ± 2.1^{b}	222.9 ± 6.4	366.6 ± 7.11^{b}
Restricted	4.2 ± 0.4^{a}	4.42 ± 0.2^{a}	6.39 ± 0.3^{a}	74.9 ± 3.3^{a}	206.5 ± 6.7	329.0 ± 8.5^{a}
Reduced litter	4.8 ± 0.6^{a}	5.29 ± 0.2^{b}	7.99 ± 0.3^{b}	73.4 ± 6.3^{a}	225.0 ± 6.4	352.4 ± 5.0^{b}

Restricted offspring were born small and reduced litter offspring were small by day 3 compared to control ($P<0.05$). Restricted offspring showed variable accelerated growth ($P<0.05$). Data are expressed as mean ± s.e.m. (n=9–10). Significant differences ($P<0.05$) across the groups are indicated by different letters; for example, 'a' is different from 'b.'

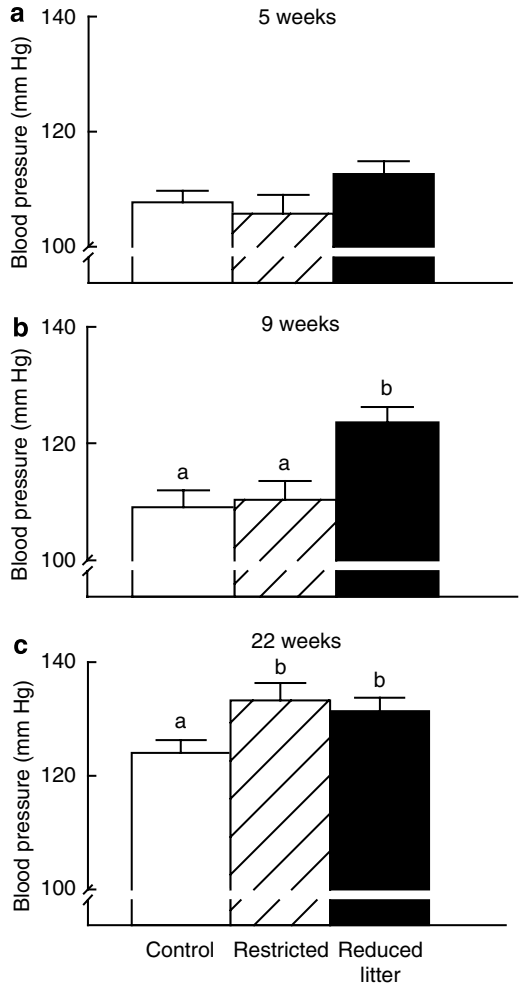

Figure 1 | Blood pressure in male offspring. Blood pressure in male offspring at (**a**) 5 weeks, (**b**) 9 weeks, and (**c**) 22 weeks of age. Open bars represent control offspring, hatched bars offspring of the restricted group, and solid bars offspring of the reduced litter group. Reduced litter male offspring at 9 weeks of age and growth-restricted males at 22 weeks had higher blood pressure than control ($P<0.05$). Data are expressed as mean ± s.e.m. ($n=8$–10). Significant differences ($P<0.05$) across the groups are indicated by different letters; for example, 'a' is different from 'b.'

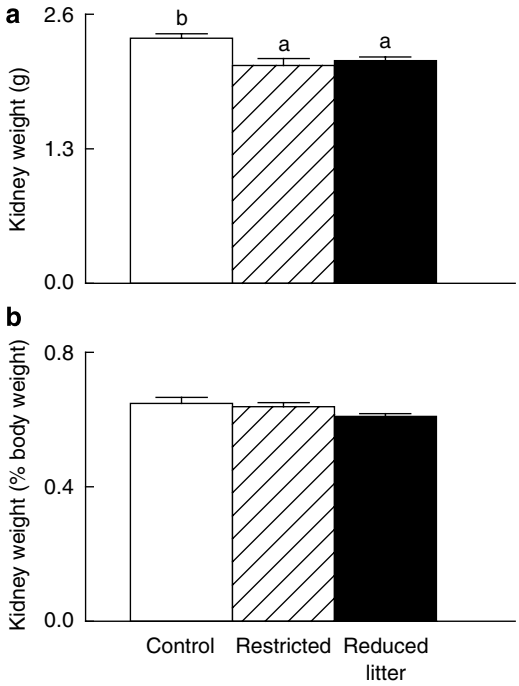

Figure 2 | Kidney weight in male offspring. (**a**) Total kidney weight (both kidneys) and (**b**) kidney weight as a percentage of body weight in male offspring at 6 months of age. Open bars represent control offspring, hatched bars offspring of the restricted group, and solid bars offspring of the reduced litter group. Absolute, but not relative, kidney weight was lower in growth-restricted offspring ($P<0.05$). Data are expressed as mean ± s.e.m. ($n=9$–10). Significant differences ($P<0.05$) across the groups are indicated by different letters; for example, 'a' is different from 'b.'

and 27%, respectively) was found in the reduced litter and restricted groups compared to controls ($P<0.05$; Figure 3a). In restricted offspring, mean individual glomerular volume was increased by 27% compared to controls ($P<0.05$; Figure 3b), whereas there also tended to be an increase in the reduced litter group (by 20%, Figure 3b). Total glomerular volume was similar across all groups (Figure 3c).

Heart and left ventricle weights

Total heart weight (as a percentage of body weight) was similar in all groups at 6 months (Figure 4a). In the restricted and reduced litter groups, left ventricle weight as a percentage of total heart weight was increased compared to controls ($P<0.05$; Figure 4b).

Renal and cardiac gene expression

The effects of perinatal and postnatal restriction on relative gene expression levels in the kidneys are shown in Table 2. There were no differences between the groups in those genes examined. Gene expression in the left ventricle was examined

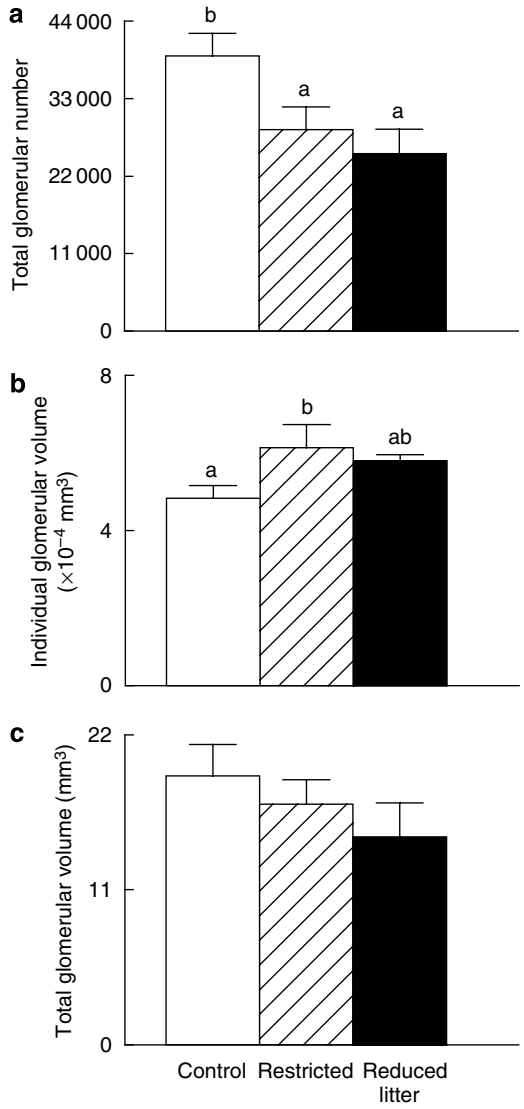

Figure 3 | Glomerular number and volume in male offspring. (**a**) Estimated total glomerular number (nephron number), (**b**) individual glomerular volume, and (**c**) total glomerular volume in male offspring at 6 months of age. Open bars represent control offspring, hatched bars offspring of the restricted group, and solid bars offspring of the reduced litter group. Growth-restricted male offspring had a nephron deficit and restricted pups had glomerular hypertrophy. Data are expressed as mean ± s.e.m. ($n = 9$–10). Significant differences ($P < 0.05$) across the groups are indicated by different letters; for example, 'a' is different from 'b' but not different from 'ab.'

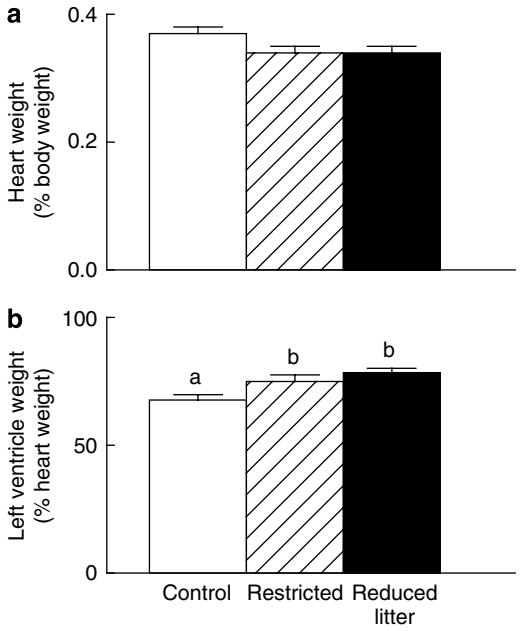

Figure 4 | Heart weight in male offspring. (**a**) Heart weight as a percentage of body weight and (**b**) left ventricle weight as a percentage of total heart weight in male offspring at 6 months of age. Open bars represent control offspring, hatched bars offspring of the restricted group, and solid bars offspring of the reduced litter group. Growth-restricted male offspring had left ventricular hypertrophy relative to body weight ($P < 0.05$). Data are expressed as mean ± s.e.m. ($n = 9$–10). Significant differences ($P < 0.05$) across the groups are indicated by different letters; for example, 'a' is different from 'b.'

Table 2 | Relative renal AT_{1A}, AT_{1B}, TIMP1, TIMP2, MMP2, MMP9, Coll1, Coll3, and TGF-β1 receptor expression for male offspring at 6 months

	Control	Restricted	Reduced litter
AT_{1A}	1.027 ± 0.092	1.197 ± 0.133	1.447 ± 0.126
AT_{1B}	1.056 ± 0.132	0.904 ± 0.134	1.148 ± 0.207
TIMP1	1.104 ± 0.184	1.230 ± 0.199	1.301 ± 0.235
TIMP2	1.110 ± 0.197	0.853 ± 0.110	1.005 ± 0.119
MMP2	1.279 ± 0.299	1.563 ± 0.289	1.871 ± 0.217
MMP9	1.135 ± 0.193	2.285 ± 0.799	2.752 ± 0.966
Coll1	1.322 ± 0.333	1.442 ± 0.270	1.725 ± 0.162
Coll3	1.186 ± 0.252	1.042 ± 0.123	1.495 ± 0.153
TGF-β1	1.190 ± 0.230	0.948 ± 0.155	0.740 ± 0.157

AT, angiotensin II type; Coll, collagen; MMP, metalloproteinase; TGF-β1, transforming growth factor-β1; TIMP, tissue inhibitor of metalloproteinase.
Values are expressed relative to a calibrator (the control group). There were no differences in renal gene expression across the groups. Data are expressed as mean ± s.e.m. (n=9–10).

owing to signs of increased left ventricular mass and hypertension at 22 weeks of age in the growth-restricted groups. A significant increase in expression of the AT_{1A} receptor was found in the left ventricle of the restricted group when compared to the control and reduced litter groups ($P < 0.05$; Figure 5a). Expression of the AT_{1B} receptor also tended to be increased in the restricted group, although no significant differences were detected. Expression of AT_2 mRNA in the kidney and heart was not detectable after 40 cycles of PCR in any of the samples examined. There was increased expression of collagen 3 in the left ventricle of the restricted group ($P < 0.05$; Figure 6f), and increased expression of TIMP1 (Figure 6a) in the left ventricle of the restricted group compared to the reduced litter group ($P < 0.05$) with the control group intermediate. Relative atrial natriuretic peptide (ANP) and transforming growth factor-β1 (TGF-β1) gene expression levels in the heart were not different between the groups. Relative ANP mRNA levels in the three groups were as follows: control, 1.0 ± 0.1; restricted, 1.1 ± 0.3; and reduced litter, 0.7 ± 0.1; and for

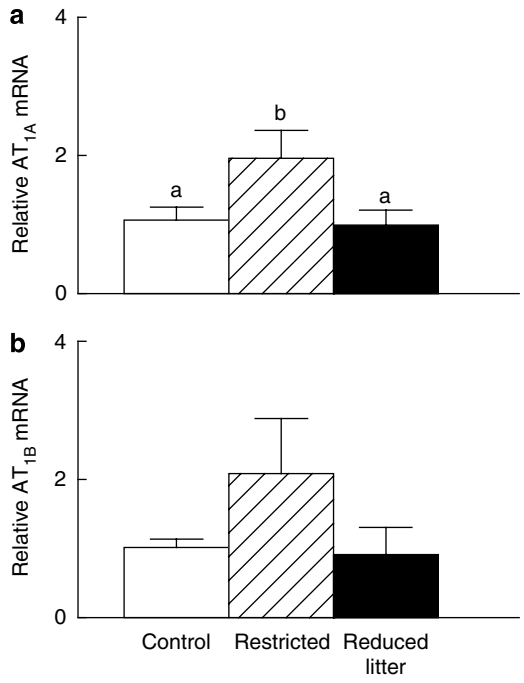

Figure 5 | AT receptor gene expression in the left ventricle. Relative gene expression of (**a**) the AT_{1A} receptor and (**b**) the AT_{1B} receptor in the left ventricle of male offspring at 6 months of age. Open bars represent control offspring, hatched bars offspring of the restricted group, and solid bars offspring of the reduced litter group. Restricted male offspring had increased expression of the AT_{1A} receptor compared to the other groups ($P<0.05$). Data are expressed as mean ± s.e.m. ($n=9$–10). Significant differences ($P<0.05$) across the groups are indicated by different letters; for example, 'a' is different from 'b.'

TGF-β1 the relative mRNA levels were as follows: control, 1.0 ± 0.1; restricted, 1.2 ± 0.3; and reduced litter, 0.8 ± 0.1.

DISCUSSION

This study demonstrates that reduction of litter size at birth, with the associated compromised nutrition and growth in the early postnatal period, not only induces a nephron deficit in adult male offspring but also results in elevated blood pressure. This occurs even though offspring are born with a normal birth weight. The nephron deficit induced by the reduction in litter size and postnatal growth restriction was similar in magnitude to that observed in animals that experienced growth restriction before and after birth due to uteroplacental insufficiency, suggesting that renal development in the rat can be profoundly affected by postnatal events. Growth restriction in the postnatal period also increased left ventricular mass, indicating that hypertension may be contributing to other aspects of disease development.

In this study, we induced postnatal growth restriction by reducing litter size at birth from approximately 10 down to 5 pups. A reduction of litter size at birth has been used in many studies to act as a 'control' group for various models (for example, maternal nutritional interventions and placental insufficiency); however, the effects on growth of the remaining offspring tend to be variable. Others have reported that a severe reduction in pup number (by approximately 70%) has been shown to result in accelerated postnatal growth, presumably due to increased nutrition to individual pups.[20–22,30,31] However, we have recently reported in our model that litter reduction removes the stimulus for increased milk production, which normally occurs after birth, resulting in lower maternal milk production and altered composition, thus slowing postnatal growth.[19] However, the postnatal growth restriction may only be partially responsible for the outcomes observed in this study, as we cannot discount that removal of pups may stimulate a stress response in the dam subjected to a reduction in the number of suckling pups, which may potentially inhibit lactation[32–34] and alter the development of offspring.

Rodents are usually born with only 10–20% of their mature glomeruli,[35] and thus postnatal influences can potentially affect a large part of nephrogenesis. A previous study inducing postnatal growth restriction by cross-fostering extra pups onto a control dam at birth[24] found a similar decrease in nephron endowment in male offspring as that reported here, even though the degree of growth restriction was greater in that study (animals were ~30% smaller than controls by day 7 compared to 10–15% by day 7 in our study). The degree of nephron deficit was also similar to that seen after prenatal growth restriction owing to maternal protein restriction[11,36] or uteroplacental insufficiency.[14] In our previous study, male rats that were born small as a result of uteroplacental insufficiency, and cross-fostered at birth onto a different dam with impaired lactation also had a significant nephron deficit that could be restored by cross-fostering the pups onto a dam with normal lactation.[14] Also of interest was our previous finding that when a litter of pups born with normal birth weight was reduced to five pups and then cross-fostered onto a control dam, nephron endowment was unaffected.[14] In our cross-foster model, pups from a reduced litter also underwent postnatal growth restriction, but in that case the slowed growth only emerged later in lactation, after nephrogenesis was complete.[14] The reasons for these differences are not clear, but the cross-foster procedure itself may impact on nephron endowment. In this study, control animals that remained with their own mother had approximately 35 000 nephrons, whereas in the cross-foster study, control pups placed onto a control dam had approximately 27 000 nephrons. These differences in nephron number cannot be accounted for by differences in animal strains or methodologies used to determine nephron endowment, as they were similar in both studies. We suggest that a reduction of litter size may produce a stress response in the dam, which is not present in the control or restricted dams in this study. In our cross-foster study,[14] all dams are subject to a similar stress with removal of their own pups before accepting a new litter.

These studies highlight that adequate nutrition during the later stages of nephrogenesis in the early postnatal period is critical for determining final nephron endowment in the rat.[14,24] In the human, these final stages of nephrogenesis

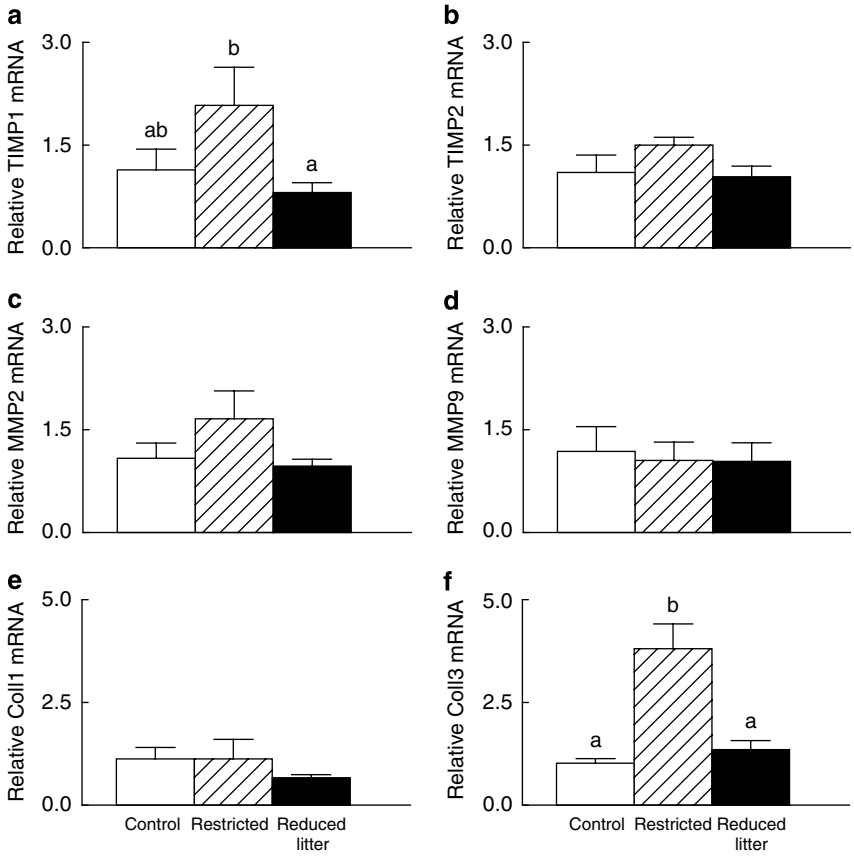

Figure 6 | Gene expression in the left ventricle. Relative gene expression of (**a**) TIMP1, (**b**) TIMP2, (**c**) MMP2, (**d**) MMP9, (**e**), collagen 1 (Coll1), and (**f**) collagen 3 (Coll3) in the left ventricle of male offspring at 6 months of age. Open bars represent control offspring, hatched bars offspring of the restricted group, and solid bars offspring of the reduced litter group. Both growth-restricted groups had increased expression of TIMP1 and Coll3 ($P<0.05$). Data are expressed as mean ± s.e.m. ($n=9$–10). Significant differences ($P<0.05$) across the groups are indicated by different letters; for example, 'a' is different from 'b.'

occur during *in utero* life and thus highlight the importance of adequate uteroplacental perfusion and nutrient delivery to the fetus during this period. Low birth weight has been associated with a reduced nephron endowment in humans.[37] However, our studies may be of greater relevance to babies born prematurely, especially those born before gestational week 36, when nephrogenesis would be continuing. No studies to date have examined the impact of nutrition on nephron endowment in premature babies.

The novel findings of this study suggest that growth restriction induced immediately after birth in the rat is associated with increased blood pressure in male offspring. This is consistent with studies in the rat showing that prenatal growth restriction results in a nephron deficit and hypertension in the adult following maternal protein restriction.[10,11] However, it should also be noted that intrauterine growth restriction even with a nephron deficit does not always result in hypertension,[38] and nor does intrauterine growth restriction resulting in hypertension always involve a nephron deficit.[39] However, to our knowledge, this is the first study demonstrating hypertension following postnatal growth restriction alone. It is of interest that the increase in blood pressure following postnatal growth restriction occurred at an earlier age than that in animals where growth restriction was induced prenatally, suggesting differences in mechanisms leading to disease development. Further studies using indwelling catheters or telemetry to measure blood pressure are needed to confirm and extend our findings, as there is evidence that some maternal perturbations result in elevated blood pressure in offspring when measured by tail cuff but not when measured chronically.[40] However, the use of tail-cuff methodology in this study has allowed sequential measurements in the same animal to identify critical time points of disease development.

Previous programming studies have reported a low nephron number and hypertension, in association with an upregulation in AT_1 receptor mRNA expression in postnatal and adult kidney.[26,27,29,41] We have recently shown increased renal gene expression of AT_1 receptor at 6 months in male offspring following uteroplacental insufficiency and cross-fostering at birth onto a mother with impaired lactation.[14] Cross-fostering normally grown pups, either with an intact litter size or reduced litter size, onto a mother with impaired lactation also increased renal AT_1 receptor mRNA.[14] This suggested that decreased nutrition during lactation may be important for increased renal AT_1 receptor expression in

adult rat offspring. In this study, however, no such increase was detected. It may be that the cross-fostering, along with the poor lactational environment, has contributed to the increased AT_1 receptor gene expression in our previous study.[14] In addition, no effect of growth restriction on the renal gene expression of collagen 1 or 3, MMP2, MMP9, TIMP1, TIMP2, or TGF-β was found. Mesangial cell expansion, tubular fibrosis, and glomerulosclerosis often result in altered expression of one or more of these factors.[42,43] This suggests that there is no overt renal fibrosis or glomerulosclerosis in our model. In addition, although we did not specifically examine tissues for signs of fibrosis or glomerulosclerosis, there was no evidence of overt pathology in the sections examined for glomerular counting. Nevertheless, renal pathology may emerge at an older age.

An interesting finding of this study was that both restricted and reduced litter offspring had an increased left ventricular mass as a proportion of heart weight, suggesting a degree of left ventricular hypertrophy. This is likely to have resulted from sustained increases in blood pressure associated with increased hemodynamic load. Ventricular hypertrophy has been found in female rat offspring following prenatal growth restriction[44–46] as well as in sheep with sustained hypertension owing to prenatal glucocorticoid exposure.[44–46] Many factors can contribute to increased ventricular mass. In the rat model of growth restriction with associated left ventricular hypertrophy, there were increases in the relative gene expression levels of ANP in the left ventricle as well as the atrial isoform of the myosin light chain.[44–46] In our model, we found no change in ANP gene expression but did find increased expression of the AT_{1A} receptor. Although there is conflicting evidence for the role of renin–angiotensin system in the development of cardiac hypertrophy, angiotensin II binding to the AT_1 receptor can induce hypertrophy,[47] whereas the AT_2 receptor may play a cardioprotective role.[48] However, given that both the restricted and reduced litter offspring had increased left ventricular mass but that only the restricted group had increased AT_{1A} receptor mRNA, it is unlikely that a change in the cardiac renin–angiotensin system is the major factor contributing to increased left ventricular mass. We also chose to examine the expression of some extracellular matrix components, as these play crucial roles in the remodeling of the left ventricle (for review, see Gallagher *et al.*[49]). As with the AT_{1A} receptor, increased gene expression for collagen 3 and TIMP1 in the left ventricle was found only in the restricted group. This suggests that the mechanisms leading to alterations in the left ventricle may be dependent upon the timing of the growth restriction. Further studies during postnatal development are required to investigate the mechanisms and consequences of this increased left ventricular mass in our model.

Although prenatal growth restriction is consistently associated with the onset of adult disease, particularly hypertension, the effects of early postnatal growth restriction have been less clearly defined. This study demonstrates that in the rat, early growth deficits after birth can program a reduced nephron endowment, increased blood pressure, and left ventricular hypertrophy at least in adult male offspring. It should be noted that in a parallel study, adult female offspring at the same age did not exhibit alterations in blood pressure following either insult (data not shown), although whether this emerges later with aging and is related to changes in nephron endowment or other determinants is currently under investigation. These findings also emphasize the need to consider the appropriate controls in such studies and to carefully assess the consequences of the early postnatal, particularly lactation, environment on growth. This is of utmost importance when using models in which the prenatal environment is manipulated, as we may also be affecting early postnatal nutrition with additional independent effects on adult outcomes.

MATERIALS AND METHODS

Animals

This study was approved by The University of Melbourne Animal Experimentation Ethics Subcommittee before commencement. Wistar–Kyoto rats (9–13 weeks of age) were obtained from the Australian Resource Centre (Murdoch, WA, Australia) and maintained under standard conditions. Males were introduced and the presence of sperm in the vaginal smear the following morning was taken as day 1 of pregnancy.

On day 18 of gestation, pregnant rats were randomly allocated into the uteroplacental insufficiency (restricted) or sham control group (control) and surgery was (sham or bilateral uterine vessel ligation) performed.[14,18,19] All surgical procedures were performed under aseptic conditions with surgery time of approximately 10 min. Briefly, rats were anesthetized by intraperitoneal injection of a mixed solution containing ketamine (Parnell Laboratories, Alexandria, NSW, Australia; 50 mg per kg body weight) and Ilium Xylazil-20 (Troy Laboratories, Smithfield, NSW, Australia; 10 mg per kg body weight), and ligation of the uterine vessels on both the left and right sides was performed. Sham surgery for the control group was performed in the same manner, except that the uterine vessels were not ligated (control and reduced litter group). Animals were returned to individual boxes within 40 min following recovery and allowed to give birth naturally on day 22. The number of pups in each litter was recorded on day 1. In half of the animals from the control group, the litter size was reduced to five pups to generate a reduced litter group. Animals in which the bilateral uterine vessel ligation had been performed were termed the restricted group. Pups were weaned from their mothers at day 35 and male pups studied.

Weights and blood pressure

Weights of individual pups were measured at days 3, 6, and 35, as well as at 10 weeks and 6 months of age. Blood pressure was determined by tail cuff at 5, 9, and 22 weeks of age ($n = 8$–10 per group), as described previously.[14]

Postmortem and tissue collection

At 6 months of age, offspring were anesthetized (as above) and the right kidney removed, weighed, and fixed in 10% neutral buffered formalin for subsequent analysis of nephron number.[14,18] The left kidney was frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ for subsequent extraction of total RNA. The heart was removed and weighed. The left ventricle was dissected from the remainder of the

Table 3 | Primer and probe sequences, and final concentrations for use in real-time PCR

	Sequence (5′–3′)	Nucleotide position	Final concentration (nM)
Forward primer			
MMP2	TGTCTCCTGCTCTGTAG	2368–2384	300
MMP9	CGCTCTGCATTTCTTCA	1596–1612	300
TIMP1	AACCCACCCACAGACA	119–134	300
TIMP2	GTTGGAGGAAAGAAGGAA	435–452	300
Coll3	ATGGCAATCCTGATCTTC	4114–4131	300
Coll1	GCTGGTCTTCCAGGTCCTAAG	2284–2304	300
TGF-β	CATGGAGCTGGTGAAAC	256–272	300
ANP	TGTACAGTGCGGTGTCCAACA	158–176	150
Reverse primer			
MMP2	GCCACCCTCTTAAATCTG	2433–2416	300
MMP9	ACGTGCGGGCAATAAG	1687–1672	300
TIMP1	ACCCATGAATTTAGCCCTTA	179–160	300
TIMP2	CCAGGGCACAATAAAGTC	524–507	300
Coll3	GCAGTGGTATGTAATGTTC	4211–4193	300
Coll1	CGCCATCTTTGCCAGGAGAA	2362–2343	300
TGF-β	CTGGCGAGCCTTAGTT	594–579	300
ANP	TTCTCCTCCAGGTGGTCTAGCA	222–201	150
Taqman probe			
MMP2	AATCAGCCTTCTCCTTCACCTGGTG	2387–2411	100
MMP9	ACGGTCGGTATTGGAAGTTCTCGA	1616–1639	100
TIMP1	TTTCTGCAACTCGGACCTGGTTA	137–159	100
TIMP2	ATCTAATTGCAGGGAAGGCGGAA	454–476	50
Coll3	TGTCCTTGATGTACAGCTGGCC	4139–1460	100
Coll1	ACAGAGGTGATGCTGGTCCCAAA	2309–2331	100
TGF-β	AAGCGCATCGAAGCCATCCGT	275–295	75

ANP, atrial natriuretic peptide; Coll, collagen; MMP, metalloproteinase; TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of metalloproteinase.
Accession numbers: MMP2, NM_031054; MMP9, NM_031055; TIMP1, NM_053819; TIMP2, NM_021989; Coll3, NM_032085; Coll1, NM_053304; TGF-β1, NM_021578; and ANP, NM_012612.

heart and a section frozen in liquid nitrogen and stored at −80 °C for extraction of total RNA.

Estimation of glomerular number and volume

Glomerular number and volume were estimated using the physical dissector/fractionator technique as described previously.[14,41] Glomerular number and volume were determined in five male offspring from each group (one pup from each litter).

Gene expression analysis

Total RNA was extracted from the left kidney and real-time PCR was performed, as described previously,[14] for the AT_{1A}, AT_{1B}, and AT_2 receptors. In addition, we examined gene expression of collagens 1 and 3, MMP2, MMP9, TIMP1, TIMP2, TGF-β, and ANP, with two different endogenous controls (GAPDH and 18S) validated, with GAPDH chosen as the housekeeping gene. Primer and probe sequences, along with optimal concentrations for use, are shown in Table 3. Total RNA was also extracted from the left ventricle of the heart after it was determined that the growth-restricted groups showed signs of increased left ventricular mass.

Statistical analysis

For group comparisons, data were analyzed by one-way analysis of variance followed by Student–Newman–Keuls test for *post hoc* comparisons. Analysis of variance calculations were performed using SPSS-X (SPSS Inc., Encinitas, CA, USA). Results are presented as mean ± s.e.m. $P<0.05$ was taken as statistically significant. Not more than two littermates from each mother were used in each experimental group.

ACKNOWLEDGMENTS

We thank Kelli Johnson for performing the glomerular counting and Chris Chiu for assistance with gene expression analysis. We also thank the National Heart Foundation (grant to ME Wlodek and JA Owens), Channel 7 Children's Foundation South Australia (grant to JA Owens and ME Wlodek), and the University of Melbourne (joint grant to ME Wlodek and KM Moritz) for supporting this work. KM Moritz is supported by a National Health and Medical Research Council Career Development Award and AL Siebel by a National Health and Medical Research Council Peter Doherty Fellowship.

REFERENCES

1. Barker DJP, Osmond C, Golding J *et al.* Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ* 1989; **298**: 564–567.
2. Gluckman P, Hanson MA. Living with the past: evolution, development, and patterns of disease. *Science* 2004; **305**: 1733–1736.
3. Eriksson JG, Tuomilehto J, Winter PD *et al.* Catch-up growth in childhood and death from coronary heart disease: longitudinal study. *BMJ* 1999; **318**: 427–431.
4. Singhal A, Cole TJ, Lucas A. Early nutrition in preterm infants and later blood pressure: two cohorts after randomised trials. *Lancet* 2001; **357**: 413–419.
5. Eriksson JG, Forsén T, Tuomilehto J *et al.* Early growth and coronary heart disease in later life: longitudinal study. *BMJ* 2001; **322**: 948–953.
6. Eriksson JG, Forsen T, Tuomilehto J *et al.* Early adiposity rebound in childhood and risk of type 2 diabetes in adult life. *Diabetologia* 2003; **46**: 190–194.
7. Forsen TJ, Eriksson JG, Osmond C *et al.* The infant growth of boys who later develop coronary heart disease. *Ann Med* 2004; **36**: 389–392.
8. Eriksson JG, Osmond C, Kajantie E *et al.* Patterns of growth among children who later develop type 2 diabetes or its risk factors. *Diabetologia* 2006; **49**: 2853–2858.
9. Moritz KM, Dodic M, Wintour EM. Kidney development and the fetal programming of adult disease. *BioEssays* 2003; **25**: 212–220.

10. Langley-Evans SC, Welham SJ, Jackson AA. Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. *Life Sci* 1999; **64**: 965–974.
11. Woods LL, Weeks DA, Rasch R. Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. *Kidney Int* 2004; **65**: 1339–1348.
12. Moritz KM, Boon WM, Wintour EM. Glucocorticoid programming of adult disease. *Cell Tissue Res* 2005; **322**: 81–88.
13. Alexander BT. Fetal programming of hypertension. *Am J Physiol* 2006; **290**: R1–R10.
14. Wlodek ME, Mibus AL, Tan A *et al.* Normal lactational environment restores nephron endowment and prevents hypertension after placental restriction in the rat. *J Am Soc Nephrol* 2007; **18**: 1688–1696.
15. Jansson T, Lambert GW. Effect of intrauterine growth restriction on blood pressure, glucose tolerance and sympathetic nervous system activity in the rat at 3–4 months of age. *J Hypertens* 1999; **17**: 1239–1248.
16. Alexander BT. Placental insufficiency leads to development of hypertension in growth-restricted offspring. *Hypertension* 2003; **41**: 457–462.
17. Lane RH, Chandorkar AK, Flozak AS *et al.* Intrauterine growth retardation alters mitochondrial gene expression and function in fetal and juvenile rat skeletal muscle. *Pediatr Res* 1998; **43**: 563–570.
18. Wlodek ME, Westcott KT, O'Dowd R *et al.* Uteroplacental restriction in the rat impairs fetal growth in association with alterations in placental growth factors including PTHrP. *Am J Physiol* 2005; **288**: R1620–R1627.
19. O'Dowd R, Kent JC, Moseley JM *et al.* The effects of uteroplacental insufficiency and reducing litter size on maternal mammary function and postnatal offspring growth. *Am J Physiol* 2008; **294**: R539–R548.
20. Plagemann A, Harder T, Rake A *et al.* Observations on the orexigenic hypothalamic neuropeptide Y-system in neonatally overfed weanling rats. *J Neuroendocrinol* 1999; **11**: 541–546.
21. Velkoska E, Cole TJ, Morris MJ. Early dietary intervention: long-term effects on blood pressure, brain neuropeptide Y, and adiposity markers. *Am J Physiol* 2005; **288**: E1236–E1243.
22. Faust IM, Johnson PR, Hirsch J. Long-term effects of early nutritional experience on the development of obesity in the rat. *J Nutr* 1980; **110**: 2027–2034.
23. Wlodek ME, Lorenc U, O'Dowd R *et al.* Uteroplacental insufficiency impairs mammary function, milk intake and postnatal growth. *Pediatr Res* 2003; **53**: 36A.
24. Schreuder MF, Nyengaard JR, Remmers F *et al.* Postnatal food restriction in the rat as a model for a low nephron endowment. *Am J Physiol* 2006; **291**: F1104–F1107.
25. Woods LL, Ingelfinger JR, Rasch R. Modest maternal protein restriction fails to program adult hypertension in female rats. *Am J Physiol* 2005; **289**: R1131–R1136.
26. McMullen S, Langley-Evans SC. Maternal low-protein diet in rat pregnancy programs blood pressure through sex-specific mechanisms. *Am J Physiol* 2005; **288**: R85–R90.
27. Woods LL, Ingelfinger JR, Nyengaard JR *et al.* Maternal protein restriction suppresses the newborn renin–angiotensin system and programs adult hypertension in rats. *Pediatr Res* 2001; **49**: 460–467.
28. Gilbert JS, Lang AL, Nijland MJ. Maternal nutrient restriction and the fetal left ventricle: decreased angiotensin receptor expression. *Reprod Biol Endocrinol* 2005; **3**: 27.
29. Vehaskari VM, Stewart T, Lafont D *et al.* Kidney angiotensin and angiotensin receptor expression in prenatally programmed hypertension. *Am J Physiol* 2004; **287**: F262–F267.
30. Auldist DE, Carlson D, Morrish L *et al.* The influence of suckling interval on milk production of sows. *J Anim Sci* 2000; **78**: 2026–2031.
31. Babicky A, Ostadalova I, Parizek J *et al.* Onset and duration of the physiological weaning period for infant rats reared in nests of different sizes. *Physiol Bohemoslov* 1972; **22**: 449–456.
32. Tu MT, Lupien SJ, Walker CD. Measuring stress responses in postpartum mothers: perspectives from studies in human and animal populations. *Stress* 2005; **8**: 19–34.
33. Champagne FA, Meaney MJ. Stress during gestation alters postpartum maternal care and the development of the offspring in a rodent model. *Biol Psychiatry* 2006; **59**: 1227–1235.
34. Deschamps S, Woodside B, Walker CD. Pups' presence eliminates the stress hyporesponsiveness of early lactating females to a psychological stress representing a threat to the pups. *J Neuroendocrinol* 2003; **15**: 486–497.
35. Cebrian C, Herzlinger DA. Morphometric index of the developing murine kidney. *Dev Dyn* 2004; **231**: 601–608.
36. Woods LL, Douglas AW. Naturally occurring intrauterine growth retardation and adult blood pressure in rats. *Pediatr Res* 2004; **56**: 763–767.
37. Hughson M, Farris AB, Douglas-Denton R *et al.* Glomerular number and size in autopsy kidneys: the relationship to birth weight. *Kidney Int* 2003; **63**: 2113–2122.
38. Zimanyi MA, Bertram JF, Black MJ. Does a nephron deficit in rats predispose to salt-sensitive hypertension? *Kidney Blood Press Res* 2004; **27**: 239–247.
39. Bursztyn M, Gross M-L, Goltser-Dubner T *et al.* Adult hypertension in intrauterine growth-restricted offspring of hyperinsulinemic rats: evidence of subtle renal damage. *Hypertension* 2006; **48**: 717–723.
40. Tonkiss J, Trzcinska M, Galler JR *et al.* Prenatal malnutrition-induced changes in blood pressure: dissociation of stress and nonstress responses using radiotelemetry. *Hypertension* 1998; **32**: 108–114.
41. Singh R, Cullen-McEwen LA, Kett KM *et al.* Prenatal corticosterone exposure results in altered AT1/AT2, nephron deficit and hypertension in the rat offspring. *J Physiol* 2007; **579**: 503–513.
42. Schaefer L, Han X, August C *et al.* Differential regulation of glomerular gelatinase B (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in obese Zucker rats. *Diabetologia* 1997; **40**: 1035–1043.
43. Bolbrinker J, Markovic S, Wehland M *et al.* Expression and response to angiotensin-converting enzyme inhibition of matrix metalloproteinases 2 and 9 in renal glomerular damage in young transgenic rats with renin-dependant hypertension. *J Pharmacol Exp Ther* 2006; **316**: 8–16.
44. Battista MC, Oligny LL, St-Louis J *et al.* Intrauterine growth restriction in rats is associated with hypertension and renal dysfunction in adulthood. *Am J Physiol* 2002; **283**: E124–E131.
45. Dodic M, Samuel C, Moritz KM *et al.* Impaired cardiac functional reserve and left ventricular hypertrophy in adult sheep after prenatal dexamethasone exposure. *Circ Res* 2001; **89**: 623–629.
46. Battista MC, Calvo E, Chorvatova A *et al.* Intrauterine growth restriction and the programming of left ventricular remodelling in female rats. *J Physiol* 2005; **565**: 197–205.
47. Kjeldsen SE, Strand A, Julius S *et al.* Mechanism of angiotensin II type 1 receptor blocker action in the regression of left ventricular hypertrophy. *J Clin Hypertens* 2006; **8**: 487–492.
48. Anavekar NS, Solomon SD. Angiotensin II receptor blockade and ventricular remodelling. *J Renin Angiotensin Aldosterone Syst* 2005; **6**: 43–48.
49. Gallagher GL, Jackson CJ, Hunyor SN. Myocardial extracellular matrix remodeling in ischemic heart failure. *Front Biosci* 2007; **12**: 1410–1419.